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Complexity of Human Ovarian Folliculogenesis: Molecular Markers of Ovarian-Based Infertility and the Impact of Endocrine-Disrupting Chemicals

Kristine Rosenberg

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

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Inimese munasarja follikulogeneesi keerukus: munasarjapõhise viljatuse molekulaarsed markerid ja endokriinsüsteemi mõjutavate kemikaalide toime

KRISTINE ROSENBERG

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List of Publications

The list of author's publications, based on which the thesis has been prepared:

I **Roos K**, Rooda I, Keif RS, Liivrand M, Smolander OP, Salumets A, Velthut-Meikas A.
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II Bellavia A, Zou R, Björvang RD, **Roos K**, Sjunnesson Y, Hallberg I, Holte J, Pikki A, Lenters V, Portengen L, Koekkoek J, Lamoree M, Van Duursen M, Vermeulen R, Salumets A, Velthut-Meikas A, Damdimopoulou P.
Association between chemical mixtures and female fertility in women undergoing assisted reproduction in Sweden and Estonia.
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III Rooda I, Hasan MM, **Roos K**, Viil J, Andronowska A, Smolander OP, Jaakma Ü, Salumets A, Fazeli A, Velthut-Meikas A.
Cellular, Extracellular and Extracellular Vesicular miRNA Profiles of Pre-Ovulatory Follicles Indicate Signaling Disturbances in Polycystic Ovaries.
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Author's Contribution to the Publications

Contributions to the publications in this thesis are as follows:

I The author obtained ethical approvals and consent forms from the patients, collected biomaterial and clinical data, conducted experiments, analysed the results, prepared figures, and tables, drafted the manuscript, and participated in the preparation of final revisions of the manuscript before submission.

II The author obtained ethical approvals and consent forms from the patients, collected biomaterial and clinical data, analysed, and interpreted a part of the results, contributed to the writing process, revised the manuscript, and edited the final draft before submission.

III The author obtained ethical approvals and consent forms from the patients, collected biomaterial and clinical data, and contributed to manuscript editing.

Introduction

Nordic countries like Estonia, Finland, and Norway report record-low birth rates (calculated as the number of children per woman of reproductive age): 1.61 (Births | Statistikaamet, 2020), 1.35, and 1.56, respectively (Jokinen et al., 2020). This trend extends globally, with the European Union (EU) at 1.50, the United States (USA) at 1.60, China at 1.70, and Australia at 1.58, a significant decline from rates over 2.00 fifty years ago (*The World Bank, Fertility Rate, Total (Births per Woman)*, 2020). There could be many reasons for this decreasing trend, including improved contraception access, personal choices, government welfare policies (Leridon, 2006; Vollset et al., 2020), rising divorce rates (Nikolaou et al., 2002), and the choice to remain childless. Some complications interfere with people's ability to have children. Infertility, affecting approximately 186 million people worldwide, becomes a concern for couples who, despite a year of regular, unprotected intercourse, do not achieve pregnancy (National Center for Health Statistics, 2005; Ombelet, 2020).

According to the statistics on infertility cases, men and women experience infertility to a similar extent in about 35–40% of cases. In 20–25% of infertility cases, the problems are present on both sides, and in some cases, the cause remains unexplained (R. E. Jones & Lopez, 2016). Some of the frequently reported infertility aetiologies, both separately and in combination, include chromosomal abnormalities (Yahaya et al., 2021), hormone imbalances and endocrine disorders, inflammatory diseases, stress (Bendarska-Czerwińska et al., 2022), increasing maternal age (Brugo-Olmedo et al., 2001), a high body mass index (BMI) (Gesink Law et al., 2007), and exposure to certain environmental pollutants and chemicals (Conforti et al., 2018; World Health Organization et al., 2013).

Infertility clinics provide assisted reproductive technologies (ART) to help infertile couples have children. Over the course of several decades, the success rates of ART have remained modest and stable, averaging a 30% live birth (LB) rate and being lower in different aetiology groups for infertility (Amini et al., 2021). To improve LB rates at infertility clinics, it is crucial to first use high-throughput methods to describe the reproductive system in detail. Next, it is important to compare the changes caused by various pathologies to those observed in fertile women. This approach helps to better understand the underlying causes of infertility. With this knowledge, more effective ART techniques and medical therapies can be developed to overcome these challenges.

This thesis focuses on women with different aetiologies of infertility. Accordingly, the primary objective of the thesis is to characterise ovarian function and sensitivity to stimulation in different clinical patient groups by analysing the content of follicular fluid (FF), the preconception environment of the oocyte in the preovulatory follicle. The gene expression of somatic cells isolated from FF and the proportions of somatic cell types are studied in depth. Secondly, the influence of environmental chemicals on ovarian sensitivity to stimulation is investigated. Finally, the link between microRNA (miRNA) disturbances and the condition known as polycystic ovary syndrome (PCOS) is elucidated. For this purpose, a large dataset of biological material obtained from ART patients and oocyte donors was studied with multiple genome-wide methods to unravel novel potential therapy suggestions based on the differences between the gene expression profiles of somatic cells and FF samples from preovulatory follicles. Moreover, this thesis fills multiple knowledge gaps in the current epidemiological situation of reproductive health and environmental chemical exposure among Estonian and Swedish women.

Abbreviations

AFC	antral follicle count
AMH	anti-Müllerian hormone
AOP	Adverse Outcome Pathways
aromatase	aromatase cytochrome P450
ART	assisted reproductive technology
ATP	adenosine triphosphate
BMI	body-mass index
BMP-15	bone morphogenic protein 15
BPA	bisphenol A
cAMP	cyclic adenosine monophosphate
CGC	cumulus granulosa cell
CL	corpus luteum
COC	cumulus-oocyte complex
COS	controlled ovarian stimulation
CP	clinical pregnancy
DEG	differentially expressed gene
DEHP	di-2-ethylhexyl phthalate
DES	diethylstilbestrol
DF	dominant follicle
DiNP	diisononyl phthalate
E2	oestradiol, 17β-oestradiol
ECHA	European Chemicals Agency
ECM	extracellular matrix
EDC	endocrine-disruptive chemical
EFSA	European Food Safety Agency
ER	oestrogen receptor
EV	extracellular vesicle
FDR	false discovery rate
FF	follicular fluid
FOI	follicle oocyte index
FORT	follicular output rate
FREIA	Female Reproductive Toxicology of EDCs
FSH	follicle-stimulating hormone
FSHR	follicle-stimulating hormone receptor
GC	granulosa cell
GDF-9	growth differentiation factor 9
GM-CSF	granulocyte macrophage colony stimulating factor
GnRH	gonadotropin-releasing hormone
GPCR	G-protein-coupled receptor
GV	germinal vesicle

hCG	human chorionic gonadotropin
HR	hyporesponder
ICSI	intracytoplasmic sperm injection
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor-binding protein
IL	interleukin
INSL3	insulin-like peptide 3
IR	insulin resistance
IVF	*in vitro* fertilisation
IVM	*in vitro* maturation
LB	live birth
LH	luteinizing hormone
LPC	lysophosphatidylcholine
M1	primary oocyte
M2	fully mature haploid oocyte
MECPP	mono-(2-ethyl-5-carboxypentyl) phthalate
MEHHP	mono-(2-ethyl-5-hydroxyhexyl) phthalate
MEHP	mono-2-ethylhexyl phthalate
MEOHP	mono (2-ethyl-5-oxohexyl) phthalate
MEP	mono-ethyl phthalate
MGC	mural granulosa cell
miRNA	microRNA
MMP	matrix metalloproteinases
mTOR	the mammalian target of rapamycin
NR	normoresponder
OHSS	ovarian hyperstimulation syndrome
OPU	oocyte-pick up
OSI	ovarian sensitivity index
P4	progesterone
PAPP-A	pregnancy-associated plasma protein A
PB	polar body
PCOM	polycystic ovarian morphology
PCOS	polycystic ovary syndrome
PFAS	per- and polyfluoroalkyl substances
PFOA	perfluorooctanoic acid
PFUnA	perfluoroundecanoic acid
PGT	preimplantation testing
PI3K/AKT	phosphoinositide 3-kinase and protein kinase B
PIP3	phosphatidylinositol 3,4,5-triphosphate
POF	premature ovarian failure
POI	premature ovarian insufficiency
POR	poor responder

PTEN	phosphatase and tensin homolog
REACH	Registration, Evaluation, Authorization, and Restriction of Chemicals
rFSH	recombinant follicle-stimulating hormone
r-LH	recombinant luteinizing hormone
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
scRNA-seq	single-cell RNA-sequencing
SNP	single-nucleotide polymorphism
TC	theca cell
TGF-β	transforming growth factor beta
TZP	transzonal projections
VEGF	vascular endothelial growth factor
WHO	World Health Organization
ΣDEHP	the sum of DEHP metabolites: MEHP, MECPP, MEHHP, and MEOHP

1 Review of the Literature

1.1 Human folliculogenesis

The ability of a woman to achieve and carry a pregnancy and give birth to a healthy child is referred to as female fertility. It is dependent on reproductive tract organs such as the uterus, paired ovaries, and fallopian tubes, as well as fluctuating sex hormone levels. Folliculogenesis is the process by which ovarian follicles develop and become activated, with majority of them being eliminated by atresia before ovulation occurs (R. E. Jones & Lopez, 2016). The process begins during the intrauterine development of the female foetus. As early as the fifth week of gestation, the ovary of the female foetus contains as many as 1300 primordial germ cells (Figure 1). Subsequently, by the seventh week of gestation, these cells have undergone mitotic divisions with incomplete cytokinesis, forming oogonia (McGee & Hsueh, 2000).

Next, these oocytes initiate the start of meiosis but arrest in prophase I of meiosis (Grive & Freiman, 2015). When the networking between pregranulosa cells and oocytes starts, primordial follicles in the developing gonad are formed from a pool of oogonia. The primordial follicles function as a continual supply of growing follicles during the entire period of the female reproductive lifespan, forming the ovarian reserve. Hence, primordial follicles consist of an oocyte that is arrested in prophase I of meiosis, referred to as the germinal vesicle (GV) stage (as depicted in Figures 1 and 2A). This GV-stage oocyte is encircled by a basal lamina and a single layer of flattened granulosa cells (GCs). Without an oocyte, follicles cannot form (Skinner, 2005).

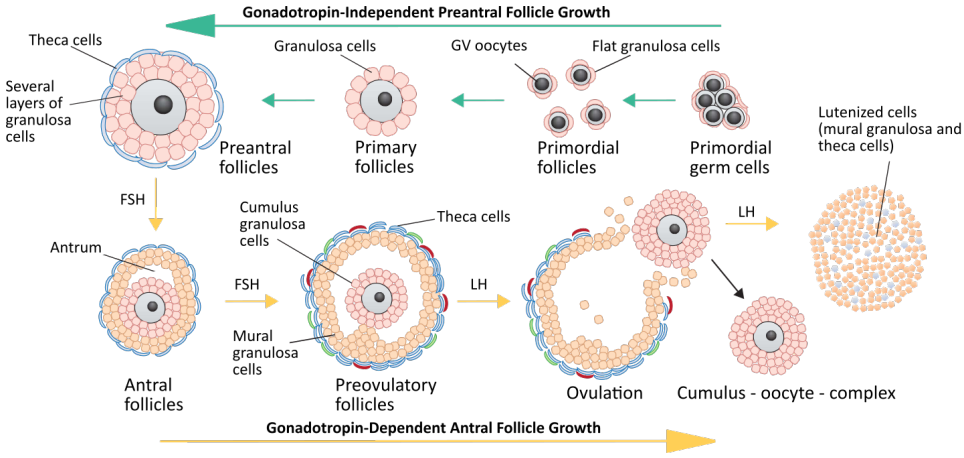


Figure 1. *The main stages of folliculogenesis. Folliculogenesis initiates with the recruitment of primordial follicles. While resting in the ovarian cortex, they form the female's entire ovarian reserve. At this stage, the main cell type, granulosa cells (GCs), begin to actively proliferate, and the theca cell (TC) layer forms to surround the follicle, producing androgens, which GCs convert to oestradiol. Gonadotropins, such as follicle-stimulating hormone (FSH) are now required for follicular growth. The antrum divides GCs into mural and cumulus granulosa cells as they proliferate. The dominant follicle is selected, and the others are eliminated by atresia. High levels of luteinizing hormone (LH) induce ovulation by causing the cumulus-oocyte-complex to be released and the TCs and mural GCs to luteinizing in order to form the corpus luteum, which secretes progesterone to sustain the pregnancy (adapted and modified from Georges et al., 2014).*

Primordial follicles stay dormant for years until a cohort of them starts development into primary follicles. However, the exact mechanism triggering this activation remains unclear. There are about 7 million primordial follicles at 20 weeks of gestation (McGee & Hsueh, 2000). By the time a female reaches puberty, only 400,000 primordial follicles are left (Gougeon et al., 1994), from which 300 to 400 oocytes mature and ovulate (Oktem & Oktay, 2008).

During folliculogenesis, there is a distinction between gonadotropin-independent and gonadotropin-dependent follicle growth, also known as the preantral and antral follicle growth phases, as well as between initial and cyclic follicle recruitment (Edson et al., 2009). Initial recruitment occurs from the formation of primordial follicles to the primary follicle transition and is independent of gonadotropins. The pivotal event is the transition of GCs from flattened to cuboidal cell morphology, which is controlled by multiple pathways including the mammalian target of rapamycin (mTOR), Hippo, and transforming growth factor beta (TGF-β) (Ernst et al., 2018; Ford et al., 2020; Grosbois & Demeestere, 2018).

However, only a minority of activated follicles reach the primary follicle stage because the majority are eliminated by atretic degeneration (Baerwald et al., 2012; Gershon & Dekel, 2020; Sathananthan et al., 2006). Apart from follicular atresia, follicular degeneration may also occur through apoptosis, triggered by factors such as inadequate hormonal support, diminished blood, and nutrient supply, disrupted oocyte-GCs communication, and genetic mutations. Moreover, exposure to external toxins can induce necrosis, leading to an inflammatory response, collectively resulting in follicle elimination (Gougeon, 2010; Regan et al., 2018).

Theca cell (TC) recruitment from the stromal-interstitial cell population, the vigorous proliferation of GCs and TCs, and follicle growth, including the increase in oocyte volume, are the following crucial events in the process of folliculogenesis (Skinner, 2005).

During the preantral stage of follicle growth, the GCs and TCs are still in the precursor stage and do not synthesise steroid hormones. Inside secondary follicles, there are several layers of GCs encircling the developing oocyte. Furthermore, in the follicle, the basement membrane binds the GCs, clearly separating them from the layer of follicular TCs. Follicles grow in size, a fluid-filled cavity forms between the GCs, and the follicle enters the antral stage (Hennet & Combelles, 2012).

Follicle growth from preantral to preovulatory stage is dependent on gonadotropin secretion, and following puberty, the activation takes place on a cyclic basis. Gonadotropic hormones have become critical in the mechanisms that control follicle development and steroid hormone secretion to achieve ovulation (Gougeon, 1996; Hillier, 2001).

A follicle-stimulating hormone (FSH) regulates and stimulates follicle growth, and the synthesis of steroid hormones from GCs, resulting in follicle dominance in the secretion of oestradiol (also named 17β-oestradiol, E2), and inhibin. During different stages of a woman's life, three key biologically active oestrogens are synthesized: E2 prevails during the reproductive period, estriol is prominent during pregnancy, and estrone becomes the dominant circulating oestrogen at the onset of menopause (Parisi et al., 2023).

Luteinizing hormone (LH) stimulates androgen synthesis in TCs, which serves as a substrate to produce ovarian oestrogens by GCs and regulates ovulation. FSH and LH function by binding to their respective receptors, FSHR and LHR, respectively (Messinis et al., 2010). The named hormones are released in pulses from the anterior lobe of the pituitary gland approximately every hour in response to hypothalamic-produced gonadotropin-releasing hormone (GnRH), which is also released in a pulsatile manner (Cox & Takov, 2022; Hennet & Combelles, 2012; F. Matsuda et al., 2012).

A gonadotropin-dependent cycle, also known as the menstrual cycle, involves the selection of a dominant follicle (DF) from a group of antral follicles, which usually results in the release of a single oocyte (Gougeon, 2010). During the recruitment of DF, a change in steroidogenesis occurs in selected follicles with diameters ranging from 8–11 mm, resulting in a significant increase in hormone levels, particularly the synthesis of E2 and progesterone (P4), upon the expression of aromatase cytochrome P450 (aromatase) enzyme. In addition, selected follicles secrete elevated levels of inhibins and insulin-like growth factor (IGF)-2, and therefore IGF binding proteins (IGFBPs) and their specific proteases are also upregulated in DF development (Kristensen et al., 2018; Son et al., 2011). Furthermore, these selected follicles exhibit increased expression of FSHR, allowing them to be more sensitive to FSH (Gougeon, 2010) and facilitating the upregulation of LHR expression, which is essential for the synthesis of E2 in the second half of the follicular phase. In DF, the higher levels of aromatase and upregulated E2 production are largely driven by decreasing levels of TGF-β family member anti-Müllerian hormone (AMH) synthesis by GCs and increasing levels of inhibins (Kristensen et al., 2018).

Along with the factors listed above, many endocrine, autocrine, juxtracrine, and paracrine factors, including TGF-β growth factors such as inhibins, activins, and IGF-2 via pregnancy-associated plasma protein A (PAPP-A), have an important effect in regulating the effect of FSH and LH on the fate of a follicle, i.e., whether it becomes a DF and undergoes ovulation or degenerates as a result of atresia (Edson et al., 2009; Kristensen et al., 2018). Furthermore, it has been reported that there is an increase in the expression levels of the hormone PAPP-A in TCs during the initial phase of follicle growth, which is then followed by a subsequent rise in the number of GCs as the follicle progresses towards the DF stage (Kristensen et al., 2018).

A preovulatory or Graafian follicle is in the last stage of antral growth. Its diameter varies with the size of the antrum, ranging from 17–25 mm. The preovulatory follicle is composed of two main types of differentiated GCs: mural GCs (MGCs) that surround the antrum and cumulus GCs (CGCs) that surround the oocyte (Palermo, 2007). A fully mature haploid oocyte (M2) is released from a preovulatory follicle and is tightly connected to CGCs, forming a cumulus-oocyte-complex (COC). The oocyte in the COC is capable of progressing to meiotic division II after being fertilised by the spermatozoon after ovulation (R. E. Jones & Lopez, 2016).

Each adult woman has a unique ovarian reserve that can be measured using AMH. Because AMH levels correlate with the number of antral follicles observed with ultrasound imaging, it is commonly used in clinical practice (van Rooij et al., 2002; Weenen et al., 2004). The AMH is involved in regulating the transition from primordial to primary follicles: while the AMH is being actively secreted by FSH-independent developing follicles, it simultaneously inhibits the development of primordial follicles, acting as an effective form of feedback regulation between the follicle pool (Skinner, 2005; Weenen et al., 2004). The number of follicles and oocyte quality vary considerably between women and decline with age (Tatone et al., 2008).

Menopause marks the end of the reproductive period, when there are still at least 100–1000 resting follicles remaining but follicle development toward ovulation is ineffective or has stopped due to declining P4 and primarily E2 levels. The low levels of E2 lead to an absence of negative feedback on the secretion of gonadotropins, thereby causing an elevation in the secretion of FSH and LH (Gougeon, 1996; R. E. Jones & Lopez, 2016; Kling et al., 2019). AMH levels vary during a woman's lifespan and in response to various pathologies (Eudy et al., 2019; C. Kim et al., 2016; Ledger, 2010). By measuring

AMH and linking it with age, the onset of menopause and other related conditions, for instance, breast cancer, could be predicted (Broer et al., 2011).

Ovarian reserve may be significantly reduced as a result of severe endometriosis, ovarian surgery, chemotherapy, smoking, chlamydia infection, pelvic inflammatory or autoimmune diseases. Also, genetic factors and chromosomal abnormalities have a significant role in both the quantity of oocyte and the rate of decline (Brugo-Olmedo et al., 2001). A decreased ovarian pool has been linked to unexplained infertility (Yücel et al., 2018) and recurrent miscarriage (Trout & Seifer, 2000). Furthermore, it is associated with unfavourable lipid levels and increased cardiovascular risk in women with normal menstrual cycles (Chu et al., 2001).

Dysregulations in the multistage folliculogenesis process cause a variety of reproductive health issues, the majority of which result in infertility diagnoses: lack of a primordial follicle pool or ovulation process, premature ovarian failure (POF), early menopause, and poor oocyte quality (Ford et al., 2020; R. E. Jones & Lopez, 2016; Oktem & Oktay, 2008). In order to improve clinical approaches, a comprehensive understanding of folliculogenesis, the recognition of follicular somatic cell types, and the detection of changes in their functions due to various aetiologies of infertility is essential (Oktem & Oktay, 2008).

1.2 The diverse environment of the preovulatory follicle

Preovulatory follicle is the final stage of folliculogenesis preceding ovulation, during which crucial oocyte functions such as growth, meiotic maturation, take place. By the preovulatory stage a human follicle has expanded from the primary stage of 30 µm to more than 17 mm, indicating that follicular cell functions and numbers have changed significantly, and molecular signalling occurs over longer distances. As a result, it consists of several components that participate in the development of the M2 in order to facilitate subsequent fertilisation and the continuation of life. These are the CGCs, MGCs in various luteinizing phases, two layers of follicle-surrounding TCs (Hennet & Combelles, 2012; Lai et al., 2015), immune cells and other somatic cell types, and FF with its various components (Gougeon, 1996).

Through bi-directional interaction, somatic cells in the ovarian follicle provide the oocyte with the nutrients, signals, and physical support it needs for growth (Eppig, 2001). During this time, the oocyte's needs for metabolites are constantly changing. Hormones made outside of the follicle, like FSH and LH, and inside the follicle, like P4, AMH, and E2, affect how signalling pathways and cell types work in the follicle before ovulation (Hennet & Combelles, 2012; R. E. Jones & Lopez, 2016). Higher levels of LH cause a change in the steroidogenic activity of the somatic cells in the preovulatory follicle: P4 production is increased while E2 production is decreased. The levels of prostaglandins and metalloproteases are also altered (Fortune et al., 2009). Because of this, each part of the follicle adds to the microenvironment and affects the oocyte within. The preovulatory FF and its cell types are more accurately characterised as a result of the implementation of newly developed methods.

The application of novel molecular, metabolic, and biochemical markers would improve *in vitro* fertilisation (IVF) results, particularly in relation to pregnancy-associated follicles, oocyte and embryo quality, implantation, and pregnancy rate. However, despite their potential benefits, the integration of these markers into routine IVF laboratory procedures remains limited (Revelli et al., 2009). Currently, the most significant indicator of IVF success is the quality of the embryo that an embryologist observes using a light microscope (Lv et al., 2020; Terriou et al., 2001).

During the oocyte pick-up (OPU) stage of the ART procedure, follicular somatic cells, FF, and its components become accessible resources. These hold potential as diagnostic tools for discerning variations in patient responsiveness to controlled ovarian stimulation (COS) and reproductive disorders like reduced ovarian reserve, endometriosis, oxidative stress, inflammation, and idiopathic infertility. Consequently, it is necessary to investigate the gene expression profiles of follicular cells, the metabolites in FF, the oocyte morphology throughout the preovulatory follicle, and the culture medium of fertilised oocytes. Undoubtedly, reliable methods will enter the IVF market (Collodel et al., 2023; Marchiani et al., 2020; Y.-T. Wu et al., 2015).

1.2.1 Constituents of the follicle

1) Oocyte

The re-entering to the meiotic cell cycle occurs after a surge in LH, which is followed by GV breakdown in primary oocytes (Figure 2A). The term refers to the dissolution of the nucleus known as the GV during the arrested stage of prophase of meiosis I developmental phase. The oocyte progresses through the first meiotic resumption and enters the primary oocyte stage (M1) (Figure 2B). This event is characterised by the extrusion of the first nonfunctional polar body (PB, Figure 2C). PBs are formed by asymmetric cytokinesis, where cytosol and organelles are mostly moved to the oocyte and a relatively small amount of cytoplasm, including a separate nucleus, ribosomes, Golgi, mitochondria, and cortical granules, is left for these PBs that end up degenerating by apoptosis and the fragments remain in the zona pellucida (M. He et al., 2021). After the first meiotic division, the oocyte proceeds to undergo the second meiotic division. M2 oocyte remains in a state of metaphase II arrest until fertilisation occurs, resulting in the development of a M2 with a second PB (Figure 2D) (Eppig, 2001).

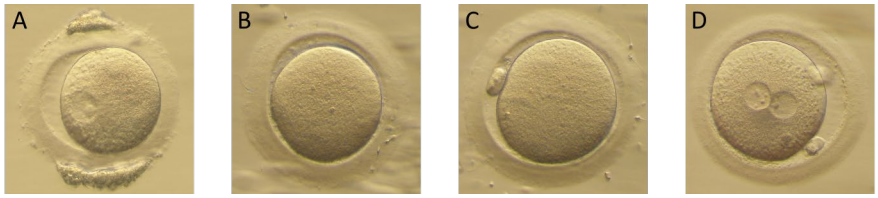


Figure 2. *Developmental stages of the human oocyte.* ***(A)*** *The germinal vesicle-stage (GV) oocyte is immature and arrested in prophase I of meiosis found inside the primordial follicle. (B) Even though the primary oocyte (M1) oocyte has passed the GV stage and has entered metaphase I, it is still an immature oocyte. It can, however, be matured in vitro. Immature oocytes, such as GV and M1, have 46 chromosomes and cannot be fertilised.* ***(C)*** *A mature haploid oocyte known as M2 has reached the second metaphase stage, has one polar body (PB), and is ready for fertilisation with sperm.* ***(D)*** *After successful fertilisation between two gametes, a zygote is formed, and the pronuclei of maternal and paternal origin and the second PB are visible after 16–18 hours. (Nova Vita Clinic phase-contrast images of human oocytes).*

The oocyte itself experiences significant morphological changes during follicular growth from small antral follicles to fully grown: its volume increases by over 13% while its diameter experiences a comparatively smaller increase of approximately 4%, as demonstrated in mice (Eppig & O'Brien, 1996). Moreover, the gene expression patterns of oocytes exhibit variations across developmental phases (Yu et al., 2020; Y. Zhang et al., 2018). These variations can occur within the same developmental phases but across women of varying ages (Ntostis et al., 2021; J.-J. Zhang et al., 2020).

Additionally, differences in gene expression are observed between *in vitro*- and *in vivo*-matured oocytes (G. M. Jones et al., 2008), particularly in the context of metabolising energy (Zhao et al., 2019).

Following successful oocyte-sperm fusion, the oocyte undergoes activation, which includes a series of biochemical and physical changes to prevent polyspermy. A sequence of intracellular Ca^{2+} oscillations caused by sperm entry into the ooplasm, rises its free calcium concentration and this triggers the second meiotic division to be completed, increases oocyte metabolism, protein, RNA, and DNA synthesis, and begins the preparation for the first mitotic division. In humans, the process of oocyte activation to sperm and oocyte pronuclear fusion takes about 12 hours and results in the formation of a zygote (Figure 2D) (M. He et al., 2021; R. E. Jones & Lopez, 2016; Swain & Pool, 2008).

Folliculogenesis influences the development and quality of oocytes. The absence of an oocyte or its poor quality are the major limiting factors in ART (Broekmans et al., 2006). In addition to sperm factors, failure of oocyte activation, encompassing alterations in the zona pellucida to hinder polyspermy and the completion of the second meiotic division, might underlie the necessity of repeated fertilisation procedures on M2s at IVF laboratories (Swain & Pool, 2008).

To overcome fertilisation failure and subsequent cleavage anomalies caused by the lack of calcium, assisted oocyte activation in IVF, which has been shown to significantly increase fertilisation, blastocyst formation, and clinical pregnancy (CP) rates, could be used (Shan et al., 2022). The method involves adding calcium ionophores to the cultivation medium, such as Calcimycin (Ebner et al., 2015) or Ionomycin (Deemeh et al., 2015), to artificially provide adequate calcium. Preis et al. have shown that oocytes that absorb a greater amount of glycose from the environment and actively convert it to lactate have the highest fertilisation potential (Preis et al., 2005).

The routine practices of the standard IVF laboratory methods do not include any decision methods for oocyte selection other than evaluating the maturity level. Likewise, embryologists assess embryos critically based on blastomere size and the amount or the number of degenerating cells attached using visual observation or an Embryoscope with software. Due to ethical reasons limiting direct examination of human oocytes, most research is conducted by examining the somatic cells from FF, which are not utilised in IVF treatment.

Another option for research involves animal models. In such cases mouse and bovine oocytes and subsequent embryos are an alternative. Therefore, several studies have examined the links between oocyte morphology, FF and its somatic cells or IVF culture medium characteristics, response to gonadotropin stimulation, and fertilisation rates or LB outcomes during IVF treatment (Appasamy et al., 2008; Bosco et al., 2017; Y. Fan et al., 2019; Kõks et al., 2010; Lukassen et al., 2003).

Also, human oocyte PBs are allowed to be used in oocyte genetic testing, where the PB is removed prior to fertilisation with little to no harm to the oocyte. As a result of the two meiotic divisions, the oocyte produces PB twice during maturation. This approach enables the detection of unequal distributions of maternal chromosomes or mutations in specific genes, along with the evaluation of oocyte quality through the analysis of mRNA (Klatsky et al., 2010).

Increasing the number and quality of oocytes during IVF treatment, as well as evaluating them or the developing embryos non-invasively and more effectively in the IVF laboratory, is a beneficial strategy that shortens the time-to-pregnancy. Considering this, preimplantation testing (PGT) of cultured embryos has become more common in

clinical settings because it provides critical insights into identifying euploid embryos and recessive genetic diseases such as cystic fibrosis or spinal muscular atrophy. Compared to PB testing, the aforementioned technique allows for the transfer of unaffected embryos, increasing the chances of a successful pregnancy and the birth of a healthy newborn (Greco et al., 2020).

Cell-free DNA released into the culture medium has significant potential to revolutionise non-invasive PGT and replace current invasive, time-consuming methods. These conventional approaches involve the physical removal of cells from oocytes or embryos, posing a risk of embryo degeneration (Huang et al., 2019). Therefore, the search for non-invasive biomarkers and technology solutions is ongoing. In the IVF market, a few techniques for improving implantation rates by trying to influence embryo viability are already available: the use of hyaluronan or granulocyte macrophage colony stimulating factor (GM-CSF) enriched cultivation media, as well as assisted hatching. Hyaluronan, a glycoprotein, facilitates embryo attachment to the endometrium by establishing a highly viscous uterine environment. Conversely, GM-CSF, a crucial protein expressed during embryo development, enhances embryo survival, growth, and implantation potential (P. Chen et al., 2021; Glatthorn & Decherney, 2022). The disruption of the zona pellucida benefits embryos with hardened zona and promotes the exchange of metabolites, growth factors, and communication between the hatched embryo and the endometrium (Carney et al., 2012; D. Li et al., 2016).

The *in vitro* oocyte maturation (IVM) method allows oocyte maturation in the lab from ovaries that have not been stimulated by gonadotropins or have only been stimulated at low levels (Edwards, 2007; Gilchrist et al., 2008). Despite some LBs, the developmental competence of IVM oocytes and IVM-culture systems is low, limiting its clinical implementation (Grynberg et al., 2022). Expertise in IVM would fill an important gap in ART outcomes for patients, for instance, with oocyte maturation errors in stimulated cycles, patients who have given their ovary tissue for preservation prior to cancer treatment and cannot perform hormone therapy, or patients with PCOS who have a high number of small antral follicles and are at risk for ovarian hyperstimulation syndrome (OHSS) (Edwards, 2007). For instance, over the course of a year, at least 23% of the total oocytes collected during IVF treatment are immature (Nova Vita statistics for 2020). A more comprehensive understanding of the molecular mechanism underlying folliculogenesis would facilitate the activation of the resting primordial follicles in the ovarian cortex into M2 oocytes by IVM (McLaughlin et al., 2018).

Enhancing our knowledge of how the follicle and its components facilitate oocyte development empowers us to supplement artificial conditions with essential substances for achieving success. To compensate for external changes affecting the oocyte, ART technology must replicate the follicular environment and comprehend the alterations the oocyte requires and regulates within the follicle (Grynberg et al., 2022).

2) Granulosa cells

GCs, the most abundant somatic cell type in the follicle undergo significant transformation and expansion during folliculogenesis to provide physical support and a microenvironment for the oocyte (Skinner, 2005). The interaction between oocyte and GCs is complex. GCs are in bidirectional communication with the oocyte through gap junctions, transzonal projections (TZP) and paracrine signalling (Hennet & Combelles, 2012). As a result, the oocyte regulates the proliferation and differentiation of GCs, which subsequently become involved in steroid and protein production (Hennet & Combelles, 2012).

During the antral follicular phase GCs are sensitive to gonadotropins and express receptors that mediate the actions of hormones and growth factors, including FSH, LH, IGF (Ipsa et al., 2019), and epidermal growth factor that impact oocyte maturation and meiosis progression (Mehlmann, 2005).

Furthermore, GCs have an important role in the oocyte's growth, differentiation, meiosis, cytoplasmic and nuclear maturation, and transcriptional activity in the oocyte genome (Eppig, 2001; Palma et al., 2012). The communication between the oocyte and follicular cells is bidirectional, with the oocyte releasing factors such as growth differentiation factor 9 (GDF-9) and TGF-β isoforms, which inhibit further growth-promoting actions of GCs (Matzuk et al., 2002). Conversely, the oocyte also regulates processes in the surrounding follicular cells.

Elevated levels of cyclic adenosine monophosphate (cAMP) influence the maintaining of the meiotic arrest of premature oocytes. This regulation involves both follicular somatic cells, particularly GCs, and the oocytes themselves. When FSH binds to FSHR on GCs, it activates adenylyl cyclases, which convert adenosine triphosphate (ATP) into cAMP. The increased cAMP levels within GCs inhibit the activity of phosphodiesterase, the enzyme responsible for breaking down cAMP. Consequently, cAMP is transferred into the oocytes, leading to elevated cAMP levels within the oocyte. Additionally, the oocyte itself generates cAMP through the stimulation of GPR3, a G-protein-coupled receptor (GPCR) pathway. This self-produced cAMP further contributes to maintaining the meiotic arrest. When inhibitory signals are diminished, and the closure of gap junctions and TZP between the oocyte and CGCs takes place, there is a further reduction in the transfer of cAMP from CGC to oocytes. Consequently, the decline in cAMP concentrations within the oocyte serves as the trigger for the resumption of meiosis (Mehlmann, 2005; Norris et al., 2009; B. Pan & Li, 2019; Shimada, 2012).

The formation of a follicular antrum divides the GC population into two main groups: CGCs and MGCs. As MGCs create the follicle's inner lining, CGCs surround the oocyte by building a tight COC, thereby some of them are in direct contact with the developing oocyte (Hennet & Combelles, 2012). E2 and P4, two of the most substantial ovarian steroids, are produced by the MGCs. MGCs respond to FSH after it binds to its receptor FSHR and activates the intracellular cAMP and protein kinase A pathway to activate *CYP19A1* gene-encoded aromatase expression, which converts testosterone to E2 (Palermo, 2007). Increased FSH and E2 levels stimulate both MGCs' and CGCs' proliferation and differentiation as well as follicular growth being facilitated by rising FSH levels, which also increase E2 production in MGCs (Ai et al., 2019). Moreover, GCs synthesise essential proteins such as inhibin, activin, and follistatin, which are involved in various cellular functions and signalling pathways that also contribute to the regulation of E2 synthesis. Inhibins produced by GCs enhance the sensitivity of TCs to LH, leading to increased androgen production, essential for subsequent E2 synthesis. Conversely, activins suppress the production of androgens by the TCs (Hennet & Combelles, 2012; Knight & Glister, 2001).

Compared to MGCs, CGCs demonstrate higher expression levels of genes that are implicated in the molecular communication between cells and extracellular matrix (ECM) formation, as well as enzymes involved in glycolysis and cholesterol biosynthesis (Hennet & Combelles, 2012; Zhuo & Kimata, 2001). CGCs, for example, must metabolise glycose into pyruvate to provide energy to the oocyte because the oocyte lacks the ability to produce it (Su et al., 2009).

The innermost layer of CGCs forms TZPs through the oocyte's zona pellucida barrier to transport small molecular weight molecules such as ions, glycose, ribonucleosides, amino acids, and signalling molecules including cAMP and cyclic guanosine monophosphate (Gilchrist et al., 2008; Hennet & Combelles, 2012; Okudaira et al., 2017).

CGCs and oocytes both express GDF-9 and bone morphogenic protein 15 (BMP-15), members of the TGF-β superfamily, which are important regulators of both GCs' subtypes and TCs' growth, differentiation, function, and apoptosis, with a significant impact on oocyte development and subsequent processes such as meiotic arrest and ovulation (M. He et al., 2021; Yang et al., 2019; Yatsenko & Rajkovic, 2019). Elevated mRNA expression levels of these named factors in CGCs have been linked to improved oocyte maturation and fertilisation rate, embryo quality, and pregnancy outcomes (Y. Li et al., 2014).

An expansion of CGCs occurs when gonadotropin levels rise to stimulate ovulation, causing CGCs to secrete hyaluronic acid that is bound to the COC by linker proteins. Water molecules attach, causing the space between CGCs to expand and form a matrix around the oocyte that is rich in long hyaluronan oligosaccharide chains cross-linked with proteins and proteoglycans. COC ECM components are critical in ensuring the COC's survival, stability, elasticity, and movement as it flows through the fallopian tubes following its release from the fully mature DF after ovulation (Robker et al., 2018a; Turathum et al., 2021).

Furthermore, the COC matrix contributes to sperm selection by operating as a physical barrier that only allows capacitated and functionally competent sperm to penetrate and reach the oocyte. CGCs release factors such as progesterone, hyaluronic acid, PGE1, PGE2, PGF2, PSSS35, PACAP, and PTX3 that cause modifications in sperm, such as stimulating sperm motility, acrosome reactions, and fertilisation (Turathum et al., 2021). Without the CGC expansion process and interaction with ECM components, the oocyte has a much lower chance to ovulate (Robker et al., 2018a) and become fertilised (Turathum et al., 2021; Zhuo & Kimata, 2001).

Following ovulation, the MGCs and TCs luteinize, marked by the acquisition of LH receptors. The subsequent LH surge causes these cells to shift their hormone production from E2 to P4, facilitating the establishment of the corpus luteum (CL) and an environment favourable for potential pregnancy (Gougeon, 1996; R. E. Jones & Lopez, 2016). CGCs are a byproduct of the ART procedure that can be used to investigate the oocyte's potential in a non-invasive manner. For example, a link between significantly higher expression of VCAN, PTGS2, PFKP, and GREM1 in CGCs from oocytes that lead to pregnancy and LBs has been revealed (Gebhardt et al., 2011).

Moreover, it is debatable whether both non-luteinized MGCs and luteinized MGCs exhibit stem cell characteristics. Studies have shown that GCs of porcine preovulatory GCs can convert into neural stem cells and differentiate into neurons (Bukovsky et al., 2008). Similarly, human-luteinized MGCs cultured *in vitro* can differentiate into neurons, chondrocytes, and osteoblasts (Kossowska-Tomaszczuk et al., 2009). Remarkably, research conducted by Peng et al. has demonstrated that oocyte-secreted factors such as GDF-9 and BMP-15 exert a considerable influence on the gene expression and characteristics of GCs. It has been observed that MGCs can transform and adopt gene expression profiles similar to those of CGCs under the influence of these oocyte-secreted growth factors. This suggests a pivotal role for oocyte-secreted factors in triggering specific gene expression patterns and functional alterations in GCs, contributing to the differentiation and specialisation of CGCs (Peng et al., 2013).

Chaffin et al. conducted a study on a rhesus monkey model, which demonstrated that after ovulation, CGCs, when separated from the oocyte, exhibited an increase in the expression of LHR and steroidogenic enzymes. These changes in gene expression suggest a shift towards a more MGC-like phenotype in the CGCs (Chaffin et al., 2012).

Furthermore, while MGCs and CGCs share a common developmental pathway, studying the differences between these cells allows us better to understand how different GC types contribute to various aspects of reproductive processes and overall fertility. Furthermore, several studies (Harris et al., 2011; H. Wu et al., 2022) have yielded compelling evidence regarding the heterogeneity of MGCs, implying the existence of multiple subpopulations within MGCs that potentially have distinct functions. Subsequently, the research of MGCs and CGCs, along with their respective subtypes is advantageous for clinical applications if these characteristics are further researched and applied.

3) Theca and stromal cells

TCs form the outer layers of the follicle and provide structural integrity. TCs are classified according to the follicle stage as early, interna, externa, and lutein TCs (Magoffin, 2005; Young & McNeilly, 2010). TCs are responsible for the formation of an extended network of blood vessels to supply the avascular multilayered follicle cells with oxygen, nutrients, and pituitary gonadotropins and for the production of androgens (Young & McNeilly, 2010). These cells have mitochondria with tubular cristae, smooth endoplasmic reticulum, and an abundance of lipid vesicles, which are all characteristics of steroid-producing cells. More specifically, theca interna cells are especially responsible for the synthesis of androstenedione, an oestrogenic precursor. Higher LH levels stimulate them to produce even more androgens from cholesterol. These androgens, including androstenedione and testosterone, diffuse across the follicle basement membrane to GCs (Oktem & Oktay, 2008).

Furthermore, as a result of LH stimulation, TCs express many steroidogenic enzymes such as CYP11A1, HSD3B1, and STAR, allowing cholesterol to be delivered to the inner mitochondrial membrane where CYP11A1 is expressed (Magoffin, 2005). Following this, TCs express CYP17A1 enzyme but not CYP19A1, which is expressed by nearby GCs for androgen-to-oestrogen conversion (Magoffin, 2005). TCs, in addition to producing androgens, also synthesise progestins, cytokines, growth regulatory factors such as BMPs, IGFs, vascular endothelial growth factors (VEGFs), and TGF-β isoforms and thus perform endocrine, structural, vascular, and immune functions during folliculogenesis (Young & McNeilly, 2010).

Due to the absence of LH receptors on theca precursor cells, TCs' differentiation is independent of gonadotropins, and TCs' layer formation has been observed in the ovaries of FSH-deficient mice (Burns et al., 2001). TCs start to express LH receptor from the secondary follicle stage (Edson et al., 2009). GCs secrete activins, a KIT ligand that upregulates TGF-α, TGF-β, keratinocyte growth factor, and hepatocyte growth factor, which all have an inhibitory effect on androstenedione production, to prevent TCs from producing androgens prior to the formation of antral follicles (Edson et al., 2009).

External TCs are essential for ovulation and vascularisation processes owing to their composition, which is rich in fibroblasts, macrophages, and smooth muscle-like cells (Hummitzsch et al., 2019). Internal and external TCs are distinguished by differential expression of cell surface markers; ANPEP is associated with internal TCs, while ENG is associated with external TCs. Luteinized TCs are characterised by the expression of the steroidogenic gene CYP17A1 (Devoto et al., 2009; Man et al., 2020).

4) Immune cells

The preovulatory follicle contains a diverse range of immune cells, such as macrophages, monocytes, mast cells, eosinophils, B- and T-cells, natural killer cells, and neutrophils (Y. Choi et al., 2023). These immune cells play an active role in follicular development, engaging in processes such as follicle wall reconstruction and rupture, angiogenesis, inflammation, tissue repair, ovulation, transformation of the ruptured follicle into the CL, and degradation of the CL during luteolysis (Brännström & Enskog, 2002; Duffy et al., 2019).

The LH-induced signalling pathway during ovulation shares similarities with inflammatory responses. It affects GCs and TCs, which not only produce steroids and prostaglandins but also secrete chemokines and cytokines. These signalling molecules stimulate both immune and non-immune ovarian cells, attracting additional immune cells to the ovary (Duffy et al., 2019). Malfunctioning of the immune system can lead to fertility-related disorders. Understanding how immune cells interact with the follicular microenvironment is critical for understanding the mechanisms underlying fertility-related diseases (Duffy et al., 2019; Z. Li et al., 2019a; Lukassen et al., 2003; T. Zhang et al., 2017).

5) Other somatic cells

The preovulatory follicle is surrounded by diverse somatic cell types, including endothelial cells, smooth muscle cells, ovarian surface epithelial cells, and fibroblasts (X. Fan et al., 2019; Kinnear et al., 2020). Endothelial and smooth muscle cells located within blood vessels are responsible for the establishment and maintenance of the blood supply, and vital for oxygenation, hormone transportation, and nutrient delivery (Kinnear et al., 2020).

Smooth muscle cells and ovarian surface epithelium cells are particularly important during the follicular cyclic phases of relaxation and contraction, including rupture and ovulation (Robker et al., 2018b). Additionally, ovarian surface epithelium cells provide crucial structural support and contribute to the post-ovulation repair process (Kinnear et al., 2020). For a long time, these cells have been linked to the most common and aggressive type of epithelial ovarian cancer, known as high-grade serous ovarian carcinomas (Schindler et al., 2017). However, recent data suggests that this cancer may actually originate from the epithelial cells of the fallopian tube (Bergsten et al., 2020).

In addition, fibroblasts secrete essential ECM proteins such as collagen and fibronectin. These proteins actively contribute to the formation of the ECM, which provides the follicle with the necessary support, scaffolding, and structural firmness (Kinnear et al., 2020). The ECM interacts with cell receptors and growth factors to regulate critical cell processes like adhesion, migration, and proliferation (Kinnear et al., 2020).

6) Follicular fluid

FF is a complex mixture of various components derived from both the bloodstream and the secretions of somatic cells within the follicle. It contains a diverse array of biomolecules, including proteins, steroids, hormones, antiapoptotic factors, amino acids, nucleotides, enzymes, electrolytes, anticoagulants, reactive oxygen species (ROS), antioxidants, extracellular vesicles (EVs), and metabolites. These biomolecules have a crucial role in mediating communication between the oocyte and its surrounding environment, facilitating the acquisition of essential molecular components necessary for oocyte maturation and subsequent embryo development (Hennet & Combelles, 2012; Revelli et al., 2009).

However, in addition to useful and necessary compounds, there are others, such as certain chemicals and ROS, that, at excessive concentrations, are toxic to cells. ROS are highly unstable and reactive oxygen-containing molecules that produce free radicals that, at elevated concentrations, can seriously impair cellular processes, damage DNA, and induce apoptosis.

ROS impact oocyte quality through various mechanisms. Elevated ROS levels accumulate in mitochondria during routine biological metabolism, leading to mitochondrial dysfunction that impairs ATP synthesis and contributes to increased meiotic spindle abnormalities. Additionally, heightened ROS levels are correlated with reduced developmental potential, aneuploidy, and telomere shortening in oocytes (Sasaki et al., 2019). Therefore, the measurement of ROS levels and assessment of the balance between ROS and antioxidants, known as oxidative stress, have emerged as important parameters in ART procedures. A noteworthy finding from an ART treatment study by Velthut-Meikas et al. indicates a positive correlation between heightened follicular oxidative stress levels and improved ovarian stimulation outcomes. Patients with elevated follicular oxidative stress displayed lower FSH usage per retrieved oocyte and higher serum E2 levels (Velthut et al., 2013). In conditions such as endometriosis-associated fertility, excessive ROS have been found to induce senescence in CGC via endoplasmic reticulum stress and mitochondrial dysfunction (X. Lin et al., 2020).

Oxidative stress also negatively affects steroid hormone production, particularly E2, in GCs, leading to altered ovarian response and reduced oocyte quality (Appasamy et al., 2008; Seino et al., 2002). Excessive ROS can interfere with chromosomal alignment during proliferation, disrupt microtubule organisation as shown in mice (W.-J. Choi et al., 2007), and inhibit oocyte maturation as shown in humans (Tamura et al., 2008).

In ART protocols, the use of potent antioxidants, such as melatonin (Tamura et al., 2020) and glutathione peroxidase (A. Fu et al., 2021; Paszkowski et al., 1995), has shown promise in reducing the effects of ROS. These antioxidants can help reduce ROS levels in women with high oxidative stress, thereby improving oocyte quality and fertilisation rates.

1.2.2 Molecular communication between cell types by miRNAs

Folliculogenesis relies on extensive cell-to-cell communication and the intricate network across various cell types and distances (Eppig, 2001). Within this process, bioactive molecules including RNAs such as miRNAs, DNA, lipids, and proteins, are encapsulated within EVs, allowing their release and transport to recipient cells over long distances (Machtinger et al., 2016).

Extracellular miRNAs as well as EVs, which can be found in blood, cerebrospinal fluid, saliva, breast milk, urine, seminal and amniotic fluid, placenta, and FF, serve as chemical messengers involved in cell signalling, regulating essential processes like growth, differentiation, and apoptosis (Machtinger et al., 2021; Tamaddon et al., 2022; Y. Xie et al., 2023). miRNAs are small, on average 22-nucleotide, stable, highly conserved noncoding RNA molecules that serve as important regulators of gene expression in tissues (D. Xu et al., 2022). EVs are structured membranous nanoparticles carrying diverse cargo and are classified into distinct subtypes such as exosomes, microvesicles and apoptotic vesicles based on their size and biogenesis. These lipid bilayer-coated vesicles have an important role in orchestrating a wide range of physiological processes while also

contributing to the development and progression of various pathological conditions (Machtinger et al., 2021; D. Xu et al., 2022).

Two mechanisms by which miRNAs can be delivered to target cells after secretion into extracellular fluids are through packaging into EVs or binding to proteins such as argonaute. The most common pathway by which miRNAs work is that one or multiple copies of complementary miRNAs bind to the target mRNA, where translational repression or mRNA degradation is initiated (Assou et al., 2013; G. Fu et al., 2013; Machtinger et al., 2016; O'Brien et al., 2018). MiRNAs exhibit greater stability compared to mRNAs and can be analysed from bodily fluids, rendering them promising potential targets for drug development or diagnostic markers. For example, miRNAs have emerged as potential diagnostic markers and therapeutic targets in conditions such as prostate and ovarian cancers (X. Chen et al., 2008; Zen & Zhang, 2012).

Our comprehension of miRNAs roles in infertility aetiologies like PCOS, endometriosis, and placental development is constantly advancing. However, further research is warranted to fully elucidate the mechanisms of miRNAs (Butler et al., 2020; G. Fu et al., 2013; Javadi et al., 2022; Marí-Alexandre et al., 2018). Shedding light on this, a study investigated miRNAs originating from introns of FSHR and CYP19A1 genes. These miRNAs exert regulatory effects on genes situated across various chromosomes, indicating that miRNA influence is not confined to the location of their genes (Rooda, Hensen, et al., 2020).

Several studies have also revealed distinct expression patterns of miRNAs in human GV-stage oocytes to eight-cell embryos (Paloviita et al., 2021), M2 oocytes, CGCs (Assou et al., 2013), and between MGCs and CGCs, emphasising their role in cumulus-oocyte communication and signalling pathways such as meiosis, TGF-β, and insulin (Velthut-Meikas et al., 2013a). Moreover, additional research exploring the involvement of EVs and miRNAs in infertility conditions like PCOS and endometriosis, as well as their impact or prediction on embryo quality and subsequent pregnancy rates in IVF, is necessary for advancing our comprehension of their underlying mechanisms and developing effective clinical strategies (Machtinger et al., 2016, 2021).

1.3 IVF treatment for infertility

In the context of a couple's infertility, numerous female and male factors have been clarified. Female fertility issues include irregular menstrual cycle, fallopian tube blockages, endometrial dysfunctions, and various pathologies such as endometriosis, PCOS, and premature ovarian insufficiency (POI). Inadequate sperm production or abnormal sperm function are frequent reasons for male infertility. Common issues include advanced age, obesity, chronic health concerns, infections, and specific genetic disorders (R. E. Jones & Lopez, 2016).

ART procedures that have been specifically developed to overcome infertility challenges include COS to obtain multiple M2s, which are fertilised in a laboratory setting using conventional IVF or intracytoplasmic sperm injection (ICSI). The conventional IVF method is used if sperm quality parameters are within World Health Organization (WHO) guidelines, and then oocytes and the purified sperm are co-incubated in a culture dish for 16–18 hours. In ICSI, on the other hand, a single sperm is chosen and injected directly into the cytoplasm of an oocyte, skipping many steps involved in the fusion of sperm and oocyte. If conventional IVF fails to result in fertilisation or sperm parameters fall below WHO standards, ICSI may be used.

Following fertilisation, zygotes begin to divide and are cultured for 2–6 days until the morphologically best embryos are transferred to the uterus and the others cryopreserved.

IVF-conceived LB was first accomplished in 1978 in England without ovarian stimulation (Steptoe & Edwards, 1978) and in 1995 in Estonia with stimulation (Allvee et al., 2014).

Thereafter, ovarian stimulation protocols, methods and mediums have been significantly modified due to new techniques, for example the efficient and safe recombinant follicle-stimulating hormone (rFSH) medications in the market and PGT for embryos prior to transfer, but the overall success rate remains modest, hovering around 25–30% for nearly 40 years (Andersen et al., 2004; Voullaire et al., 2002; Wyns et al., 2022). According to statistics, a range of ART techniques, with a particular focus on IVF, are utilised approximately 2.5 million times annually. It's noteworthy that these numbers are part of a growing trend, indicating an increasing reliance on ARTs worldwide (B. C. Fauser, 2019). In Estonia, for instance, ART procedures were performed 3288 times, resulting in the birth of 680 children, accounting for 5.9% of all LBs in 2022 (Tervise Arengu Instituut, 2022).

1.3.1 COS and OPU

The ability of the ovaries to respond to the exogenous gonadotropin stimulation is the key to the success of ART procedures in achieving pregnancy (Akande et al., 2002). In contrast to the natural cycle, in which one or two DFs typically start to develop and ovulate, gonadotropin stimulation is required to produce multiple M2s that grow in a cohort (Gougeon, 2010).

The conventional COS consists of three main stages: 1) exogenously administered rFSH to induce folliculogenesis and production of steroid hormones; 2) use of GnRH agonist or antagonist to down-regulate the patient's own pituitary activity in order to avoid LH surge and subsequent follicles' premature ovulation; 3) finally the triggering the oocyte meiotic maturation 36h prior OPU with an injection of human chorionic gonadotropin (hCG). This classical agonist or antagonist approach is appropriate for the majority of patients, but not for all.

Aside from the previously described conventional stimulation protocols, treatments based on the course of natural cycle hormonal changes are also provided, such as: 1) full natural cycle, also known as spontaneous, where no medication is used, 2) modified natural cycle IVF, which uses only hCG to trigger ovulation, and 3) mild IVF, which is similar to the most commonly used conventional method known as standard or routine IVF but limits all medication dosing. The expected oocyte yield then rises from 1–2 to 7 in the order of the protocols mentioned. Natural or semi-natural stimulations are considered less stressful than standard ones, with shorted duration, fewer visits and no unpleasant side effects or multiple injections and could therefore be easily repeated in consecutive months (Nargund, Fauser, et al., 2007).

Conventional COS is associated with endometrial disruptions, increased chromosomal abnormalities in oocytes and embryos (Baart et al., 2007; McCulloh et al., 2019), and high hormone levels with the risk of OHSS (Fernández-Sánchez et al., 2019; Nargund, Hutchison, et al., 2007). However, a controlled weekly schedule with low cancellation and a high pregnancy rate, attributed to a greater number of achieved oocytes, provides benefits for the standard IVF protocols in clinics (Polyzos et al., 2018; Venetis et al., 2019). Natural or modified natural cycles are preferred for cancer patients or those with a family history of oestrogen-sensitive malignancies, poor responders (PORs), women with previous cases of failed implantation, severe endometriosis, or those who do not want or cannot afford medicines (Matsuura et al., 2008; Nargund et al., 2001, 2017).

The conventional GnRH antagonist protocols, which were first introduced in 2002 and are widely used worldwide, have seen limited significant changes. As the IVF market continues to grow and holds significant financial value in the billions of dollars, there is a growing demand for innovative and effective treatment protocols and medications to address various causes of infertility and improve outcomes (Patrizio et al., 2022). To enhance success rates, several additional treatment steps have been introduced alongside standard fertility treatments. While these interventions have shown promising effects, further comprehensive research is needed to fully understand their impact. Although the current market offers products for these purposes, there is still a clear need for extensive research on optimal protocols and dosages for different patient groups.

Adjuvants used in combination with fertility treatments include heparin, micro aspirin, Estrofem and Viagra for endometrium thickening, prednisolone, dexamethasone, intralipids, anti-TNF, G-CSF cytokines, growth hormones, dehydroepiandrosterone (DHEA), melatonin, coenzyme Q10, metformin, and others. These adjuvants offer various benefits, such as reducing the risk of preeclampsia, improving blood flow to the uterus and ovaries, enhancing oocyte quality, stimulating the endometrium, promoting trophoblast differentiation and invasion, modulating the immune response, and providing antioxidant effects. In addition to adjuvants, certain procedures, like platelet-rich plasminogen activator injections into the ovaries, have been utilised to increase the quantity and quality of oocytes, promote uterine lining thickness (Nardo & Chouliaras, 2020), and improve endometrial receptivity (Altmäe & Aghajanova, 2019).

Various markers have been proposed over the past few decades to assess ovarian responsiveness during IVF treatment, including age, AMH, antral follicle count (AFC), basal FSH, E2, inhibin B, and dynamic tests like the Clomiphene Citrate Challenge Test or exogenous FSH ovarian reserve test (Broekmans et al., 2006; Broer et al., 2011; La Marca et al., 2010; Mutlu et al., 2013). Among these markers, AMH is the most commonly utilised parameter in ART (Ledger, 2010), as it can predict poor and hyper responses (Nelson et al., 2007) as well as the LB rate in IVF (La Marca et al., 2011). However, the availability and cost of the AMH assay are limited to certain IVF clinics (Biasoni et al., 2011). Therefore, identifying more precise and cost-effective factors that can predict the outcome of ART procedures, increase efficacy, and reduce complications such as OHSS is of significant importance. Such knowledge could be immediately applied to improve and personalise treatment protocols, develop pharmaceutical products, or refine laboratory practices related to oocytes, sperm, and embryos (Biasoni et al., 2011).

1.3.2 Ovarian aetiologies of infertility – low ovarian response to COS

The declining quantity and quality of oocytes with age in the ovarian follicular pool has been well-documented (Baerwald et al., 2012; Broekmans et al., 2007). However, there are cases where patients, regardless of their age, produce only a few or no oocytes despite receiving adequate COS. These individuals are commonly referred to as having a low ovarian response to COS, also known as hyporesponse, suboptimal response, or poor response. The prevalence of patients in this category ranges from 9% to 24%, with some studies reporting rates as high as 33% (Ferraretti et al., 2011; Ubaldi et al., 2014). Additionally, PORs often face a higher likelihood of treatment cancellation, which ranges from 15% to 40% (Al-Azemi et al., 2011; Lamazou et al., 2012).

One countermeasure for poor response is to elevate the gonadotropin dosage and extend the stimulation period. Nonetheless, this approach has not proven effective in addressing the low birth rate outcome (Baker et al., 2015; Drakopoulos et al., 2020).

It has been suggested that switching from the conventional stimulation protocol to a shorter agonist or antagonist protocol can be a strategy to enhance stimulation effectiveness. However, it should be noted that this approach may not always result in improved outcomes (Akman et al., 2000; Ferraretti et al., 2011; Kdous et al., 2014; Murillo et al., 2023).

Since the oocyte yield of PORs is low regardless of stimulation intensity (≤7), the search for targeted stimulation doses and novel adjuvants for modified treatments continues (Datta et al., 2020, 2021; Montoya-Botero et al., 2021). Despite the fact that donated oocytes are the most effective therapy for PORs, POF or POI (Blumenfeld, 2011, 2020), there is a strong desire to find alternatives and assist the most unfortunate poor response cases before accepting the donated oocyte cell cycle in order to have a healthy child who is also the genetical successor.

Causes for different responses to IVF therapy are multifactorial and can include both patient-related factors and treatment-related factors. Genetic mutations, for example single-nucleotide polymorphism (SNP) in gonadotropins and their receptors have been associated with hyporesponse as a potential cause (Alviggi et al., 2013, 2016; Perez Mayorga et al., 2000). It is definitely informative to perform genetic testing of FSH and LH receptors or genome-wide SNP panel for recurrent IVF patients (Tang et al., 2015). More specifically, the polymorphisms of THR307Ala and Asn680Ser in FSHR are associated with elevated FSH levels and higher rate of poor response compared to carriers of other genotypes (Yan et al., 2013).

Unlike poor ovarian response, decreased ovarian reserve is more precisely associated with genetic abnormalities, particularly those predominantly located on the long arm of the X chromosome. Examples of such abnormalities include Turner syndrome (47, XXX), mosaics, deletions, inversions, and balanced translocations (X, autosomes) (Di-Battista et al., 2020; Man et al., 2022). Furthermore, suboptimal response to COS is associated with high age (Ron-El et al., 2000) and BMI (Buyuk et al., 2011). Unhealthy lifestyle choices, including smoking and excessive alcohol consumption, can negatively impact ovarian response in IVF stimulation protocols, potentially leading to hyporesponse (Firns et al., 2015; Salih Joelsson et al., 2019).

The current lack of understanding regarding the underlying mechanism responsible for poor response necessitates a comprehensive evaluation of ovarian sensitivity and other dysregulations associated with inadequate stimulation outcomes. By unravelling the aetiology of hyporesponse, there is a potential to refine and optimise treatment strategies, leading to improved outcomes in these cases (Grynberg & Labrosse, 2019).

Accurately identifying PORs and evaluating their ovarian response prior to commencing IVF therapy is of utmost importance for its success. One of the effective approaches involves analysing patients' distinctive characteristics and grouping them based on various parameters, such as age, AMH levels, AFC, or their previous IVF history. This concept is widely employed in the identification of PORs, as defined by the Bologna criteria (Grisendi et al., 2019) and the Poseidon criteria (Esteves et al., 2022). These criteria provide guidelines for classifying patients with suboptimal ovarian response, facilitating informed counselling regarding potential IVF outcomes (Drakopoulos et al., 2020). It is important to highlight that there is a lack of unanimous consensus regarding the definition and suggested cut-offs of PORs in the field of ART. To date, more than 41 different definitions have been proposed (Polyzos & Devroey, 2011), reflecting the complexity and ongoing debate surrounding this topic. Suboptimal response to COS remains a significant challenge in everyday clinical practice (Drakopoulos et al., 2020).

In addition to patient identifying and grouping, the direct evaluation of ovarian responsiveness before an IVF cycle enables a thorough assessment of a patient's ovarian function and estimated treatment response. Such an assessment enables the calculation of appropriate gonadotropin dosing or other modifications to enhance the chances of success, or to make the decision to cancel the procedure (Mutlu et al., 2013).

To evaluate a patient's ovarian responsiveness, multiple calculations have been introduced, each considering various measurable parameters with slightly different approaches. Three such calculations are the follicular output rate (FORT) (Genro et al., 2011), follicle oocyte index (FOI) (Alviggi et al., 2018), and ovarian sensitivity index (OSI) (Huber et al., 2013) and exact calculation formulas may vary among studies (Figure 3).
FORT and FOI have emerged as indexes that offer enhanced insights into the dynamic process of follicular growth in response to exogenous gonadotropins during IVF treatment. In contrast, OSI has been recognised as a measure that provides a more comprehensive assessment of the ovarian potential to produce oocytes (Alviggi et al., 2018; Revelli et al., 2020).

$$\text{FORT} = \frac{\text{Preovulatory follicle count}}{\text{Antral follicle count}} \qquad \text{FOI} = \frac{\text{Retrieved oocyte count}}{\text{Antral follicle count}}$$

$$\text{OSI} = \frac{\text{Retrieved oocyte count}}{\text{Total gonadotropins dose}} \qquad \text{OSI}_{\text{m}} = \ln\left(\frac{\text{Retrieved oocyte count}}{\text{Total gonadotropins dose}} * 1000\right)$$

Figure 3*. Calculation formulas for follicular output rate (FORT), follicle-oocyte index (FOI), and ovarian sensitivity index (OSI), along with example of OSI modification (OSI_m) to improve normal distribution of the outcome (Bellavia et al., 2023).*

The calculation methods of various indexes and their predictive capabilities for IVF outcomes have been extensively investigated. Notably, Cesarano et al. conducted a comparison of these indexes within the context of IVF treatment outcomes, including M2 rate, embryo quality, and transfer outcome. The study revealed that among the three indexes, the OSI exhibited the strongest correlation and the highest accuracy (Cesarano et al., 2022). Furthermore, in a retrospective study conducted by Hu et al., it was discovered that the OSI values exhibited a significant correlation with LB outcomes. This finding suggests that OSI could serve as an independent indicator for predicting the chances of achieving a successful LB in ART treatments. On the other hand, the study did not observe any notable differences in LB outcomes related to the FORT. This implies that FORT may not be as indicative of treatment success when compared to OSI (F. Hu et al., 2023).

Furthermore, in the study by Revelli et al., 2020, the predictive ability of the OSI for CP outcomes was demonstrated, outstanding the prognostic value of the total number of oocytes. Notably, the study showed the consistent performance of OSI even when there were modifications to the COS protocol in repeated IVF cycles. These findings emphasise the enduring strength and dependability of OSI as a valuable predictor of CP, unaffected by variations in the COS protocol (Revelli et al., 2020). Other authors (H. W. R. Li et al., 2014; Weghofer et al., 2020; Yadav et al., 2019) have also validated OSI and agreed with its strong correlation to predictive capacity with other IVF ovarian response parameters such as age, AMH, AFC and the duration of stimulation. Besides this, there can be considerable variability in OSI values observed among women with similar ovarian reserve levels (Huber et al., 2013).

Future research about ovarian sensitivity will continue to focus on gene expression profiles of FF cells and the patient's genome in order to identify genetic biomarkers associated with ovarian response (Altmäe et al., 2011; García-Jiménez et al., 2018).

Another area of investigation is the relationship between these biomarkers and FF proteins. Additionally, efforts are being made to create a predictive score that incorporates IVF stimulation data for greater accuracy (Chalumeau et al., 2018). These ongoing studies aim to improve OSI accuracy and practical application in clinical practice.

1.3.3 Ovarian aetiologies of infertility – PCOS and polycystic ovarian morphology

PCOS is the most common heterogeneous endocrinopathy at reproductive age that leads to infertility, affecting approximately up to 26% of women (Tamaddon et al., 2022). PCOS is diagnosed in adults according to the most commonly used Rotterdam criteria if at least two elements are present from the list of ovulatory dysfunction such as oligo-ovulation or anovulation, hyperandrogenism, and polycystic ovarian morphology (PCOM) on ultrasound (The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004).

PCOM can impact either a single ovary or both. It may entail several features: there is an elevated count (at least 20) of follicles in each ovary, these follicles display uniformity in size ranging from 2 to 9 mm in diameter or they are arranged at the periphery of the ovary, which creates a distinctive “string of pearls” appearance. Additionally, there is an observable overall enlargement of the ovary or an increased ratio of stromal area to the total ovarian area (Dewailly et al., 2014). In addition to that previously mentioned, PCOS usually includes many clinical concerns such as hyperinsulinism, insulin resistance (IR), obesity, hirsutism, diabetes and depression (Goodarzi et al., 2011). According to research, PCOS is also associated with chronic low-grade inflammation, including elevated levels of leukocytes, endothelial dysfunction, and proinflammatory cytokine disruption (Benson et al., 2008; Mobeen et al., 2016).

PCOS is caused by a number of disturbances, and while genetic, hormonal, and environmental factors all play a role in PCOS aetiology, the underlying risk factors remain unknown. Furthermore, no successful treatment plans have been established (B. Fauser et al., 2004). However, it is known that PCOS patients have a high LH:FSH ratio and a higher frequency of GnRH pulses, which stimulate TCs to produce androgens in excess (Sadeghi et al., 2022). Besides this, hypomethylation of TC LH/choriogonadotropin receptor leads to higher gene expression and sensitivity to LH (Ibáñez et al., 2017). Additionally, many studies have shown bisphenol A (BPA) induced negative effects: induced androgen secretion in TCs and inhibition of testosterone catabolism, dysregulation of 17-beta-hydroxylase, cholesterol side-chain cleavage enzyme and steroidogenic acute regulatory protein that again results in the overproduction of androgens by TCs (Rutkowska & Diamanti-Kandarakis, 2016).

In addition, abnormal expression of specific miRNAs, including hsa-miR-32, hsa-miR-34c-5p, hsa-miR-135a, hsa-miR-18b, and hsa-miR-9, has been implicated in the pathogenesis of PCOS (Roth et al., 2014). Conversely, decreased expression of hsa-miR-132 and hsa-miR-320 has also been observed (Sang et al., 2013). These and numerous other studies have highlighted the significance of miRNAs as important regulators of E2 secretion, insulin signalling processes and overall steroidogenesis (Luo et al., 2021; Tamaddon et al., 2022). The emphasis on identifying and quantifying miRNA expression in various compartments like peripheral blood, FF, and follicular cells has grown. Specific miRNAs show potential as biomarkers for predicting and diagnosing reproductive disorders such as PCOS, endometriosis, cervical cancer, or poor ovarian response in women. As a result, there has been a substantial increase in miRNA profiling studies conducted on FF, aiming to uncover valuable insights in these areas (Javadi et al., 2022; Tamaddon et al., 2022).

1.3.4 Current additional treatments during COS with rFSH for PCOS patients

Metformin continues to be the primary treatment for PCOS patients, both before and during IVF and early pregnancy, owing to its capacity to regulate insulin and its positive effects on androgen levels and ovulation in PCOS patients (Johnson, 2014).

Studies have revealed that metformin triggers the upregulation of DICER1, c-MYC, IRS-2, and HIF1α, leading to shifts in the patterns of miRNA expression. These changes play a crucial role in establishing lipid metabolism and enhancing glucose uptake in insulin-sensitive tissues (Blandino et al., 2012). Furthermore, it has been demonstrated that three miRNAs, namely hsa-miR-122, hsa-miR-223, and hsa-miR-229, show potential as candidates for biological vectors in PCOS. This is based on their significant reduction in PCOS patients when treated with metformin (Udesen et al., 2020).

Furthermore, the co-administration of metformin with medications like clomiphene citrate or letrozole can lessen the risk of OHSS, which is more prevalent in PCOS cases. This combination has been shown to enhance the likelihood of ovulation and increase LB rates among PCOS patients. Additionally, this combination assists in regulating LH and FSH levels while also improving endometrial receptivity (Jiang et al., 2022; Mejia et al., 2019).

Clomiphene citrate acts as an oestrogens antagonist, improving gonadotropin hormone secretion and ovarian follicular development by blocking oestrogen receptors (Mejia et al., 2019; Mitwally & Casper, 2001). Letrozole is a nonsteroidal aromatase inhibitor of the third generation. It lowers the levels of androgens by stopping their production and conversion to oestrogens, which makes the follicles more responsive (Jiang et al., 2022). With a 40% higher pregnancy rate and a shorter time-to-pregnancy, letrozole is currently favoured over clomiphene citrate in PCOS patients (Amer et al., 2017).

To conclude, addressing IR and optimising ovulation are key considerations in IVF treatment for PCOS patients. Medications like metformin, clomiphene citrate, and letrozole, when used in combination, can help improve the chances of successful IVF outcomes in women with PCOS. These current treatment add-ons aim to regulate hormonal imbalances, enhance ovulation, and increase the likelihood of achieving a healthy pregnancy.

The limited use of pathway inhibitors in PCOS management (T. Li et al., 2017) highlights the considerable untapped potential in this regard. miRNAs could serve as specific and reliable biomarkers for diagnosing PCOS. This underscores the exciting prospects for future research and innovation in the field of PCOS treatment.

1.4 Endocrine-disrupting chemicals

In addition to naturally occurring elements, our surrounding air, soil, and water contain substances of artificial origin – chemicals – that have become a part of our everyday lives. Man-made chemicals enter the environment through industrial processes, pesticides in agriculture, and the use of household items such as electronic devices and kitchen cookware as well as personal care products, pharmaceutical medicines and plastic materials (Giulivo et al., 2016; Yilmaz et al., 2020). Chemicals are used to improve the durability and quality of products by enhancing characteristics such as nonstick and waterproof properties, strength, and odour.

We are constantly exposed to a wide range of chemical mixtures as they are an inevitable element of daily life. The most general routes of exposure to man-made chemicals are respiratory, oral, and skin contact; other ways include intravenous and intramuscular administration, placental transfer, and breastmilk (Giulivo et al., 2016; J. H. Kim et al., 2020).

Chemicals that interfere with the hormonal systems of living organisms are called endocrine-disrupting chemicals (EDCs) and are among the most dangerous (Giulivo et al., 2016). There are more than a thousand chemicals with endocrine-acting properties and they are grouped by the combination of type, structure, key characteristics, use, and sources (La Merrill et al., 2020; Yilmaz et al., 2020).

One of their harmful characteristics is their ability to imitate, block or interfere with the action of steroid hormones in the body, allowing them to gain access to receptor-mediated signalling and thus alter the real hormone production, levels, transport, signalling pathways, and function (Crellin et al., 2001; Diamanti-Kandarakis et al., 2009; X. Yao et al., 2023; You et al., 2001). Another way is inducing epigenetic modifications and changing the hormone responsiveness (Ho et al., 2012; Rissman & Adli, 2014). EDCs, being lipophilic, tend to accumulate in adipose tissue, resulting in a prolonged half-life within the body.

The timing of exposure is a critical factor, as developing foetuses and newborns are highly susceptible to endocrine disruption. This susceptibility can result in diverse pathologies emerging later in life or even impacting future generations (La Merrill et al., 2020; Vinnars et al., 2023; Yilmaz et al., 2020). EDCs have been linked to the development of numerous health issues, including various cancers, weight problems, behavioural disorders, learning disabilities, thyroid gland dysfunction, and infertility. These effects can even occur at extremely low doses. As a result, EDCs present a significant global challenge to both public health and ecological balance (La Merrill et al., 2020).

1.4.1 Association of EDCs with ovarian function and female reproductive health. Mechanisms of action

Exposure to EDCs has been implicated in various infertility-related pathologies, including decreased number of primordial germ cells, menstrual irregularities, PCOS, endometriosis, breast cancer, and early menopause resulting from premature ovarian insufficiency or failure (Giulivo et al., 2016; X. Yao et al., 2023). Furthermore, EDC exposure has also been linked to an increase in the occurrence of uterine fibroids (Bariani et al., 2020).

The EDC mechanism of action is retrieved by specifically targeting various nuclear receptors, including oestrogen receptors (ERs), androgen receptors, mineralocorticoid receptors, P4 receptors, glucocorticoid receptors, thyroid receptors, and peroxisome proliferator-activated receptors. EDCs can alter the function of enzymes involved in hormone metabolism and interfere with receptor activity. As a result, they can impact the levels and effects of active hormones such as androgens and oestrogens (Diamanti-Kandarakis et al., 2009; Giulivo et al., 2016). By disturbing different biochemical pathways, EDCs can have a wide array of consequences, such as inhibiting or mimicking transcriptional or post-transcriptional mechanisms, impacting the metabolism of steroid hormones, and epigenetic changes such as impact the modification of DNA and histone proteins and non-coding RNA expression such as miRNAs (Diamanti-Kandarakis et al., 2009; Giulivo et al., 2016; La Merrill et al., 2020).

Examples of widely produced EDCs that have been shown to act on ovaries include bisphenols, phthalates, parabens, pesticides, and pollutants such as per- and polyfluorinated substances (PFAS). Bisphenols, particularly the well-known derivative called BPA, have been extensively used in the production of plastic materials. They exert their effects by actively interfering with hormone-receptor mediated pathways, including the aromatase pathway (X. Yao et al., 2023). BPA has been found to have detrimental effects on various endocrine signalling systems, such as androgenic, thyroid, P4, and

insulin pathways. Although BPA has been removed from the market, other bisphenol family members, unfortunately, exhibit similar negative effects and have replaced it (Giulivo et al., 2016).

Phthalates, which are widely studied synthetic chemicals used as plasticizers, possess anti-androgenic and anti-oestrogenic properties (Rodprasert et al., 2023). Due to their low molecular weight and the absence of covalent bonds that link them to the plastics they are mixed with, phthalates can easily volatilize and become freely present in the surrounding air (Giulivo et al., 2016; Hannon & Flaws, 2015). Their widespread distribution in biological samples emphasises their importance as major environmental pollutants that can accumulate in the human body.

Di-2-ethylhexyl phthalate (DEHP) is a widely utilised phthalate compound, commonly found in various consumer products. Upon entering the human body, DEHP undergoes metabolism within the gastrointestinal tract, resulting in the formation of metabolites. These metabolites include mono-2-ethylhexyl phthalate (MEHP), mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), and mono (2-ethyl-5-oxohexyl) phthalate (MEOHP) (Hannon & Flaws, 2015).

Parabens, on the other hand, are commonly utilised due to their low cost as antimicrobial agents and preservatives in food, cosmetics, and medicine (Giulivo et al., 2016). Parabens, phenols, and phthalates share the characteristic of undergoing relatively rapid metabolism and excretion, often within a timeframe of less than 24 hours. This quick elimination process categorises them as non-persistent compounds (Anderson et al., 2001; Moos et al., 2016; Völkel et al., 2002).

Compared to the aforementioned, PFASs possess a long half-life, and are extensively employed in waterproof textiles and fire-fighting foams. The disruption of oocyte maturation by PFAS chemicals, resulting in alterations in embryo development, has been demonstrated (Hallberg et al., 2019; Hallberg, Persson, et al., 2021). Furthermore, these chemicals cross the placenta during pregnancy and accumulate in foetal tissues, particularly in higher quantities within male foetuses than in females (Mamsen et al., 2019). In males later in life, these substances have been linked to adverse associations with semen parameters, suggesting potential adverse impacts on male reproductive health. Pesticides find widespread usage in agricultural and domestic environments, primarily to eradicate insects, fungi, and undesirable vegetation (Vessa et al., 2022).

The study of the effects of EDCs on the ovary and ovulation is an expanding field. EDCs have been extensively studied in relation to their effects on male reproductive health (Diamanti-Kandarakis et al., 2009; Kumar & Singh, 2022; La Merrill et al., 2020; Rodprasert et al., 2023; Sharma et al., 2020), whereas the research on their association with female reproductive health is relatively limited and further investigation is necessary (Hannon & Flaws, 2015; Vinnars et al., 2023).

Indeed, recent research has uncovered a significant link between EDCs and adverse outcomes in IVF, as well as fertility in general. This includes the influence of EDCs on folliculogenesis, reduced ovarian reserve, extended time-to-pregnancy, and elevated risks of infertility (Beck et al., 2024; Björvang et al., 2021, 2022; T. Li et al., 2023; Y. Pan et al., 2019; X. Yao et al., 2023). Table 1 summarises the adverse effects of EDC groups on human ovarian function, gamete development, and overall fertility.

Table 1. *An overview of the known influence of endocrine-disrupting chemicals on fertility, IVF outcomes, and reproductive cells*

Chemicals	Ovarian effects	Effects on components of the antral follicle	Fertility and IVF results
Bisphenols	disrupted follicular development, anovulation, lower AFCs, irregular cycles (Land et al., 2022), lower E2 levels (Ehrlich et al., 2012); disturbance in androgen, gonadotropin, and SHBG levels (Green et al., 2021)	reduced cell viability, lower E2 and P4 in IVF-derived GCs, limiting COC expansion (Land et al., 2022); increased GC autophagy and apoptosis (M. Lin et al., 2021)	reduced total and mature IVF oocyte yield, poor fertilisation, higher miscarriage risk (Vessa et al., 2022), low embryo quality, implantation, increased infertility (Green et al., 2021; Land et al., 2022), diminished sperm concentration, motility and vitality (Green et al., 2021)
DEHP	altered menstrual cycle, follicular development, ovulation; reduced E2, P4, testosterone; ovarian hormone disruption, lower FSH, LH; fewer AFCs in women (Land et al., 2022); higher SHBG in women (Green et al., 2021)	mice: more CGC apoptosis, less oocyte maturation (Land et al., 2022), reducing imprinted gene methylation in oocytes and causing more spindle abnormalities in mature oocytes *in vitro* (X. Yao et al., 2023)	fewer IVF oocytes (Hauser et al., 2016; Machtinger et al., 2018; Vessa et al., 2022); poor quality embryos, reduced implantation, pregnancies, and births (Machtinger et al., 2018; Mínguez-Alarcón et al., 2019); impaired sperm motility, DNA, and protein changes (Land et al., 2022)
MEHP, a metabolite of DEHP	lower P4 level (Land et al., 2022)	reduced mitochondrial membrane polarisation, disrupted ER modelling in COC, decreased COC expansion, (Land et al., 2022)	elevated ROS, reduced oocyte fertilisation rate, reduced embryonic maturation, changes in gene and protein expression in 7-day blastocyst centred on metabolic pathways, changes in DNA and histone methylation (Land et al., 2022); higher pregnancy loss (Green et al., 2021)
PFAS	no LH surge, irregular cycles, lowered E2 and P4 levels, inhibits ovulation (Land et al., 2022)	inhibited oocyte meiotic maturation and expansion of COC (Land et al., 2022)	fewer IVF oocytes, delayed pregnancy, fertility decline (Land et al., 2022); embryo development changes (Hallberg et al., 2019; Hallberg, Persson, et al., 2021); compromised semen (Vessa et al., 2022); poor sperm motility, high DNA damage (Y. Pan et al., 2019)
Parabens	human: hormone disruption, lower E2, decreased AFCs (Jurewicz et al., 2020), shorter menstrual cycles. Rats: reduced E2, P4 levels. Mice: Altered cycles, atretic follicles, inhibited antral growth (Land et al., 2022)	porcine: it inhibits COC expansion and oocyte maturation (Land et al., 2022), altering oocyte morphology (Barajas-Salinas et al., 2021)	impaired fertility (Jurewicz et al., 2020; Land et al., 2022); higher FSH doses per retrieved oocyte during IVF (Bellavia et al., 2023); decreased M2 count (Radwan et al., 2023)
Pesticides	menstrual irregularity, impaired ovulation, diminished ovarian reserve, premature ovarian insufficiency (Vabre et al., 2017)	lower sperm quality (Vabre et al., 2017), oocyte and GCs chromosomal abnormalities, decrease in oocyte maturation rates (X. Yao et al., 2023)	decreased oocyte fertilisation, poor embryo quality, lower IVF birth rates, longer time-to-pregnancy (Vessa et al., 2022), increase in oxidative stress levels (X. Yao et al., 2023)

AFC – antral follicle count, CGC – cumulus granulosa cell, COC – cumulus oocyte complex, E2 – oestradiol, EDC – endocrine-disrupting chemical, FF – follicular fluid, FSH – follicle stimulating hormone, GC – granulosa cell, IVF – *in vitro* fertilisation, LH – luteinizing hormone, M2 – mature oocyte, MGC – mural granulosa cell, P4 – progesterone, ROS – reactive oxygen species, SHBG – sex hormone binding globulin

These effects include disruptions in follicular development, hormonal imbalances, and reduced fertility. This underscores the urgency of further research on EDCs' impact on reproductive health and highlights the importance of addressing this issue in clinical practice.

The handling of a hazardous chemical must not endanger human life or health, but accidents have occurred due to insufficient testing and component analysis. Diethylstilbestrol (DES) is a transplacental EDC that caused a significant catastrophe. This compound is a nonsteroidal, synthetic oestrogen prescribed for various medical purposes. It was previously used as a contraceptive or emergency contraceptive pill before and during pregnancy to prevent miscarriages or premature deliveries (Vinnars et al., 2023). Additionally, DES was prescribed to treat conditions such as vaginitis and menopausal symptoms caused by oestrogen deficiencies. It was even used to suppress postpartum lactation. However, the use of DES led to severe consequences.

In roughly 60 years, after nearly 10 million people had been exposed to DES, it was banned as a Group 1 human carcinogen that causes abnormalities or cancers of the genital tract and breast, neurodevelopmental alterations, immune, pancreatic, and cardiovascular disorders, and problems associated with sociosexual behaviour (Zamora-León, 2021). Neonatal exposure to DES modifies DNA methylation and decreases histone deacetylase expression in the uterus (Jefferson et al., 2013).

In addition to its use on humans, DES was administered to food-producing animals to promote growth, and it was observed in high concentrations in baby foods, meat, milk, fish, and river water in Italy. The third generation is still negatively affected by DES due to intergenerational inheritance, and data collection continues (Newbold, 2004; Zamora-León, 2021).

Another drug toxicity tragedy involved the sedative thalidomide, which was prescribed to pregnant women for nausea relief from 1955 to 1965 and resulted in severe birth defects such as limb reduction anomalies, congenital heart disease, malformations of the ear compartments and ocular abnormalities (J. H. Kim & Scialli, 2011). All of these tragedies have demonstrated the unavoidable need for more effective and systematic testing protocols. It is unacceptable for such tragedies to occur again, highlighting the risks associated with EDCs (Vinnars et al., 2023).

1.4.2 Regulations are needed

In the EU, the regulation of EDCs is supported by the Regulation on Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH). REACH was established in 2006 and it obligates responsibilities for manufacturers, importers, and users of EDCs, guaranteeing the systematic evaluation and proper handling of these substances within the European market (Kassotis et al., 2020). In the EU, the European Chemicals Agency (ECHA) and the European Food Safety Agency (EFSA) play key roles in enforcing chemical regulations. They offer essential databases and resources for the identification and management of chemicals, including those classified as EDCs.

Furthermore, various sectors like cosmetics, plant protection products, and pesticides have specific regulations and directives tailored to address distinct categories of EDCs. These sector-specific regulations, in conjunction with the overarching role of the ECHA and EFSA, contribute to a comprehensive system of chemical regulation in Europe (Demeneix, 2019; Kalofiri et al., 2021; Kassotis et al., 2020).

EDCs in the US are regulated through a multi-agency approach, similar to that of the EU. The Environmental Protection Agency and the Food and Drug Administration play

key roles in the supervision of EDCs control, operating under a range of laws and regulations (Kassotis et al., 2020). Similarly, other countries and regions adopt diverse strategies for testing and managing EDCs. International organisations like the United Nations Environment Programme and the WHO provide significant input to global EDC management, working to assess the prevalence of these chemicals in the environment. As a result, regulations and approaches to EDCs can vary widely, reflecting the distinct priorities and guidelines established by different countries and regions (Kalofiri et al., 2021; Kassotis et al., 2020).

Although chemicals are tested and analysed beforehand on animals and models, and multiple agencies exist worldwide to manage them, there is still a need for standardised and validated approaches with higher specificity. Regulatory agencies employ their own particular methods, which may lead to different hazard evaluation results and delay consensus and jurisdictional conclusions (Kalofiri et al., 2021; La Merrill et al., 2020; OECD, 2018). There have been tragedies in the past that cannot be repeated. Therefore, deeper research is needed even if there are results of the absence of instant toxicity. Due to the fact that EDCs may have different doses for achieving a toxic effect, they may have a lengthy lag time before the effect is observed, and EDCs may have unique sensitive windows of susceptibility, which necessitates the collection of a significant amount of data (Schug et al., 2011; Vandenberg, 2014; Vandenberg et al., 2012). There are numerous studies, but no widely accepted systematic database (La Merrill et al., 2020).

In response to these concerns, various scientific projects such as the Adverse Outcome Pathways (AOPs) and the Female Reproductive Toxicology of EDCs (FREIA) project have emerged to mitigate the aforementioned issues. The AOPs framework is a practical tool that identifies biologically significant events, stressors, and their adverse outcomes, and as a result, it encompasses a comprehensive database of all the data that could be captured and supplemented by other researchers. AOPs could be used to evaluate chemical toxicity risk strategies more efficiently (Ankley et al., 2010). On the other hand, the existing literature on AOPs associated with female fertility is limited in scope, emphasising the need for additional research to identify and characterise further AOPs in this field (Johansson et al., 2020). Currently, most AOPs are developed and maintained on the AOP-Wiki (https://aopwiki.org) (Martens et al., 2022).

The FREIA project was launched to enhance understanding of the impact of EDCs on women's health, with a specific focus on ovarian development, functions, and their implications for female fertility. The project aims to develop more efficient test strategies, such as computer, cell culture, or animal models, for assessing the effects of EDCs on female fertility. Additionally, the project seeks to identify and prohibit hazardous EDCs while providing sustainable options for disease prevention and health-related behaviours (Duursen et al., 2020). Within the FREIA project, Estonian researchers are investigating whether chemicals in IVF patients' FF affect IVF outcomes.

For instance, a concerning fact is that among the 100,000 chemicals available in the EU market, only 500 have undergone comprehensive risk assessments (Vinnars et al., 2023). With over 40,000 chemicals in commercial use in the US alone, there is an urgent need for biomonitoring to address health risks and their effects (Pellizzari et al., 2019).

2 Aims of the Study

The specific aims of the thesis are as follows:

- To identify molecular pathways differentially regulated between hypo- (HR) and normoresponder (NR) patient groups and elucidate the mechanisms associated with reduced ovarian responsiveness during IVF.
- To analyse the cellular composition profile of preovulatory follicles in patients categorised as either NRs or HRs and compare the proportions of distinct somatic cell clusters within FF between NR and HR patients, aiming to elucidate their contributions to varying outcomes during gonadotropin stimulation for IVF.
- To assess the relationships between various EDCs and IVF outcomes, including measures such as the OSI, which serves as an indicator of ovarian response to stimulation with rFSH.
- To investigate the correlation between EDC concentrations in FF samples and the rates of CP and LB in IVF.
- To investigate the miRNA profiles in different ovarian follicle components of fertile women.
- To compare the ovarian cellular and extracellular miRNA profiles of fertile women with those of women diagnosed with PCOS to identify differences in ovarian follicle function.

3 Materials and Methods

The following methods were used during the study:

- Collection of FF samples (Publications I, II, III) and cellular material from the FF samples (Publications I, III)
- RNA extraction and quality control (Publications I and III)
- Library preparation and bulk RNA sequencing (RNA-seq) (Publication I)
- Library preparation and single-cell RNA-seq (scRNA-seq) (Publication I)
- Bioinformatic analysis of RNA-seq data (Publications I and III)
- Differential gene expression analysis (Publications I and III)
- Gene ontology analysis of targeted pathways (Publications I and III)
- Cell-cluster deconvolution of bulk RNA-seq datasets based on scRNA-seq data (Publication I)
- Quantitative analysis of 59 chemicals by isotope dilution liquid chromatography with tandem mass spectrometry from the FF samples (Publication II)
- Isolation of EVs from the FF samples (Publication III)
- Nanoparticle tracking analysis, Western blot, and transmission electron microscopy analysis of EVs (Publication III)
- Small RNA extraction and quality control (Publication III)
- Library preparation and small RNA-seq (Publication III)
- Bioinformatic prediction of miRNA targets (Publication III)
- Statistical data analysis (Publications I, II, III)

4 Results and Discussion

4.1 Participant selection and cohort compilation: investigating ovarian responsiveness, environmental chemicals, and PCOS in IVF treatment

The functionality of the ovaries is significantly influenced by factors such as female age, hormonal milieu, and various lifestyle and environmental elements, including smoking, diet, and exposure to chemicals (Broekmans et al., 2007; ESHRE Capri Workshop Group, 2005; Hoyer, 2005; Nelson et al., 2013a; Secomandi et al., 2022). However, there is a notable lack of research regarding the characteristics of preovulatory follicles in individuals with different aetiologies, such as ovarian hyporesponsiveness to gonadotropins and PCOS. Furthermore, the search for environmental influences on these conditions remains insufficiently explored. Bridging this research gap is crucial to gaining a comprehensive understanding of the complex relationship between environmental factors and the development or occurrence of specific aetiologies, thereby advancing knowledge in the field of reproductive health and fertility.

In particular, it is essential to evaluate the associations between ovarian responsiveness to stimulation with hormones and its impact on somatic cell types within the follicle, altered molecular pathways, chemical mixtures found in the ovarian preovulatory FF, and the repertoire of miRNAs in the PCOS ovary.

Our focus of further investigation was on OSI, given the various indexes and formulas available, such as FORT (Genro et al., 2011), FOI (Alviggi et al., 2018), and OSI (Huber et al., 2013), all of which predict ovarian responses to gonadotropins during IVF.

When considering a woman's first IVF cycle, attention to current health parameters like age, AFC, and AMH levels can help adjust medication doses, minimise the risk of OHSS, and provide appropriate counselling on potential outcomes and alternatives. On the other hand, ongoing IVF cycle results, such as the ratio of total preovulatory follicle number to retrieved M2s, could be used.

However, the reliability and independent predictive capacity of the OSI formula in IVF treatment requires further evaluation and assessment. The OSI parameter, which can be calculated using various methods, is based on the total amount of rFSH consumed and the number of retrieved oocytes (Biasoni et al., 2011). It can be determined after the OPU in IVF. In publication I, where we examined the variation in genome-wide RNA expression levels of preovulatory FF somatic cells in patients with hyporesponsive results, we calculated OSI as the division of the total dosage of exogenous gonadotropins by the number of retrieved oocytes (OSI = rFSH IU/oocyte). Publication II aimed to explore the impact of chemical exposures on ovarian response during IVF treatment and its success rate. In this study, the OSI parameter was used with a different variation: (OSI = ln((number of oocytes retrieved)/(total rFSH dose (IU)) × 1000)). The formulas are presented in Figure 3.

A total of 277 women from Nova Vita Clinic in Tallinn, Estonia, and 185 women from Carl von Linnékliniken in Uppsala, Sweden, were recruited for publications I, II, and III. Table 2 presents the clinical characteristics of the women in the study cohorts, including previous births, causes of infertility, ovarian responsiveness, and other relevant parameters observed during IVF cycles, along with the corresponding success rates.

Table 2. *Characteristics of the recruited study participants by publication*

	Publication I	Publication II	Publication III
Year(s) collected	2019–2020	2016–2019	2019
Number of participants, n	80	333	46
Location	Nova Vita Clinic	Nova Vita Clinic and Carl von Linnékliniken	Nova Vita Clinic
Age (years), mean (SD)	32.9 (4.8)	34.8 (4.5)	30.7 (5.0)
BMI (kg/m²), mean (SD)	22.3 (3.1)	23.4 (3.5)	23.0 (2.9)
Parity, n (%)			
0	55 (68.8)	202 (60.7)	34 (73.9)
≥1	25 (31.2)	131 (39.3)	12 (26.1)
Cause of infertility, n (%)			
Female factor	23 (28.8)	132 (39.6)	14 (30.4)
Oocyte donor	18 (22.5)	/	15 (36.6)
Male factor	18 (22.5)	75 (22.5)	16 (34.8)
Combination of both	15 (18.8)	35 (10.5)	1 (2.2)
Unexplained	6 (7.5)	91 (27.3)	/
Administered rFSH (IU), mean (SD)	1931.3 (753.4)	2351.7 (1045.5)	1632.0 (1165.9)
Retrieved oocytes, mean (SD)	12.8 (8.8)	10.8 (6.9)	15.7 (8.6)
OSI (rFSH IU/oocyte), mean (SD)	338.0 (503.3)	370.3 (514.1)	160.1 (222.7)
Metaphase II oocyte number, mean (SD)	10.0 (7.1)	8.7 (5.8)	12.7 (7.3)
Treatment type, n (%)*			
conventional IVF	28 (45.2)	153 (45.9)	8 (25.8)
ICSI	34 (54.8)	180 (54.1)	23 (74.2)
Cumulative live birth %, mean (SD)*	37.9 (46.7)	37.4 (44.3)	54.8 (49.8)

*Oocyte donors (Publication I, n=18; Publication III, n=15) are excluded from the calculation.
The cumulative live birth % was calculated as the total number of deliveries (>24 weeks of gestation) divided by the total number of performed embryo transfers, including all fresh and the subsequent frozen-thawed cycles.

BMI – body mass index, ICSI – intracytoplasmic sperm injection, IVF – *in vitro* fertilisation, IU – international units, OSI – ovarian sensitivity index, rFSH – recombinant follicle-stimulating hormone, SD – standard deviation

Patients were selected based on study goals, focusing on two distinct groups: fertile women with normal ovarian reserve and oocyte donors (reference group), and women with different responses to stimulation with rFSH (Publication I) or those diagnosed with PCOS (Publication III). In publications I and III, which focused on hyporesponse and PCOS, respectively, the age of the patients was below 41 years. In publication II, which examined environmental chemicals, the patients were under 44 years of age. However, all selected patients shared certain characteristics, like the presence of both ovaries and the absence of relevant pathologies, including conditions such as ovarian tumours, ovarian cysts, and chronic inflammatory conditions.

Despite the cohorts being from different years and locations, we ensured comparable collection and processing of biomaterials and data, thereby ensuring consistency and reliability in our research findings. However, variations in the size and composition of the data-sets presented some limitations.

In all three publications, we adjusted the women's metadata for age. Additionally, in publication II, the Estonian cohort's metadata was further adjusted for potential confounders such as BMI, parity, and the number of previous IVF cycles. The Swedish cohort's metadata was also adjusted for infertility duration, smoking, fatty fish consumption, and the use of personal care products, as the required information was available. Moreover, the components of the OSI formula were expressed differently in

publications I and II. These investigations are crucial for comprehending the observed changes and identifying strategies to enhance current therapies and increase the rate of successful LBs. Given the significance of female age in determining the success rate of IVF procedures (Tatone et al., 2008) and the need to examine multiple pathologies, it was necessary to ensure large sample sizes to establish comparable cohorts of women and detect significant variations.

4.1.1 Preovulatory FF somatic cells from HR patients express several genes at different levels compared to NRs (Publication I)

To investigate the effect of hyporesponse and the consequently increased use of gonadotropins during IVF treatment, the OSI cutoff of ≥200 IU of rFSH per retrieved oocyte was applied for the classification of the patient as HR. 150 IU of rFSH per day is considered mild and the standard stimulation approach (Leijdekkers et al., 2020; Popovic-Todorovic et al., 2003). On the other hand, higher doses create a more intense stimulation to achieve oocyte development, increasing the risk of OHSS. Therefore, it is essential to minimise the risk of OHSS as much as possible (Datta et al., 2021). Besides this, increased rFSH doses during stimulation are associated with lower rates of oocyte fertilisation and embryo quality, and thus lower LB outcomes (Tian et al., 2019).

In our pursuit of comprehending the intricate connection between OSI parameters and the alterations in genome-wide RNA expression. Our focus was on the follicular somatic cells of HR and NR patients and our primary objective was the identification of statistically significantly differentially expressed genes (DEGs). These DEGs exhibited modified expression patterns and were investigated for their enrichment into Reactome pathways.

Bulk RNA-seq analysis was conducted on pooled ovarian follicular somatic cells from a total of 18 patients, comprising 9 HR and 9 NR individuals. This approach allowed us to explore the intricate interplay of gene expression across the entirety of the patients' transcriptomes.

Without age as a confounder, the transcriptome data revealed that the expression levels of 895 genes were significantly changed between HR and NR (Figure 4A) and enriched into 12 Reactome pathways (Figure 4B). The identified pathways encompassed steroid and lipid metabolism, cholesterol biosynthesis, as well as of cell junction organisation and muscle contraction, among others.

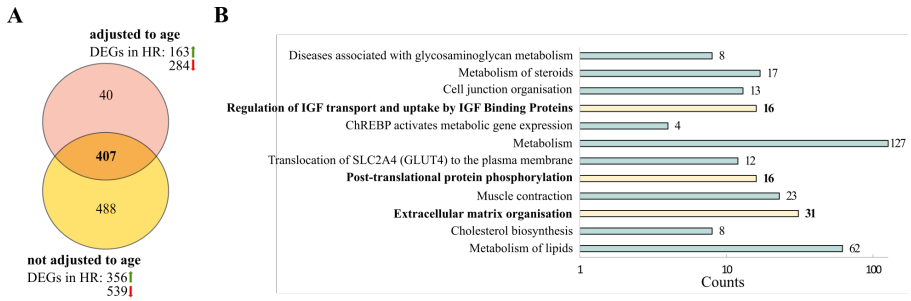


Figure 4. *Differential expression analysis of preovulatory follicular fluid somatic cell genes in hypo- (HRs) and normoresponders (NRs) using bulk RNA-seq.* ***(A)*** *Venn diagram depicting the differentially expressed genes (DEGs) between study groups, considering both the age-adjusted and non-adjusted analyses.* ***(B)*** *Enrichment analysis of Reactome pathways utilising DEG data from HR and NR groups (FDR<0.05), organised in descending order of statistical significance. Emphasised in bold and accompanied by yellow bars are the molecular pathways that sustain significant enrichment following age adjustment. Adapted and modified from Roos et al., 2022.*

The impact of disrupted steroid and lipid metabolism, along with cholesterol biosynthesis, in oocytes and/or GCs during folliculogenesis and its IVF outcomes, such as oocyte quality, embryo development, and subsequent pregnancy success, have been acknowledged (Arias et al., 2022; T. Liu et al., 2022). However, the specific mechanisms governing ovarian responses to hormones remain less understood. This research could transform the field by providing new insights into the molecular signalling disruptions within ovarian follicles of HR patients, thereby paving the way for enhanced treatment approaches and optimised medication utilisation.

Moreover, the metabolism of lipid pathway exhibited a greater number of upregulated DEGs compared to downregulated ones (36 vs. 26) in HR patients. An excess accumulation of lipids can significantly disrupt ovarian reproductive function by initiating oxidative stress and inflammation in the ovaries. It can also interfere with oocyte meiosis and harm the surrounding follicular somatic cells (T. Liu et al., 2022). Mu et al. also found potential biomarkers in human serum during COS, like the combination of glycine, acetylglycine, and lipids, that could predict different responses to ovarian stimulation (Mu et al., 2022). This reinforces the importance of metabolic changes in the context of ovarian responsiveness.

Cataldi et al. found that the levels of certain phosphocholines decreased in the FF of patients who had poor ovarian responses. These phosphocholines could be used as biomarkers to identify these patients (Cataldi et al., 2013). A positive correlation between quantitative detection of blood and FF lipoprotein lysophosphatidylcholine (LPC) and the ovarian stimulation parameter FORT was described by suggesting it could be used as an indicator of ovarian reactivity (Yang et al., 2022).

Remarkably, the LPC pathway shares common genes with the cholesterol synthesis pathway, suggesting their connection within lipid metabolism (Cha et al., 2018). Furthermore, most of the DEGs in the Reactome cholesterol pathway also show heightened activity in the mevalonate metabolic pathway, indicating a close intertwining of these pathways (Guerra et al., 2021). The mevalonate metabolic pathway comprises multiple steps culminating in the production of farnesyl pyrophosphate, a crucial precursor for molecules like cholesterol and coenzyme Q10 (Dallner & Sindelar, 2000). These findings reinforce the alignment of our transcriptomic results with existing research, underscoring the significance of metabolic changes in influencing ovarian sensitivity.

Age serves as a fundamental parameter that is likely to play a pivotal role in shaping ovarian response, potentially setting off a cascade of changes (Ottolenghi et al., 2004). These changes may encompass various aspects, including the alterations in lipid and cholesterol metabolism. Optimal fertility starts to decline after 30 years and sharply starts to diminish after 35 years of age (Dunson et al., 2002). In our study cohort, OSI was correlated with patient age ($p<0.0001$). Maternal age-related changes in the ovary are detrimental, resulting in decreased sensitivity to hormones. As age increases, higher doses of FSH are needed to complete folliculogenesis, increasing the likelihood of ovulatory problems, aneuploidy in oocytes, more miscarriages, reduced fertility, and adverse pregnancy outcomes (Nelson et al., 2013b). Given the direct influence of age on fertility parameters, it is standard practice in infertility research to analyse results by age group. We adopted this approach in our study to precisely explore the intricate connection between age and ovarian function.

Our research revealed that gene expression changes correlated with patient age from 34 years and above (Supplementary Figure 2, Publication I) and therefore we used this age as a cut-off to segregate patients between age groups. To make sure our data analysis

was accurate, we used age group as covariate in further analyses. After adjusting for age, the HR and NR groups were characterised by 447 DEGs. We found that biological pathways, including ECM organisation, post-translational protein phosphorylation, and regulation of IGF transport and uptake by IGFBPs were associated with 407 common DEGs regardless of age (false discovery rate (FDR)<0.05), the majority of which were downregulated in HR group (Figure 4A and B; Supplementary Tables 1 and 2, indicated in bold, Publication I). These 407 genes contribute to molecular processes associated with decreased responsiveness to gonadotropins, regardless of age.

For instance, DEGs shared between age-adjusted and non-adjusted models demonstrate different gene expression patterns. Those showing increased expression in the HR group are highlighted with underlined formatting, while those with decreased expression in HR are presented in **bold** compared to the NR group. This association holds direct implications in the context of fertility (Supplementary Table 3, Publication I).

- associated with ovarian function and follicular development: ***ACAN*** (Tola et al., 2017); ***PDGFA*** (Pinkas et al., 2008); ADAMTS metalloproteases family members such as ***ADAMTS1***, ***ADAMTS9***, and ***ADAMTS14*** (Russell et al., 2015); ***MMP19*** (Goldman & Shalev, 2004)
- associated with TGF-β bioavailability and/or function: ***LTBP1*** (Drews et al., 2008; Hatzirodos et al., 2011), ***FBN1*** (Kielty et al., 2002), ***FBLN5*** (Manders et al., 2018)
- GCs differentiation or functional regulation: ***ITGA2***, ***FN1*** (Kulus et al., 2019; Ożegowska et al., 2019), ***ADAM9*** (Hatzirodos et al., 2014); ***ADAMTS1*** (Brown et al., 2010)
- responsible for COC expansion: *FGG* (Poulsen et al., 2020); ***CAPN2*** (Kawashima et al., 2012); ***PDGFA*** (Yerushalmi et al., 2014); ***ADAM10*** (Caixeta et al., 2013); ***ADAMTS1*** (Robker et al., 2018b)
- related to oocyte growth, maturation, and quality: ADAM and ADAMTS metalloproteases family members such as ***ADAMTS1***, ***ADAMTS9***, ***ADAM9***, ***ADAM10*** (GohariTaban et al., 2019; Poulsen et al., 2020; Robker et al., 2018b; Yung et al., 2010);
- related to ovulation: *FGG* (Burnik Papler et al., 2015), ADAM and ADAMTS metalloproteases family members such as ***ADAMTS1***, ***ADAMTS9***, ***ADAM9***, ***ADAM10*** (Brown et al., 2010; Caixeta et al., 2013; GohariTaban et al., 2019; Hatzirodos et al., 2014; Poulsen et al., 2020; Yung et al., 2010); ***SERPINE1*** (Y.-X. Liu et al., 2004); ***MMP19*** (Goldman & Shalev, 2004)

In HR group, various genes crucial for ovarian function and follicular development exhibit downregulation in their expression. This dysregulation leads to disruptions in key events preceding ovulation, affecting processes such as folliculogenesis, the TGF-β signalling cascade, ECM remodelling, and the differentiation of GCs and TCs. Consequently, these conditions may contribute to the nuclear and cytoplasmic immaturity of oocytes, potentially resulting in oocyte aneuploidy and reduced LB rates (McGinnis & Albertini, 2010; Roberts et al., 2005).

The LH surge serves as the catalyst for ovulation, orchestrates ECM reorganisation alongside concurrent post-translational modifications (Russell & Robker, 2007). Essential for both the structural integrity of the ovary and signal transmission within the preovulatory follicle, ECM proteins have an important role (Russell et al., 2015; Russell & Robker, 2007).

Matrix metalloproteinases (MMPs) are crucial for ECM function in diverse tissues and organs. These enzymes, particularly collagenases, actively participate in collagen metabolism (Curry & Osteen, 2003). The abnormal expression of collagen and its fragments has been linked to pregnancy complications (Knöfler et al., 2019). Furthermore, studies in mouse models have indicated that P4 fosters the production of collagen and elastic fiber proteins, while E2 governs the expression of genes linked to elastin production, collagen generation, and the processing and assembly of elastic fibers. Consequently, these steroid hormones play a direct role in modulating pathways involved in the organization of the ECM (Nallasamy et al., 2017).

MMPs are particularly vital for ovarian follicle development, ovulation, subsequent CL formation and regression during every reproductive cycle (Curry & Osteen, 2003). Studies in mice have shown that *MMP19* gene expression increases in GCs as follicles mature towards ovulation (Nalvarte et al., 2016). Moreover, hCG stimulation induced a 5–10-fold upregulation of *MMP19* on both granulosa and thecal-interstitial cells in large preovulatory and ovulating follicles (Goldman & Shalev, 2004).

On the other hand, studies in ERβ knockout mice have demonstrated that *MMP19* expression decreases when ERβ is overexpressed. This downregulation is attributed to the binding of ligand-bound activated ERβ to a specificity protein-1 binding site, as evidenced in human MCF-7 cells. Consequently, reduced MMP19 levels lead to increased fibrosis surrounding maturing follicles and a diminished blood supply (Nalvarte et al., 2016). Given that GCs play a crucial role in follicle structure, these alterations can negatively impact oocyte development and disrupt various signalling pathways, potentially contributing to infertility. Considering the crucial role of MMPs, including MMP19, in ovarian follicle development, ovulation, and ECM function, the observed *MMP19* gene expression downregulation may contribute to ovulatory issues, compromised follicular maturation, in our HR patient population. Notably, MMPs are regulated by PDGF and TGF-β, both of which were downregulated in our study findings about HR patients. This downregulation indicates their potential role in improper follicle and oocyte development and ovarian pathologies if dysregulated (Goldman & Shalev, 2004).

In addition to MMPs, during the preovulatory stage among patients with hyporesponse, there is a notable deficiency in crucial proteins, as indicated by the reduced gene expression of key members within the ADAM and ADAMTS metalloprotease families – specifically, *ADAMTS1*, *ADAMTS9*, *ADAM9*, and *ADAM10*. These proteins are pivotal in processes like folliculogenesis, cell adhesion, signalling, COC expansion, and meiotic division and consequently also leading to diminished oocyte number, maturation, and quality (Brown et al., 2010; GohariTaban et al., 2019; Hatzirodos et al., 2014; Russell et al., 2015; Tola et al., 2017).

Members of the TGF-β superfamily, including BMPs, activins, inhibins, AMH, and growth and differentiation factors, play crucial roles in gonadal development, folliculogenesis, and angiogenesis (Patton et al., 2021). Knockout studies in mice have shown that the absence of the TGF-β family leads to embryonic or perinatal lethality (Pangas & Matzuk, 2004). Given that GCs are abundant in TGF-β1 and its receptors, recent evidence indicates that TGF-β1 stimulates the expression of CYP19A1 through the SMAD2 and ERK1/2 signalling pathways. This stimulation promotes the active synthesis of E2 in human GCs (Cheng et al., 2021). Therefore, TGF-β1 levels are not only present in the serum but are also detectable in FF. TGF-β1 has been shown to correlate with β-hCG, P4, and oestrogen levels (J. Xie & Cao, 2019), as well as with good embryo quality

(Gao et al., 2012), suggesting a potential association with favourable pregnancy outcomes. Due to varying study results, it is however necessary to further assess the levels and functions of TGF-β1.

The role of TGF-β extends to androgen action, coordinating with various growth factors, including IGF-1 (Franks & Hardy, 2018). IGF-1, expressed in the ovaries and uterus, actively participates in the activation of primordial follicles, folliculogenesis, steroidogenesis, oocyte maturation, and later embryo implantation. Research has demonstrated that IGF-1 plays a role in facilitating the transition of GCs and TCs into luteinized cells (Ipsa et al., 2019). Investigation further suggests that the cooperation between IGF-1, FSH, and LH contributes to the enlargement of GCs and TCs. This may affect steroidogenesis by changing gonadotropic receptors. Additionally, IGF-1 may contribute to the survival of selected follicles by decreasing the incidence of follicular atresia (Ipsa et al., 2019).

Contrary to PCOS, hyporesponse is characterised by a reduction of TGF-β superfamily ligands, resulting in changes in ovarian hormones and morphology. In PCOS, elevated TGF-β levels cause thickening of the ovarian capsule and stroma due to increased collagen deposition and the accumulation of fibrotic tissue (Raja-Khan et al., 2014). This implies that in the context of hyporesponse, lower TGF-β levels might yield an opposing effect.

Our discoveries also point to a significant diminishment in the expression of *LTBP1* gene, a member of the LTBP family, among individuals with HR. Studies in mice reveal that the absence of LTBP1 substantially reduces the expression of genes linked to TGF-β activity (Drews et al., 2008). Moreover, mice deficient in the short alternatively spliced isoform of LTBP1 exhibit compromised ovarian function, including a susceptibility to ovarian cyst formation and decreased serum levels of P4 and oestrogen (Dietzel et al., 2017).

Moreover, regardless of age, patients with hyporesponse displayed significant disruptions with the biological pathway involving the addition of phosphate groups to proteins through post-translational phosphorylation, along with its related genes. In cases of hyporesponse, obstacles may arise in the activation of FSH and LH receptors belonging to the superfamily of GPCRs via phosphorylation cascades, potentially affecting the appropriate receptors' responsiveness to ligands of GCs and TCs. LHR and FSHR undergo a spectrum of post-translational modifications, encompassing glycosylation, palmitoylation, phosphorylation, and ubiquitination. Of particular significance, phosphorylation occurs subsequent to the ligand-receptor interaction, playing a pivotal role in facilitating receptor endocytosis (Menon et al., 2005).

In summary, our study demonstrates that a higher OSI is associated with a decrease in the expression of key genes related to ovarian function and follicular development. Furthermore, we identified disruptions in post-translational phosphorylation and other critical signalling pathways. This complex interplay ultimately leads to disturbances in folliculogenesis, impacting hormone levels and compromising oocyte maturation, resulting in a significant reduction in oocyte count and diminished LB rates. Our analysis underscores the impact of increased gonadotropin stimulation, as defined by the OSI parameter, on preovulatory follicular somatic cells. Additionally, we uncovered distinct molecular pathways that could serve as promising prognostic indicators in the clinical management of ovarian hyporesponse, providing valuable insights for patient care.

4.2 The human preovulatory FF contains 14 distinct cell clusters (Publication I)

Moving forward, our study will investigate the cellular aspects of hyporesponse to rFSH. We aimed to explore if changes in cell cluster proportions were also contributing factors. To identify cell clusters in the human preovulatory FF, we employed scRNA-seq technology. This approach involved analysing 25,957 isolated follicular somatic cells obtained from 3 patients undergoing COS and OPU. These patients were fertile women, including 2 with male-factor infertility and 1 oocyte donor. They were all classified as NR due to their use of a mild stimulation protocol, with a daily dose of rFSH of ≤150 IU (Datta et al., 2021) and a retrieved oocyte yield of 23±12.5. We implemented strict raw sequencing data filtering criteria to ensure high quality (Supplementary Data 3, Publication I). This resulted in a total of 24,213 single cells suitable for clustering and mapping the cell clusters within the preovulatory follicle. It is noteworthy that the distribution of discovered cell clusters, cells, and cell cycle phases was relatively consistent across patients in the merged dataset (Supplementary Figure 3, Publication I).

The scRNA-seq has been used in ovarian-related studies, including the analysis of various regions within whole ovarian tissue (A. S. K. Jones et al., 2024), cell populations within the adult human ovarian cortex (Wagner et al., 2020), investigations into different small antral follicles with diameters ranging from 1–2 mm to 2–5 mm (X. Fan et al., 2019), mapping of oocytes in ovaries of murine, *Drosophila*, and zebrafish models (Y. Liu et al., 2022; Morris et al., 2022; Slaidina et al., 2020), exploring ovarian cancer (Izar et al., 2020) and comparing oocytes from patients with different aetiologies, such as PCOS (Q. Liu et al., 2016; Qi et al., 2020). Prior to our study, Wu et al.'s (H. Wu et al., 2022) research paper was the only one to examine human preovulatory FF somatic cells by using scRNA-seq.

ScRNA-seq method is revolutionary in transcriptomics because it enables to sort cells *in silico* based on their transcriptional similarities, eliminating the need for prior cell sorting methods like flow cytometry. The advantage of employing techniques with minimal pre-processing before testing is evident: reducing manipulation preserves the mRNA integrity of the original microenvironment, ensuring a more authentic acquisition of results. Therefore, scRNA-seq is an effective tool for studying heterogeneity in previously unknown pools of cells or cells at various stages of differentiation, whereas bulk RNA-seq measures the average transcript expression in a cell population (Kashima et al., 2020).

Our primary finding is the presence of 14 distinct cell clusters in the preovulatory FF (Figure 5A). Examining the expression patterns of DEGs within identified cell clusters (Supplementary Table 4, Publication I) using markers from the existing literature and the Human Protein Atlas database, we identified 10 non-immune cell lineages (PTPRC-) and 4 immune cell lineages (PTPRC+). As expected, 87.5% of total cells were GCs (*SERPINE2*), which divide into CGCs (*CYP19A1*) and seven GC subclusters that express both shared and distinct GC markers.

Other non-immune somatic cells were theca/stroma (*GNG11*) and epithelial cells (*KRT17*). Among the 4 leukocyte lineages were neutrophils (*CXCL8*), T cells (*IL7R*), M1- (*CD86*), and M2- (*CD163*) macrophages (Figure 5B)**.** Immune cells in the follicle have been linked to normal physiology as well as various reproductive problems (Brännström & Enskog, 2002; Z. Li et al., 2019b; R. Wu et al., 2004). Theca/stroma, and epithelial cells were located in the collected samples, probably due to a technical artifact of the OPU procedure: once the needle penetrates the follicular structure, it fractures the overlying

padding cells and suction-draws in cells. Several studies have also identified TCs and epithelial cells in FF samples (Ai et al., 2019; Lai et al., 2015).

Our analysis revealed that MGCs divide into 7 clusters, each exhibiting unique gene expression patterns and enriched molecular pathways identified through Reactome annotations (Supplementary Tables 5 and 6, Publication I). Accordingly, we were able to identify GC clusters that were mostly involved in P4 production, post-translational protein phosphorylation, active interleukin (IL)4 and IL13 signalling, insulin receptor recycling and glyconeogenesis, synthesis of very-long-chain fatty acyl-CoAs, signalling via NTRK1 and ILs, or in the process of apoptosis.

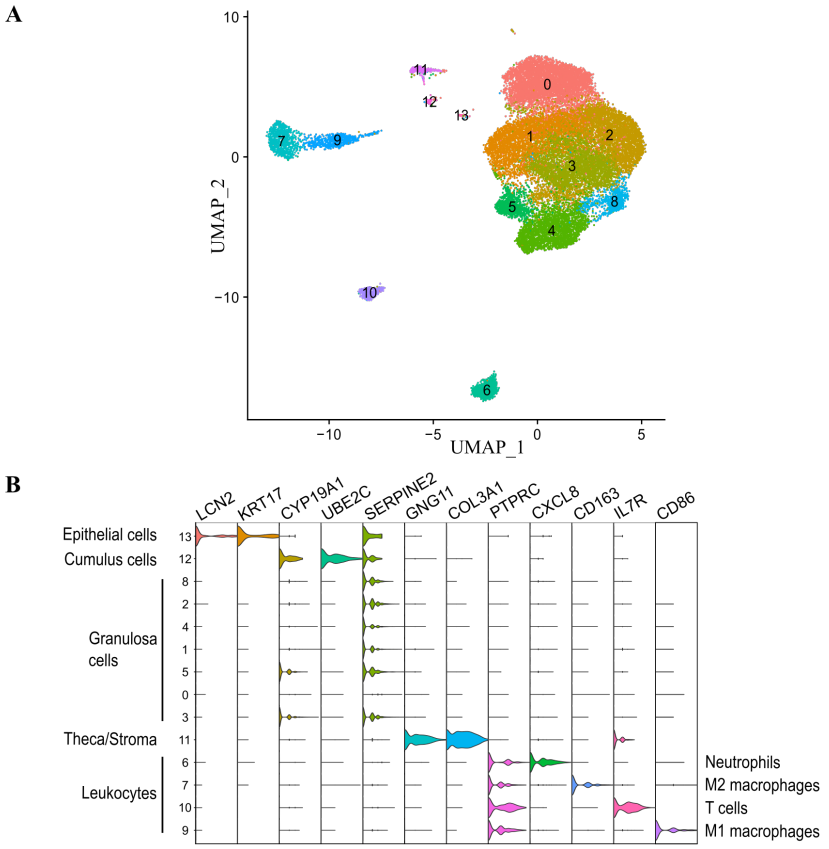


Figure 5. *Representation of identified cell clusters within the human preovulatory follicle using single-cell RNA-seq data.* ***(A)*** *Visualisation of individual cell clusters, identified through the original Louvain algorithm, presented on a UMAP plot.* ***(B)*** *Annotated UMAP plot utilising distinctive gene expression patterns, delineating 4 leukocyte cell clusters, epithelial cells, theca/stroma cells, cumulus cells, and granulosa cells. Adapted and modified from Roos et al., 2022.*

At the time of this study, there was no previously collected scRNA-seq-based data for GC clusters. Currently, two similar studies, one by Wu et al. (H. Wu et al., 2022) and the other by Choi et al. (Y. Choi et al., 2023), have been conducted using FF from women undergoing IVF procedures.

One notable strength of our work is that we identified similar MGC clusters to Wu et al., although they characterised more MGC clusters than we did. Additionally, we both

observed that some of these clusters share similarly expressed genes, suggesting that they function more actively in processes like P4 synthesis, angiogenesis, apoptosis, and ECM remodelling, compared to others. Choi et al. delineated a smaller number of GC clusters compared to ours, totalling 5, and identified cluster of TCs. These findings further emphasise and outline the importance of heterogeneity in GC populations in human follicular aspirates. However, it is worth highlighting that our study uniquely identified a CGC cluster that was not characterised in Wu et al.'s or Choi et al.'s research.

The observed variations between our study and two others may be attributed to multiple factors. One such factor is the difference in the number of study participants. Additionally, aspects that are both data-driven and hypothesis-driven have an impact on scRNA-seq analysis. Therefore, differences in the results between different research groups may also arise due to limitations in data quality parameters, such as the number of cells analysed, genes detected, data dimensions, and the specific bioinformatics approaches employed. These variations highlight the complex nature of scRNA-seq analysis and the importance of considering multiple factors that can influence the outcomes. In comparison to bulk RNA-seq data, the multidimensional data obtained by the scRNA-seq method has a high level of technical noise, necessitating the use of a statistical algorithm, making it as computational as it is experimental (Kashima et al., 2020).

4.2.1 Changes in FF cell cluster proportions of patients with hyporesponse (Publication I)

The discovery of distinct functional GC clusters is a significant finding for comprehending the composition of the preovulatory follicle. It paves the way for a deeper understanding between GC cluster dysregulations and pathological folliculogenesis and subsequent infertility. One of our goals was to find differences between the cell cluster proportions of HR and NR patients' preovulatory follicles. Therefore, we utilised a computational method known as deconvolution, which uses the cell cluster-specific gene expression data obtained from the scRNA-seq study to predict the cell cluster proportions in bulk RNA-seq data between patient groups.

We discovered a significant difference between HR and NR patients in the proportions of 3 preovulatory follicular cell clusters, including the *ARGLU1*+ and *SEMA3A*+ GC clusters, and theca/stroma cell clusters (adjusted to age). All these clusters were present at a lower proportion in the HR group relating to lower gene expression values of the matching marker genes during hyporesponse to stimulation (Figure 4B, Publication I).

The *ARGLU1*+ GC cluster exhibits diverse functions, encompassing insulin receptor recycling, interactions with laminin, steroid hormone metabolism, and glycogenesis. Insulin receptor cycling not only facilitates the clearance of insulin but also amplifies short-term insulin signalling (Y. Chen et al., 2019). The binding of insulin to its receptor triggers multiple signalling pathways in the ovarian tissues, including those associated with glucose uptake and metabolism, phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) and mitogen-activated protein kinase as well as the IGF system. These pathways, in turn, exert indirect influences on steroid metabolism and LH responsiveness in human GCs (Dupont & Scaramuzzi, 2016). In cases of ineffective insulin receptors, elevated levels of insulin may occur, contributing to various issues such as anovulation and increased ovarian androgens, specifically testosterone (Jamnongjit & Hammes, 2006). Reduced availability of insulin receptors is correlated with IR (Y. Chen et al., 2019).

Moreover, insulin inhibits the activity of IGFBP-1, resulting in increased IGF-1 availability. This dual effect significantly impacts ovarian function, leading to decreased

sex hormone binding globulin levels and, consequently, increased androgen availability (Zhong et al., 2023). Additionally, the ARGLU1 protein plays a central role in the transcription of genes mediated by the ER (D. Zhang et al., 2011).

In conclusion, the multifaceted role of the *ARGLU1*+ GC cluster, particularly its involvement in insulin-related pathways, underscores its potential significance in influencing steroidogenesis and hormonal responsiveness in human GCs in HR patients.

Within the cluster of *SEMA3A*+ GCs, two distinctions stand out: the expression of *TECRL*, which is involved in lipid metabolism, and *ADAMTS1*, which is a key factor in regulating angiogenesis. These genes are both significantly up-regulated and play essential roles in the ordinary human ovulatory cascade (Wissing et al., 2014; Yung et al., 2010). ADAMTS1, mainly produced by MGCs, undergoes sharp upregulation post-LH surge through transactivation by the P4 receptor. Its role in ovulation extends to influencing the morphogenesis of the ovulating follicle wall and COC matrix, ensuring successful fertilisation. Following fertilisation, ADAMTS1 facilitates versican catabolism, essential for COC matrix degradation (Brown et al., 2010).

While additional research and experiments are needed to fully understand the roles of *ARGLU1*+ and *SEMA3A*+ GC subtypes in HR patients, a clearer definition exists for TCs and their insufficiency. TCs, among their functions, are notable for expressing insulin-like peptide 3 (INSL3), and their receptor levels increase as antral follicles develop. The INSL3 signalling pathway is fundamental for the production of androgens. Additionally, INSL3 has been shown to stimulate the oocyte maturation in rat studies (Esteban-Lopez & Agoulnik, 2020). Notably, female knockout mice lacking INSL3 display deficiencies in antral follicles, encounter challenges in corpora lutea development, and consequently produce smaller litters (Spanel-Borowski et al., 2001). In human studies, reduced INSL3 levels have been specifically observed in individuals with low ovarian reserve and following menopause (Esteban-Lopez & Agoulnik, 2020), as well as in the evaluation of POI (Zhu et al., 2021). Integrating INSL3 levels with other markers associated with ovarian response could be a valuable tool for evaluating TC function and predicting a patient's responsiveness to ART. Even in healthy ovaries, the responsiveness of TCs to hCG/LH declines in the early thirties, as does androgen secretion (Piltonen et al., 2003). This deficiency in this vital estrogens precursor holds significant importance in the context of endometrial development and receptivity as well, ultimately leading to a negative impact on pregnancy rates (Parisi et al., 2023). Our findings suggest that a decline in TC layers may contribute to ovarian response issues.

To uncover potential associations among the identified genes, specific cell clusters, and hyporesponse, we have conducted an in-depth analysis of the single-cell transcriptomes derived from preovulatory follicles in the NR group. However, in order to further elucidate these connections and their clinical implications, a specialised genetic investigation, such as a genome-wide association study or focused gene expression analyses, is warranted. This approach would provide a more comprehensive understanding of the underlying mechanisms. Our findings indicate a correlation between hyporesponsiveness and the proportions of 3 preovulatory follicle cell clusters, shedding light on how gene expression levels change at the molecular level. It is important to acknowledge that the influence of well-known adjuvants used in ART may significantly impact on some of these changes.

Moving forward, it becomes evident that addressing these findings through a personalised approach is essential. This approach can not only optimise the response to ovarian stimulation with gonadotropins but also enhance the chances of a successful

pregnancy. In the subsequent chapter, we will explore potential clinical solutions and recommendations that arise from our research, paving the way for more effective interventions in cases of suboptimal ovarian response.

4.2.2 Hyporesponse during IVF necessitates a modified COS protocol, contrary to the use of rFSH alone (Publication I)

Our findings emphasise the importance of customising COS approaches to effectively address hyporesponse during IVF treatment. Androgens play a pivotal role in follicular development, particularly in their connection to TCs, which are responsible for the production of sufficient androgen levels, subsequently converted to E2 by GCs (Oktem & Oktay, 2008). In HR patients, we observed a significant decline in TC numbers, potentially leading to decreased androgen levels, coupled with dysregulations in IGF transport and uptake by IGFBPs.

To counter this, considering adjuvant treatments like testosterone or DHEA is warranted. Such treatments show promise in promoting follicle growth, enhancing oocyte quality, and improving insulin sensitivity. Additionally, they might elevate FSH receptor expression in GCs, leading to improved follicle recruitment and differentiation (S.-N. Chen et al., 2019; Chern et al., 2018; Jirge et al., 2014; L. Xu et al., 2019). In particular, the administration of DHEA has been linked to potential reductions in gonadotropin doses and the duration of COS (Kotb et al., 2016).

In our study, the average age of our entire cohort was 30.6 ± 4.5 years. Data from Estonia in 2022 revealed that women aged 35 and older underwent a significantly higher number of IVF cycles compared to women aged 34 and younger (1939 vs. 1349) (Tervise Arengu Instituut, 2022). Furthermore, fertility treatment statistics from England reported an average age for IVF treatment of 35.3 in 2018. It is evident that the predominant group of women receiving ART treatments typically falls into the over-35 age range, and this proportion is increasing (Pierce & Mocanu, 2018), a trend on the rise consistent with data from the Nova Vita Clinic's database. This is further underscored by the fact that 30% of the OPUs were performed on women aged 40 and above, highlighting the growing prevalence of older age groups seeking ART. This underscores the growing significance of androgen supplementation, potentially improving ovarian responsiveness to rFSH stimulation and enhancing ART outcomes for patients, regardless of their infertility diagnosis.

In addition, recombinant LH (r-LH) has the potential to decrease the number of immature oocytes, enhance the success of embryo transfer (Pezzuto et al., 2010), and reduce apoptosis in CGCs or MGCs (Ruvolo et al., 2007), thus contributing to better IVF outcomes. These findings underscore the capability of r-LH to enhance ovarian responses, especially in individuals with previous suboptimal responses. Moreover, r-LH could be a valuable inclusion in COS protocols, particularly for PORs. It might elevate implantation and pregnancy rates, particularly among older patients and those with insufficient ovarian responses (De Placido et al., 2005; Hill et al., 2012). LHRs initially present on TCs become active in FSH-stimulated GCs as follicular development progresses. Incorporating r-LH is often more beneficial than increasing rFSH doses in PORs. This approach stimulates FSHR expression, intensifying treatment sensitivity (Di Guardo et al., 2023). While LH/hCG-containing medications at the start of stimulation can impact E2 and P4 production, the outcomes exhibit variability across research results (Barberi et al., 2012; Smitz et al., 2007). r-LH also influences TGF-β production by GCs

(Barberi et al., 2012), impacting IGFs and IGFBPs, which are vital for ovarian function, including lipid metabolism, cell growth, and differentiation (Allard & Duan, 2018).

Furthermore, administering growth hormone during IVF for patients with a poor prognosis has demonstrated a positive impact on folliculogenesis (Haahr et al., 2018), resulting in an increased yield of retrieved oocytes (Sood et al., 2021) and improved LB rates (Keane et al., 2017, 2018). However, there is an inconsistency in the available data on growth hormone supplementation, underlining the need for further research to clarify the precise role of growth hormone in this context. Customising treatment based on individual patient characteristics remains crucial for optimal outcomes.

4.3 Determining EDC levels in the FF and their association with IVF outcomes (Publication II)

In the preceding chapter, we studied ovarian sensitivity to gonadotropins, uncovering variations among patients and the factors contributing to them. As we move forward from exploring ovarian sensitivity, it is crucial to broaden our perspective beyond individual ovarian responses. Apart from variations in ovarian sensitivity and other fertility-affecting factors, such as limited oocyte reserve, hormonal changes, and conditions like PCOS, we must also acknowledge the profound influence of synthetic environmental compounds on female fertility. This chapter embarks on an investigation into the pivotal role of environmental chemicals in shaping the fertility challenges faced by women, with a specific focus on measuring EDC levels in FF and their significant associations with IVF outcomes.

Currently, our comprehension of EDC contamination within the ovary and its effects on ovarian reserve, fertility parameters, and IVF treatment outcomes remains limited (Green et al., 2021). Shifting our focus to the epidemiological aspect of the study, we turn our attention to the measurement of 59 chemicals in preovulatory FF collected from IVF patients in Estonia and Sweden, constituting a total of 333 women. Remarkably, 11 of these 59 chemicals were detected in more than 90% of women, including 3 phthalate metabolites, 4 DEHP metabolites, 1 paraben metabolite, and 6 PFAS metabolites (Table 2, Publication II). The presence of these chemicals prompts us to consider their potential impact on ovarian health.

The identification of a substantial presence of 4 DEHP metabolites in FF is particularly exciting. This finding deepens our understanding of how this widely used plasticizer, DEHP, from the phthalate group, with a global production of up to 4 million tonnes annually, may adversely affect reproductive health, especially ovarian health (Giulivo et al., 2016). In rat models, it was found that exposure to DEHP can lower serum E2 levels, halt ovulation, cause polycystic ovaries, and lead to smaller preovulatory GCs (Davis et al., 1994; Lovekamp & Davis, 2001). In mouse models, it was observed that DEHP inhibits the growth of antral follicles and disrupts steroidogenesis and the proliferation of GCs (Hannon et al., 2023). Liu et al. (J.-C. Liu et al., 2021) conducted a study to investigate the transgenerational effects of DEHP exposure by analysing the ovarian slices of offspring naturally exposed to DEHP through the maternal milk of mothers exposed to DEHP. It is estimated that humans consume DEHP metabolites at a rate of 3–30 ng/kg per day, but potentially more (Hannon & Flaws, 2015), and lactating mice were treated with similar doses. Transcriptome analysis revealed that DEHP primarily affects pathways related to ovarian hormone secretion and oxidative stress. The decrease in E2 levels due to dysfunctional steroidogenesis-related enzymes leads to reduced GC

proliferation. Additionally, oxidative stress and DNA damage activate the Caspase9 pathway, increasing apoptosis levels, resulting in diminished oocyte quality and a decrease in overall oocyte count, ultimately impacting antral follicle development (J.-C. Liu et al., 2021).

Due to its environmental sources and processing methods, DEHP is prevalent even in bovine milk, poultry, eggs, and their products (Giulivo et al., 2016). While DEHP has been replaced with diisononyl phthalate (DiNP) in certain products due to health concerns, prenatal DiNP exposure has been linked to male genital birth defects in children and reproductive dysfunction in adult males. It is important to note that DEHP and DiNP share similar antiandrogenic properties (Bornehag et al., 2015). Our discovery regarding DEHP is a significant contribution to the body of knowledge, highlighting the importance of further research into its potential impacts on reproductive health.

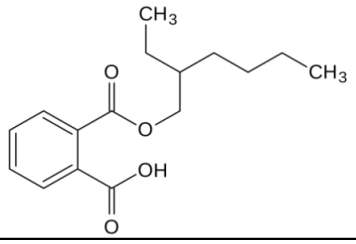
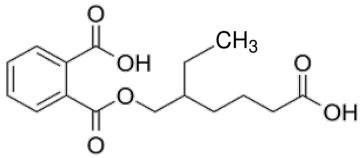
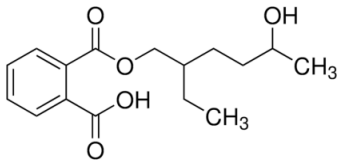
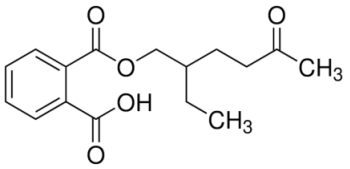
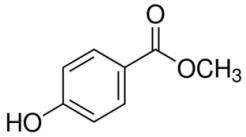
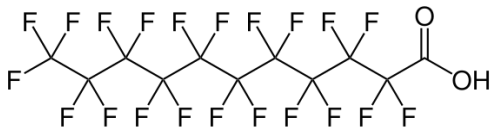
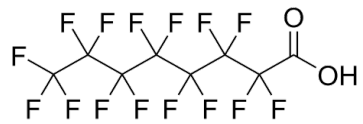
Furthermore, the chemical composition in FF samples from Estonian and Swedish women exhibited variations, hinting at differences in their consumption habits. Focusing on the 11 distinct chemicals present in both cohorts, the correlation patterns of DEHP diverged significantly. In the Estonian cohort, DEHP correlated positively with three chemicals: cxMiNP, methylparaben and PFHxS. In the Swedish cohort, no correlation with DEHP was found (unpublished data). These distinctions imply potential variations in chemical exposure and interactions, warranting further investigation into their impact on female fertility.

Interestingly, correlating these chemicals with IVF parameters, including OSI and CP rate, we uncovered significant associations. Specifically, we observed a substantial association between a higher concentration of the sum of 4 DEHP metabolites (ΣDEHP): MEHP, MECPP, MEHHP, and MEOHP, and reduced ovarian sensitivity in the Swedish cohort. In contrast, in the Estonian cohort, methylparaben exhibited a significant association (Table 3, Publication II). Altogether, we identified seven compounds that exhibited an inverse relationship with the OSI parameter. The chemical structures of these seven EDCs, among the 59 chemicals investigated, are provided in Table 3. These findings underscore the disruptive potential of these substances on ovarian sensitivity to gonadotropins, signalling the need for further research.

It is intriguing to note that women often exhibit higher levels of phthalate and paraben metabolites in their urine compared to men (CDC, 2021; Green et al., 2021). Phthalates, parabens, and bisphenols, along with their respective metabolites, have all been identified as ovarian toxins. When these chemicals are present together, they have been shown to alter gene expression, protein levels, and enzyme activity in the developing ovary (La Merrill et al., 2020). Phthalates are not covalently bound to products, allowing them to leak into the surrounding environment, found in food, water, indoor air, and dust (Giulivo et al., 2016; Wang & Qian, 2021). Additionally, various phthalates, including DEHP, have demonstrated a direct negative correlation with circulating testosterone levels, affecting both males and females, including pregnant women (Meeker & Ferguson, 2014; Sathyanarayana et al., 2014).

The vulnerability of expectant mothers and their unborn children is a matter of concern due to the distinct changes in pregnant women's ability to metabolise, detoxify, and eliminate chemicals and toxins (Vinnars et al., 2023). These alterations make them more susceptible to the adverse effects of substances like DEHP. However, it is crucial to emphasise that further research and confirmation in larger study cohorts is needed to fully understand the extent of DEHP's impact on ovarian sensitivity and its potential role in infertility.

Table 3. *Chemical structures of 7 endocrine-disrupting chemicals with inverse relationship to ovarian sensitivity index*

Name		Structure of Compound
Metabolites of di-2-ethylhexyl phthalate (DEHP)	Mono-2-ethylhexyl phthalate (MEHP)	
	Mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP)	
	Mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP)	
	Mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP)	
Methylparaben		
Perfluoroundecanoic acid (PFUnDA)		
Perfluorooctanoic acid (PFOA)		

Source: adapted and modified from PubChem (S. Kim et al., 2023).

In line with this, research has uncovered that phthalates, particularly DEHP, possess the capacity to suppress the expression of INSL3 mRNA in mice (Ivell et al., 2022; Song et al., 2008). INSL3 production primarily occurs within the ovarian TCs in females (Esteban-Lopez & Agoulnik, 2020). This link between DEHP exposure and the

downregulation of INSL3 highlights the intricate interplay between environmental factors and ovarian sensitivity. Importantly, this discovery aligns with our observations in publication I, where we noted a reduction in the number of TCs in women with a diminished response to ovarian stimulation. This reduction could potentially lead to diminished INSL3 production – a phenomenon influenced by environmental chemicals as well.

Furthermore, DEHP exposure has been associated with a lower likelihood of achieving CP and LB after ART procedures (Hauser et al., 2016; Messerlian et al., 2016; Panagiotou et al., 2021), which is consistent with our research on reduced OSI. It is noteworthy that our study did not find significant associations between DEHP concentrations in FF and CP or LB outcomes. However, several factors should be considered to interpret this observation. These factors encompass the limited dataset of pregnancies, a relatively small cohort size that may lack statistical power, and a relatively short follow-up duration, which may not accurately capture cumulative effects.

In addition, Barnett-Itzhaki et al. reported that they found 7 phthalates in the FF of more than 74% of their study population. These phthalates were linked to EV-miRNA profiles, which affect oocyte development, maturation, and fertilisation (Barnett-Itzhaki et al., 2021). Also, during IVF treatment, a negative association between urinary phthalate levels and the total number of oocytes, M2 count, fertilisation rate, and embryo quality has been observed (Hauser et al., 2016). However, the specific mechanisms of action of phthalates and other EDCs in this context remain unexamined.

Furthermore, both parabens and phthalates can cross the placental barrier, posing potential risks to foetal development (Giulivo et al., 2016; Wang & Qian, 2021). Foetuses are particularly vulnerable to the reproductive, anatomical, and functional developmental effects of EDCs, such as phthalates and parabens, which can lead to the development of diseases later in life (Giulivo et al., 2016; Vinnars et al., 2023).

The widespread use of parabens in personal care and food products, pharmaceuticals, and cosmetics is due to their antifungal and antibacterial properties, as well as their affordability (Giulivo et al., 2016). In the US, Canada, and the EU, the legal limits for parabens in cosmetics are regulated to be at a maximum of 0.4% for a single ester and 0.8% for a mixture of parabens (Kolatorova et al., 2018). Studies have shown that parabens can lead to changes in ovarian histology, ovarian weight, and hormone levels when administered to prepubescent female rats. Parabens bind to ERs and exert oestrogenic effects (Vo et al., 2010). Overall, food is considered a primary source of phthalate and paraben exposure (Kolatorova et al., 2018; Vessa et al., 2022).

While numerous studies have investigated various parabens in relation to IVF outcomes, limited research has specifically addressed the association with methylparaben (Jurewicz et al., 2020; Mínguez-Alarcón et al., 2019; Radwan et al., 2023) as observed in our study. Furthermore, Smith et al. reported that methylparaben was detected in over 99% of IVF patients' urine samples (Smith et al., 2013). Additionally, higher paternal concentrations of methylparaben were linked to reduced LB rates in intrauterine insemination procedures (Dodge et al., 2015). Exposure to methylparaben was found to alter porcine oocyte morphology and hinder the expansion of CGC, ultimately affecting the oocyte maturation process (Barajas-Salinas et al., 2021).

The findings from our comprehensive study reveal potential inverse associations between concentrations of perfluoroundecanoic acid (PFUnDA) and OSI in both the combined population and the Estonian subgroup. Within the Estonian population (Table 4,

Publication II), there is a notable potential correlation indicating that higher concentrations of perfluorooctanoic acid (PFOA) may be associated with lower OSI.

PFAS chemicals, particularly PFOA, have previously been detected in the FF of women undergoing IVF, with up to 98% of samples testing positive (Kang et al., 2020; Y. R. Kim et al., 2020). These compounds have been linked to elevated cholesterol (Steenland et al., 2009) and androgen levels (Heffernan et al., 2018). Given that PFAS substances modify the activity of nuclear receptors implicated in steroid metabolism (Green et al., 2021), bind to ERs, and activate peroxisome proliferation-activated receptors, they have the potential to disrupt ovarian function.

Specifically, PFAS chemicals are amphiphilic and primarily bind to plasma proteins like albumin. This binding alters albumin's conformation, affecting its normal function. For instance, reduced metal-binding capacity in albumin leads to the formation of a variant known as ischemia-modified albumin, a recognised marker of oxidative stress prevalent in women with PCOS (Forsthuber et al., 2020; Guven et al., 2009; Hughes et al., 2021). Moreover, oxidative stress has been demonstrated to modify the conformation of other proteins, increasing their permeability across the blood-follicular barrier. Effectively managing oxidative stress can help mitigate the levels of EDCs that negatively impact ovarian health (Luddi et al., 2020).

Notably, albumin is a common macromolecule used in cell culture media, including IVF solutions, to aid in the fertilisation and development of gametes. Albumin, crucial as a carrier for various substances in the bloodstream, including steroid and thyroid hormones, fatty acids, enzymes, and growth factors, also plays a significant role in the capacitation process during fertilisation. During the capacitation process, albumin has been shown to capture cholesterol released by the sperm plasma membrane (Hughes et al., 2021).

Unfortunately, purified human serum albumin has been found to contain DEHP metabolites, potentially exposing oocytes to chemically contaminated FF in laboratory settings (Hughes et al., 2021). Further research is essential to confirm the observed environmental toxins' adverse effects, such as increased oocyte apoptosis, reduced cell quantity, and altered gene expression during the blastocyst stage (Hughes et al., 2021).

High plasma concentrations of PFOA have been associated with a decrease in the number of oocytes and M2s, as well as lower embryonic quality (Ma et al., 2021). Elevated maternal concentrations of PFAS chemicals are associated with an extended time-to-pregnancy, reduced fertility (Vélez et al., 2015) and low birth weight (Green et al., 2021). In our findings, we observed that a higher concentration of PFOA was associated with reduced chances of achieving a CP (Figure 3, Publication II). It is important to note that the manufacture of PFOA was prohibited in the EU and the US starting in 2020 and 2015, respectively (Zuccaro et al., 2022). These findings highlight the need for stricter regulations and sustainable practices to reduce chemical exposure and improve fertility rates.

Differing results between the Estonian and Swedish cohorts can be attributed to various factors, emphasising the importance of cautious interpretation and diverse research. These factors encompass population variations, geographical location, differences in energy production methods, other environmental factors, the choice of statistical methodologies, and the relatively small sample size. All of these elements can contribute to the variations in the impact of chemicals on female fertility. It is crucial to highlight that the effects of EDCs often exhibit dose-dependent variability.

Therefore, we also examined dose-dependency within the combined population by categorising the chemicals as exposures through quartile analysis. Furthermore, the Swedish cohort had a brief lifestyle questionnaire, whereas the Estonian cohort lacked this information, making it challenging to precisely assess the potential dose and reach of these chemicals. This underscores the need for cautious interpretation and comprehensive research in diverse populations.

This study leads the way in assessing the impact of EDC mixtures on ovarian function and female fertility, offering valuable insights. Ongoing efforts in the future aim to unravel the complex ways in which EDCs affect fertility and to develop new tests for detecting disruptions in infertility mechanisms. Currently available test protocols and methods lack the specificity required for identifying chemicals that disrupt hormonal processes. These disruptions can have lasting effects, influencing embryonic development through various life stages and ultimately impacting reproductive functioning (Duursen et al., 2020). While our study primarily concentrated on female IVF material, it is essential to comprehend the effects on both genders. Consequently, evaluating IVF outcomes, including CP and LB rates, in conjunction with paternal information is crucial to gaining a comprehensive understanding.

Further validation of the impact of EDCs is needed, including *in vitro* and *in vivo* toxicity studies, computational approaches, and studies involving larger and more diverse cohorts, spanning various diagnoses, age groups, and genders (Björvang & Damdimopoulou, 2020; Green et al., 2021). Additionally, measuring EDC levels in different body fluids and tissues for comparative purposes, potentially requiring validation, is crucial (Green et al., 2021). The main route for the elimination of most chemicals and their byproducts from the body is through urine (Giulivo et al., 2016). However, examining the immediate environment of the germ cells or the specific tissue under investigation provides insights into how chemicals accumulate and impact their functions. While our studies employed FF, which has been shown to correlate with chemicals in serum (Björvang et al., 2022; Hallberg, Plassmann, et al., 2021; Heffernan et al., 2018), further research into our findings in serum and urine is still warranted. For instance, a study was conducted on PFAS substances and compared the levels of PFAS, specifically PFOA and PFUnDA, in both serum and semen. The results showed a correlation between these compounds, although their concentrations in semen were notably lower (Y. Pan et al., 2019).

Our study's results provide compelling evidence regarding the adverse effects of EDCs on reproductive health. These findings underscore the urgent need for improved testing and proactive measures. Specifically, stricter regulations governing the use of harmful chemicals across industries are warranted. Simultaneously, we must promote sustainable and eco-friendly practices. Moreover, it is crucial to enhance public awareness of the inherent risks linked to chemical exposure.

In summary, our research highlights the importance of strict regulations, and heightened public awareness to address the detrimental impacts of EDCs on reproductive health. While associations between EDCs and OSI have been established, further research is needed to elucidate their precise impact on cellular populations. It is evident that EDCs influence ovarian function, but the precise underlying mechanisms necessitate elucidation. Our findings have shed light on additional reasons why ovarian stimulation may not be as effective as anticipated. Additionally, investigating potential strategies to mitigate the impact of EDCs on ovarian function could be a promising avenue for further exploration in this field.

These ongoing research efforts promise to advance our understanding of the intricate relationship between EDCs and fertility, offering potential insights for more effective interventions and safeguards. Our and other authors' findings have the potential to reshape how we approach reproductive health, ensuring a healthier future for generations to come (Green et al., 2021; Vinnars et al., 2023).

4.4 miRNA profiles of patients with PCOS (Publication III)

Now, we turn our attention to miRNA expression patterns within various segments of ovarian follicles. Our upcoming research involves individuals diagnosed with PCOS, enabling a comparative miRNA expression analysis between two patient groups: fertile individuals and those with PCOS. Despite the extensive research on PCOS, our study aims to identify specific miRNA profiles in 3 different preovulatory FF compartments from the same patients, providing valuable insights into intrafollicular signalling. Notably, prior studies have not simultaneously examined miRNA profiles in all 3 compartments of IVF patients.

We hypothesise that differences exist in miRNA profiles, both cellular and extracellular, between fertile women and those diagnosed with PCOS. To address this, we collected 3 matched sample types from a single preovulatory follicle in 8 fertile women without a PCOS diagnosis and 7 patients diagnosed with PCOS. These sample types include follicular somatic cells, cell-depleted FF, and EVs purified from cell-depleted FF. Follicular somatic cells, mainly composed of MGC, are consistently implied as MGC in both the article and these paragraphs. Figure 6A schematically illustrates the sampling collection and separation process.

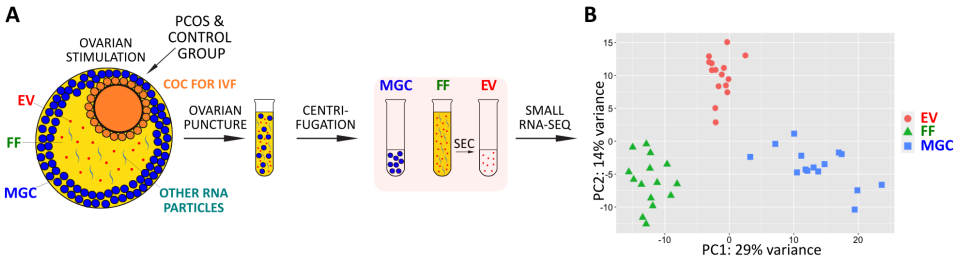


Figure 6. *Sample collection and miRNA analysis in polycystic ovary syndrome (PCOS) vs. fertile control group patients. (A) A visual overview of preovulatory follicle sample collection and processing. (B) Principal component analysis utilising expressed miRNAs across different sample types. Adapted and modified from* Rooda, Hasan, et al., 2020. *PCOS – polycystic ovary syndrome, COC – cumulus oocyte complex, EV – extracellular vesicles, FF – cell-depleted follicular fluid, MGC – mural granulosa cells, SEC – size-exclusion chromatography.*

Subsequently, we conducted whole-genome small RNA-seq using the extracted small RNA samples from each sample type. This method enables us to quantify miRNA expression levels and discover novel miRNA sequences when utilised in conjunction with the miRDeep2 algorithm (Friedländer et al., 2012; Morin et al., 2008).

Initially, we analysed the miRNA profiles of three follicular compartments from IVF procedures in fertile women and those diagnosed with PCOS. The principal component analysis effectively distinguished these sample types, as depicted in Figure 6B. MiRNA profiles were clustered clearly according to their origin, with MGCs exhibiting the most substantial distinctions from the other sample types (Figure 3A and B, Publication III).

It is important to mention that the FF and EV samples were anticipated to exhibit close clustering, considering that the EVs were isolated from the corresponding FF samples. Our findings revealed significant variations in miRNA content among these 3 follicle compartments, with 1381 miRNAs detected in MGCs, 1060 in FF, and 658 in EVs.

The exclusive presence of 124 miRNAs in MGC samples demonstrates the distinctiveness of MGCs' miRNA profiles, according to our research (Figure 4A, Publication III). This observation indicates the regulatory pathway within the follicle, suggesting that not all cellular miRNAs are secreted into the FF. Instead, specific mechanisms, involving both EVs and non-EV pathways, carefully orchestrate the release of these miRNAs. Several sorting mechanisms selectively package miRNAs into EVs, including sequence-dependent and sequence-independent sorting, indicating that EV-carried miRNAs may deliver targeted and efficient messages for specific tasks (D. Xu et al., 2022). For instance, regarding EV-pathways, it has been observed in dendritic cells that the miRNAs contained in exosomes vary according to maturity, and they are released into the target dendritic cell cytoplasm via fusion mechanisms (Montecalvo et al., 2012). Similarly, in a study of somatic follicular cells from women over 38 years old, researchers noted an elevated release of small EVs. They identified certain miRNAs that were specifically expressed and associated with the regulation of EV release, particularly through pathways like protein processing in the endoplasmic reticulum, p53, and mTOR signalling (Battaglia et al., 2020). Additionally, it has been demonstrated that the release of miRNAs associated with lipoproteins is influenced by dietary and metabolic changes, as shown in studies of cultured hepatocytes (Vickers et al., 2011). An increasing number of studies have emerged and continue to emerge focusing on the characterization of miRNA release mechanisms in specific cell types and disease conditions.

Furthermore, the distinct miRNA content across these compartments prompts further exploration of their functional roles. Previous studies have extensively examined RNA-seq data separately from FF (Feng et al., 2015; Martinez et al., 2018; Sang et al., 2013), MGCs (Velthut-Meikas et al., 2013b), GCs and oocytes (da Silveira et al., 2015), and EVs (Neyroud et al., 2022; Sang et al., 2013; Soares et al., 2023) derived from human follicles. Additionally, research has investigated miRNAs in the context of various conditions, including poor ovarian response (Zhao et al., 2021) and IVF outcomes (Martinez et al., 2018). This extensive body of work underscores the pivotal role of RNA mapping in detecting early RNA-level changes, thereby offering a promising avenue for early disease diagnosis and pathological condition development. The rise of RNA immunotherapies, RNA vaccines, and the exploration of RNA biomarkers highlight the growing importance of various RNA subtypes in modern medicine, signalling significant advancements and ongoing research efforts in this field (Tamaddon et al., 2022).

A total of 175 miRNAs identified in EVs are involved in regulating 436 distinct signalling pathways (Figure 4C, Supplementary Table S4B, Publication III). Conversely, 11–16 miRNAs found in FF but not within EVs are associated with only 3 general signalling pathways, lacking specificity in their ovarian function. Circulating miRNAs exhibit remarkable resilience in body fluids, regarding their interactions with argonaute proteins, high-density lipoproteins, and encapsulation within EVs (D. Xu et al., 2022).

Additionally, the trafficking of EVs toward specific cell types appears to depend on the physiological conditions of both the producing and receiving cells (Fritz et al., 2016). This intricate crosstalk involves the transportation of essential cargo, such as mRNAs, proteins, and various types of non-coding RNAs, from donor cells to recipient cells (D. Xu et al., 2022). This molecular exchange significantly impacts the fate and signalling

pathways of the recipient cells, ultimately serving as a pivotal mechanism in maintaining the normal cellular reproduction process (Y. Xie et al., 2023). As EV cargo can influence gene expression in recipient cells, these findings hold significance for understanding long-distance signalling within follicles during IVF under different conditions (Berumen Sánchez et al., 2021). EV miRNAs are anticipated to have a higher degree of stability compared to EV-free extracellular miRNAs, enabling them to serve as highly efficient agents of intercellular communication (Machtinger et al., 2016).

Among the 436 EV-mediated targeted pathways revealed were those related to oestrogen-mediated signalling, signalling by nuclear receptors, signalling by the TGF-β receptor complex, and its family members. The presence of miRNAs involved in the regulation of nuclear receptors such as oestrogen, testosterone, and the TGF-β pathway aligns with their established pivotal roles in ovarian folliculogenesis and function.

Furthermore, our study detected miRNAs with the potential to target pathways where phosphatidylinositol 3,4,5-triphosphate (PIP3) activates AKT signalling, signalling by receptor tyrosine kinases (RTKs), phosphatase and tensin homolog (PTEN) regulation, and negative regulation of the PI3K/AKT network.

The PI3K/PTEN/AKT signalling pathways, crucial in regulating primordial follicle activation and oocyte growth, are initiated by FSH and various growth factors like insulin, VEGF, and fibroblast growth factor, which activate PI3K downstream of RTKs and/or GPCRs (De Felici & Klinger, 2021; T. Li et al., 2017; S. Matsuda et al., 2013). PTEN negatively regulates this pathway by dephosphorylating PIP3, governing follicle growth initiation and protecting against premature follicle depletion. Balancing PI3K and PTEN is vital for correct folliculogenesis and reproductive health (De Felici & Klinger, 2021). PTEN inhibitors show promise in clinical settings for promoting follicle activation and fertilisable oocyte production (S. Matsuda et al., 2013). Human GCs in large preovulatory follicles exhibit higher PTEN levels compared to those in small follicles, potentially affecting the proliferation and differentiation of GCs via the PI3K/AKT pathway (Goto et al., 2007, 2009). Matsuno et al. (Matsuno et al., 2019) conducted a study on porcine FF EV mRNA transcriptome analysis. They identified pathways such as PI3K-AKT, MAPK, and various metabolic pathways predicted to be influenced by these mRNA transcripts. These findings align with our observations regarding miRNA expression profiles, indicating that the molecular pathways identified in follicular fluid extracellular vesicles are conserved across species.

Additionally, we identified 113 miRNAs that were shared exclusively between MGCs and FF. Based on our findings, it is likely that these miRNAs originate from specialised secretory compartments with distinct surface markers separate from EVs, suggesting an alternative mechanism for their secretion into the extracellular space. However, the presence of 172 miRNAs in all 3 sample types indicates that a sizable portion of miRNAs, specifically, exhibit relatively unrestricted cell entry and exit.

Specifically, we identified differences in 30 miRNAs in MGCs and 10 miRNAs in FF of PCOS women compared to fertile control samples, with an FDR threshold of less than 0.1 (Figure 6A and B, Supplementary Table S7A and B, Publication III). Surprisingly, no EV miRNA met the same FDR cutoff. The elevated variability noted in EV samples likely arises from the extensive processing undergone by EV samples in comparison to FF and MGC samples. The present findings suggest that distinct responses occur within these compartments among individuals with PCOS subjects versus fertile controls (Figure 3A, Publication III). It is important to acknowledge that miRNAs can regulate multiple target mRNAs, while conversely, the 3’ untranslated region of a singular mRNA can be subject

to regulation by multiple miRNAs (O'Brien et al., 2018). This complexity contributes to the multifaceted role of miRNAs in PCOS pathophysiology.

One of the pivotal outcomes of this study is the delivery of clear lists of differentially expressed miRNAs for each studied sample type. These lists underscore significant disparities among MGCs, cell-depleted FF, and EVs. This emphasises the possible effects of PCOS and the unique changes that are happening in these compartments, which need more research.

Notably, hsa-miR-200c-3p was upregulated in both cell-depleted FF and EV samples, and it was found to be involved in more than one Reactome pathway. Prior studies have also reported elevated levels of hsa-miR-200c-3p in PCOS patients' peripheral blood (De Nardo Maffazioli et al., 2022), FF (L. Yao et al., 2018) and GCs (T. He et al., 2018; T. He, Sun, et al., 2019). The miR-200c family has been associated with the development of IR, a hallmark of PCOS (Belgardt et al., 2015).

Furthermore, our analysis revealed PCOS-related alterations in Reactome pathways, with 25 pathways identified in FF, 20 pathways in MGCs, and 13 pathways in EVs (Figure 7, Supplementary Table S8, Publication III). Certain upregulated miRNAs in MGCs were associated with transcriptional regulation, IL signalling, nuclear receptors, oestrogen signalling receptor, and cell cycle pathways.

Conversely, miRNAs specific to the FF demonstrated enrichment in vital pathways, including those associated with TGF-β receptors and their family members, RTK signalling, apoptosis pathways, and the AKT signalling cascade. Notably, among the critical downstream pathways of AKT, mTOR plays a central role, regulating cell growth and coordinating metabolic reactions in response to various stimuli, including nutrients, growth factors, and diverse extracellular signals (De Felici & Klinger, 2021). Additionally, we observed that EV-specific miRNAs displayed upregulation in IGFR1 signalling, ERBB2 signalling, and related pathways. Immune-related pathways were identified in both MGCs and cell-depleted FF.

Our results highlight the significance of hsa-miR-200c-3p and other miRNAs in targeting pathways such as PI3K/AKT, RTKs, ILs, and cytokine signalling, underscoring their crucial role in PCOS development. These miRNAs can potentially impact metabolic activities, cellular processes, inflammation, and follicular growth.

It is crucial to emphasise that even a single alteration in miRNA can profoundly impact these critical pathways. For instance, modifications in the PI3K-AKT signalling pathway are strongly associated with IR, diabetes, and metabolic disorders. This holds particular metabolic significance as downstream molecules of the PI3K-AKT signalling pathway play a role in lipid metabolism. Therefore, this pathway is considered to be implicated in the development of PCOS (Gong et al., 2020; T. Li et al., 2017).

Numerous studies are currently investigating dysregulations within the IGF1R pathway (Geng et al., 2019; Luo et al., 2021; Mao et al., 2018; Tamaddon et al., 2022), inflammatory processes (Cirillo et al., 2019; Tamaddon et al., 2022), and the role of various miRNAs in the context of PCOS (Butler et al., 2020; Cirillo et al., 2019; J. Hu et al., 2020; Javadi et al., 2022; Roth et al., 2014; Sang et al., 2013).

Dysregulations in the IGF1R pathway play a critical role in promoting androgen synthesis by ovarian TCs, contributing to an increased susceptibility to PCOS development. Furthermore, aberrations in IGF1R signalling have a restricting effect on steroidogenesis within the maturing oocyte. This pathway assumes paramount significance in mediating the AKT activation induced by FSH and orchestrating the transition of human CGCs from their pre-antral to preovulatory states (Baumgarten et al., 2014). As a result, the IGF1R

pathway has garnered attention as a promising therapeutic target for managing PCOS symptoms, particularly those related to androgen excess and IR (Baumgarten et al., 2014; T. He, Liu, et al., 2019; Seymen et al., 2021).

In the review by Tamaddon et al. (Tamaddon et al., 2022), various miRNAs linked to PCOS were outlined, and our study substantiates the importance of some of these findings. Our comparison between donors and women with PCOS revealed variations in expression that were evident in MGCs for the hsa-miR-7b family, hsa-miR-155-5p, hsa-miR-27b, and hsa-miR-486-5p, while distinctions in FF were noted for the hsa-miR-509, hsa-miR-223 families, and the specific hsa-miR-146-5p. As miRNAs often organise into families, our investigation successfully pinpointed representatives from these families, aligning with the aforementioned review. This identification of miRNAs provides a potential avenue for adjusting the levels of entire miRNA families or co-regulated miRNAs, both collectively and individually.

Several ongoing clinical trials focus on miRNAs in obesity and related metabolic disorders, with potential benefits for PCOS patients. In addition to exploring miRNAs, ongoing research delves into small interfering RNA therapies, anti-miRNA oligonucleotides, and miRNA mimics (Singh et al., 2023). In summary, numerous miRNAs have surfaced as promising clinical biomarkers in PCOS-related research, and active exploration in this field continues. In light of our findings and those of other studies, there are additional pathways and associated miRNAs that warrant closer investigation. Exploring these pathways and miRNAs holds promise for the future management of PCOS. Additionally, our findings shed light on the potential improvement of IVM techniques to support PCOS patients during IVF treatment and enhance its results.

5 Conclusions

The main conclusions of this thesis are as follows:

- Patients with reduced sensitivity to gonadotropins exhibit distinct gene expression profiles. Hyporesponse to ovarian stimulation with rFSH is associated with dysregulation of key pathways related to steroid and lipid metabolism, cell junction organisation, and cholesterol biosynthesis. Additionally, age-independent processes involving ECM organisation, post-translational protein phosphorylation, and regulation of IGF transport and uptake by IGFBPs contribute to these observed changes. These findings provide valuable insights into the molecular basis of reduced ovarian responsiveness during IVF.
- Our analysis revealed that preovulatory follicles consist of 14 somatic cell clusters, including four confirmed leukocyte lineages (neutrophils, T-cells, M1- and M2-macrophages), as well as ten nonimmune cell lineages. These nonimmune lineages encompass epithelial cells, theca/stroma cells, cumulus GCs, and seven specific clusters of GCs.
- In hyporesponsive patients, compared to normoresponsive patients, significantly reduced cell counts were observed in 3 specific cell clusters: *ARGLU1*+, *SEMA3A*+ GCs, and TC clusters. These findings underscore the potential significance of these particular cellular components in influencing the different responses to gonadotropin stimulation in IVF.
- We confirmed the presence of EDCs in FF, with 11 of the 59 measured chemicals detected in over 90% of women's FF samples. These chemicals comprised 3 phthalate metabolites, 4 DEHP metabolites, 1 paraben metabolite, and 6 PFAS metabolites.
- Strong associations were found between the levels of certain EDCs, notably 4 DEHP metabolites and methylparaben, in FF and reduced ovarian sensitivity. These findings indicate that environmental chemicals could play a significant role in diminishing female fertility.
- While DEHP, methylparaben, and PFAS compounds were linked to ovarian sensitivity, no significant associations were found with CP and LB rates. This underscores the need for larger, more diverse studies to comprehensively understand the impact of endocrine disruptors on female fertility and IVF outcomes.
- The variation in miRNA profiles among different follicular compartments: cells, FF and EVs underscores their distinct expression patterns and suggests their potential functions in cell-to-cell communication.
- PCOS has altered the miRNA expression profiles of MGCs, cell-depleted FF, and EVs purified from the FF.
- Significant differences in miRNA expression patterns were identified between these two groups, with specific miRNAs playing key roles in pathways associated with PCOS, such as the IGF1R pathway, inflammatory processes, and metabolic regulation.

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Abstract

Complexity of Human Ovarian Folliculogenesis: Molecular Markers of Ovarian-Based Infertility and the Impact of Endocrine-Disrupting Chemicals

Reproductive system disorders and environmental effects have a significant impact on the global decline in birth rates. While *in vitro* fertilisation (IVF) methods have improved and the causes of infertility are being more effectively addressed, some female patients still face a low IVF success rate (<30%). This presents a significant challenge to fertility specialists.

The ovary plays a key role in understanding the causes of female infertility. The ovary responds to hormones produced by the pituitary gland, creating a suitable environment in the follicle for the development and maturation of the oocyte. The follicle is a structural component of the ovary, containing various cell types such as mural granulosa cells (MGC), cumulus granulosa cells, theca cells, and a richly composed follicular fluid (FF) containing numerous metabolites, proteins, nucleic acids, and steroids. All of these follicular somatic cells and compounds support normal ovarian function and are interconnected through miRNAs and extracellular vesicles (EVs).

In the final stages of folliculogenesis, a mature oocyte is prepared for ovulation, where it can be fertilised and develop into an embryo. However, disruptions in folliculogenesis and the communication between follicular cell types can lead to infertility.

This doctoral thesis focuses on the molecular changes occurring in the preovulatory follicles of infertile women. The study primarily involves IVF patients, particularly those whose ovaries did not respond adequately to hormonal stimulation and those diagnosed with polycystic ovary syndrome (PCOS). Additionally, it examines the presence of endocrine-disrupting chemicals (EDCs) in FF and their impact on IVF outcomes. EDCs are known to be harmful to the functioning of the endocrine system, negatively affecting hormone production, transportation processes, and their biological effects. Exposure to these chemicals has been associated with decreased fertility and other health problems.

In the first part of the study, genome-wide gene expression and pathway enrichment analysis were linked to the ovarian sensitivity index at the RNA level. This index observed the relationship between the amount of recombinant follicle-stimulating hormone (rFSH) used in IVF and the number of oocytes that were retrieved (with a threshold of 200 IU rFSH per oocyte), dividing patients into normo- and hyporesponse groups. The analysis revealed changes in 12 molecular signalling pathways, including cholesterol biosynthesis and steroid metabolism. Interestingly, some of these alterations were independent of patient age. Furthermore, changes in the proportions of cell types within the follicle were investigated. Specifically, among the 14 different cell types found in preovulatory follicles, certain subsets of MGCs, such as *ARGLU1*+ and *SEMA3A*+ granulosa cells and theca cells, were underrepresented in patients with hyporesponse.

The second part of the study determined the presence of EDCs in FF, identifying 11 hazardous chemicals, which were present in more than 90% of patients. These chemicals could affect the microenvironment for oocyte development and, consequently, fertility. Some chemicals, such as di-2-ethylhexyl phthalate metabolites and methylparaben, were associated with ovaries being less responsive to hormonal stimulation. This underscores the need for improved monitoring and analysis in regulating the everyday use of these chemicals.

In the third part, the study compared miRNA profiles in different follicular compartments (MGCs, FFs, and EVs) between PCOS-diagnosed women and a control group. The results highlighted the distinct impact of PCOS on miRNA content and, subsequently, various molecular pathways in each examined sample type when compared to fertile women. Specifically, the signalling of oestrogen in MGCs, the signalling of IGF1R in EVs, and the signalling of the TGF-β receptor and its family members in FF were all changed.

Together, this doctoral thesis contributes valuable insights into understanding how multiple factors, including changes in preovulatory follicular cell types, molecular pathways, miRNA profiles, and exposure to environmental chemicals, significantly influence ovarian sensitivity and the microenvironment for oocyte development. Understanding factors affecting female fertility can lead to the development of more effective treatments for IVF patients, ultimately improving live birth outcomes.

Lühikokkuvõte

Inimese munasarja follikulogeneesi keerukus: munasarjapõhise viljatuse molekulaarsed markerid ja endokriinsüsteemi mõjutavate kemikaalide toime

Reproduktiivsüsteemi häired ja keskkonna faktorid avaldavad olulist mõju ülemaailmsele sündimuse langusele. Hoolimata kehavälise viljastamise (*in vitro* fertilisation, IVF) meetodite täiustustest ja viljatuse põhjuste täpsemast diagnoosimisest, kogevad siiski mõned naispatsiendid endiselt madalat IVF-i edukust (<30%). See on tõsine väljakutse viljatusspetsialistidele.

Munasari on naise viljatuse põhjuste mõistmisel võtmerollis. Nimelt reageerib munasari ajuripatsi poolt toodetud hormoonidele, luues sobiva keskkonna munaraku arenguks ja küpsemiseks folliikulis. Folliikul on munasarja struktuur, kus leiduvad eri rakutüübid nagu muraalsed (MGC) ja kumuluse granuloosrakud, teeka rakud ning rikkaliku koostisega follikulaarvedelik (FF), kus on esindatud paljud metaboliidid, valgud, nukleiinhapped ja steroidid. Kõik need rakud ja ühendid toetavad normaalset munasarja funktsiooni, olles seotud üksteisega nii mikroRNAde kui ka rakuväliste vesiikulite (EV) kaudu.

Follikulogeneesi lõppfaasis on küps munarakk valmis ovulatsiooniks, et viljastuda ning areneda embrüoks. Kuid häired follikulogeneesis ja folliikuli rakkude omavahelises suhtlemises võivad põhjustada viljatust. Vaatamata eelnenud uuringute rohkusele, ei ole siiski veel selge, milliseid muutusi erinevad viljatuse diagnoosid võivad põhjustada munasarja folliikulites.

Käesolev doktoritöö keskendub viljatute naiste munasarjade ovulatsioonieelse folliikulis toimunud muutustele. Uuringud hõlmasid IVF-patsiente, kelle munasarjad ei reageerinud piisavalt hormoonstimulatsioonile, ning patsiente, kellel diagnoositi polütsüstiliste munasarjade sündroom (PCOS). Samuti uuriti endokriinsüsteemi kahjustavate kemikaalide (EDC) sisaldust FF-s ning nende mõju IVF tulemustele. EDC-d on teadaolevalt kahjulikud endokriinsüsteemi funktsioonile, mõjutades negatiivselt hormoonide tootmist, transporti ning nende bioloogilist toimet. EDC-dega kokkupuudet on seostatud viljakuse vähenemisega ja teiste terviseprobleemidega.

Uuringu esimeses osas seostati ülegenoomset geeniekspressiooni ja signaaliradade rikastusanalüüsi tulemusi RNA tasemel munasarjade tundlikkuse indeksiga. Viimati mainitu hõlmas endas IVF-is kasutatud ravidoosi suhet munaraku saagikuse kohta (piirmäär 200IU rFSH/munarakk) ning võimaldas jagada patsiendid ebapiisava ja piisava hormoonstimulatsiooni tulemustega gruppideks. Leiti, et muutused esinesid 12 molekulaarses signaalrajas, näiteks kolesterooli biosünteesis ja steroidide ainevahetuses. Mõned toimunud muutused ei sõltunud sealjuures patsientide vanusest. Samuti uuriti muutusi ovulatsioonieelses folliikulis esinevate rakutüüpide proportsioonides, kus leiti, et ebapiisava hormoonvastusega patsientidel 14 erinevast rakutüübist olid mõned rakugrupid alaesindatud. Nendeks olid granuloosrakkude alamtüüpidest ARGLU1+ ja SEMA3A ning teeka rakud.

Teises osas määrati EDC-de sisaldust FF-s ja tuvastati 11 ohtlikku kemikaali, mis esinesid enam kui 90% patsientidest. Need kemikaalid võivad mõjutada munaraku kasvukeskkonda ning seeläbi viljakust. Teatud kemikaalide, näiteks bis(2-etüülheksüül)ftalaadi metaboliitide ja metüülparabeeni, esinemine seostus ka hormoonstimulatsioonile vähemtundlike

munasarjadega, rõhutades nende kemikaalide igapäevaseks kasutamiseks efektiivsema jälgimise ja ohutuse analüüsi vajadust.

Väitekirja kolmandas osas võrreldi PCOS diagnoosiga naiste miRNA profiile erinevates folliikuli osades: MGC, FF-is ja EV-des. Tulemused näitasid, et PCOS mõjutab miRNA sisaldust eri folliikuli osades erinevalt ning seeläbi ka mitmeid molekulaarseid radu võrreldes viljakate naistega. Täpsemalt tuvastati, et mõjutatud oli östrogeeni vahendatud signaaliülekanne MGC-des, IGF1R signaaliülekanne EV-des, ning TGF-β retseptori ja selle pereliikmete signaaliülekanne FF-is.

Kokkuvõttes annab see doktoritöö väärtusliku panuse arusaamisele sellest, kuidas mitmed tegurid, sealhulgas ovulatsioonieelse folliikuli rakutüüpide muutused, molekulaarsed rajad, miRNA-de profiil ning keskkonnakemikaalide mõju muudab munasarjade tundlikkust ja munaraku kasvukeskkonda. Naise viljakust mõjutavate tegurite mõistmine ning nende poolt põhjustatud muutunud molekulaarsete mehhanismide täpne tundmine aitab kaasa tõhusamate ravimeetodite väljatöötamisele IVF-patsientidele, et parandada elussünni tulemusi.

Appendix 1

Publication I

Roos K, Rooda I, Keif RS, Liivrand M, Smolander OP, Salumets A, Velthut-Meikas A. Single-cell RNA-seq analysis and cell-cluster deconvolution of the human preovulatory follicular fluid cells provide insights into the pathophysiology of ovarian hyporesponse. Front Endocrinol (Lausanne). 2022 Oct 21;13:945347. doi: 10.3389/fendo.2022.945347. PMID: 36339426; PMCID: PMC9635625.

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Single-cell RNA-seq analysis and cell-cluster deconvolution of the human preovulatory follicular fluid cells provide insights into the pathophysiology of ovarian hyporesponse

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Reduction in responsiveness to gonadotropins or hyporesponsiveness may lead to the failure of *in vitro* fertilization (IVF), due to a low number of retrieved oocytes. The ovarian sensitivity index (OSI) is used to reflect the ovarian responsiveness to gonadotropin stimulation before IVF. Although introduced to clinical practice already years ago, its usefulness to predict clinical outcomes requires further research. Nevertheless, pathophysiological mechanisms of ovarian hyporesponse, along with advanced maternal age and in younger women, have not been fully elucidated. Follicles consist of multiple cell types responsible for a repertoire of biological processes including responding to pituitary gonadotropins necessary for follicle growth and oocyte maturation as well as ovulation. Encouraging evidence suggests that hyporesponse could be influenced by many contributing factors, therefore, investigating the variability of ovarian follicular cell types and their gene expression in hyporesponders is highly informative for increasing their prognosis for IVF live birth. Due to advancements in single-cell analysis technologies, the role of somatic cell populations in the development of infertility of ovarian etiology can be clarified. Here, somatic cells were collected from the fluid of preovulatory ovarian follicles of patients undergoing IVF, and RNA-seq was performed to study the associations between OSI and gene expression. We identified 12 molecular pathways differentially regulated between hypo- and normoresponder patient groups (FDR<0.05) from which extracellular matrix organization, post-translational protein phosphorylation, and regulation of Insulin-like Growth Factor (IGF) transport and uptake by IGF Binding Proteins were regulated age-

independently. We then generated single-cell RNA-seq data from matching follicles revealing 14 distinct cell clusters. Using cell cluster-specific deconvolution from the bulk RNA-seq data of 18 IVF patients we integrated the datasets as a novel approach and discovered that the abundance of three cell clusters significantly varied between hypo- and normoresponder groups suggesting their role in contributing to the deviations from normal ovarian response to gonadotropin stimulation. Our work uncovers new information regarding the differences in the follicular gene expression between hypo- and normoresponders. In addition, the current study fills the gap in understanding the inter-patient variability of cell types in human preovulatory follicles, as revealed by single-cell analysis of follicular fluid cells.

KEYWORDS

ovarian sensitivity index, IVF, hyporesponse, preovulatory follicle, bulk RNA-seq, single-cell RNA-seq, granulosa cells, deconvolution

Introduction

The mechanisms of suboptimal ovarian response remain unsolved at the molecular level for many women up to 40 years of age who are undergoing assisted reproductive treatment (ART) with exogenous gonadotropins. These women experience low success rates with IVF due to a low number of retrieved oocytes (1, 2). Up to 30% of patients are affected by this clinical phenomenon of hyporesponse to ovarian stimulation (3). Moreover, excessive use of gonadotropins in stimulation may result in ovarian hyperstimulation syndrome, a potentially lethal condition characterized by the increased permeability of the vasculature and the development of ascites. In severe cases enhanced hemoconcentration leading to oliguria can be diagnosed (4). Therefore an optimal and safe stimulation regimen is crucial for all IVF patients (5). Various studies have aimed to assess the hyporesponsiveness by investigating patients' ovarian sensitivity or resistance to gonadotropin stimulation (6, 7), screening the single nucleotide polymorphisms in the gonadotropin hormone receptors (8–10), and measuring serum concentrations of anti-FSH antibodies (11). The ovarian sensitivity index (OSI) has been launched as a parameter describing a patient's reproductive potential to produce oocytes as a response to exogenous gonadotropin stimulation. OSI is a function that connects the total amount of exogenous gonadotropins used and the number of oocytes retrieved as a result of hormone stimulation (12). Although OSI correlates with many ovarian responsiveness biomarkers, like age, antral follicle count, and anti-Müllerian hormone (13, 14), to confirm OSI reliability for hyporesponders, the relationship with other factors must be thoroughly studied. Knowledge of the contributing factors affecting OSI has great importance for the improvement of the success rate of IVF treatment for the hyporesponders.

The gonadotropins follicle-stimulating hormone (FSH), and luteinizing hormone (LH) are essential for the expansion of the preovulatory follicle and ovulation. The diameter of a preovulatory follicle reaches up to 25 mm and it is surrounded by the basal membrane that separates theca cells from the internal structure with a fluid-filled cavity, consisting mostly of different types of granulosa cells (GCs) as well as a minority of other somatic cell types (15). The fluid-filled antrum divides the GCs into two major populations: mural and cumulus cells, each with distinct roles and RNA profiles (16, 17). Cumulus GCs are in direct contact with the oocyte and are responsible for the trafficking of metabolites between the two cell types (18) as well as for the meiotic resumption of the oocyte (19).

GCs in large preovulatory follicles express both FSH and LH/hCG receptors, whereas theca cells primarily express LH/hCG receptors (20, 21). Accordingly, both cell types play a vital role in regulating gonadotropin responses in the ovarian follicle. GCs proliferate actively to lead to the expansion of the follicle (22), liquid infiltration, vascularization (23), and the production and transport of hormones and metabolites into the preovulatory follicle to accomplish the meiotic maturation of the oocyte (24, 25). Furthermore, various publications have demonstrated morphological differences between individual GCs suggesting that GCs may contain multiple subpopulations with potentially distinct functional properties (26–28). Besides, it has been shown in model organisms that the mitotic activity and steroidogenesis of GCs are also affected by their location in the follicle and distance from the oocyte (29). Molecular communication shuffling by extracellular microRNA molecules between somatic cell types has been recently proposed to have importance to normal follicular function (30). Taken together, these cells play a key regulatory role in ovarian function, and a

shift in their gene expression may affect their responsiveness to gonadotropins.

So far, studies have identified novel ovarian somatic cell clusters from preantral follicles (31), cumulus-oocyte complex (32), whole ovarian tissue (33), ovarian cortex (34), and also from the preovulatory follicular fluid (35), where the differentiation of somatic cells has culminated before ovulation. However, there is a knowledge-gap regarding the characterization of GCs, specifically from the preovulatory follicular fluid of patients classified as hyporesponders based on OSI. Here, we combine bulk RNA sequencing (RNA-seq) with single-cell RNA-seq (scRNA-seq) to explore changes in gene expression and the proportions of individual somatic cell clusters between well-characterized hypo- and normoresponders, by analyzing the cellular content of the follicular fluid. As a result, we highlight the molecular alterations that could potentially help to improve the outcome of hormone stimulation.

Materials and methods

Ethics statement

The study was approved by the Research Ethics Committee of the University of Tartu (approval no 289/M-8). Signed informed consent was obtained from all participants.

Patients and sample collection

Female patients and oocyte donors undergoing IVF at the Nova Vita Clinic were enrolled in the study from September to December 2019. Patients were classified as hyporesponders (HR) based on hormone stimulation if they administered ≥200 IU of recombinant FSH (rFSH) to receive an oocyte. Of the recruited 80 patients, 46 were found to adequately respond to stimulation (normoresponders, NR), and 34 were HR. The average age of recruited patients was 32.9 ± 4.8 years (range 22-40) and the BMI was 22.3 ± 3.1 kg/m^2 (range 17.0-34.5). All the recruited women had two ovaries. Nineteen women were eligible for RNA-seq analysis due to the availability of a sufficient number of follicular cells and the high quality of the extracted RNA (see below). The final cohort recruited for RNA-seq consisted of 10 NR and 9 HR patients. The NR group consisted of 4 oocyte donors and 6 patients with male factor infertility. The HR group included 1 oocyte donor and 8 patients with different infertility diagnoses. The causes of infertility among all eligible participants were distributed as follows: male factor only (n=6), tubal factor only (n=4), combination of tubal and male factor (n=1), multiple female factors (n=2), and unexplained (n=1), while five women were oocyte donors. Oocyte donors were excluded from the analyses regarding IVF and embryo transfer outcome, as all their oocytes were frozen. In the remaining NR group, 10 embryo transfers were performed resulting in 5 successful deliveries. In the HR group, the numbers were 10 and 4, respectively. All the 19 women satisfied the following criteria: age ≤ 40; BMI between 17-33; antral follicle count ≥ 5; and nonsmokers. Women with polycystic ovary morphology and other ovarian morphological abnormalities detected by ultrasound examination were excluded. Preovulatory follicle count was performed two days before the oocyte retrieval.

Ovarian stimulation

All patients were treated with gonadotropin-releasing hormone (GnRH) antagonist (Cetrotide, Merck, Darmstadt, Germany) protocol and the ovarian stimulation was thereafter accomplished by administering rFSH (Gonal-f®, Merck or Puregon, N.V. Organon, Oss, The Netherlands) at a daily dose. Ovulation was triggered with 0.2 mg human chorionic gonadotropin (hCG) (Ovitrelle®, Merck, or Diphereline®, Ipsen Pharma Biotech, Paris, France) if at least two leading follicles reached 18 mm in diameter. Ovum pick-up (OPU) was scheduled 36 hours later and follicular fluid from preovulatory follicles with diameters >18 mm was aspirated. Only material visibly clear of blood contamination was used in the study.

OSI calculation

OSI was calculated by dividing the total administered rFSH dose in IU by the total number of oocytes retrieved at OPU, thus obtaining the rFSH-to-oocyte ratio.

Isolation and fixation of cells from the follicular fluid

Following the removal of the oocyte-cumulus complex, the follicular fluid was centrifuged for 10 minutes at 300g to isolate all cells. The cell pellets from multiple follicles were pooled to obtain enough follicular cells from every patient. Next, the cell pellets were washed with 1x DPBS + 0.04% BSA (DPBS/BSA) (DPBS, Corning Life Sciences, Tewksbury, California, USA; BSA, MilliporeSigma, Burlington, Massachusetts, USA), and the erythrocytes were lysed with Red Blood Cell lysis buffer (150 mM NH4Cl, MilliporeSigma; 10 mmol NaHCO3, and 1.3 mM EDTA, both Amresco Inc, Solon, Ohio, USA). The remaining cell mixture was centrifuged and resuspended in DPBS/BSA. Cells were treated with 200 µL hyaluronidase (FertiPro NV, Beernem, Belgium) and 5U of DNase I (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 30 minutes to break the extracellular matrix surrounding the cells, filtrated through a 40 µm filter (pluriSelect Life Science, Leipzig, Germany) to remove cell clumps, washed with DPBS/BSA,

and fixed in 80% methanol (Naxo Ltd., Tartu, Estonia). Cell counting was performed with a hemacytometer (The Paul Marienfeld GmbH & Co, Lauda-Königshofen, Germany). Cells from each patient were divided into two parts: at least $5x10^4$ cells were used for bulk RNA-seq and >$2.5x10^4$ cells for scRNA-seq. Cells were stored at -80°C until further processing.

Bulk RNA extraction and quality control

Methanol-fixed cells were equilibrated to 4°C, centrifuged for 5 minutes at 750g, rehydrated in Wash-Resuspension Buffer (0.04% BSA MilliporeSigma; 1mM DL-Dithiothreitol Solution, Invitrogen, Waltham, Massachusetts, USA; 0.2 U/µl Protector RNase Inhibitor, Thermo Fisher Scientific; 3x SSC Buffer, Naxo Ltd.), and lysed in 700µL QIAzol solution (Qiagen, Germantown, Maryland, USA). Methanol fixation and rehydration were performed according to the protocol approved by 10X Genomics (36). Bulk RNA was extracted from pooled cells of individual patients, with miRNeasy Micro kit (Qiagen), according to the manufacturer's instructions. The quality and concentration of purified RNA were evaluated on 2100 Bioanalyzer with the RNA 6000 Pico kit (Agilent Technologies, Santa Clara, California, USA). Samples with RNA integrity number (RIN) ≥ 7 were considered eligible for further analysis. In total, the cells from 9 NR and 9 HR patients were used for further bulk RNA-seq.

Bulk RNA-seq and data analysis

Sequencing libraries from purified RNA were prepared with the QuantSeq 3' mRNA-Seq Library Prep Kit FWD (Lexogen GmbH, Vienna, Austria). Samples were indexed to allow for multiplexing. Library quality and size range was assessed using 2100 Bioanalyzer with the DNA 1000 kit (both Agilent Technologies). The libraries were diluted to a final concentration of 2 nM and subsequently sequenced on an Illumina HiSeq4000 platform. Single-end reads of 50 bp length were produced with a minimum of 2M reads per sample. Quality control of raw reads was performed with FastQC v0.11.7 (37). Adapters were filtered with ea-utils fastq-mcf v1.05 (38). Using HiSAT2 (39), split-aware alignment was accomplished against the human reference genome hg19. Reads mapping to multiple loci in the reference genome were discarded. The resulting BAM files were handled with Samtools v1.5 (40). The reads per gene were quantified with HT-seq Count v2.7.14 (41). Count-based differential expression (DE) analysis was done with the R-based Bioconductor package DESeq2 version 1.34.0 (42). Reported p-values were adjusted for multiple testing with the Benjamini-Hochberg procedure (43), which controls the false discovery rate (FDR). Principal component analysis was used to inspect sample- and group-specific variation with the R package DESeq2 (42) using the top 500 most variable genes across all samples. Surrogate variable analysis with the Bioconductor package sva version 3.42.0 (44) was used to determine the age groups to perform relevant age adjustment in DE analysis. Raw sequencing data is available at the European Nucleotide Archive, accession no PRJEB50778.

Single-cell RNA-seq and data analysis

The follicular cells from 3 NR patients were used for scRNA-seq analysis (Supplementary Data 1). Two of the patients overlapped with the bulk RNA-seq dataset described above. Methanol-fixed cells were equilibrated to 4°C, centrifuged for 5 minutes at 750g, rehydrated in Wash-Resuspension Buffer, passed through 40µm Flowmi Cell Strainer (SP Bel-Art, Wayne, New Jersey, USA), counted, and finally adjusted to a concentration of 1000 cells per microliter. The single-cell suspension was loaded onto the Chromium Controller (10x Genomics, Pleasanton, California, USA) and scRNA-seq libraries were generated by using the Chromium Controller Single-cell 3' Kit v3.1 (10x Genomics) according to the manufacturer's protocol. At least 6 000 cells were aimed to be analyzed per sample. The library quality and size range were assessed using a Bioanalyzer (Agilent Technologies) with the High Sensitivity kit. Illumina Library Quantification Kit (KAPA Biosystems Inc., Wilmington, Massachusetts, USA) was used for the final library quantification. Libraries were sequenced on an Illumina NovaSeq6000 platform according to the manufacturer's recommendations. Pair-end reads of 28bp Read 1 for cell barcode and UMI, 8bp I7 index for sample index, and 91bp Read 2 for transcript were produced with a minimum of 250M reads per sample. BCL files produced by Illumina sequencers for each flowcell were demultiplexed based on the sample index and converted into FASTQ files using the Cell Ranger mkfastq function of Cell Ranger version 3.0.2 (10X Genomics). Using Cell Ranger count, the FASTQ files were aligned against the human reference genome (hg19) and annotated with the corresponding GTF file (release 93). Raw sequencing data is available at the European Nucleotide Archive, accession no PRJEB50778.

The filtering process and cell-cluster annotation were performed using Seurat version 4.0.5 (45). Cells were retained for further analysis in cases 1) the number of detected genes was 200-6000, 2) the proportion of mitochondrial genes was <10%, and 3) the proportion of hemoglobin genes was <5%. After filtering, 24 213 cells in total were subjected to further analysis. Batch effects between the patients were eliminated using the Harmony package (46). For normalization and scaling of the data, the scTransform (47) function was used, and cell clusters were identified using the FindClusters function (resolution 0.5, with 16 dimensions, original Louvain algorithm) and visualized using 2D uniform manifold approximation and projection (UMAP).

BAM files of the sequencing data are available at the European Nucleotide Archive, project accession no PRJEB50778, sample accession numbers ERR8521472, ERR8521473, and ERR8521474.

Determining the differentially expressed genes in cell clusters and functional enrichment analysis

The FindAllMarkers function in Seurat was used to list the statistically significant differentially expressed genes (DEG) for each cell cluster and the FindMarkers function was used to compare the selected clusters. As a result, cell clusters were annotated according to the DEGs using information from The Human Protein Atlas database (48) as well as from the literature. Pathway enrichment analysis was performed with DEGs found in bulk RNA-seq and scRNA-seq analysis of obtained clusters. Queries of DEGs of interest were loaded into g:Profiler (Ensembl version 104, Ensembl Genomes version 51) (49) for the Reactome analysis to study the potential functions. Pathways for which the adjusted p-value [Benjamini-Hochberg FDR (43)] was <0.05 were considered statistically significantly enriched.

Estimating cell fractions and imputing cell-cluster-specific gene expression

CIBERSORTx (50) was used to estimate the proportions of cell clusters identified by scRNA-seq from the bulk RNA-seq samples of individual patients. A signature single-cell expression matrix of each cell cluster was generated with S-batch correction that removed variances between different library preparation protocols. The permutation value for obtaining statistical results was set to 1000. The relative abundance of cell cluster fractions was calculated as an average of 7 runs (7000 total permutations) and cell cluster differences between the study groups were analyzed with a linear regression model adjusted for age.

CIBERSORTx group-mode was implied to impute a single representative gene expression profile of cluster 1 vs other clusters from a group of HR and NR bulk RNA-seq mixture samples. As a result, unfiltered cell-cluster-specific gene expression values were obtained for both study groups. Gene expression values of "0" were replaced with the existing minimum value and expression level differences between the HR and NR groups were analyzed on $\log_2$-transformed data.

Statistical analysis

The patients' clinical characteristics were described as mean ± standard deviation (SD). The continuous variables of OSI were log-normalized, and normal distribution was checked using the Shapiro-Wilk test (51). For comparisons between the characteristics of HR and NR patient groups, Student's t-test was used. Metaphase II (MII) oocyte rate was calculated as the number of MII oocytes per retrieved oocytes. Fertilized oocyte rate was normalized for the number of MII oocytes. Good-quality embryos were defined as those where either 1) ≥6 blastomeres were present and embryo fragmentation was <50% on day 3; or 2) the size and the assessment of the inner cell mass and trophectoderm development were graded ≥1BB on day 5 or 6. The good-quality embryos meeting these criteria were either transferred and/or vitrified. The good-quality embryos were vitrified using VitriFreeze ES and thawed in VitriThaw ES (both FertiPro NV). The good-quality embryo rate was calculated according to the number of successfully fertilized oocytes. The cumulative live birth rate was calculated as the total number of deliveries (>28 weeks of gestation) divided by the total number of performed embryo transfers, including all fresh and the subsequent frozen-thawed cycles. The delivery of a singleton, twin, or other multiples was considered as one delivery. Linear regression was used to analyze the impact of age and OSI on different IVF cycle outcomes and the estimated cell fractions, except for cumulative live birth rate, where the Wilcoxon rank-sum test was used. Pearson correlation analysis was used to correlate clinical characteristics and estimated cell fractions. All statistical analyses were conducted using R software version 4.0.1 (52) in Windows 10 operating system. P-value <0.05 was considered statistically significant except for RNA-seq studies, where Benjamini-Hochberg FDR (43) <0.05 was used as a cut-off for reporting statistically significant results.

Results

Higher age is associated with reduced sensitivity to stimulation

OSI links the number of retrieved oocytes to the units of administered rFSH, reflecting the intensity of the hormone stimulation response during the IVF treatment. Despite the dose adjustments, a significant proportion of patients exhibit lower responses. The condition is caused by multiple factors that require further clarification. Advanced age, as one of the factors, has been known to be directly related to poor stimulation outcomes (53). Therefore, we first assessed the characteristics of OSI and age in all 80 study participants stratified into HR and NR groups. The HR group was defined by a threshold value of OSI as ≥200 IU (log-transformed ≥2.301) of administered rFSH per oocyte. rFSH dosage of 150 IU per day during ovarian stimulation has been considered as the standard normal dosage by multiple studies (54, 55). Since there are no standardized criteria for the OSI formula and the threshold value for an impaired ovarian response, we decided to use the OSI ≥200 IU of rFSH per oocyte as a cut-off to define hyporesponsiveness in

our study cohort. Such an approach connects higher-than-standard doses to oocyte yield. Unsurprisingly, in our study population, there was a positive correlation (Figure 1) determined between age and OSI (R=0.673, p=8.227*10^{-12}). However, the correlation was statistically significant only in the NR (R=0.610, p=6.923*10^{-06}) and not in the HR group (R=0.272, p=0.119). This leads to the hypothesis that age is not the only underlying factor in the ovarian response to rFSH hyperstimulation.

Table 1 presents the characteristics and IVF outcome parameters of the recruited patients. The HR group was 5.5 years older on average ($p<0.05$) than the NR group. As expected, the HR group received increased dosages of administered rFSH, while the number of preovulatory follicles and retrieved oocytes were lower (all with $p<0.05$). The rFSH dose at the starting day of the ovarian stimulation was comparable between groups. The higher total rFSH amounts used for HR women derive from an increase in the dosage during days 5-10 and longer overall stimulation length (both $p<0.05$, Supplementary Figure 1) due to the weaker ovarian responsiveness of this patient group. The mean OSI in the HR group was 6.6 times higher ($p<0.05$) than in the NR group. Remarkably, the HR group had a higher rate of total good-quality embryos ($p<0.05$), but there was no statistically significant difference in the rate of metaphase II oocytes and fertilized oocytes between the study groups. The cumulative live birth rate was in a strong negative correlation with the OSI after adjustment with age (p=0.007, Table 2). On average the HR patients achieved live birth nearly twice less frequently than the NR women, but due to omitting oocyte donors from this comparison, this difference was not statistically significant (p=0.103, age-adjusted Wilcoxon rank-sum test, Table 1).

Age was positively correlated to OSI, whereas the preovulatory follicle count was observed to be negatively correlated to OSI with statistical significance (Table 2). All the above-mentioned correlations are strongly linked to indicators of ovarian potential.

Hyporesponder and normoresponder patients exhibit different gene expression profiles in their preovulatory follicular fluid cells

Among the 18 patients selected for further RNA-seq experiments the above-described differences between the characteristics of the study groups remained valid (Supplementary Data 2). RNA-seq of pooled cells isolated from follicular fluid (bulk RNA-seq) was performed for each patient to determine molecular mechanisms underlying the ovarian response to stimulation.

As demonstrated above, the underlying molecular processes behind hyporesponse to ovarian stimulation are affected and potentially masked by the effect of age. Furthermore, aging has a significant impact on the gene expression of follicular somatic cells (56, 57). In our bulk RNA-seq data, we found that the hidden source of variation in genome-wide gene expression correlates with age from 34 years and above (Supplementary Figure 2). As a result, age was treated as a binary parameter (≤33 years and older) in the relevant subsequent analyses.

Indeed, the principal component analysis demonstrated a clear separation between the bulk RNA expression profiles of HR and NR groups that was not explained by age difference alone (Figure 2A). To effectively comprehend the degree of age effect

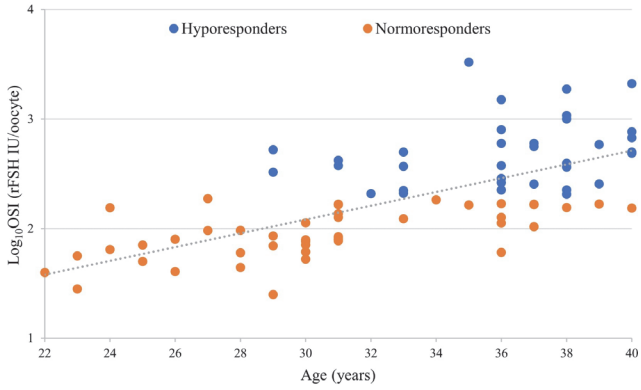

FIGURE 1
Correlation between the ovarian sensitivity index (OSI), a variable related to ovarian responsiveness, and patient's age displayed a positive linear relationship (overall p<0.0001, overall R=0.673, Pearson correlation).

TABLE 1 Characteristics of the recruited study participants (n=80).

	Normoresponders (n = 46)				Hyporesponders (n = 34)				Age-adjusted p
	Mean	SD	MIN	MAX	Mean	SD	MIN	MAX	
Age (years)	30.6	4.5	22	40	36.1	3.2	29	40	**<0.001**
BMI (kg/m²)	21.6	2.4	17.0	27.7	23.2	3.8	18.7	34.5	0.190
Administered rFSH (IU)	1504.1	414.0	950.0	2925.0	2510.6	736.3	1125.0	4201.8	**<0.001**
Preovulatory follicle count (n)	19.8	9.6	7	50	7.6	3.8	1	14	**0.001**
Retrieved oocytes (n)	17.9	8.0	8	40	5.8	3.3	1	16	**<0.001**
OSI (rFSH IU/oocyte)	100.3	46.9	25.0	187.5	659.7	652.5	206.3	3300.0	**<0.001**
Metaphase II oocyte rate (%)	77.9	15.3	44.4	100.0	79.2	27.7	0.0	100.0	0.640
Fertilized oocyte rate (%)*	66.0	24.5	0.0	100.0	65.9	31.4	0.0	100.0	0.721
Good-quality embryo rate (%)*	40.2	24.2	0.0	85.7	57.5	33.9	0.0	100.0	**0.017**
Cumulative live birth rate (%)*	53.9	48.8	0.0	100.0	23.6	41.2	0.0	100.0	0.103

*Oocyte donors (n=18) are excluded from the calculation.
The metaphase II oocyte rate calculation was adjusted for the number of retrieved oocytes, fertilized oocyte rate for the number of metaphase II oocytes, and good-quality embryo rate for the number of fertilized oocytes. The cumulative live birth rate was calculated as the total number of deliveries (>28 weeks of gestation) divided by the total number of performed embryo transfers, including all fresh and the subsequent frozen-thawed cycles.
BMI, body mass index; rFSH, recombinant follicle-stimulating hormone; IU, international units; OSI, ovarian sensitivity index.
Values in bold imply statistically significant results between groups, age-adjusted p-value <0.05.

on gene expression variations between HR and NR groups, differential gene expression analysis of bulk RNA-seq data was conducted by using two statistical models: 1) without any adjustments and 2) with age adjustments in previously described groups.

Accordingly, if no age adjustments were done, 895 genes were found to have significantly different expression levels between the HR and NR groups (Supplementary Table 1; Figure 2B). After adjusting to age, 447 DEGs with statistical significance remained (Supplementary Data 2), with 407 genes shared by both statistical models (Supplementary Tables 1 and 2 indicated in bold; Figure 2C). We conclude that these genes are involved in molecular processes that underlie hyporesponsiveness to gonadotropins age-independently.

The genes that were differentially expressed between the ovarian somatic cells of HR and NR groups were enriched into 12 Reactome pathways including lipid and steroid metabolism, as well as cholesterol biosynthesis, and cell junction organization (FDR<0.05). Importantly, three pathways remained significantly enriched regardless of age: extracellular matrix (ECM) organization, post-translational protein phosphorylation, and regulation of IGF transport and uptake by IGF Binding Proteins, the latter two sharing the DEG list (Figure 2D, Supplementary Table 3). The majority of the DEGs that regulate ECM organization such as *LAMA3*, *ITGA2*, *ACAN*, *ADAM9*, *ADAM10*, *FN1*, *PRKCA*, *FBN1*, and *SERPINE1* were downregulated in the HR group (Figure 2E). The upregulation of FGG in HR patients may be one of the explanations for the reduction of functions for several other pathway members as *FN1* and its downstream targets (Figure 2E). In conclusion, significantly altered gene expression affects the organization of the ECM and pathways related to IGF signaling in women with diminished response to gonadotropins.

TABLE 2 Correlation between the ovarian sensitivity index (OSI) and other clinical factors (age-adjusted) of the recruited study participants (n=80).

	Coefficient	Adjusted R^2	Age-adjusted p
Age (years)	7.195	0.446	**<0.001**
BMI (kg/m²)	1.759	0.068	0.088
Preovulatory follicle count (n)	-13.576	0.690	**<0.001**
Metaphase II oocyte rate (%)	0.085	-0.025	0.991
Fertilized oocyte rate (%)*	2.836	-0.025	0.781
Good-quality embryo rate (%)*	9.619	-0.019	0.391
Cumulative live birth rate (%)*	-45.888	0.234	**0.007**

*Oocyte donors (n=18) are excluded from the calculation.
The metaphase II oocyte rate calculation was adjusted for the number of retrieved oocytes, fertilized oocyte rate for the number of metaphase II oocytes, and good-quality embryo rate for the number of fertilized oocytes. The cumulative live birth rate was calculated as the total number of deliveries (>28 weeks of gestation) divided by the total number of performed embryo transfers, including all fresh and the subsequent frozen-thawed cycles. Values in bold imply statistically significant results between groups, age-adjusted p-value <0.05.
BMI, body mass index; rFSH, recombinant follicle-stimulating hormone; IU, international units; OSI, ovarian sensitivity index.

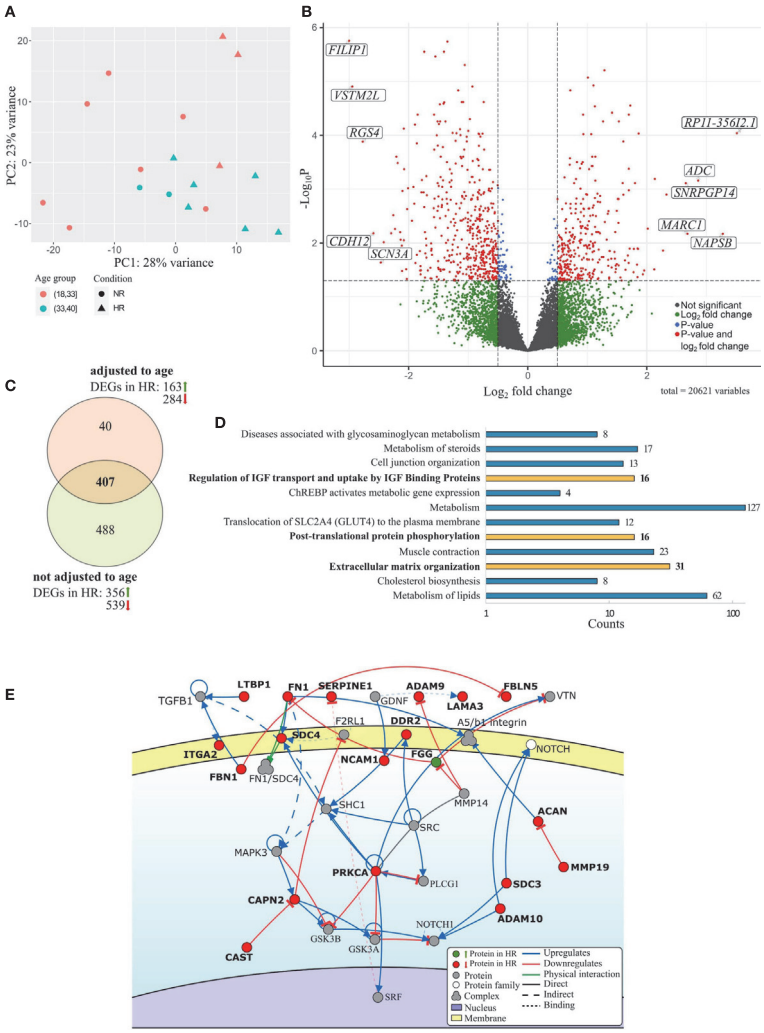

FIGURE 2
Overview of differentially expressed genes (DEGs) between the preovulatory follicular fluid somatic cells of hypo-(HR) and normoresponders (NR) identified by bulk RNA-seq. **(A)** Principal component analysis of the gene expression data from the follicular cells of HR (triangles) and NR patients (circles). Age groups (18-33 and 34-40 years) are marked in color. **(B)** Volcano plot highlighting top 5 upregulated and downregulated statistically significant DEGs between the HR and NR group, based on the fold change. **(C)** Venn diagram of DEGs between study groups with and without age adjustment. **(D)** Enrichment analysis of Reactome pathways based on DEG data between HR and NR groups (FDR<0.05) and presented in the order of decreasing statistical significance. Molecular pathways that remained significantly enriched after age adjustment are depicted by bold text and yellow bars. **(E)** A section of the major extracellular matrix organization pathway is shown schematically [adapted from Signor (58)]. Genes depicted in bold were differentially expressed between the follicular fluid somatic cells of HR and NR patients. The direction of gene expression difference between study groups is denoted by color: green indicating upregulation and red indicating downregulation in the HR group. Genes illustrated in grey were not differentially expressed.

Somatic cell clusters in the preovulatory follicle

The gene expression dissimilarities in the follicular somatic cells revealed between HR and NR patients may be explained by the variation in the proportions of infiltrated immune cells or the unsimilar rate of differentiation/luteinization of GCs. Hence, we aimed to generate a single-cell transcriptome map of the preovulatory follicular cells from the follicular fluid based on fertile women from the NR group that can be further used for cell cluster deconvolution from bulk RNA-seq datasets of larger patient groups.

Single follicular somatic cells from 3 NR patients, including 2 women with male-factor infertility and 1 oocyte donor, were sequenced. The mean age of the patients was 31.0 ± 4.6, OSI 65.7 ± 27.4, and the cumulative live birth rate was 100% (Supplementary Data 1). No segregation was observed according to the cell cycle phase or the source of cells from individual patients in the merged dataset (Supplementary Figure 3). In total, 24 213 single cells passed the quality filtering (Supplementary Data 3) and subsequently separated into 14 clusters (Figure 3A). To trace the distinct cell clusters and characterize their gene expression patterns, cluster-specific DEGs were obtained (Supplementary Data 4). Of note, some differences in the cell proportions across detected clusters were observed between individual patients (Supplementary Table 4).

We first identified 4 leukocyte lineages (PTPRC+, alternatively known as CD45+) that accounted for 11.3% of total cells, including neutrophils, T-cells, M1, and M2 macrophages. Next, 10 non-immune cell lineages (PTPRC-) were identified, including epithelial cells and theca/stroma cells, which together accounted for 1.2% of total cells, cumulus cells, and 7 clusters of GCs that accounted for 87.5% of total cells. Cell clusters were annotated based on known expressed markers summarized from literature and the Human Protein Atlas database (48): *CXCL8*, *MX2*, and *CSF3R* for neutrophils; *IL7R* for T cells, *CD86* for M1 macrophages; *CD14* and *CD163* for M2 macrophages (59); *CD46*, *KRT17* and *LCN2* for epithelial cells; *COL3A1*, *IGFBP5*, *GNG11*, *COL1A1* and *COL1A2* for theca/stroma cells (33, 60, 61); and *VCAN*, *CYP19A1*, and *UBE2C* for cumulus cells (33, 62) (Figures 3B, C). The remaining 7 unidentified clusters (numbers 0-5 and 8) formed a major group of cells marked by high expression of *SERPINE2*, *HSD17B1*, *CD59*, *FST*, *CDH2*, *PLA2G16*, and *AKIRIN1* (33), and a lack of markers for epithelial and cumulus cells. These clusters were collectively termed as GCs (Figure 3C) and studied further in more detail.

Gene expression dynamics of granulosa cell clusters

The identified GC subtypes were characterized using two methods (1): highly expressed DEGs were identified for each GC cluster separately (Supplementary Table 4); and (2) each GC cluster was compared to the pooled dataset of the remaining GC clusters (Supplementary Table 5). Each GC cluster exhibited a unique set of highly expressed genes. Reactome enrichment analysis resulted in 368, 23, 16, 8, 331, 60, and 190 terms for GC clusters 0, 1, 2, 3, 4, 5 and 8, respectively (Supplementary Table 6). Though the GC clusters varied in size, they were detected in all analyzed patient samples (Supplementary Figure 4).

It is crucial to be able to characterize the features of GC clusters to understand their impact on ovarian responsiveness to gonadotropin stimulation. We therefore carefully examined the gene expression profiles of all these clusters (Figure 3D). Cluster 0 was distinguished from other GC clusters due to the high expression of apoptotic markers (such as *RPS29*, *UBB*, *UBC*, *UBA52*, and *RPS27A*) and a vast number of ribosomal genes. The DEGs are involved in the control of apoptosis, ubiquitination, and p53 signaling according to the Reactome pathway analysis.

GC cluster 1 was recognized by *FDX1* and *HSP90AB1* expression as well as high levels of *STAR*, *HSD3B2*, and *CYP11A1* – genes for the key enzymes in progesterone production. STAR initiates the transfer of cholesterol from high-density lipoproteins into mitochondria, where it is converted to pregnenolone by CYP11A1 and progesterone by HSD3B2. DEGs of cluster 1 participate in the metabolism of steroid hormones, cholesterol biosynthesis, and estrogen-dependent nuclear events downstream of ESR-membrane signaling. In GC cluster 2, *PTX3*, *MT2A*, *CTSC*, *CYB5A*, *INHA*, and *HSD17B1* were among the most highly expressed genes and showed features in the metabolism, post-translational protein phosphorylation, regulation of IGF transport, and uptake by IGF Binding Proteins, and VLDL assembly.

DEGs from GC cluster 3 such as *SMARCA1*, *PRKAR2B*, *PTGES*, *VCAN*, *INSR*, and *CALM1* are associated with Interleukin (IL)4 and IL13 signaling. Highly expressed *NEAT1*, *MALAT1*, *ARGLU1*, *TSHZ2*, *ADAMTS9*, and *LAMA3* in cluster 4 were linked to active participation in insulin receptor recycling and laminin interactions, metabolism of steroid hormones, and gluconeogenesis. Cluster 5 was distinguished by *SEMA3A*, *TECRL*, *INHBA*, and *ADAMTS1* expression as well as *ITGA2* which were related to the cell-extracellular matrix interactions and synthesis of very-long-chain fatty acyl-CoAs. Altogether, the findings of clusters 4 and 5 DEGs confirm that they have a relevant role in ECM remodeling and response to gonadotropin surges. GC cluster 8 displayed high expression of *FOS*, *JUN*, *DNAJB1*, *EGR1*, and *PTGS2* which are involved in signaling *via* NTRK1 (TRKA) and ILs.

Taken together, we were able to identify and characterize 7 GC clusters and named these accordingly: Apoptotic GCs (cluster 0), Progesterone-producing luteinized GCs (cluster 1, P4-producing GCs), *PTX3*+ GCs (cluster 2), *SMARCA1*+ GCs (cluster 3), *ARGLU1*+ GCs (cluster 4), *SEMA3A*+ GC (cluster 5), and *FOS*+ GC (cluster 8) (Figures 3D, E).

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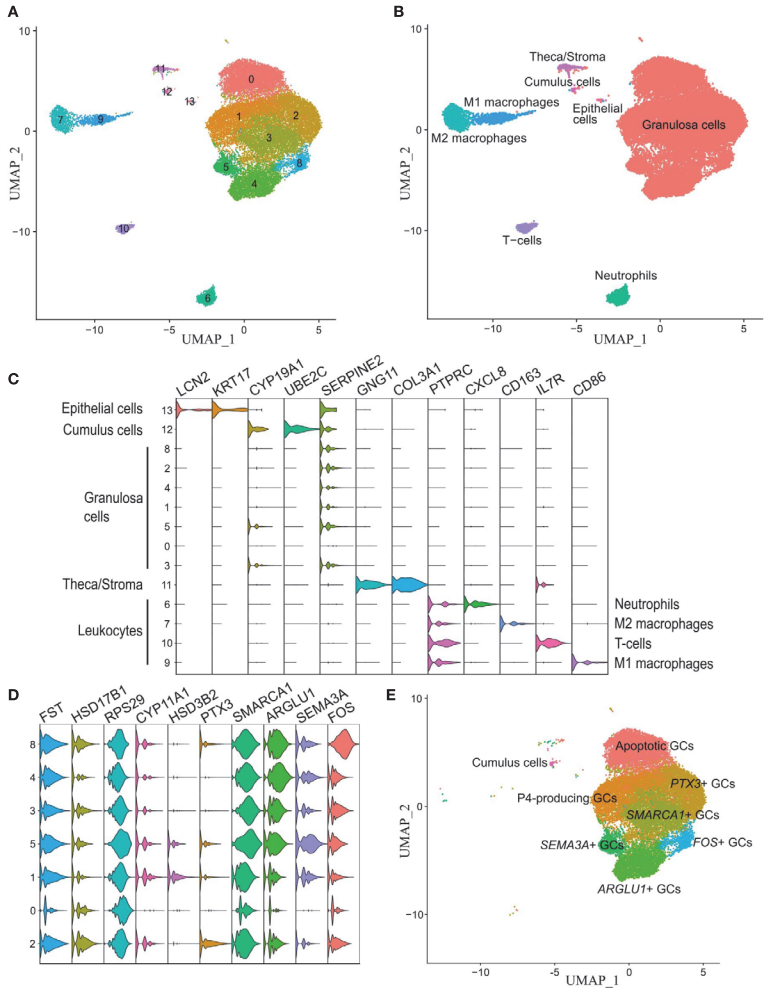

FIGURE 3
Unraveling cell clusters in the human preovulatory follicle. **(A)** Individual cell clusters detected from the single-cell RNA-seq dataset and analyzed with the original Louvain algorithm were visualized on UMAP. **(B)** The expression of known marker genes for each cell cluster group. **(C)** Annotated UMAP of the identified 4 leukocyte cell clusters, epithelial cells, theca/stroma cells, cumulus cells, and granulosa cells (GCs). **(D)** The presence of GC clusters was confirmed by the expression of known GC-specific markers (*FST*, *HSD17B1*). **(E)** Annotated clusters of GCs in the preovulatory follicle.

Preovulatory follicles of hyporesponder patients contain fewer *ARGLU1*+ GCs, *SEMA3A*+ GCs, and theca/stroma cells

With the single-cell analysis, we identified follicular cell clusters according to their gene expression profiles in the human preovulatory follicular fluid. Prognosis or manifestation of hyporesponse to gonadotropin stimulation in patients undergoing IVF treatment may be caused by either the variation in the proportion of these cell clusters or the gene expression differences in individual cell clusters without a change in cell proportions. To better understand the heterogeneity of individual cell clusters between patients and study groups, the CIBERSORTx computational framework was used on patients with available bulk RNA-seq data (n=18) to deconvolute the proportions of 14 cell clusters that were

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previously identified by scRNA-seq (Figure 4A). Importantly, fewer *ARGLU1*+ GCs (p=0.018), *SEMA3A*+ GCs (p=0.005), and theca/stroma cells (p=0.021) were identified in the samples of HR patients in comparison to the NR group when adjusted to age (Figure 4B). In addition, we observed that the marker genes of these three GC clusters (Supplementary Table 4) were downregulated in the bulk RNA-seq data of the HR patients (Figure 4C; Supplementary Table 1). This observation confirms that a change in a cell cluster proportion is partly underlying the gene expression differences verified by bulk RNA-seq.

Individual cell clusters contribute to the gene expression disturbances in hyporesponder patients

We further aimed to understand if the change in gene expression levels between HR and NR patients observed in bulk RNA-seq data is a contribution of individual cell clusters without a change in the cluster proportion. We observed that 9 marker genes of the P4-producing luteinizing GCs were differentially expressed between HR and NR patients and coincided with 8 Reactome pathways that were disturbed in correlation with the significant change in OSI values (Figure 2D; Supplementary Tables 3 and 4).

To evaluate if the expression difference of these 9 genes between HR and NR patients is indeed specific for the P4-producing luteinized GCs, we recreated the gene expression profile of the P4-producing cluster in comparison to all other clusters from our study groups using the CIBERSORTx group-mode analysis. In conclusion, we observed that the expression of 8 genes out of 9 in the P4-producing luteinized GCs were consistent with those reported in bulk RNA-seq data (Figure 5A). Only the expression of *TXNRD1* in the P4-producing luteinized GC cluster was not consistent with the direction of expression differences observed in the bulk RNA-seq dataset between HR and NR patients. This observation suggests that the differential expression of *TXNRD1* in bulk RNA-seq results from expression in other cell clusters. Concordance of the other 8 genes implies that their overall downregulation in HR patients is affected by their fundamental shift of gene expression levels in the P4-producing luteinized GCs. Each of the nine evaluated genes is linked to a Reactome pathway *via* a chord diagram to characterize the affected biological processes (Figure 5B).

In summary, the integration of bulk RNA-seq data with scRNA-seq through a deconvolution-based model has provided a novel understanding of the underlying mechanisms of hyporesponse. Our results suggest that variation in follicular cell cluster composition and altered gene expression in P4-producing luteinized GC clusters correlate with OSI values.

Discussion

The consideration of high OSI as a predictive marker of ovarian hyporesponsiveness in IVF still needs to be clarified. The

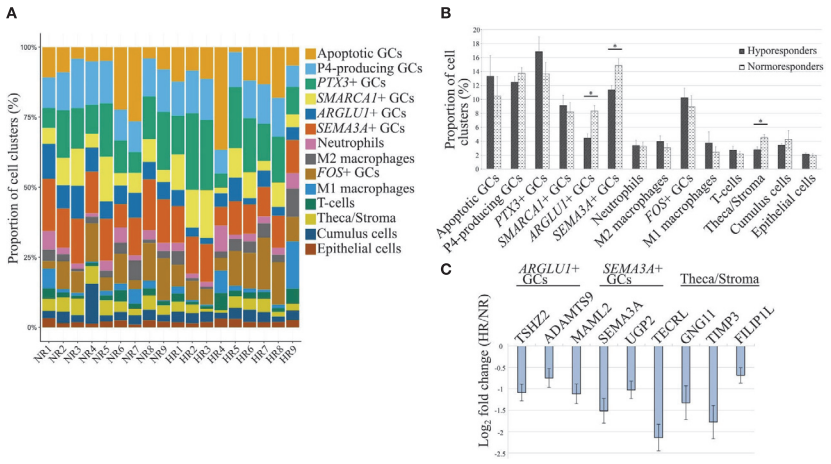

FIGURE 4
Changes in cellular proportions across identified clusters between hypo- and normoresponder patients (HR and NR, respectively). **(A)** The proportions of 14 cell clusters in 18 individual patients. **(B)** Cellular deconvolution of cell cluster proportions compared between the study groups. *- p-value <0.05 (age-adjusted linear regression). **(C)** The fold change of expression levels of 3 marker genes corresponding to the cell clusters with a statistically significant difference in their proportions between the HR and NR groups that are depicted in **(B)**.

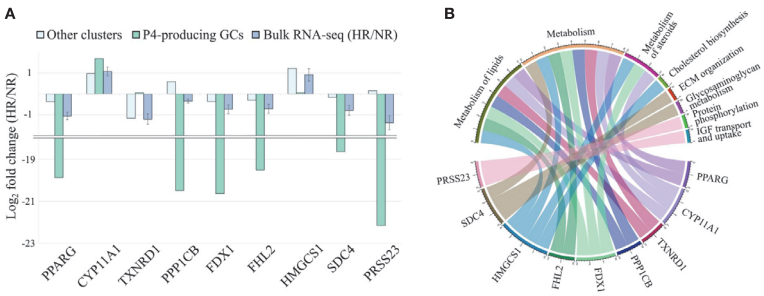

FIGURE 5
Influence of the P4-producing GC cluster (progesterone producing luteinized granulosa cells) on gene expression changes between hypo- (HR) and normoresponder (NR) groups. **(A)** Comparison of nine P4-producing GC-specific gene values in bulk RNA-seq and deconvoluted datasets. **(B)** Distribution of the nine P4-producing GC-specific genes between Reactome pathways affected by increasing OSI values.

OSI parameter may be particularly helpful in counselling patients during their IVF treatment because high OSI expresses gonadotropin dosage amount, which can lead to complications such as ovarian hyperstimulation syndrome. Moreover, the OSI parameter defines the response to hormone stimulation by the number of oocytes retrieved that is linked to many IVF cycle outcomes such as embryo quality and thus live birth rate (63, 64). Unfortunately, the OSI value of the patient's current IVF cycle becomes available only after the procedure. Therefore, expanding the described OSI concept by investigating contributing factors other than those previously mentioned, such as age, antral follicle count (13), and anti-Müllerian hormone (12) benefits to classify patients with lower ovarian response to hormone stimulation before they start treatment, enabling to adapt the doses of the stimulation drugs to be used.

Our work is the first to profile the direct association between the OSI parameter and genome-wide RNA expression level variations of the preovulatory follicular fluid cells of patients undergoing IVF.

Furthermore, we integrated scRNA-seq and bulk RNA-seq datasets of the isolated cells to dissect the differences in the gene expression and proportions of somatic cell clusters between HR and NR patients.

The OSI parameter consists of two variables: exogenous gonadotropin dose and the number of oocytes retrieved. Different formulas for OSI calculations and thresholds for determining hyporesponse have been used without a clear consensus. We calculated OSI as the total exogenous gonadotropin dosage used divided by the number of retrieved oocytes. The use of OSI ≥200 IU of rFSH per oocyte as a cut-off to define hyporesponsiveness was based on the knowledge that rFSH 150 IU per day is considered a standard treatment dose (54, 55) and hence, administrating ≥200 IU of rFSH per oocyte describes also a higher dose treatment for oocyte retrieval during IVF cycle. For instance, Huber et al. (65), used the OSI formula in which the number of recovered oocytes was multiplied by 1000 and divided by the total amount of administered rFSH. Patients were divided into three response groups based on the OSI levels: poor, normal, and high. Their cut-off level for a poor response was much higher in comparison to our study: it was defined as OSI <1.697/IU, which corresponded to 11 patients with OSI ≥600 in our study. Our analysis demonstrates that the somatic cells in the preovulatory follicle are affected already by milder gonadotropin stimulation excess than proposed by Huber et al.

We found no differences in the rates of metaphase II and fertilized oocytes between study groups, but the HR group had a higher rate of good-quality embryos and a lower cumulative live birth rate. Some studies show a decrease in oocyte quality, increased aneuploidy, and therefore a lower live birth rate in women with a poor response (66, 67), while others conclude the opposite (68, 69). One of the likely reasons for the contentious results is the accompanying influence of ovarian aging, which is difficult to eliminate due to the small number of women recruited for studies. Secondly, the inclusion criteria are different between studies: while some consider the stricter Bologna (70) or Poseidon (71) criteria for poor response, the hyporesponse is determined according to the efficiency of gonadotropin stimulation only. Regardless of this, it has been demonstrated that using low doses of rFSH during IVF improves the cycle outcomes, such as the rate of fertilization, embryo quality, and live birth, as well as the patient's comfort during the therapy (72).

It is well established that a higher age contributes to lower ovarian response, increasing the rFSH dosage (73, 74) and resulting in a decline in reproductive potential (75, 76). Likewise in our study, age positively correlated with OSI; moreover, the correlation was statistically significant only in the NR group. Therefore, our study hypothesizes that if age is not currently the main indicator for HR, other contributing factors must also be assessed.

First, the study confirms that the gene expression profiles of the preovulatory follicular fluid cells between HR and NR are significantly different. We revealed three biological pathways that are significantly affected, regardless of age. These are ECM organization, post-translational protein phosphorylation, and regulation of IGF transport and uptake by IGF Binding Proteins. In the HR group, most of the genes in these detected pathways are downregulated. The latter finding is especially interesting as IGF-1 has been proposed as a potential target for individualized controlled ovarian stimulation strategy. Increasing IGF expression by using growth hormone as a supplement during ovarian stimulation may be useful for activating folliculogenesis in poor responder patients (77), as IGF-1 has synergistic effects with gonadotropins (78). However, more randomized clinical trials are still needed to prove the concept.

ECM is responsible for ovarian morphology as well as the signal transduction within the preovulatory follicle. The LH surge stimulates ovulation in GCs by activating numerous ECM re-organizing processes (79), including the signal transduction mechanism *via* binding of Sp1/Sp3 transcription factors (80). This process contributes to successful ovulation by occurring concurrently with active post-translational phosphorylation and glycosylation (81). The reduced expression of several members of the ADAM and ADAMTS metalloprotease families, such as *ADAMTS9*, *ADAM9*, and *ADAM10*, indicates a significant shortage of essential ovulatory mediators and lowered oocyte quality in HR patients (82, 83). Even more so, downregulated *ADAMTS-1*, a key gene in ECM organization, is an important mediator of LH and progesterone effects during ovulation (84), and its functional form is selectively localized on cumulus complex cell surfaces (85). Cumulus cells encircle oocytes and provide metabolites *via* gap junctions, influencing oocyte maturation and developmental competence (86). Likewise, post-translational protein phosphorylation is required in cumulus-oocyte complexes to mediate nuclear and cytoplasmatic maturation (87), while the disturbances of this process may contribute to aneuploidy or abnormal oocyte (88). These findings suggest that the development of a mature oocyte during folliculogenesis is highly dependent on these identified pathways. The application of bulk RNA-seq on the preovulatory follicular fluid cells from HR and NR groups delineated certain molecular alterations associated with HR. However, the bulk RNA-seq results cannot exclusively indicate whether differences shown in gene expression levels are the main reason for hyporesponsiveness. Alternatively, changes in preovulatory follicle cell cluster proportions may also contribute to this condition. Accordingly, we have described the single-cell transcriptomes of NR preovulatory follicles. Using the scRNA-seq dataset and the following deconvolution analysis, allowed us to investigate the cellular composition of HR preovulatory follicles and potentially reveal the underlying factors for the impaired response.

One of the main findings of our study is the identification of 14 cell clusters from the preovulatory follicles by their unique cell cluster-specific marker genes. In follicular fluid samples, we were able to identify four types of CD45+ leukocytes, theca/stroma cells, epithelial cells, cumulus cells, and 7 subtypes of GCs. The presence of epithelial cells (89) has been described previously, as also multiple types of immune cells have been found in ovarian follicular fluid (90–92). While the number of macrophages, T-lymphocytes, and NK cells in the follicular fluid have been associated with several aetiologies of infertility, no significant variability in the proportions of leukocyte clusters were observed in our study.

The role of GCs deserves to be explored in detail because disturbances in their function could be the cause of different female reproductive disorders. By determining the transcriptomes of the GC clusters, we were able to propose their molecular functions. The GCs are vigorously producing essential steroid hormones such as progesterone and estrogen, and we were able to characterize different intensities of steroidogenic capability in the GC clusters. Elevated activity of steroid synthesis was observed in clusters 1, 2, and 5. Notably, cluster 1 expressed all of the key enzymes required for progesterone production at detectable levels: STAR for transporting cholesterol from the outer to inner mitochondrial membrane; and CYP11A1 and HSD3B2 for converting cholesterol into progesterone (93), which is enhanced by the electron donor FDX1 (94). From the bulk RNA-seq data, we highlighted Reactome pathways, like the metabolism of lipids and steroids, as well as cholesterol biosynthesis altered in HR. Adding the scRNA-seq dataset layer allowed us to demonstrate for the first time that differences in the expression of the genes enriched into these pathways are specific for distinct GC clusters.

However, it needs to be emphasized that the scRNA-seq method does not reveal the transcriptome level at a comparable depth as bulk RNA-seq methods. Hence, our results do not claim that steroidogenic pathways are not present in other identified GC clusters, rather they were not observed at the current detection limit.

Focusing on transcriptomic patterns that distinguish GC clusters, we identified an apoptotic GCs cluster (cluster 0). An increased proportion of apoptotic GCs has a negative impact on the developmental potential of the oocyte and the subsequent embryos (95), by limiting the supply of metabolites and interfering with cell communication. Some studies have attempted to evaluate the apoptosis rate of the mural and cumulus GCs collected during OPU using several apoptosis markers staining and flow cytometry analysis to estimate the chance of IVF failure (96, 97). A high expression of *PTX3* and *CD24* was found in cluster 2 cells, suggesting that these cells participate in the reorganization of the hyaluronan matrix to initiate ovulation. Furthermore, the anti-inflammatory and angiogenesis-promoting features of *PTX3* indicate that these GCs act as a protective layer in the preovulatory follicle by regulating the inflammatory milieu and maintaining a balanced microenvironment (98, 99). Cluster 4 expresses *ARGLU1*, which

is required for estrogen receptor-mediated gene transcription (100), as well as *LAMA3*, a laminin family member that participates in ovarian follicle ECM and cell junction organization, increasing GCs proliferation and survival as demonstrated in sheep (101). They provide enzymatic activity to the GCs in response to gonadotropin surges (102). *SEMA3A*, which has been shown in mice to play an important role in mediating luteinization processes following an LH surge (103), and *INHBA*, which has been shown in sheep to promote GCs proliferation, hormone synthesis, and inhibit apoptosis (104), are both highly expressed in cluster 5. By expressing a high level of *FOS* and *JUN*, cluster 8 shows features that participate in periovulatory processes with metabolic activities such as prostaglandin synthesis and cholesterol biosynthesis (105). These above-mentioned genes are involved in the downstream regulation of progesterone production and transport across GCs as well as EGF-signalling (106).

Interestingly, Wu et al. (35) have recently shown that they identified nine different functional clusters of GCs from follicular fluid cells. As there were no prior datasets available for GC clusters, we are both among the first to confirm that GCs divide into multiple clusters with different functions. Some GC clusters that appeared in both studies have similarly expressed genes. Additionally, we were able to identify cumulus cell cluster as well. Variations between studies may have arisen due to heterogeneity between the patients because of the small number of samples (3 vs 6) utilized. In addition, some technical differences were present between the two studies involving some details in sample processing and scRNA-seq library preparation protocols.

Understanding the difference in the proportions of cell clusters between HR and NR helps in the identification of cellular targets to improve IVF therapy and its outcomes. In this study, a computational approach combining data from scRNA-seq and bulk-RNA-seq, as well as cell deconvolution method CIBERSORTx (50) was used to reveal the different proportions of cell clusters between patient groups. We discovered that the proportions of three clusters: *ARGLU1*+ and *SEMA3A*+ GC clusters, along with theca/stroma cells are significantly under-represented in HR, coinciding with the lower gene expression values of the corresponding marker genes in the case of ovarian hyporesponse.

While the importance of *ARGLU1*+ and *SEMA3A*+ GC clusters in the development of hyporesponse needs further studies, there is already previous indication on the role of theca cells on the response to controlled ovarian stimulation (107). Although there is a lack of comprehensive assessment of theca cell proportions related to the ovarian stimulation response, it has been proposed that patients with theca cell shortage have decreased follicle structural support, and LH-stimulated androgen production (108). Serum androgen level is correlated with AFC, anti-Müllerian hormone, and thus to ovarian response to rFSH (109). Furthermore, it has been demonstrated that theca cells have a reduced capacity to respond to hCG/LH and the production of androgens decreases from the age of 30 (110). Authors of the latter study propose that this result derives from the change in the proportions of theca and granulosa cells during aging. Our findings reveal that patients with high OSI, regardless of age, have a lower number of theca cells.

In this study, we were able to demonstrate variations in the overall distribution of cell clusters and cell cluster-specific gene expression levels between the HR and NR groups. We confirmed that the deconvoluted data from bulk RNA-seq included our described marker genes retrieved by scRNA-seq verifying the reliability of such bioinformatic approach. Similar cell cluster-specific deconvolution analyses from bulk RNA-seq data have also been used in other biological systems: in onco-immunology the immune and cancer cell fractions have a major impact on the survival prognosis (111), and on the response to immunotherapy (112), or in unraveling novel cell types from whole tissue samples (113). The combination of affordable bulk RNA-seq data with the reference scRNA-seq datasets by cell cluster deconvolution method offers a cost-efficient approach for performing transcriptomic investigation on a large number of samples (114, 115). The type of analyses used in the current study allows for the generation of extensive novel information and clinically relevant associations from the datasets previously published in data repositories.

There are some limitations to our study. All our results were obtained from the bioinformatic analysis and experimental validation regarding the functionality of the identified cell clusters should be performed in the future. The presented datasets were generated by RNA-seq and the validation of the cell cluster markers at the protein level was not performed. The small study group size is another drawback. Further research on the additional clinical application is still needed. Nonetheless, patients, their data, and performed analyses serve as a foundation for investigating the differences between HR and NR at a single-cell level.

Collectively, the evidence proposed in this paper demonstrates that suboptimal results to ovarian stimulation could be associated with an altered cell-cluster composition or cell-cluster-specific gene expression changes in the preovulatory follicle. We have revealed molecular pathways which serve as potential prognostic biomarkers in the clinical management of ovarian hyporesponse. Investigating the underlying cause of the insensitivity of follicles to stimulation would allow identifying the suitable therapeutic targets to treat HR: either by modifying dysfunctional molecular pathways or by promoting cell cluster-specific differentiation potential. The findings of this study identify novel reasons for ovarian stimulation failure and propose directions for future research.

Data availability statement

The raw data underlying the study is publicly available at the European Nucleotide Archive with the project accession number PRJEB50778.

Ethics statement

The studies involving human participants were reviewed and approved by the Research Ethics Committee of the University of Tartu. The patients/participants provided their written informed consent to participate in this study.

Author contributions

AV-M was responsible for the study's conception and conduct. AV-M, AS and O-PS were responsible for study funding. KR was responsible for the sample collection. KR, IR, R-SK, ML, and AV-M analyzed and interpreted the data. KR and AV-M wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.945347/full#supplementary-material

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Appendix 2

Publication II

Bellavia A, Zou R, Björvang RD, **Roos K**, Sjunnesson Y, Hallberg I, Holte J, Pikki A, Lenters V, Portengen L, Koekkoek J, Lamoree M, Van Duursen M, Vermeulen R, Salumets A, Velthut-Meikas A, Damdimopoulou P.
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Association between chemical mixtures and female fertility in women undergoing assisted reproduction in Sweden and Estonia

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ABSTRACT

Objective: Women of reproductive age are exposed to ubiquitous chemicals such as phthalates, parabens, and per- and polyfluoroalkyl substances (PFAS), which have potential endocrine disrupting properties and might affect fertility. Our objective was to investigate associations between potential endocrine-disrupting chemicals (EDCs) and female fertility in two cohorts of women attending fertility clinics.

Methods: In a total population of 333 women in Sweden and Estonia, we studied the associations between chemicals and female fertility, evaluating ovarian sensitivity index (OSI) as an indicator of ovarian response, as well as clinical pregnancy and live birth from fresh and frozen embryo transfers. We measured 59 chemicals in follicular fluid samples and detected 3 phthalate metabolites, di-2-ethylhexyl phthalate (DEHP) metabolites, 1 paraben, and 6 PFAS in >90% of the women. Associations were evaluated using multivariable-adjusted linear or logistic regression, categorizing EDCs into quartiles of their distributions, as well as with Bayesian Kernel Machine Regression.

Results: We observed statistically significant lower OSI at higher concentrations of the sum of DEHP metabolites in the Swedish cohort (Q4 vs Q1, β = -0.21, 95% CI: −0.38, −0.05) and methylparaben in the Estonian cohort (Q3 vs Q1, β = -0.22, 95% CI: −0.44, −0.01). Signals of potential associations were also observed at higher concentrations of PFUnDA in both the combined population (Q2 vs. Q1, β = −0.16, 95% CI -0.31, −0.02) and the Estonian population (Q2 vs. Q1, β = −0.27, 95% CI -0.45, −0.08), and for PFOA in the Estonian population (Q4 vs. Q1, β = −0.31, 95% CI -0.61, −0.01). Associations of chemicals with clinical pregnancy and live birth presented wide confidence intervals.

Conclusions: Within a large chemical mixture, we observed significant inverse associations levels of DEHP metabolites and methylparaben, and possibly PFUnDA and PFOA, with OSI, suggesting that these chemicals may contribute to altered ovarian function and infertility in women.

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1. Introduction

The prevalence of reproductive health problems is increasing globally, with up to one in six women of reproductive age experiencing difficulties conceiving or carrying a pregnancy to term (Boivin et al., 2007; Mascarenhas et al., 2012). Despite the increasing use of assisted reproductive technologies (ARTs) among people with decreased fecundity, success rates of live birth have remained similar (Sunderam et al., 2019). Infertility is defined as an inability to conceive after 12 months of regular unprotected intercourse and it can be caused by female, male, mixed female/male factors, or unexplained mechanisms. Ovarian disorders account for infertility in about 1 in 4 infertile couples (Azziz et al., 2016; Luborsky et al., 2003). Considering the importance of hormones in ovarian function during development as well as in adult life, it is reasonable to assume that human-made chemicals that disrupt the endocrine system contribute to rates of infertility (Gore et al., 2015; Mínguez-Alarcón and Gaskins, 2017).

Endocrine disrupting chemicals (EDCs) are defined as "exogenous substances or mixtures that alter the functions of the endocrine system and consequently cause adverse effects in an intact organism, or its progeny, or subpopulations" (Kortenkamp et al., 2011; Zoeller et al., 2012). Although hundreds of chemicals have been flagged as suspected EDCs, only a few have been classified as such in the European Union (EU) (Demeneix and Slama, 2019). Particular attention has been given to known or suspected EDCs such as phthalates, bisphenols, and per- and polyfluoroalkyl substances (PFAS), which have been linked to clinical outcomes of fecundity and fertility (Mínguez-Alarcón and Gaskins, 2017; Hammarstrand et al., 2021; Rashtian et al., 2019). These chemicals are widespread and ubiquitously found in daily consumed products including personal care and household items, as well as in contaminated environments. Due to their potential to disrupt the endocrine system, they may adversely affect multiple health aspects, including reproductive health (Mínguez-Alarcón and Gaskins, 2017; Hammarstrand et al., 2021; Rashtian et al., 2019).

Our current knowledge of the potential effects of environmental chemicals on female fertility, however, is hampered by several factors. Firstly, most studies have investigated EDCs as they relate to clinical outcomes that involve both paternal and maternal factors, making it hard to disentangle the effects of potential EDCs on female fecundity. The identification of possibly modifiable factors that specifically relate to female infertility will represent an important step in providing recommendations to women seeking to improve fertility and informing chemical safety legislation. As such, it is important to evaluate how these chemicals relate to highly predictive biomarkers of female fertility such as the ovarian sensitivity index (OSI). OSI is a measurement of ovarian competence reflecting the response to the exogenous follicle-stimulating hormone (FSH) stimulation during ART, and it qualifies among a large number of variables as a major predictor of live birth (LB) (Huber et al., 2013; Vaegter et al., 2017, 2019). OSI correlates with established markers of ovarian reserve such as anti-Müllerian hormone (AMH), antral follicle count (AFC) and basal FSH levels, but it is more closely associated with LB rate probably because it reflects not only the remaining pool of oocytes but also a functional aspect of the ovaries (Weghofer et al., 2020; Revelli et al., 2020). Secondly, women are exposed to a mixture of several chemicals that act as a complex environmental exposure that can interact in the human body (Mínguez-Alarcón and Gaskins, 2017; Billionnet et al., 2012), but most studies have so far focused on the effects of individual chemicals, failing to capture this complexity. Switching the focus to mixture approaches accrues several advantages and has long been advocated from both a methodological as well as a biological standpoint (Dominici et al., 2010; Taylor et al., 2016; Kortenkamp, 2007; Drakvik et al., 2020). Thirdly, most of the current evidence on chemicals and fertility comes from single-centered studies, limiting the generalizability of research findings.

To address these issues, we conducted an epidemiological study including 333 women undergoing ART procedures at two fertility clinics in Sweden and Estonia. Associations were investigated between a mixture of known and suspected EDCs, including both non-persistent chemicals such as phthalates and parabens, and persistent chemicals such as PFAS, and indicators of fertility in assisted conception that include the OSI as a female-specific index, and established clinical outcomes such as live birth (LB) and clinical pregnancy (CP). This study was performed as part of the EU-funded project FREIA (van Duursen et al., 2020).

2. Methods

2.1. Study population

Embedded in two European cohorts in Sweden and Estonia, this study included a total of 333 women undergoing ART treatment. Participants from the Swedish cohort were recruited at the Carl von Linnékliniken in Uppsala from April to June 2016. Out of 244 eligible women (age: 21–43) 190 were recruited. Five declined while 185 accepted and were included in the study. The Swedish study was approved by the Swedish Ethical Review Authority (original license dnr 2015/798–31/2, amendments 2016/360–32 and 2016/1523–32). The Estonian cohort consisted of 148 women (age: 23–43) recruited at Nova Vita Clinic AS in Tallinn between February and November 2019. Out of 195 eligible women, 182 were recruited, and a final cohort of 148 women was selected based on the amount of follicular fluid expected to be required for all chemical measurements (>2 ml). The Estonian study was approved by the Research Ethics Committee of the University of Tartu (approval no 289/M-8). In both cohorts, women were provided with oral and written information about the study, and they signed an informed written consent form in accordance with the Declaration of Helsinki. In addition, women in the Swedish cohort filled in a short questionnaire regarding their lifestyle. Samples and data were pseudonymized with random codes and processed by relevant regulations (the Swedish data protection law PUL, the Swedish law on biobanking in healthcare, General Data Protection Regulation, and the Estonian data protection law).

2.2. Sample collection

In the Swedish cohort, follicular fluid containing all cellular material and without visible blood contamination was collected from the follicles. The first aliquot was always discarded to avoid possible dilution by the flushing medium left in the needle. The samples were pooled per patient, and centrifuged at 500g for 15 min. The Estonian cohort samples were also collected from the follicles and centrifuged at 300g for 10 min and then at 2000g for 10 min. The flushing medium was removed from the needle and the hose prior to the ovarian puncture to avoid any sample dilution. The cell-free follicular fluid samples were aliquoted, and delivered to the university on ice within 2 h and frozen at −80 °C. To control for possible contamination from the IVF laboratory environment, 33 blank samples were collected and subjected to chemical analysis. In the Swedish cohort, the blank samples consisted of G-Rinse (Vitrolife, Stocholm, Sweden) medium that was used to flush the needle (Wallace Single Lumen Oocyte Recovery System 17G, CooperSurgical Fertility and Genomics, Målov, Denmark) prior to ovum pick-up, and such blank samples were collected at multiple time points during the sample collection period. The blank sample for the Estonian cohort was an unused flushing medium with 10 IU/ml heparin (ORIGIO, CooperSurgical Fertility and Genomics) that was passed through an unused single lumen ovum aspiration needle and hose (Cook Medical LLC, Bloomington, IN, USA).

2.3. Exposure assessment

Quantitative analyses of all chemicals were conducted in

laboratories using two methodologies for i) bisphenols, parabens, and phthalate metabolites and ii) PFAS. Tables S1 and S2 in the Supplementary Material present a complete list of all assessed chemicals including the isotopically labelled internal standards. For both methods, isotope dilution liquid chromatography with tandem mass spectrometry (LC-MS/MS) was used. Additional details can be found in the Supplementary Material.

To quantify phthalates and parabens, a deconjugation step was carried out before solid-phase extraction (SPE). The conjugated metabolites in the follicular fluid samples (200 μl) were hydrolyzed by β–glucuronidase for 180 min at 37 °C. After concentration and washing, the target compounds were eluted from 10 mg Oasis MAX cartridges with 1 ml 2% formic acid in methanol. The extracts were measured on an LC (ExionLC, Sciex) coupled with a Turbo V Ion source (ESI) operating in the negative ion mode prior to triple quadrupole mass selective detection (6500+, Sciex). The compounds were separated on a Phenyl-hexyl column (Kinetex, 100 × 2.1 mm, 1.7 μm, Phenomenex) by applying a gradient of 0.2 mM NH4F and 0.2 mM NH4F in acetonitrile.

To quantify PFAS, aliquots of 200 μl follicular fluid were extracted with SPE using 10 mg Oasis WAX cartridges (Waters). The obtained extracts were analyzed on the same LC-MS/MS system, using an XBridge BEH C18 XP Column (2.5 μm, 2.1 × 150 mm) with 2 mM NH4CHOO and methanol – acetonitrile as gradient solvents. PFAS data from the Swedish cohort were previously quantified in a different laboratory (Björvang et al., 2022), and we, therefore, re-analyzed 10 samples with the current methods to ensure comparability, detecting negligible differences.

Our exposure assessment covered 59 chemicals (10 bisphenols, 6 parabens, 16 phthalate metabolites, and 27 PFAS) in the Estonian cohort and 40 (10 bisphenols, 6 parabens, 16 phthalate metabolites, and 8 PFAS) in the Swedish follicular fluid samples. Because of the very high correlation levels between the four metabolites [mono-2-ethylhexyl phthalate (MEHP), mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono (2-ethyl-5-oxohexyl) phthalate (MEOHP)] of di-2-ethylhexyl phthalate (DEHP) (Fig. S1) and same parental compound, their sum (ΣDEHP) was created by dividing each metabolite concentration by its molecular weight and then summing for statistical analysis. Geometric mean values were used for women who had two plasticizers assessments (n = 16) or two PFAS assessments (n = 15). After inspecting the distribution of all exposures, 11 chemicals with less than 10% of samples below the limit of quantification (LOQ) were evaluated in primary analyses. These included 4 phthalates: ΣDEHP, MEP, cxMiNP (the only secondary metabolite of DiNP with high detection levels; primary metabolites were not quantified), MOHiBP (a secondary metabolite of MiBP; primary metabolites were not quantified); methylparaben; and 6 PFAS: PFHxS, PFOA, PFOS, PFNA, PFUnDA, and PFDA. In addition, cxMiNCH was detected in high proportions only among Swedish women, and propylparaben only among Estonian women. Therefore, these two chemicals were only examined in stratified analyses.

2.4. Outcomes assessment

For both cohorts, data on reproductive health at baseline and treatment outcomes were retrieved from electronic health records. To assess female fertility, we used OSI as a continuous measure of ovarian response to stimulation (Huber et al., 2013), as well as the fertility treatment endpoints of CP and LB from fresh and cumulative (i.e., fresh and frozen) embryo transfers, evaluated as binary outcomes (yes/no). In both cohorts, AMH and FSH were assessed from blood taken during the infertility investigation, before any fertility treatment, while the follicular fluid was taken during ovum pick-up. The follow-up times for Swedish and Estonian cohorts, used to examine CP and LB rates, were 5 years and 2.5 years, respectively. The two cohorts followed similar procedures for outcome measurements assessment, albeit the hormonal stimulation protocol differed between the centers and is described below.

2.4.1. Ovarian stimulation and ovarian sensitivity index

In the Swedish cohort, basal AFC (bAFC), which is the total number of follicles with a size of 2–10 mm, was determined via ultrasound before stimulation. In addition, the concentration of AMH in serum was measured. Participants underwent either gonadotropin-releasing hormone (GnRH) agonist protocol (82%) using Suprecur (Suprecur, Cheplapharm Arzneimittel GmbH, Greifswald, Germany) or Synarela (Synarela, Pfizer, New York City, New York, USA) where the pituitary was desensitized starting the luteal phase, or GnRH antagonist protocol (18%) where GnRH antagonist Orgalutran (Orgalutran,N.V. Organon, Oss, The Netherlands) was given on Day 6 of menses. To stimulate follicle growth and oocytes maturation, recombinant FSH (rFSH, Gonal-F or Fostimon, Bemfola, Gedeon Richter Plc., Budapest, Hungary) and/or human menopausal gonadotropin (Menopur, Ferring Pharmaceuticals Ltd,Saint-Prex, Switzerland) were given from day 3 of menses. Once there were at least three follicles of ≥17 mm, human chorionic gonadotropin (hCG) was given. After 36–37 h, oocytes were retrieved through the transvaginal ultrasound-guided ovarian puncture.

In the Estonian cohort, ovarian hormonal stimulation was conducted according to the GnRH antagonist (Cetrotide, Merck, Darmstadt, Germany) protocol with the administration of rFSH (Gonal-F®, Merck; Bemfola, Gedeon Richter Plc). AFC was measured after stimulation (sAFC) 2–3 days before ovum pick-up, and AMH was only measured among participants demonstrating potentially diminished ovarian reserve. All patients underwent oocyte retrieval 36 h after hCG administration (Ovitrelle®, Merck) if at least two follicles were observed with a diameter of ≥18 mm.

OSI was calculated by taking the natural logarithm (*ln*) of the previously described formula to improve the normal distribution of the outcome (Huber et al., 2013):

$$\text{OSI} = ln((\text{number of oocytes retrieved})/(\text{total rFSH dose (IU)}))\times 1000)$$

2.4.2. Clinical pregnancy and live birth

One to two embryos were transferred into the uterus per cycle. The remaining embryos were frozen and thereafter preserved in liquid nitrogen. CP was defined by confirming the presence of a gestational sac and fetal heartbeat by ultrasound 4 weeks after positive human chorionic gonadotropin detection from blood. LB was defined as the birth of a live baby after at least 24 weeks of gestation. Both measures were evaluated as binary indicators of success from only fresh transfers as well as both fresh and frozen transfers.

2.5. Other variables

Using a direct acyclic graph (DAG) (Tennant et al., 2021), presented in Fig. S2, we identified a set of potential confounders to be evaluated in primary analyses, and other covariates that might lie on the exposure-outcome pathway (i.e., potentially mediators) that we evaluated in secondary analyses. Potential confounders available for participants from both cohorts included age, body mass index (BMI), parity, previous in-vitro fertilization (IVF) cycles (IVF/intracytoplasmic sperm injection), and their outcome, and infertility causes. These variables were retained in the primary models if their inclusion changed the exposure's coefficients by at least 10%. Women participating in the Swedish cohort also completed a self-administrated questionnaire with additional lifestyle questions, thus allowing us to evaluate whether additional potential confounders, as well as sources of exposure, were independently associated with fertility outcomes in this subpopulation. Specifically, we assessed smoking, fish intake, personal care product (PCP) use, infertility duration, alcohol consumption, and menstrual cycle regularity (assessed from patient records), using the same

change-in-estimate criterion. PCP use was assessed using the average score of the 4-point Likert scale assessing the frequency of use of 6 products. Other variables related to female fertility, such as AMH concentration, basal AFC, endometrial thickness, and thyroid-stimulating hormones (only available in the Swedish cohort), possibly lie on the exposure-outcome causal pathway (Fig. S2) and were therefore evaluated as potential mediators in secondary analyses.

2.6. Statistical analysis

Descriptive statistics were presented as mean (standard deviation, SD) or number (percentage) in the combined population and per cohort. We also conducted a correlation analysis on the selected chemicals by calculating Spearman correlation coefficients.

To examine the associations between potential EDCs and female fertility outcomes, we first used linear regression (for OSI, which was normally distributed) and logistic regression (for CP and LB), to independently evaluate each chemical in separate models adjusted for potential confounders. All models were evaluated in the combined population and stratified by cohort. Covariates that met the inclusion criterion were age, BMI, parity, and previous IVF. For the Swedish cohort we further adjusted for infertility duration, smoking, fatty fish intake, and PCP use. Smoking was not adjusted for in the analysis of the Estonian cohort due to the very small number of smokers. When analyzing the combined population, we used two approaches to account for structural differences between the Swedish and Estonian cohorts: 1) controlling for the cohort as an additional covariate in regression, and 2) using a linear mixed model with cohort as a random intercept. Analyses using these two approaches yielded consistent results and only the results from the first approach are therefore presented. To relax assumptions of linearity in dose-response associations, chemicals were evaluated as categorical exposures by calculating quartiles in the combined population. By only selecting chemicals with minimal levels of non-quantification, we did not use any imputation technique and evaluated all models using complete-cases analysis. To test the robustness of the findings, we also considered the possibility that MEHP might not be a biologically formed metabolite in the follicle but rather derived from unspecific hydrolysis during sample collection and processing, and we, therefore, conducted a sensitivity analysis excluding MEHP from ΣDEHP. In another sensitivity analysis, we excluded cases of infertility due to male causes as these might not be associated with exposure levels of EDCs in follicular fluid in this study. Finally, we conducted a secondary exploratory analysis further adjusting for covariates that might also act as mediators.

Next, to account for co-exposure confounding within chemicals and to address potential mixture effects, we jointly evaluated all chemicals as a chemical mixture. We first mutually adjusted for multiple chemicals in one single regression model. This approach, however, can be subject to substantial bias in the presence of high correlation (i.e., multicollinearity), which can be quantified by variance inflation factors (VIFs). To address this issue, we applied Bayesian Kernel Machine Regression (BKMR), a statistical approach specifically designed to evaluate complex mixtures of correlated chemicals. BKMR is a supervised non-parametric method that incorporates a variable selection approach within the estimation of individual dose-response associations as well as the overall effect of the chemical mixture and flexibly accounts for potential non-linear relationships and interaction effects (Bobb et al., 2015, 2018). We used the hierarchical version of BKMR (Bobb et al., 2015), which allows for informing the model of clusters of chemicals (i.e., phthalates and parabens versus PFAS). BKMR also allows for estimating an overall mixture effect that can be interpreted as the change in the outcome while jointly increasing each chemical by percentiles.

All analyses were performed with the statistical software R version 4.1 (R Foundation for Statistical Computing, Vienna, Austria). All tests were two-tailed, and p-values <0.05 were conventionally used to indicate statistically significant associations.

3. Results

3.1. Population characteristics

Table 1 reports descriptive statistics of covariates, in the combined population and stratified by cohort. There were some differences in the distribution of several variables between cohorts. Estonian were more frequently infertile due to female causes than the women in the Swedish cohort. In addition, lower endometrial thickness and AMH were observed in the Estonian cohort, even though AMH data were only available in 35 women with an indication of low ovarian reserve in the Estonian cohort. In total, we observed 106 CP from fresh, 155 CP from fresh/frozen, 93 LB from fresh, and 135 LB from fresh/frozen transfers. The Swedish and Estonian cohorts had similar OSI (mean values of 0.62 and 0.63, respectively) as well as probabilities of CP (36% and 38%, respectively) and LB (32% and 34%, respectively).

Out of the measured chemicals [10 bisphenols, 6 parabens, 16 phthalate metabolites, and 27 PFAS (8 PFAS in Swedish cohort)], 11 (3 phthalate metabolites, the DEHP metabolites, evaluated as a molar sum, 1 paraben, and 6 PFAS) were quantified in >90% of women and used for statistical analyses. Distributions of these chemicals are presented in Table 2. The LOQs and distributions for all chemicals analyzed can be found in Table S3. PFAS concentrations were generally higher in the Swedish cohort, and the range of reported values was much wider than in the Estonian samples. No substantially different patterns in phthalates and parabens distributions were observed between the Estonian and Swedish cohorts. Fig. 1 presents the correlation matrix of the 11 evaluated chemicals. We observed a strong correlation structure ($r > 0.5$) between the six PFAS chemicals, whereas phthalates and parabens were largely uncorrelated. Analysis of the blank samples suggests minimal contamination from the embryo laboratory environment.

3.2. Phthalates, parabens, and fertility outcomes

The associations between phthalates, parabens, and OSI are presented in Table 3. In the combined population, we observed lower OSI at higher chemical concentrations, with generally broad confidence intervals. Within the 2 individual cohorts, significant differences were observed for ΣDEHP in the Swedish cohort (Q4 vs Q1, $\beta = -0.21$, 95% CI: -0.38, -0.05) and methylparaben in the Estonian cohort (Q3 vs Q1, $\beta = -0.22$, 95% CI: -0.44, -0.01). Evaluating chemicals as a mixture by mutually adjusting for phthalates and parabens in the same model did not affect the results (Table S4). In addition, consistent results were obtained from the analyses further adjusting for potential mediators (Table S5), in the sensitivity analysis excluding MEHP from ΣDEHP (data not shown), and excluding cases of infertility due to male causes (Table S6).

Fig. 2 presents associations between phthalates and parabens, evaluated independently, and clinical outcomes. Higher cxMiNP concentration was associated with lower odds of CP (Q4 vs. Q1, OR = 0.48, 95% CI 0.23, 0.94) in the combined population. No other significant associations between phthalates and parabens and clinical outcomes were observed.

3.3. PFAS and fertility outcomes

Table 4 presents the associations of PFAS with OSI, where all chemicals were mutually adjusted for in one regression model because of their high correlation. Higher concentrations of PFAS were generally related to lower OSI, with statistically significant associations for PFUnDA in both the combined population (Q2 vs. Q1, $\beta = -0.16$, 95% CI -0.31, -0.02) and the Estonian population (Q2 vs. Q1, $\beta = -0.27$, 95% CI -0.45, -0.08), and for PFOA in the Estonian population (Q4 vs. Q1, $\beta = -0.31$, 95% CI -0.61, -0.01). Analysis using individual regression models for each chemical showed consistent results for PFUnDA (Table S7). Further adjusting for potential mediators (Table S8) and

Table 1
Characteristics of the study population, overall and by cohort.

	Combined population (n = 333)	Sweden (n = 185)	Estonia (n = 148)
Variables available in both cohorts			
Age, mean (SD)	34.8 (4.5)	34.4 (4.7)	35.2 (4.2)
BMI, mean (SD)	23.4 (3.5)	23.5 (3.5)	23.2 (3.6)
Smoking, n (%)			
Never	265 (79.6)	162 (87.6)	103 (69.6)
Former/Current	30 (9.0)	23 (12.4)	7 (4.7)
Missing	38 (11.4)	0	38 (25.7)
ICSI or conventional IVF, n (%)			
ICSI	180 (54.1)	94 (50.8)	86 (58.1)
Conventional IVF	153 (45.9)	91 (49.2)	62 (41.9)
Parity, n (%)			
0	202 (60.7)	106 (57.3)	96 (64.9)
≥1	131 (39.3)	79 (42.7)	52 (35.1)
Infertility cause, n (%)			
Both male and female	35 (10.5)	14 (7.6)	21 (14.2)
Female	132 (39.6)	54 (29.2)	78 (52.7)
Male	75 (22.5)	44 (23.8)	31 (20.9)
Unexplained	91 (27.3)	73 (39.5)	18 (12.2)
Previous IVF, n (%)			
No	192 (57.7)	91 (49.2)	101 (68.2)
Yes	141 (42.3)	94 (50.8)	47 (31.8)
Previous IVF children, n (%)			
No	281 (84.4)	153 (82.7)	128 (86.5)
Yes	52 (15.6)	32 (17.3)	20 (13.5)
Regularity of menses, n (%)			
Regular	260 (78.1)	160 (86.5)	100 (67.6)
Irregular	36 (10.8)	25 (13.5)	11 (7.4)
Missing	37 (11.1)	0	37 (25.0)
Other variables			
Infertility duration, mean (SD)	\	2.3 (1.6)	
Personal Care Products score[a], mean (SD)		2.9 (0.5)	\
Alcohol, n (%)			
Daily/weekly	\	43 (23.2)	\
Monthly		63 (34.1)	\
Seldom/never	\	76 (41.1)	\
Missing	\	3 (1.6)	\
Fatty fish, n (%)			
Daily/weekly		89 (48.1)	\
Monthly		71 (38.4)	\
Seldom/never	\	19 (10.3)	\
Missing	\	6 (3.2)	\
Anti-mullerian hormone (AMH) concentration,[b] mean (SD)	3.0 (2.9)	3.3 (3.0)	1.7 (1.7)
Thyroid stimulating hormones (TSH) concentration, mean (SD)	\	1.6 (0.8)	
Basal antral follicle count (bAFC),[c] mean (SD)	\	19.4 (11.9)	\
Stimulated antral follicle count (sAFC), mean (SD)		\	12.4 (9.6)
Endometrial thickness,[d] mean (SD)	11.1 (2.3)	11.6 (2.4)	10.5 (2.0)

[a] Calculated as a score of several personal care products usage.
[b] AMH in the Estonian cohort was only calculated for n = 35 (23.6%) women who demonstrated problems with ovarian reserve.
[c] bAFC had n = 21 (11.4%) missing values in the Swedish cohort.
[d] Endometrial thickness had n = 20 (13.5%) missing values in the Estonian cohort.

excluding cases of infertility due to male causes (Table S6) did not substantially affect the results (Table S8).

Fig. 3 presents associations between PFAS, mutually adjusted in one logistic regression model, and clinical outcomes. PFHxS was associated with lower odds of LB from fresh transfer (Q2 vs. Q1, OR = 0.35, 95% CI 0.12, 0.98; Q3 vs. Q1, OR = 0.31, 95% CI 0.10, 0.94) and lower odds of LB (Q2 vs. Q1, OR = 0.23, 95% CI 0.09, 0.76) from fresh/frozen transfers. In addition, a higher concentration of PFOA was related to lower odds of CP from the fresh/frozen transfers (Q4 vs. Q1: OR = 0.31, 95% CI 0.10, 0.92). Interestingly, we also noted a positive relation between PFUnDA concentration and LB from the fresh transfer (Q2 vs. Q1: OR = 3.18, 95% CI 1.11, 9.97). In both linear and logistic regression models mutually adjusting for PFAS, VIFs (range: 3.0 to 10.0 for the overall population) suggested that these results are likely affected by multicollinearity, thus mixture modeling, presented in the next subsection, was required to validate the results.

3.4. Bayesian Kernel Machine Regression (BKMR) analysis

Hierarchical BKMR was performed in a subsample of the combined population where complete data on all exposures (i.e., phthalates/parabens and PFAS) and confounders were available (n = 283). Since BKMR requires evaluating chemicals as continuous covariates, concentrations of chemicals were log-transformed prior to analysis. In addition, we fitted a BKMR model without the hierarchy (all exposures) and containing an option to allow for within-cohort differences to account for structural population differences. Negligible differences in the results were observed, and we, therefore, presented only results from hierarchical BKMR without random effects for cohort.

Dose-response associations between selected chemicals (i.e., those showing some signal in regression and mixture modeling) and OSI are presented in Fig. 4, showing overall inverse associations. However, all estimates presented broad credible intervals that included the null associations. The overall mixture effect is presented in Fig. 5, suggesting an inverse association between OSI and the chemical mixture. BKMR

Table 2
Distributions and levels of quantification of chemicals evaluated in primary analyses (ng/mL).

	Sweden (n = 185)	Estonia (n = 148)	Combined population (n = 333)
MEHP			
Mean (SD)	1.04 (1.65)	0.98 (0.40)	1.02 (1.28)
Median [Min, Max]	0.78 [0.48, 21.00]	0.9 [0.68, 3.60]	0.85 [0.48, 21.00]
Non-quantified, n (%)	25 (13.5%)	33 (22.3%)	58 (17.4%)
MECPP			
Mean (SD)	0.39 (1.85)	0.30 (0.21)	0.35 (1.38)
Median [Min, Max]	0.19 [0.06, 25.00]	0.23 [0.09, 1.60]	0.21 [0.06, 25.00]
Non-quantified, n (%)	3 (1.6%)	3 (2.0%)	6 (1.8%)
MEHHP			
Mean (SD)	0.08 (0.35)	0.06 (0.02)	0.07 (0.26)
Median [Min, Max]	0.04 [0.02, 4.70]	0.06 [0.04, 0.13]	0.05 [0.02, 4.70]
Non-quantified, n (%)	5 (2.7%)	8 (5.4%)	13 (3.9%)
MEOHP			
Mean (SD)	0.05 (0.25)	0.05 (0.02)	0.05 (0.21)
Median [Min, Max]	0.02 [0.01, 3.30]	0.05 [0.04, 0.17]	0.03 [0.01, 3.30]
Non-quantified, n (%)	10 (5.4%)	90 (60.8%)	100 (30.0%)
ΣDEHP[a]			
Mean (SD)	0.004 (0.002)	0.004 (0.002)	0.004 (0.002)
Median [Min, Max]	0.004 [0.0003, 0.014]	0.004 [0.0005, 0.014]	0.004 [0.0003, 0.014]
Non-quantified, n (%)	4 (2.2%)	1 (0.7%)	5 (1.5%)
MEP			
Mean (SD)	0.98 (1.10)	1.02 (1.54)	1.00 (1.31)
Median [Min, Max]	0.74 [0.29, 10.00]	0.6 [0.28, 12.00]	0.71 [0.28, 12.00]
Non-quantified, n (%)	4 (2.2%)	8 (5.7%)	12 (3.6%)
cxMiNP			
Mean (SD)	2.52 (7.03)	2.99 (6.62)	2.72 (6.85)
Median [Min, Max]	0.61 [0.10, 70.00]	0.55 [0.16, 37.00]	0.58 [0.10, 70.00]
Non-quantified, n (%)	3 (1.6%)	5 (3.4%)	8 (2.4%)
MOHiBP			
Mean (SD)	0.07 (0.05)	0.04 (0.02)	0.06 (0.04)
Median [Min, Max]	0.05 [0.02, 0.41]	0.04 [0.02, 0.16]	0.05 [0.02, 0.41]
Non-quantified, n (%)	13 (7.0%)	3 (2.0%)	16 (4.8%)
Methylparaben			
Mean (SD)	152.69 (653.76)	58.14 (39.08)	110.19 (487.43)
Median [Min, Max]	7.9 [0.13, 6000.00]	54 [0.16, 230.00]	30 [0.13, 6000.00]
Non-quantified, n (%)	5 (2.7%)	1 (0.7%)	6 (1.8%)
PFHxS			
Mean (SD)	1.15 (1.69)	0.23 (0.23)	0.74 (1.34)
Median [Min, Max]	0.60 [0.01, 13.00]	0.17 [0.04, 2.40]	0.33 [0.01, 13.00]
Non-quantified, n (%)	5 (2.7%)	1 (0.7%)	6 (1.8%)
PFOA			
Mean (SD)	1.35 (0.90)	0.73 (0.40)	1.07 (0.78)
Median [Min, Max]	1.16 [0.17, 8.05]	0.63 [0.19, 3.20]	0.85 [0.17, 8.05]
Non-quantified, n (%)	4 (2.2%)	1 (0.7%)	5 (1.5%)
PFOS			
Mean (SD)	4.09 (2.43)	2.44 (1.65)	3.35 (2.27)
Median [Min, Max]	3.54 [0.17, 15.05]	1.9 [0.52, 12.00]	2.69 [0.17, 15.05]
Non-quantified, n (%)	4 (2.2%)	2 (1.3%)	6 (1.8%)
PFNA			
Mean (SD)	0.58 (0.35)	0.46 (0.33)	0.52 (0.35)
Median [Min, Max]	0.51 [0.02, 3.16]	0.37 [0.08, 2.30]	0.44 [0.02, 3.16]
Non-quantified, n (%)	4 (2.2%)	1 (0.7%)	5 (1.5%)
PFUnDA			
Mean (SD)	0.23 (0.13)	0.11 (0.08)	0.18 (0.12)
Median [Min, Max]	0.21 [0.04, 0.68]	0.09 [0.03, 0.55]	0.14 [0.03, 0.68]
Non-quantified, n (%)	13 (7%)	6 (4.1%)	19 (5.7%)
PFDA			
Mean (SD)	0.28 (0.15)	0.18 (0.15)	0.24 (0.16)
Median [Min, Max]	0.26 [0.06, 1.09]	0.14 [0.03, 1.00]	0.19 [0.03, 1.09]
Non-quantified, n (%)	5 (2.7%)	1 (0.7%)	6 (1.8%)

[a] Molecular sum of MEHP, MECPP, MEHHP, MEOHP with the unit of mol/mL.

analysis on CP and LB demonstrated little evidence for associations despite an overall inverse trend (Fig. S3).

4. Discussion

This study is one of the first to evaluate the impact of mixtures of known and suspected EDCs on ovarian function and subsequent fertility in women. We observed significant associations of high levels of DEHP metabolites and methylparaben, and possibly PFUnDA and PFOA, with lower OSI, suggesting that these chemicals may interfere with ovarian sensitivity in women.

The potential link between exposure to EDCs and fertility has been the focus of several recent studies and literature reviews (Mínguez-Alarcón and Gaskins, 2017; Björvang et al., 2022; Kahn et al., 2020; Björvang and Damdimopoulou, 2020; Karwacka et al., 2019). Although male factors, like semen quality and sperm DNA damage, have been thoroughly studied and commonly used as a marker for male fecundity in chemical risk assessment (Meeker et al., 2010; Pant et al., 2008; European Food Safety Authority, 2018; EFSA Panel on Food Contact Materials et al., 2019), limited information is however available

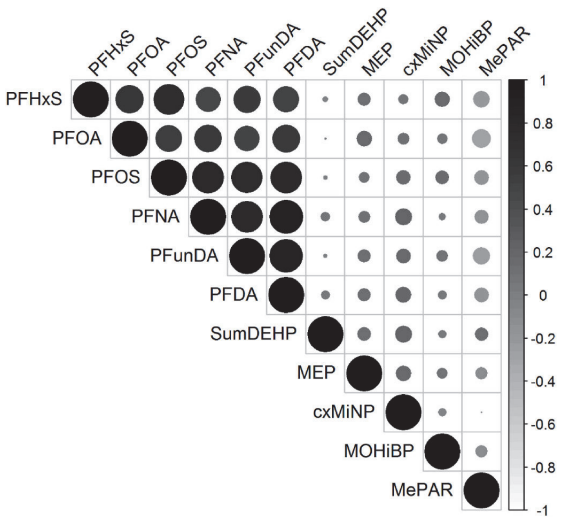

Fig. 1. Correlation of chemical measurements for the compounds detected with >LOQ in >90% of samples. Darker color and larger sizes indicated higher correlation coefficients.

on the potential effects of EDCs on female fertility and fecundity. A recent review on EDCs and female fecundity highlighted some potential causes of previous inconsistency, including heterogeneity in study population selection, exposure assessment, and co-exposure to other chemicals (Mínguez-Alarcón and Gaskins, 2017). Similarly, a review paper specifically focusing on IVF outcomes concluded that the evidence supporting an association between EDC exposures and ovarian reserve or IVF outcomes in humans remains limited (Karwacka et al., 2019).

Several studies conducted on women visiting fertility clinics have documented associations between higher levels of EDC exposure and less favorable success measures, such as lower rates of CP or LB (Björvang et al., 2022; Karwacka et al., 2019; Mínguez-Alarcón et al., 2015, 2019). Nevertheless, solely focusing on these clinical outcomes might fail to distinguish between paternal and maternal causes of infertility. Little attention has been paid to the associations between EDCs and female hormonal indicators, thus limiting our understanding of the potential effects of these widespread chemicals on women's fecundity and fertility (Karwacka et al., 2019). Here, we focus on OSI to specifically consider the associations of EDC exposure to indicators of ovarian function. OSI has been shown to be a good biomarker of female fertility and predictor of success in ART. As a marker of ovarian responsiveness to exogenous gonadotrophin stimulation, OSI is a predictor of IVF/ICSI outcome that has been shown to be superior to baseline FSH or AMH in predicting pregnancy as well as the total number of oocytes (Vaegter et al., 2017, 2019; Weghofer et al., 2020; Revelli et al., 2020). OSI is a measurement of ovarian competence

Table 3
Associations of phthalates and parabens concentrations with Ovarian Sensitivity Index, evaluated with individual regression models for each chemical.[a].

Plasticizer concentrations	Combined population (n = 333)[b]			Sweden (n = 185)[c]			Estonia (n = 148)		
	n	Beta	95% CI	n	Beta	95% CI	n	Beta	95% CI
ΣDEHP									
Q1 [0.0003, 0.0031]	82	Ref	–	51	Ref	–	31	Ref	–
Q2 (0.0031, 0.0039]	82	−0.05	(-0.16, 0.07)	51	−0.08	(-0.23, 0.06)	31	−0.05	(-0.24, 0.14)
Q3 (0.0039, 0.0049]	82	−0.05	(-0.16, 0.07)	40	−0.09	(-0.24, 0.07)	42	−0.05	(-0.22, 0.13)
Q4 (0.0049, 0.0144]	82	−0.07	(-0.19, 0.04)	39	**−0.21**	**(-0.38–0.05)**	43	−0.01	(-0.18, 0.16)
MEP									
Q1 [0.29, 0.51]	82	Ref	–	23	Ref	–	59	Ref	–
Q2 (0.51, 0.71]	88	−0.06	(-0.18, 0.06)	59	0.06	(-0.13, 0.25)	29	−0.10	(-0.27, 0.07)
Q3 (0.71, 0.98]	73	−0.02	(-0.14, 0.11)	56	0.09	(-0.11, 0.28)	17	−0.08	(-0.29, 0.12)
Q4 (0.98, 12.00]	78	−0.02	(-0.14, 0.10)	43	0.03	(-0.18, 0.24)	35	−0.01	(-0.17, 0.15)
cxMiNP									
Q1 [0.10, 0.30]	85	Ref	–	42	Ref	–	43	Ref	–
Q2 (0.30, 0.58]	79	0.00	(-0.11, 0.12)	46	−0.03	(-0.20, 0.13)	44	−0.05	(-0.22, 0.11)
Q3 (0.58, 1.80]	81	−0.04	(-0.15, 0.07)	54	−0.07	(-0.23, 0.09)	27	−0.15	(-0.33, 0.03)
Q4 (1.8, 70.00]	80	−0.08	(-0.19, 0.04)	40	−0.06	(-0.23, 0.11)	40	−0.13	(-0.30, 0.03)
MOHiBP									
Q1 [0.02, 0.03]	87	Ref	–	28	Ref	–	59	Ref	–
Q2 (0.03, 0.05]	120	0.05	(-0.06, 0.15)	65	−0.05	(-0.21, 0.12)	55	0.10	(-0.04, 0.24)
Q3 (0.05, 0.06]	40	0.11	(-0.03, 0.25)	26	0.02	(-0.18, 0.22)	14	0.17	(-0.05, 0.39)
Q4 (0.06, 0.41]	70	0.01	(-0.11, 0.13)	53	−0.15	(-0.33, 0.02)	17	0.13	(-0.08, 0.34)
Methylparaben									
Q1 [0.13, 5.30]	84	Ref	–	71	Ref	–	13	Ref	–
Q2 (5.30, 30.00]	80	0.03	(-0.09, 0.14)	60	0.06	(-0.07, 0.19)	20	−0.11	(-0.37, 0.15)
Q3 (30.00, 71.00]	83	−0.11	(-0.23, 0.02)	14	−0.02	(-0.25, 0.20)	69	**−0.22**	**(-0.44, -0.01)**
Q4 (71.00, 6000.00]	80	−0.05	(-0.17, 0.07)	35	−0.08	(-0.24, 0.08)	45	−0.08	(-0.31, 0.14)
cxMiNCH[d]									
Q1 [0.01, 0.04]		Ref	–	70	Ref	–	\	Ref	–
Q2 (0.04, 0.08]	\	\	\	33	0.03	(-0.12, 0.19)	\	\	
Q3 (0.08, 0.15]	\	\	\	29	−0.11	(-0.28, 0.06)	\	\	
Q4 (0.15, 16.00]	\	\	\	43	−0.14	(-0.30, 0.01)	\	\	
Propylparaben[e]									
Q1 [0.07,0.35]		Ref	–	\	Ref	–	6	Ref	–
Q2 (0.35, 6.90]	\	\	\	\	\	\	32	−0.22	(-0.54, 0.11)
Q3 (6.90, 15.00]	\	\	\	\	\	\	55	−0.23	(-0.55, 0.08)
Q4 (15.00, 110.00]	\	\	\	\	\	\	54	−0.13	(-0.44, 0.19)

[a] Adjusted for age, BMI, parity, and previous IVF.
[b] Further adjusted for cohort to account for structural differences between cohorts.
[c] Further adjusted for infertility duration, smoking, fatty fish consumption, and PCP use.
[d] High detection only in the Swedish population.
[e] High detection only in the Estonian population.

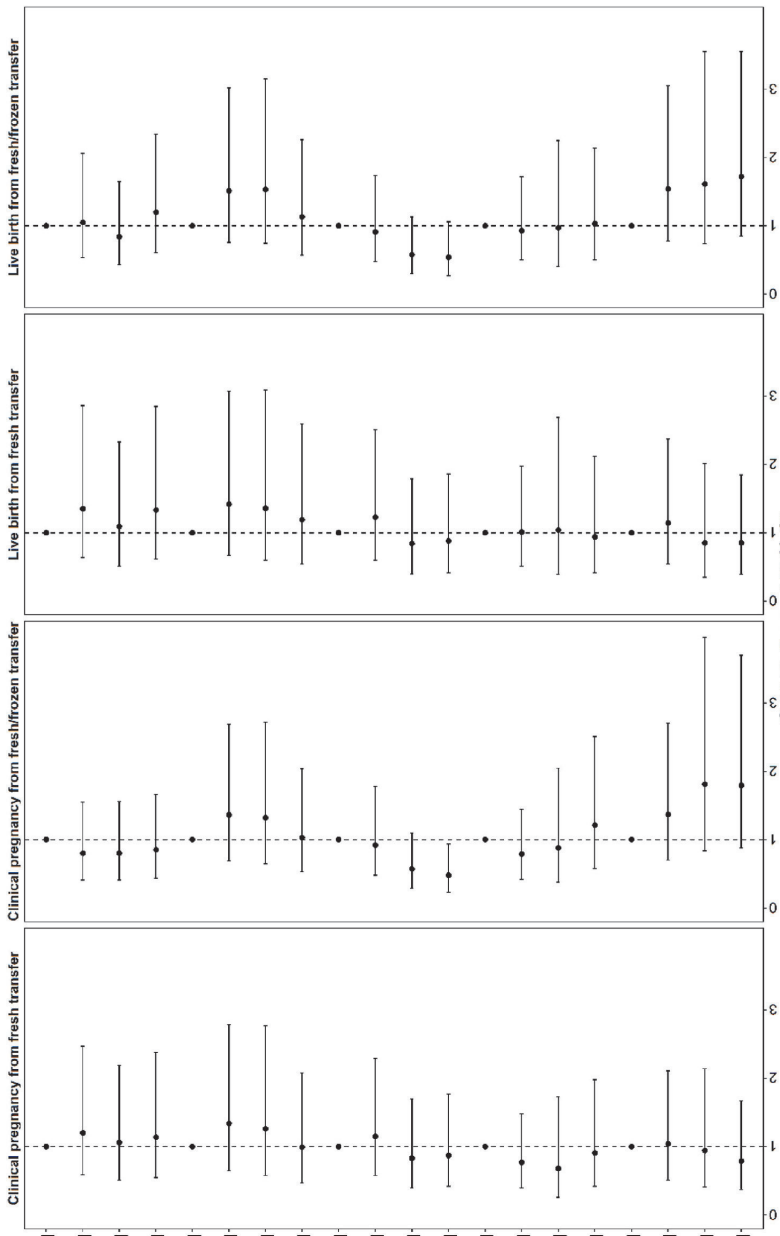

Fig. 2. Associations of phthalates and parabens concentrations with clinical pregnancy and live birth, assessed with individual logistic regression models adjusted for age, BMI, parity, previous IVF, and cohort, in the overall sample of Swedish and Estonian women. Chemicals were evaluated as categorical variables by quartiles of their distribution. Quartiles ranges are reported in parentheses. Results were based on observed 106 CP from fresh, 155 CP from fresh/frozen, 93 LB from fresh, and 135 LB from fresh/frozen transfers.

Table 4
Associations of PFAS concentrations with Ovarian Sensitivity Index, evaluated with a single regression model, mutually adjusting for all chemicals.[a].

PFAS concentrations	Combined population (n = 333)[b]			Sweden (n = 185)[c]			Estonia (n = 148)		
	n	Beta	95% CI	n	Beta	95% CI	n	Beta	95% CI
PFHxS									
Q1 [0.01, 0.17]	84	Ref	–	11	Ref	–	73	Ref	–
Q2 (0.17, 0.33]	80	−0.07	(-0.22, 0.09)	33	−0.03	(-0.34, 0.28)	47	−0.06	(-0.26, 0.13)
Q3 (0.33, 0.69]	81	0.00	(-0.17, 0.18)	58	0.00	(-0.31, 0.31)	23	0.05	(-0.18, 0.29)
Q4 (0.69, 13]	82	−0.002	(-0.20, 0.20)	78	0.02	(-0.30, 0.35)	4	0.10	(-0.32, 0.51)
PFOA									
Q1 [0.17, 0.60]	82	Ref	–	17	Ref	–	65	Ref	–
Q2 (0.60, 0.85]	83	0.10	(-0.03, 0.23)	38	0.14	(-0.12, 0.40)	45	0.10	(-0.05, 0.26)
Q3 (0.85, 1.33]	81	0.10	(-0.05, 0.25)	55	0.24	(-0.00, 0.49)	26	−0.01	(-0.22, 0.21)
Q4 (1.33, 8.05]	82	0.03	(-0.15, 0.21)	71	0.15	(-0.13, 0.43)	11	**−0.31**	**(-0.61, -0.01)**
PFOS									
Q1 [0.17, 1.84]	82	Ref	–	15	Ref	–	67	Ref	–
Q2 (1.84, 2.69]	82	0.01	(-0.16, 0.19)	44	0.17	(-0.15, 0.49)	38	−0.01	(-0.23, 0.22)
Q3 (2.69, 4.34]	81	−0.10	(-0.30, 0.10)	58	0.03	(-0.30, 0.36)	23	−0.05	(-0.34, 0.24)
Q4 (4.34, 15.10]	82	0.03	(-0.20, 0.26)	64	0.23	(-0.12, 0.58)	18	−0.24	(-0.66, 0.17)
PFNA									
Q1 [0.02, 0.30]	82	Ref	–	24	Ref	–	58	Ref	–
Q2 (0.3, 0.44]	82	0.15	(-0.01, 0.31)	47	0.15	(-0.12, 0.42)	35	0.14	(-0.09, 0.36)
Q3 (0.44, 0.64]	82	0.05	(-0.16, 0.26)	51	0.10	(-0.25, 0.45)	31	−0.02	(-0.30, 0.27)
Q4 (0.64, 3.16]	82	0.11	(-0.14, 0.35)	59	0.08	(-0.31, 0.47)	23	0.23	(-0.18, 0.64)
PFUnDA									
Q1 [0.03, 0.08]	82	Ref	–	15	Ref	–	67	Ref	–
Q2 (0.08, 0.14]	75	**−0.16**	**(-0.31, -0.02)**	33	0.05	(-0.21, 0.31)	42	**−0.27**	**(-0.45, -0.08)**
Q3 (0.14, 0.25]	78	−0.05	(-0.23, 0.13)	51	0.03	(-0.25, 0.31)	27	−0.16	(-0.41, 0.09)
Q4 (0.25, 0.68]	79	−0.08	(-0.31, 0.15)	73	0.06	(-0.26, 0.39)	6	−0.33	(-0.77, 0.10)
PFDA									
Q1 [0.03, 0.13]	82	Ref	–	18	Ref	–	64	Ref	–
Q2 (0.13, 0.19]	82	−0.10	(-0.26, 0.06)	43	0.04	(-0.28, 0.36)	39	−0.03	(-0.25, 0.18)
Q3 (0.19, 0.31]	81	−0.03	(-0.24, 0.18)	54	0.17	(-0.22, 0.55)	27	0.05	(-0.24, 0.33)
Q4 (0.31, 1.09]	82	−0.11	(-0.36, 0.13)	65	−0.03	(-0.44, 0.38)	17	0.30	(-0.07, 0.67)

[a] Adjusted for age, BMI, parity, and previous IVF.
[b] Further adjusted for cohort to account for structural differences between cohorts.
[c] Further adjusted for infertility duration, smoking, fatty fish consumption, and PCP use.

reflecting the response to exogenous FSH stimulation. The reason for its introduction in reproductive endocrinology was the observation that although the total number of oocytes retrieved at ovum pick-up correlates to pregnancy rate, these correlations are much stronger if account is also taken of the dose of FSH and human menopausal gonadotropin (hMG) given (Huber et al., 2013). Physiologically, this factor is thus describing a stimulus-response observation, and the most proper way to measure it is as the number of oocytes retrieved divided by the total dose of FSH/hMG used. The high prediction potential likely relates to the fact that OSI reflects not ony the number of oocytes left in the reserve, but also functional aspects of ovaries, ultimately associated with the likelihood of retrieving euploid oocytes at ovum pick-up (Huber et al., 2013; Weghofer et al., 2020). Based on these data, we selected OSI as an appropriate outcome variable to reflect the female side specifically in ART.

Potential EDCs that we evaluated include phthalates, parabens, and PFAS, which can interfere with the endocrine system through a variety of complex biological mechanisms thus acting as disruptors of the endocrine system and potentially affecting biological systems related to reproductive health (La Merrill et al., 2020). Despite the growing evidence supporting a potential endocrine-disrupting the role of phthalates, parabens, and PFAS, very few chemicals are currently classified as EDCs by the EU. Svingen et al., 2022 Our results suggested the presence of inverse associations between some specific chemicals concentrations and OSI. We observed associations for phthalates, with a significant signal for DEHP metabolites among Swedish women, and parabens, with a significant signal for methylparaben among Estonian women. Both these results were robust to a set of sensitivity analyses. Higher molar sum of DEHP metabolites was previously associated with lower bAFC (Messerlian et al., 2016), and a lower probability of CP or LB following ART (Souter et al., 2013; Hauser et al., 2016). The current study suggested an inverse association between the molar sum of DEHP and OSI, which is consistent with most of the previous literature and strengthens the evidence on the role of this group of metabolites in the development of adverse reproductive outcomes (Panagiotou et al., 2021). Several mechanisms of action have been hypothesized to explain the potential association between DEHP and fertility as documented in animal studies, including the disruption of ovarian functioning and inhibiting the growth of antral follicles through reduced 17-beta oestradiol (E2) production (Panagiotou et al., 2021; Craig et al., 2014; Gupta et al., 2010; Hannon et al., 2014; Lovekamp-Swan and Davis, 2003). Interestingly, DEHP did not associate with CP or LB. The chemicals were measured only in the follicular fluid, and represent a snapshot of a time when oocytes were picked up. Follow-up studies need to assess potential associations between serum/urine DEHP and CP/LB during embryo transfer and early pregnancy. Previous studies have also reported associations of higher paraben concentration with lower rates of LB and poorer embryo quality (Dodge et al., 2015; Sabatini et al., 2011), while another study reported null associations (Mínguez-Alarcón et al., 2016). Mechanisms of actions for parabens are less clear and include oestrogenic activities such as their ability to bind with both oestrogen receptor ER-α and ER-β (Gomez et al., 2005; Okubo et al., 2001). Gonadotropin sensitivity is another indicator strictly related to OSI, which should also be further investigated (Björvang et al., 2022; Biasoni et al., 2011).

Evidence on PFAS and human fertility outcomes are sparse, and a recent review identified only two studies that documented either null associations or associations with higher androgen levels (Björvang and Damdimopoulou, 2020; Petro et al., 2014; Heffernan et al., 2018). This illustrates the complexity of assessing fertility in women and highlights the importance of studying large cohorts with multiple well-defined outcomes to identify sensitive endpoints for endocrine disruption (van Duursen, 2020). In contrast to phthalates and parabens, where we mostly observed inverse associations robust to different modeling and sensitivity analyses, our results for PFASs were less robust, and we only

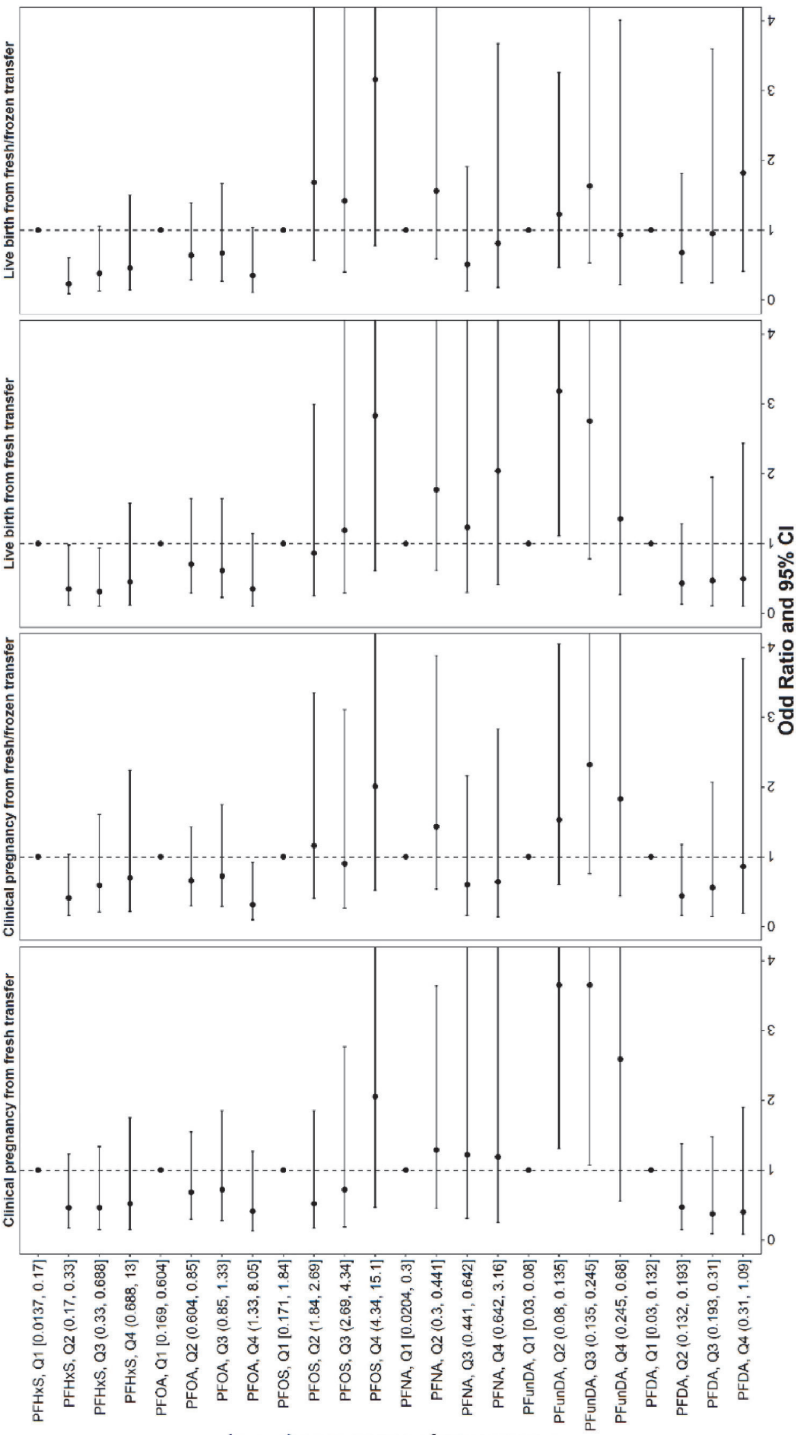

Fig. 3. Association of PFAS concentrations with clinical pregnancy and live birth, assessed with one multiple regression model adjusted for age, BMI, parity, previous IVF, and cohort, in a combined cohort of Swedish and Estonian women. PFAS were evaluated as categorical variables by quartiles of their distribution. Quartiles ranges are reported in parentheses. Results were based on observed 106 CP from fresh, 155 CP from fresh/frozen, 93 LB from fresh, and 135 LB from fresh/frozen transfers.

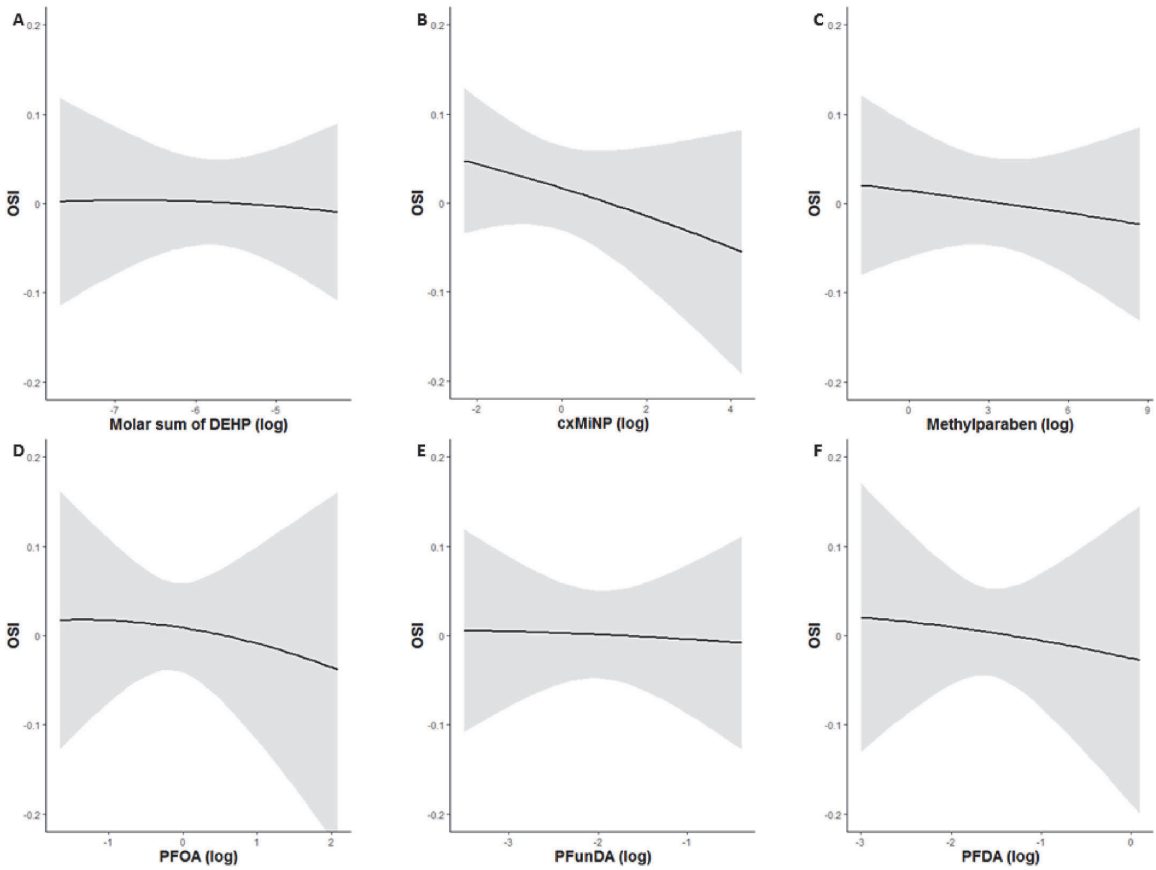

Fig. 4. Dose-response associations of DEHP (A), cxMiNP (B), methylparaben (C), PFOA (D), PFUnDA (E), and PFDA (F) with Ovarian Sensitivity Index, adjusted for age, BMI, parity, previous IVF, estimated using hierarchical Bayesian Kernel Machine Regression in a subsample with complete information (n = 283). The grey areas indicate 95% credible intervals.

observed a signal for PFUnDA and PFOA in multivariable models where associations were found both with OSI and CP/LB. These results were likely affected by high levels of collinearity and were not confirmed by the BKMR analysis. While the complex correlation structure reported among PFAS compounds could certainly play a role, these results also suggest that the association between these compounds and fertility outcomes is less consistent. An earlier study on the Swedish cohort that focused on chemicals discovered associations of PFAS with bAFC and embryo quality, but not with OSI, CP, or LB (Björvang et al., 2022). Identification of critical effects to various types of chemicals in humans remains a high-priority task, and our data suggest that within the fertility domain, multiple targets may be included with varying sensitivity to chemical exposures.

We also evaluated the associations of individual chemicals and chemical mixtures with clinical outcomes such as CP and LB. These analyses, however, were severely hampered by the low number of CP and LB available in our populations. Specifically, we only had data on 155 clinical pregnancies and 135 live births, which limited the statistical power of the logistic regression analysis. Larger cohorts or a longer-follow up time might enable higher power. Although we still observed some significant associations of higher PFHxS and cxMiNP concentrations with lower odds of CP and LB, we did not detect associations with common chemicals that have been previously associated with CP and LB, such as DEHP (Mínguez-Alarcón and Gaskins, 2017). As such, these results should be interpreted with caution.

Our study attempted to address some of the methodological challenges described by the previous literature and, as such, has several strengths (Mínguez-Alarcón and Gaskins, 2017). Firstly, we conducted our analysis within two separate cohorts, which improved the generalizability of our findings. Results on phthalates and parabens were robust in the stratified analysis as well as when using methods that account for between-cohorts variability. Secondly, to our knowledge, this is one of the first studies to investigate associations between EDCs and OSI. Future studies should investigate the role of OSI and other female fertility measures within the causal pathways leading from EDCs exposure to clinical outcomes, and evaluate the role of these early pregnancy female indicators in the associations between chemical exposures and clinical outcomes. Moreover, our results also inform toxicological studies about a potential key role of OSI, which could be evaluated as an endpoint in animal studies to test its connection to EDCs and its ability to independently predict female fecundity (van Duursen, 2020). Thirdly, we measured chemical concentrations from follicular fluids, which might enrich the ability to account for direct exposure to the maturing follicle (Hallberg et al., 2021). Previous studies, including a recent assessment in the Swedish cohort, have shown that levels of exposure assessed in the blood correlated with those assessed from the follicular fluid (rho 0.64–0.99) (Björvang et al., 2022). Finally, we conducted a thorough examination of EDC exposures as chemical mixtures. While it

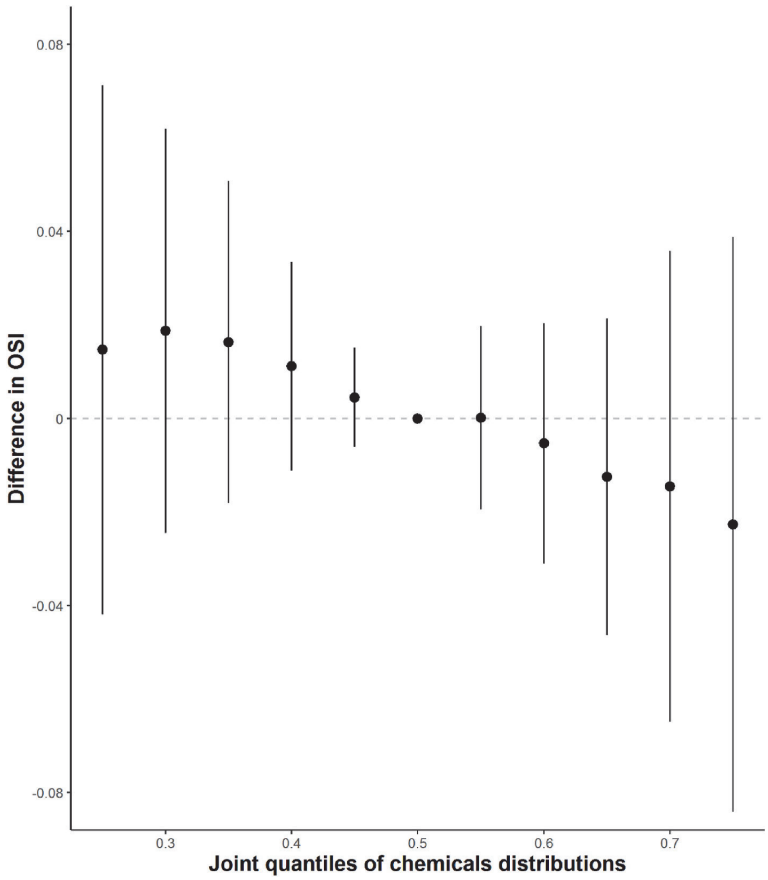

Fig. 5. Overall mixture effect of chemicals on Ovarian Sensitivity Index (OSI), adjusted for age, BMI, parity, previous IVF, estimated using hierarchical Bayesian Kernel Machine Regression in a subsample with complete information (n = 283). For each quintile (x-axis) point estimates and credible intervals represent the change in OSI when each chemical is set to that quintile of their distributions, as compared to when they are all set at their median.

is recognized that environmental exposures are present in the world as a complex mixture, most epidemiologic studies fail to integrate the presence of co-exposures into the analyses, which has been recognized as one of the potential causes of inconsistency among previous studies (Mínguez-Alarcón and Gaskins, 2017). The switch to a mixture framework has been repeatedly indicated as a priority in the field (Dominici et al., 2010; Taylor et al., 2016; Kortenkamp, 2007; Drakvik et al., 2020), and several analytical techniques to achieve this goal have been described and presented (Stafoggia et al., 2017; Hamra and Buckley, 2018; Gibson et al., 2019). In this study, we examined the structure of the mixture in terms of correlation clusters and described the ability of regression methods to characterize the data. To allow further flexibility and relax regression assumptions, we also sought to strengthen our findings with BKMR. Despite the relatively small sample size, this method allowed for estimating the overall effect of the chemical mixture, suggesting the presence of an inverse cumulative trend.

Due to the relatively small sample size, the power of our analysis was limited, especially with regards to the binary outcomes of CP and LB. Because of the small sample size, together with the high number of evaluated models, we cannot exclude that our results might arise due to a chance component. Nevertheless, results from the mixture modeling, which incorporates all chemicals in a single statistical model, mostly confirmed results obtained from regression models. In addition, the finding of an inverse association between the overall mixture and OSI strengthens the interpretation of our chemical-specific results. Nevertheless, BKMR does not consider absolute values of exposure levels or toxicology of each chemical and does not therefore provide straightforward clinical interpretations. The relatively small sample size also prevented us from conducting stratified analysis to evaluate whether associations differ by relevant factors such as the cause of infertility. Another limitation is that, while PFAS are persistent chemicals, phthalates and parabens are quickly metabolized and their levels in biological samples are known to fluctuate. Therefore, the phthalates and parabens levels likely only reflect recent exposure rather than the typical levels during follicle growth and oocyte maturation, which takes approximately half a year in humans. Finally, while the combination of two cohorts provides additional strengths, a drawback was that we could not adjust for the whole set of confounders in all analyses. Despite evaluating several potential confounders and defining criteria for covariates inclusion, residual confounding might still be present and hamper the generalizability of our findings, which should be validated in other populations.

In conclusion, this study provided additional evidence supporting the presence of an inverse association between DEHP metabolites and female fertility and identifying additional chemicals such as methylparaben, and possibly PFUnDA and PFOA, that can be involved in the

biological processes causing female infertility via disruption of ovarian function. By accounting for the complexity of the chemical exposures and by directly evaluating a critical marker of female infertility, this study adds robust evidence to the literature to support the adverse effects of EDCs on reproductive health, with potential implications for public health interventions and recommendations.

Credit author statement

Andrea Bellavia: Formal analysis, Data Curation, Writing – Original Draft, Review and Editing; Runyu Zou: Formal analysis, Data Curation, Writing – Original Draft, Visualization; Richelle D. Björvang: Investigation, Data Curation, Writing – Original Draft; Kristine Roos: Formal analysis, Investigation, Resources, Data Curation, Writing – Original Draft; Ylva Sjunnesson: Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition; Ida Hallberg: Methodology, Investigation; Jan Holte: Resources; Anne Pikki: Investigation; Virissa Lenters: Methodology, Writing – Reviewing & Editing; Lützen Portengen: Methodology; Jacco Koekkoek: Investigation, Formal Analysis; Marja Lamoree: Validation, Supervision, Writing – Review and Editing; Majorie Van Duursen: Conceptualization, Project administration, Funding acquisition, Writing – Original Draft, Review and Editing; Roel Vermeulen: Conceptualization, Writing – Reviewing & Editing, Funding Acquisition; Andres Salumets: Conceptualization, Writing – Reviewing & Editing, Funding Acquisition; Agne Velthut-Meikas: Conceptualization, Resources, Data Curation, Writing – Original Draft, Review and Editing, Supervision, Funding Acquisition; Pauliina Damdimopoulou: Conceptualization, Resources, Writing – Original Draft, Review and Editing, Supervision, Project Administration, Funding Acquisition. All authors read, reviewed and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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Appendix 3

Publication III

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Cellular, Extracellular and Extracellular Vesicular miRNA Profiles of Pre-Ovulatory Follicles Indicate Signaling Disturbances in Polycystic Ovaries

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Abstract: Cell-free RNAs have the potential to act as a means of gene expression regulation between cells and are therefore used as diagnostic markers describing the state of tissue environment. The origin and functions of such RNAs in human ovarian follicle, the environment of oocyte maturation, are unclear. The current study investigates the difference in the microRNA profiles of fertile women and polycystic ovary syndrome (PCOS) patients in three compartments from the same preovulatory follicle: mural granulosa cells (MGC), cell-free follicular fluid (FF), and extracellular vesicles (EV) of the FF by small RNA sequencing. In silico analysis was used for the prediction and over-representation of targeted pathways for the detected microRNAs. PCOS follicles were distinguished from normal tissue by the differential expression of 30 microRNAs in MGC and 10 microRNAs in FF (FDR < 0.1) that commonly regulate cytokine signaling pathways. The concentration of EV-s was higher in the FF of PCOS patients ($p = 0.04$) containing eight differentially expressed microRNAs ($p < 0.05$). In addition, we present the microRNA profiles of MGC, FF, and EV in the fertile follicle and demonstrate that microRNAs loaded into EVs target mRNAs of distinct signaling pathways in comparison to microRNAs in FF. To conclude, the three follicular compartments play distinct roles in the signaling disturbances associated with PCOS.

Keywords: extracellular vesicles; human ovarian follicle; granulosa cells; follicular fluid; polycystic ovary syndrome; PCOS; miRNA; intercellular communication

1. Introduction

Disturbances in normal ovarian physiology cause subfertility or infertility leading to prolonged effort or inability for a woman to conceive. Polycystic ovary syndrome (PCOS) is a common hormonal disturbance affecting up to 20% reproductive age women worldwide [1]. PCOS is a complex syndrome with reproductive, metabolic and psychological features and is characterized by hyperandrogenism, obesity, insulin resistance, polycystic ovarian morphology (PCOM) and/or anovulation [2,3]. The phenotype varies broadly depending on the genotype, ethnicity, and environmental factors [1]. Heterogeneity of PCOS is a challenge for diagnostics causing delayed detection and dissatisfaction with care [4]. The current knowledge regarding molecular mechanisms behind the dysfunction of PCOS is still incomplete.

The human ovarian follicle is a dynamic structure that supports oocyte maturation, ovulation, and steroid hormone synthesis. Granulosa, theca, and follicular immune cells are the somatic cell populations ensuring the flawless performance of the above-mentioned processes crucial for female fertility. By the pre-ovulatory stage, the follicle diameter expands above 20 mm and is filled with follicular fluid (FF) [5]. This fluid-filled environment enables long-distance cell communication between different cell populations via cell-secreted (lipo)proteins, ribo-protein complexes (RBPs), and extracellular vesicles (EVs) containing nucleic acids and proteins from the secreting cells [6]. The possible disorders in long-distance intercellular signaling in human polycystic ovaries have not been thoroughly investigated.

EVs are lipid bilayer-coated nanoparticles in varying size range [7]. Based on their size and mode of biogenesis EVs are classified into three major subtypes; exosomes (40–100 nm), microvesicles (100–500 nm), and apoptotic bodies (500 nm–2 um) [8]. The release of EVs and RBPs has been extensively studied and attributed to all cell types in the human body. Moreover, cell-free RNAs in RBPs and EVs have been detected in all investigated body fluids, including FF [9,10]. Small RNAs in EVs have caught more attention, however more than 90% of circulating miRNAs are present outside of EVs associated with AGO2, nucleophosmin 1, or high-density lipoprotein, among other proteins [6]. The secretion of RNA molecules via EVs is at least partly controlled by the releasing cells and the RNA content of EVs is cell specific [11]. However, AGO2-miRNA complexes may also be released non-specifically into extracellular space following cell death [6]. The RNA content of both EVs and RBPs can be taken up by recipient cells from the same or another cell population and potentially modulates signaling pathways in the recipient [12]. Examples of significance of such long-distance communication can be drawn from cancer studies and immunology [13], among other fields. In reproductive studies, fluorescently labelled EVs isolated from FF were taken up by ovarian granulosa cells in an equine in vitro model, suggesting that the exchange of RNA is potentially an important mean of communication also in the normal ovarian physiology [14]. Up to now, mainly miRNAs have been widely studied as the constituents of the follicular EVs due to their well-known molecular function. However, also other types of long and small RNAs have been described as the components of EVs and RBPs: mRNA, lncRNA, SRP RNA, circRNA, snRNA, snoRNA, vault RNA, Y RNA, piRNA, tRNA, and rRNA fragments [6]. The extracellular RNA content has been proposed as a diagnostic tool for disease states, as several cell populations have been demonstrated to change the repertoire of released cell-free RNAs upon external stimulus or disease [15,16].

The current study hypothesizes that there are differences in the cellular and extracellular miRNA expression levels between the ovarian follicles of healthy and PCOS patients indicating molecular signaling disturbances at preovulatory stage. We set out to investigate in a genome-wide manner the cellular and extracellular miRNA profile of three matched sample types collected from single follicles of healthy fertile women versus PCOS patients comprising of granulosa cells (MGC), cell-depleted FF and EVs purified from the FF. Such combined dataset for the human follicle is unique. The obtained information will provide new avenues for therapeutic approaches for PCOS patients, e.g., the development of new ovarian stimulation as well as in vitro oocyte maturation protocols for improving their infertility treatment outcomes.

2. Results

Intrafollicular communication differences between the fertile and PCOS ovaries were modelled by analyzing material from three distinct sources, each collected from the same follicle: small RNA from MGC, all cell-free small RNA populations from FF, and small RNA in EVs purified from the FF. The rationale of sample collection and compartmentalization is outlined in Figure 1.

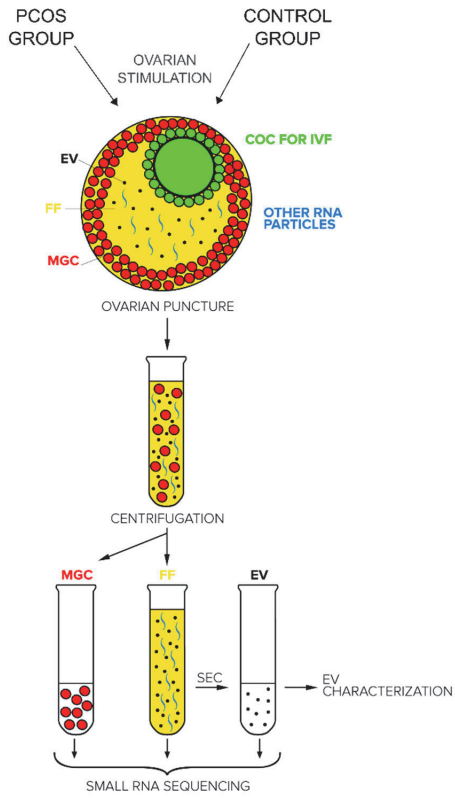

Figure 1. Schematic description of sample collection and processing from human pre-ovulatory follicles of polycystic ovary syndrome (PCOS) patients and fertile control group. COC—cumulus oocyte complex (green), FF—cell-free follicular fluid (yellow) containing extracellular vesicles (EV, black) and non-EV-bound RNA (blue), IVF—in vitro fertilization, MGC—mural granulosa cells (red).

EVs were characterized for their size, concentration, and surface markers. All three sample types underwent small RNA sequencing, and the results were validated by real-time quantitative PCR (RT-qPCR). The main characteristics of patients and the number of analyzed samples according to each method are presented in Table 1.

Table 1. General characteristics of study participants by used method.

		Age (Mean, Years)	SD (Years)	BMI (Mean, kg/m^2)	SD (kg/m^2)
Extracellular vesicle characterization:					
PCOS	n = 15	32.7	4.3	23.5	3.1
Oocyte donors	n = 15	25.8	3.2	22.3	2.8
p-value		<0.001		0.313	
Small RNA sequencing:					
PCOS	n = 7	34	4.8	22.7	2.6
Oocyte donors	n = 8	26.9	2.2	22.7	3.6
p-value		0.002		0.999	
miRNA expression validation (RT-qPCR):					
PCOS	n = 15	32.7	4.3	23.5	3.1
Control group	n = 16	33	4	23	3
p-value		0.626		0.831	

2.1. Characterization of Nanoparticles Isolated from Human Follicular Fluid as Extracellular Vesicles

FF-derived EVs from SEC fractions 6–9 (Supplementary Figure S1) were characterized by three independent methods: NTA, TEM, and Western blot (WB) analysis (Figure 2). According to the NTA size profile analysis (Figure 2A), most of the nanoparticles were under 200 nm in diameter with a large population range within 75–165 nm, which is a typical EV size range [17]. We observed a 4-nm difference between healthy women (mean 138.6± SEM 0.2 nm) and PCOS patients (142.7 ± 0.2 nm) FF-derived EVs ($p < 2.2 \times 10^{-16}$, Supplementary Figure S2). Secondly, the PCOS FF samples contain higher EV concentration compared to the control group (p = 0.04, Figure 2B).

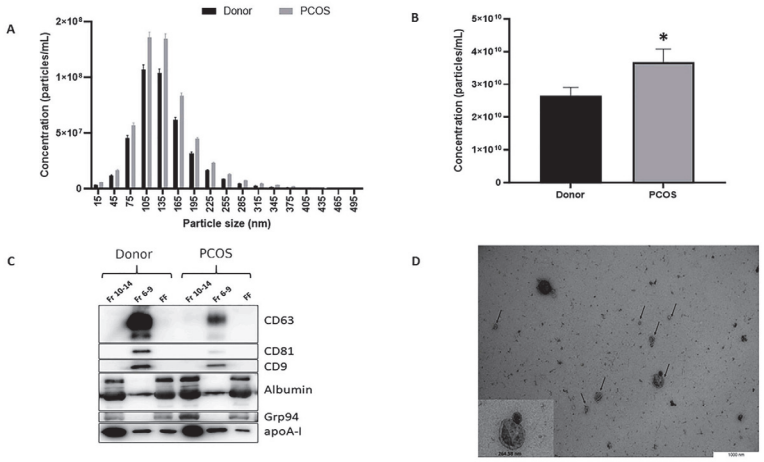

Figure 2. Characterization of extracellular vesicles (EVs) isolated from cell-free follicular fluid (FF). (**A**) Size profile of EVs in study groups (mean ± SEM). (**B**) Concentration of EVs in polycystic ovarian syndrome patients (PCOS) and control samples (mean ±SEM). (**C**) Positive signals of EV markers CD63, CD81, CD9 were detected from EV samples (Fr 6-9), while undetectable from protein fractions (Fr 10-14) and FF samples before EV isolation (FF). Albumin, Grp94, and apoA-I were used as markers of negative selection demonstrating diminished signal intensity in EV samples compared to the protein fraction and FF samples. (**D**) Transmission electron microscopy analysis of purified EVs, indicated by arrows. Data in (**A**,**B**) is demonstrated as mean ± SEM. * p = 0.04 Student's t-test.

Tetraspanins CD63, CD81, and CD9, considered as positive EV markers, were used to verify the presence and enrichment of extracellular vesicles in EV preparations. Based on WB analysis (Figure 2C), all tetraspanins were enriched in EVs, whereas the protein fractions of FF and non-purified

FF had undetectable levels of the studied EV markers. Endoplasmic reticulum protein Grp94, which is expected to be absent or under-represented in smaller EVs, notably exosomes [18], was indeed absent from EV samples, whereas protein fractions and non-purified FF were positive for Grp94. The purity of EVs and the efficiency of SEC was also tested by analyzing the presence of albumin and apolipoprotein A-I (apoA-I) as these proteins are often co-isolated with EVs. Strong signals were detected in FF samples, and although both of these proteins were also detectable in EV samples, the largest quantities were enriched in later fractions (Fr 10–14) corresponding to protein enrichment (Figure 2C). TEM analysis also confirmed the presence of EVs in the studied samples (Figure 2D).

2.2. Small RNA Profile of Granulosa Cells, Cell-Free Follicular Fluid and Extracellular Vesicles

Whole-genome small RNA sequencing was performed for all three sample types (MGC, FF and EV) to model intrafollicular signaling for eight oocyte donors and seven PCOS patients (Table 1). The sequencing depth and mapping efficiency for each sample type are represented in Supplementary Table S1. Analysis of sequencing read size distribution after adapter trimming demonstrated different patterns of small RNA sequence lengths between sample types (Supplementary Figure S3) referring to distinct variability in their content of small RNA populations. A prevalent size peak at 17–24 nt corresponding to the length of miRNAs appears in all samples. In addition, MGC and FF samples contain small RNAs of 28–36 nt length not detected in EV samples. Average sequence length is also shorter in EV samples compared to FF and MGC samples ($p < 0.005$, Supplementary Table S1). The current study focuses further on the miRNA content of each sample type.

2.3. miRNAs in Granulosa Cells, Cell-Free Follicular Fluid and Extracellular Vesicles

In total 1525 unique miRNAs were detected by at least one read: 658 miRNAs were observed in EVs, 1060 in FFs and 1381 in MGCs. All sample types share a large proportion of the most abundant miRNAs among the top 20 most represented sequences in every sample type (EV and FF share 15, FF and MGC share eight out of 20, Supplementary Table S2).

Clustering analysis revealed significant differences between sample types according to their miRNA content (Figure 3A,B). As expected, EV and FF samples cluster closer to each other in comparison to MGC samples (Figure 3A), as EV samples are a sub-compartment purified from the corresponding FF samples.

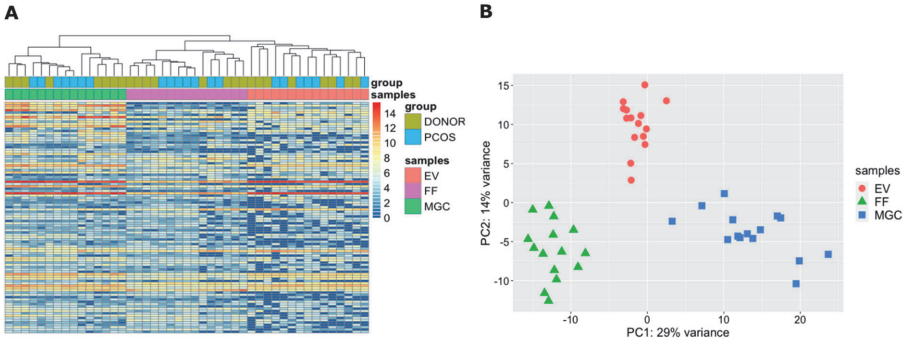

Figure 3. Cluster analysis of investigated samples and study groups. (**A**) Hierarchical clustering of top 100 most variable miRNAs across all samples. Results are depicted by DESeq2 normalized counts on $\log_2$ scale. (**B**) Principal component analysis based on expressed miRNAs per sample type. EV—extracellular vesicles, FF—cell-free follicular fluid, MGC—mural granulosa cells, PCOS—polycystic ovary syndrome.

2.4. Cellular and Extracellular miRNAs in the Healthy Ovarian Follicle

The analysis of samples from oocyte donors ($n = 8$) representing the healthy ovary revealed that in total 172 miRNAs are common to all three sample types (>5 reads observed in >50% of samples per

sample type). A set of 124 miRNAs were only present in MGC samples indicating that these are not secreted out of the cells (Figure 4A, Supplementary Table S3).

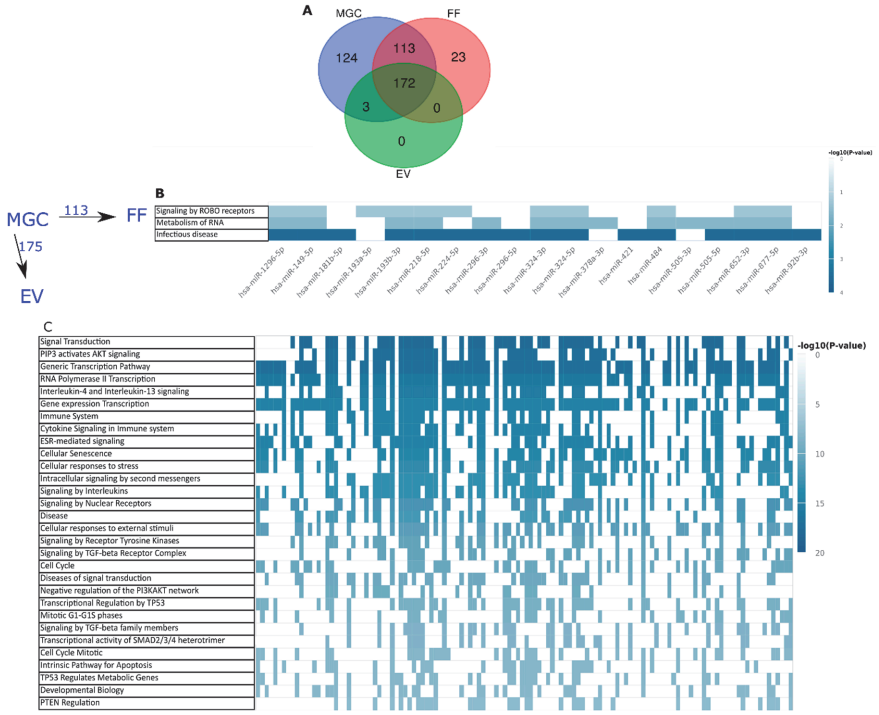

Figure 4. Cellular and extracellular miRNAs observed in ovarian follicles of fertile women. (**A**) Distribution of observed miRNAs (>5 reads in >50% of samples) between sample types. (**B**) Pathways over-represented by the targets of 113 miRNAs secreted by mural granulosa cells (MGC) into follicular fluid (FF) outside of extracellular vesicles (EV). (**C**) Top 30 pathways potentially regulated by 175 miRNAs secreted into FF in EVs. Each column in (**B**,**C**) corresponds to one miRNA regulating a pathway, if marked in blue.

EVs did not contain any unique miRNAs compared to FF or MGC. Three miRNAs (hsa-miR-374a-5p, hsa-miR-190a-5p and hsa-miR-196a-5p) were shared only between EV and MGC samples and not present in FF. We hypothesize that these miRNAs are specifically enriched into EVs. In the FF samples that contain RNA molecules present also in other forms these miRNAs remain below detection limit.

Moreover, MGC samples share 285 miRNAs with FF. Twenty-three miRNAs are exclusive to FF samples. Comparison to a recent study [19] analyzing female serum and plasma miRNAs extracted with the same protocol as in the current paper revealed that 10 of the miRNAs observed uniquely in FF potentially derive from plasma infiltrating into the follicle from perifollicular capillaries (Supplementary Table S3).

A list of 175 miRNAs were detected in EVs indicating that these miRNAs are secreted into the follicular space in vesicular form. At the same time, 113 miRNAs were common to MGC and FF only (Figure 4A), suggesting that these are secreted from cells in complexes other than EVs with characteristic surface markers. By comparing these two lists we were interested to test, if the secretion of miRNAs in EVs serves a different signaling purpose in comparison to other mechanisms of miRNA secretion. We investigated, if the miRNAs inside or outside of EVs have a potential to regulate overlapping signaling pathways. Indeed, only three pathways were commonly targeted by 11–16 FF miRNAs residing in FF outside of EVs (Figure 4B, Supplementary Table S4A). On the other hand, 436 pathways

were over-represented for miRNAs in EVs, the top 30 of the pathways were common to 30-94 miRNAs (Figure 4C, Supplementary Table S4B). The 23 miRNAs observed only in FF were omitted from the pathway over-representation analysis, as these are not probably secreted by MGC as mentioned above.

Significant enrichment depicting RNA secretion from cells to FF and the difference between miRNAs loaded into EVs in comparison to all cell-free miRNAs was analyzed by differential expression (DE) analysis in control group samples (FF vs. MGC and EV vs. FF, respectively, as depicted in Figure 5A).

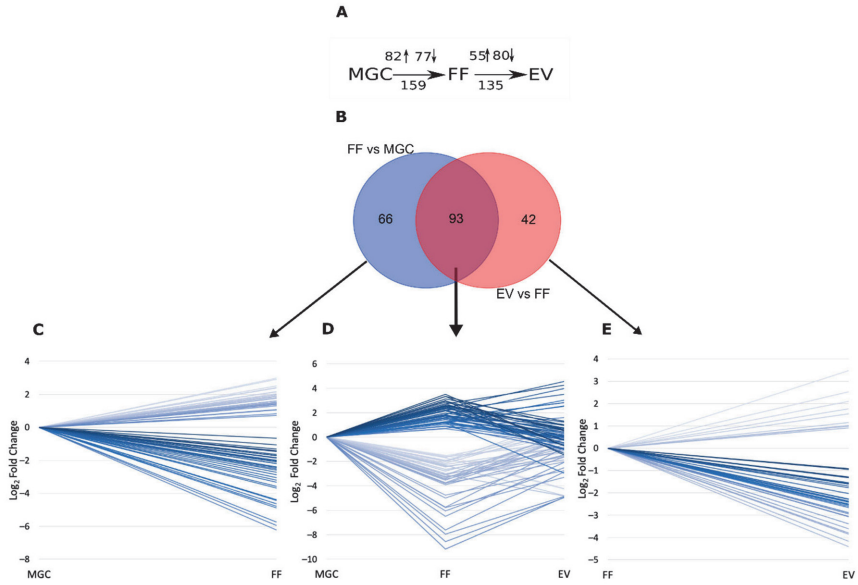

Figure 5. Comparison of cellular and cell-free miRNA expression levels. (**A**) Schematic representation of miRNA expression level comparisons performed between sample types and the number of differentially expressed (DE) results obtained. Arrows depict the number of upregulated (↑) or downregulated (↓) genes in each comparison. (**B**) Number of distinct and shared DE miRNAs between comparisons. (**C**) DE results unique to FF vs. MGC comparisons (**D**) DE results common for FF vs. MGC and EV vs. FF comparisons. (**E**) DE results unique to EV vs. FF comparisons. Each line in panels (**C**–**E**) depicts the statistically significant (FDR < 0.05) expression level difference between sample types of one miRNA. EV—extracellular vesicles, FF—cell-free follicular fluid, MGC – mural granulosa cells.

Comparison between FF and MGC samples, resulted in 159 differentially expressed miRNAs (FDR < 0.05, Figure 5A, Supplementary Figure S4A, Supplementary Table S5), while the DE analysis of EV and FF samples demonstrated the statistically significant expression of 135 miRNAs (FDR < 0.05, Figure 5A, Supplementary Figure S4B, Supplementary Table S6). From all differentially expressed miRNAs 93 were common to both comparisons: FF vs. MGC and EV vs. FF (Figure 5B). Figure 5D illustrates the expression level changes of those 93 miRNAs throughout MGC, FF and EV samples. Figure 5C,E illustrates miRNA expression changes of 66 and 42 miRNAs differentially expressed between FF vs. MGC or EV vs. FF, respectively. Significant miRNA expression changes between the analyzed compartments indicate potentially different secretion mechanisms: miRNAs with constantly increasing levels from MGC to FF and EV are more probably packed specifically into EVs. In contrast, miRNAs with the highest abundance in FF have a higher probability to be secreted into extracellular space in other macromolecular complexes.

2.5. Differences in miRNA Expression between PCOS Patients and Oocyte Donors

A comparison of samples between patient groups revealed that 30 and 10 miRNAs in MGC and FF, respectively, were differentially expressed between PCOS and oocyte donor patients (FDR <0.1, Figure 6A,B, Supplementary Table S7A,B). Due to a higher variation of miRNA expression levels across patients in EV samples (Figure 3A), no miRNAs reached the same FDR cut-off level. However, seven miRNAs were differentially expressed in EV samples between the two groups without considering the FDR (p-value < 0.05, Figure 6C, Supplementary Table S7C). Hsa-miR-200c-3p was the only commonly up-regulated miRNA in the extracellular samples FF and EV. All other DE miRNAs between the patient groups were unique to each sample type.

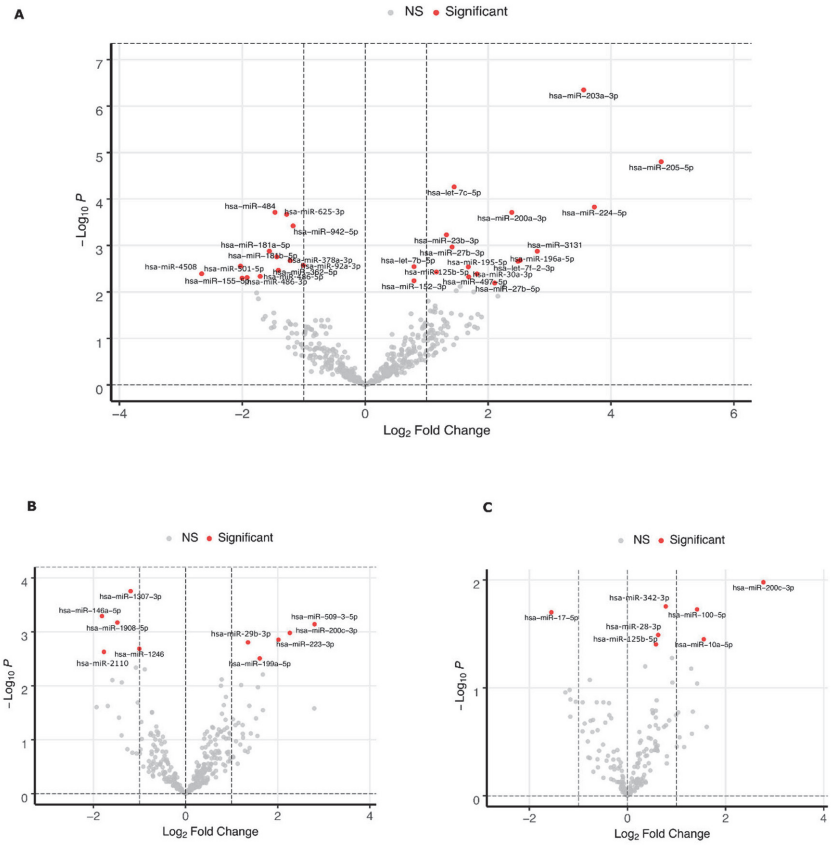

Figure 6. Differentially expressed miRNAs between oocyte donors and PCOS patients in mural granulosa cell samples (**A**), cell-free follicular fluid samples (**B**) and extracellular vesicle samples **(C)**. Statistical significance cut-off is FDR < 0.1 (A and B) or p < 0.05 (**C**).

Validation of the RNA sequencing results was performed by RT-qPCR for miRNAs with the highest fold change and that have been previously related to ovarian functions (Table 2). Since oocyte donors are generally young women the average age difference between the two study groups in RNA sequencing experiment was statistically significant (p-value = 0.002, Table 1). As there is previous evidence that the expression of some miRNAs can be affected by age [20,21], a validation cohort of age-matching women undergoing IVF due to male-factor infertility was added to the oocyte donor samples used for RNA sequencing. The average age difference between the PCOS and RT-qPCR validation control group was therefore not statistically significantly (p-value = 0.626, Table 1).

From five of the validated miRNAs in MGC samples the expression change direction was confirmed with statistical significance for hsa-let-7c-5p, hsa-miR-196a-5p and hsa-miR-203-3p. In FF samples four of the five validated miRNAs presented same directional change, out of which two (miRNAs hsa-miR-509-3-5p and has-miR-200c-3p) were also statistically significant. All three validated miRNAs from EVs displayed the same directional change without reaching the statistically significant level (Supplementary Figure S5).

Table 2. Differentially expressed miRNAs between PCOS patients and oocyte donor samples selected for RT-qPCR validation.

A) MGC PCOS vs. donors:		
miRNA	**Log_2 Fold Change**	**miRNA role in ovary**
hsa-miR-205-5p	4.82	Expression is upregulated in MI oocytes upon IGF-1 treatment [22]. Upregulated in ovarian cancer (OC) cells compared to control group and is associated with poor survival rates. Proposed miRNA targets are SMAD4 and PTEN [23].
hsa-miR-203a-3p	3.56	Expression levels are higher in granulosa cells of young women with normal ovarian reserve compared to young women with diminished ovarian reserve [24].
hsa-miR-196a-5p	2.49	Detectable in bovine granulosa cells at day 3 but not at day 7 of the estrous cycle [25].
hsa-let-7c-5p	1.45	Expressed higher in human CGC compared to MGC cells. miRNA expression is decreased in granulosa cells of early and progressive atretic follicles and in case of premature ovarian failure syndrome (measured from plasma) [26].
hsa-miR-181a-5p	−1.56	Expressed higher in human CGC compared to MGC [27]. In mouse granulosa cells miR-181a-5p targets ACVR2A (Activin Receptor IIA) and inhibits granulosa cell proliferation [28]. In oxidative stress conditions miRNA expression is upregulated in mouse granulosa cells and mediates granulosa cell apoptosis [29].
B) FF PCOS vs. donors:		
miRNA	**Log_2 Fold Change**	**miRNA role in ovary**
hsa-miR-509-3-5p	2.80	Expression is higher in FF of PCOS patients compared to controls [30].
hsa-miR-200c-3p	2.26	Expression is higher in granulosa cells [31] as well as in FF samples [32] of PCOS patients compared to control group.
hsa-miR-223-3p	2.02	EVs obtained from FF show expression of hsa-miR-223-3p [33]. miRNA expression is decreased in cumulus cells of PCOS patients [34].
hsa-miR-1908-5p	−1.48	Low expression predicts poor prognosis for ovarian cancer [35].
hsa-miR-146a-5p	−1.81	Expression is higher in human MGC samples compared to CGC [36].
C) EV PCOS vs. donors:		
miRNA	**Log_2 Fold Change**	**miRNA role in ovary**
hsa-miR-200c-3p	2.77	Expression is higher in granulosa cells samples [31] as well as in FF [32] obtained from PCOS patients compared to control group.
hsa-miR-100-5p	1.42	Associated with cell proliferation regulation [37]. Downregulated in young women with diminished ovarian reserve compared to normal ovarian reserve [37].
hsa-miR-17-5p	−1.55	Expression is downregulated in granulosa cells and FF of PCOS women compared to controls [38]. miRNA expression is detected in EVs obtained from FF [33].

2.6. Distinctive Functions Are Dysregulated in Each Analyzed Follicular Compartment of PCOS Patients

As one miRNA may target several genes and one gene may be targeted by several miRNAs, we subsequently aimed to detect any common pathways changed in PCOS patients by the differentially expressed miRNAs. Reactome pathway over-representation was performed for all differentially expressed miRNA-s according to RNA sequencing in each sample type. Lists demonstrating higher and lower miRNA expression levels in PCOS group compared to controls were analyzed separately.

miRNAs that are significantly upregulated in the PCOS group regulate in total 20 pathways in MGC, 25 pathways in FF and 13 pathways in EV (Figure 7, Supplementary Table S8). Transcription regulation and cell cycle pathways constitute the majority of the over-represented terms in MGC.

In addition, miRNAs regulating signaling by estrogen receptors (ESR) and nuclear receptors in general were more abundant in the MGC of PCOS patients.

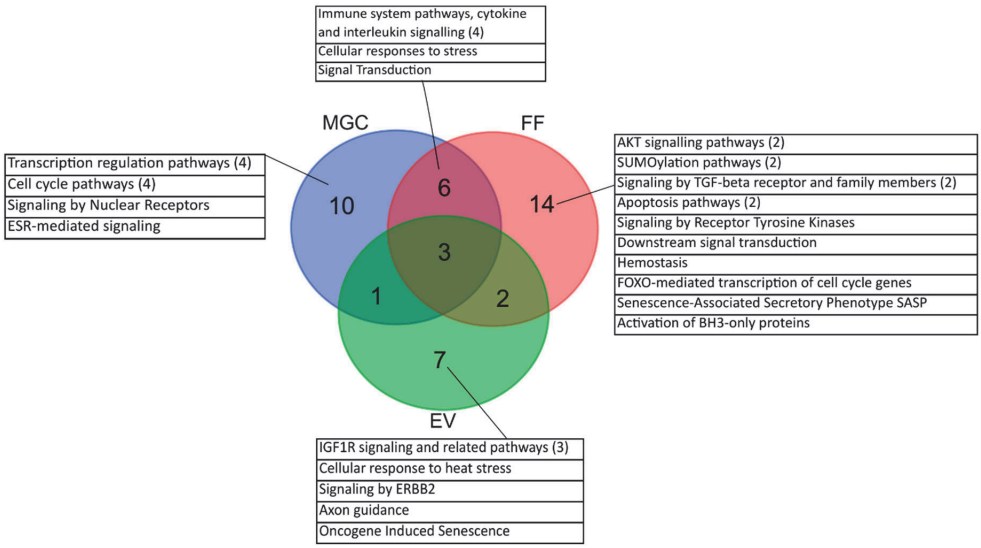

Figure 7. Number of Reactome pathways over-represented for miRNAs that are more abundantly expressed in PCOS patients compared to the fertile control group in each sample type. Numbers in brackets refer to combined pathways with similar outcome. EV—extracellular vesicles, FF—cell-free follicular fluid, MGC—mural granulosa cells.

Over-represented miRNAs in FF regulate several signal transduction pathways: AKT, TGF-beta, as well as pathways related to apoptosis and protein modification by SUMOylation appeared as the most frequent terms. The most common terms for miRNA-s that were significantly more abundant in the EVs of PCOS women were related to IGF1R signaling pathways.

In addition, miRNAs up-regulated in the MGC and FF samples of PCOS women target common immune system related pathways that are not apparent predicted targets for miRNAs in the EVs.

miRNAs that were less abundant in the MGC of PCOS patients are involved in pathway "Cellular responses to external stimuli". No over-representation of pathways was achieved for miRNAs with low abundance in FF and EV in the PCOS group (Supplementary Table S8).

2.7. Potential Novel miRNA as Marker for Follicular EVs

RNA sequencing data can be useful for predicting novel, yet unannotated miRNAs. After filtering candidate novel miRNA sequences suggested by miRDeep2 algorithm, we propose one potential new miRNA (mature sequence: CCUGGGCAUGGGACUGG, predicted stem-loop sequence in Figure 8A) that was expressed in 12 different patients and in all three sample types (EV, FF and MGC). It was most frequently detected in EV samples (in nine EV samples, four FF, and five MGC samples) demonstrating significantly higher expression levels compared to FF and MGC samples (Figure 8B). Moreover, validation with RT-qPCR demonstrated significantly higher levels of this sequence in EVs compared to FF samples (Figure 8B). The expression levels of the novel miRNA did not differ between PCOS and donor group in any of the sample types (data not shown). Five previously annotated miRNAs share a similar seed sequence (nucleotides 2–8 from 5′ end) with the potential novel miRNA (Figure 8C). miRDB predicted 1430 potential targets for the novel miRNA that were further enriched into 82 terms in the ontology domain of biological processes (FDR < 0.05, Supplementary Table S9). These were further reduced by semantic similarity analysis to three

largest categories: regulation of cell communication, cell junction organization, and nervous system development (Supplementary Figure S6).

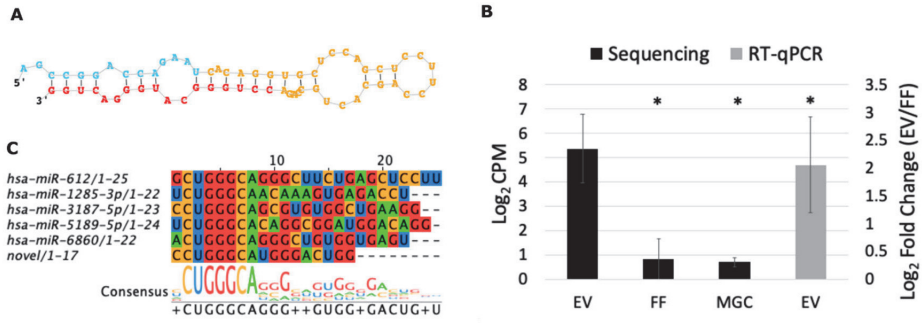

Figure 8. Novel miRNA detected by small RNA sequencing of single follicle components. (**A**) Predicted stem-loop sequence by miRDeep2. (**B**) Novel miRNA expression levels in the extracellular vesicles of follicular fluid (EV), cell-free follicular fluid (FF) and in granulosa cells (MGC) according to RNA sequencing displayed as a mean of count per million (CPM) ±SEM on $\log_2$ scale (n = 15). RT-qPCR validation result is displayed as fold change ±SEM on $\log_2$ scale (n = 15). (**C**) Novel miRNA aligned to previously annotated miRNAs with similar seed sequence. * $p < 0.05$, Student's t-test.

3. Discussion

A key aspect of cellular and organismal homeostasis in the higher mammals is intercellular communication, where cells are required to communicate with each other in order to maintain the vital functions of the body. Some of the important mediators of this cell-cell communication are cell-free RBPs and nanoparticles, including EVs that contain molecules from a plethora of RNA biotypes [6]. All body fluids, including the follicular fluid, are rich sources of cell-free nucleic acids and EVs [39]. EVs are heterogeneous, and their subtypes; exosome and microvesicles share a similar size range, which imposes a challenge in their efficient isolation, purification, and separation [40]. There are several different methods used for EVs isolation, and every method has its own limitations towards the purity of EVs from other RNA-containing particles. According to the Minimal Information for Studies of Extracellular Vesicles 2018 guidelines (MISEV) [39], purification methods should be chosen based on the downstream application of EVs. In the current study the MISEV-approved SEC method that gives the flexibility to separate the EVs according to their size ranges was used to acquire pure and functional EVs with reasonable recovery rates [41–43].

Several studies suggest that EVs are involved in intercellular communication in both normal physiology and pathological condition [44]. A recent study presented the higher blood plasma concentration of platelet-derived microparticles in PCOS patients as compared to healthy women [45]. Our study additionally detected significantly more EVs in the FF of PCOS women compared to oocyte donors with normal ovarian morphology.

We have, for the first time, analyzed matched cellular and extracellular small RNA profiles from individual pre-ovulatory follicles by whole-genome small RNA sequencing. In addition, the extracellular small RNAs were analyzed in two fractions: small RNAs loaded into EVs and total cell-free small RNAs present in FF. While separate RNA sequencing studies of MGC [27], FF [9] and EV [9] samples from the human follicle are available, combining the data from the three compartments of the follicle as a closed biological system is unique to the current study. The functionality of extracellular RNAs can roughly be divided into three: known function, predicted function and unknown function [46]. MiRNAs belong into the known function group and therefore we continued to investigate the miRNA profiles of the samples. We observed that the miRNA profile clearly distinguishes the three sample types indicating that the miRNAs are released in EV- and non-EV-mediated mechanisms serve distinct functional purposes. To support our results, it has been

demonstrated that the cell-free and EV-associated miRNAs also have different profiles in matching plasma samples [47,48].

There are many possible sorting mechanisms proposed for loading miRNAs into EVs: sequence characteristics, post-transcriptional modifications, subcellular location and intracellular concentration have all been shown to pose an effect to the segregation of miRNAs into EVs [6]. The heterogeneity of EVs (exosomes, microvesicles or apoptotic bodies) additionally plays a role in the nucleic acid content [49]. An even larger proportion of extracellular small RNAs is secreted outside of EVs in composition of other macromolecular complexes, e.g., non-vesicular RBPs. The mechanisms by which proteins interact with miRNAs before secretion remain unclear and may depend on the particular protein in the complex, but the export of miRNAs via these pathways has been shown to be an energy-dependent process [6].

According to our study, 172 miRNAs were present in all sample types, indicating that those miRNAs are non-selectively secreted from cells via both methods (EVs and/or non-vesicular RBP pathway). Moreover, 113 miRNAs were detected only in cells or FF, meaning those miRNAs are likely secreted out of the cells in RBP, while 175 miRNAs were observed also in the EVs. The over-representation analysis of the EV and FF miRNA targets indicate that miRNAs loaded into EVs carry specific molecular signals. It is considered that cargo segregation into EVs is not a random event: for example the human bone marrow- and adipose-mesenchymal stem cell-derived exosomes contain distinctive small RNA molecules linked to their differentiation status [50]. Moreover, studies in the equine and bovine models have demonstrated that follicular fluid EVs were taken up by granulosa cells in vitro and this process affected the expression of genes involved in follicle development [14,51]. Furthermore, some miRNAs in follicular EVs may also regulate oocyte growth, as alterations in their expression were observed between follicles with different oocyte maturation stages [52]. All these studies lead to a conclusion that EVs in follicular fluid most likely have important regulatory roles.

The miRNAs in EV samples potentially target the following Reactome terms: "ESR-mediated signaling", "Signaling by Nuclear Receptors", and "PTEN Regulation", among others. Estrogen receptors belong to the nuclear receptor family and together with other members, like the androgen receptors, are involved in follicle development and ovulation [53,54]. PTEN participates in follicle activation and growth, higher PTEN levels being associated with poor oocyte competence [55,56]. To summarize, miRNA profile analysis of samples from fertile women indicates specific miRNA segregation into vesicles with various targeted pathways downstream. At the same time, the selection of miRNAs for secretion by non-EV mediated pathway tends to be a relatively random event in the follicle.

One may argue whether all the miRNAs detected in extracellular space participate in cell-to-cell signaling and what the relevant concentration of EV-, or protein-bound miRNAs would be to have a physiologically significant function. Chevillet et al. have calculated that EVs contain less than 1 copy of miRNAs per EV [57]. In sequencing experiments the small RNA cargo is analyzed in bulk, therefore there is no information whether all individual EVs are equally loaded with miRNA molecules or if there is a certain distribution of cargo into different EVs. A specific segregation of miRNA molecules may significantly raise the copy number of individual miRNAs per EV as it is proposed with low-occupancy/high-miRNA concentration distribution model indicating that rare EVs in population contain many copies of a certain miRNA [57].

As the main goal of this study, we were interested, whether the ovarian miRNA expression, secretion, or segregation into EVs are compromised by PCOS. We observed that each studied follicular compartment is affected differently by PCOS, since the miRNA profiles are dissimilar in MGC, FF and EV also in the fertile ovary. Therefore, separate pathways are affected by PCOS in MGC and in the potential recipient cells for the extracellular miRNA in the FF and EV.

Several miRNAs have been shown to be differentially expressed in the granulosa cells and/or FF between fertile and PCOS women [9,30,58,59]. In the current study, the highest number of differences in miRNA expression were detected from cellular material. At single follicle level the EV samples

diverged the least between the two groups, although some differences could be detected without multiple testing adjustment. Fewer differences in EV may be caused by the more complex processing of EV samples before RNA extraction compared to MGC and FF that may affect the results.

We identified several miRNAs differentially expressed between the patient groups which have not been previously associated with PCOS but are involved in the regulation of gene expression in follicles or in other ovary-related disorders. For example, hsa-miR-224-5p [60], which was up-regulated in the MGC of PCOS women in our study, downregulates SMAD4, which is involved in the regulation of apoptosis of granulosa cells [61]. It has been shown that hsa-miR-203a-3p, hsa-miR-195-5p, hsa-miR-486-3p, and hsa-miR-484 levels are altered in the granulosa cells of women with diminished ovarian reserve [24]. Interestingly, all the four mentioned miRNAs are expressed in the MGC of our PCOS group according to the same pattern as in normal ovarian reserve (NOR) patients: hsa-miR-203-3p and hsa-miR-195-5p are both more abundant in NOR and our PCOS group, while hsa-miR-486-3p and hsa-miR-484, are less abundant in NOR as well as in our PCOS samples. PCOS women have been shown to have a slower age-related decline in antral follicle count (AFC) compared to non-PCOS patients [62] and AFC is considered to be a reliable marker for ovarian reserve evaluation [63]. Slower decline in AFC in PCOS women may also explain our miRNA expression results.

The expression differences of hsa-miR-486-5p [64] in MGC have been previously associated with PCOS compared to women undergoing IVF due to male factor infertility with same directional expression as in our study. Moreover, hsa-miR-200a-3p [34] and hsa-miR-30a-3p [32] have been previously linked to PCOS, but in other follicular compartments. In these studies, hsa-miR-200a-3p in cumulus granulosa cells and hsa-miR-30a-3p in FF were less abundant in PCOS women which is opposite to our results. Differential expression of hsa-miR-509-3-5p [30] and hsa-miR-200c-3p [30,31] in FF has been previously associated with PCOS with mutual expression direction to our results. Hsa-miR-1307-3p [65] and hsa-miR-223-3p [34] are also altered in cumulus granulosa cells of PCOS patients, but with an opposite direction to our result. These comparisons demonstrate the dependence of miRNA expression disturbances depending on the cellular environment. By the pre-ovulatory follicular stage, when the samples have been obtained, cumulus granulosa cells have differentiated from MGC and have significant dissimilarities in gene expression and post-transcriptional regulation patterns [27,66]. From EV samples hsa-miR-200c-3p [30,31], hsa-miR-17-5p [38] have been previously shown to be altered in PCOS women in line with the results of our study.

Our results clearly demonstrate that the effects of miRNA expression differences brought upon PCOS lead to different molecular outcomes depending on the investigated sample type. For example, cytokine-mediated signaling was affected in the cellular compartment and by non-EV-mediated RNA secretion, while EV-mediated signaling potentially affects the IGF1R pathways in PCOS patients. Those results emphasize the importance of studying the follicle as a system to better understand inter-cellular signaling and possible molecular disturbances in the PCOS ovary.

In conclusion, the current study proposes novel miRNAs and their regulated signaling pathways, underlying the infertility of patients with PCOS.

4. Materials and Methods

4.1. Ethics Statement

The study was approved by the Research Ethics Committee of the University of Tartu, Estonia on January 21st, 2019 with the approval number 289/M-8. Written informed consent was obtained from all participants.

4.2. Patients and Sample Collection

FF and MGCs were collected from women undergoing ovarian stimulation and oocyte pick-up by ovarian puncture (OPU). Ovarian hormonal stimulation was conducted according to the gonadotropin-releasing hormone (GnRH) antagonist (Cetrotide, Merck Serono, Darmstadt, Germany)

protocol with the administration of recombinant follicle-stimulating hormone (Gonal-F, Merck Serono, or Puregon, Merck Sharp & Dohme Corp., Whitehouse Station, NJ, USA). All patients underwent OPU if at least two follicles were ≥18 mm in size 36 h after human chorionic gonadotropin administration (Ovitrelle, Merck Serono).

Samples were collected from two groups of women: PCOS patients (n = 15) and fertile women (IVF patients from couples with male factor infertility (n = 16) and oocyte donors (n = 15)). General characteristics of study participants are presented in Table 1. The PCOS group was formed according to the Rotterdam Consensus [67] with PCOM observed by ultrasound being the primary criterion for recruitment. Control group consisted of women with regular menstrual cycles, without any infertility diagnosis nor PCOM. All recruited women were <40 years of age.

FF containing all cellular material was collected from the first aspirated follicle visibly clear of blood contamination. The sample was first centrifuged 10 min at 300 g to remove whole cells. The supernatant was subsequently centrifuged 10 min at 2000× g to remove cell debris. The final cell-free FF was stored at −80 °C until further analysis. The remaining cell pellets of MGCs from the first centrifugation were lysed using QIAzol Lysis Reagent (QIAGEN, Hilden, Germany) and stored at −80 °C until RNA extraction.

4.3. Isolation of Extracellular Vesicles from Follicular Fluid Samples

Five hundred μL of each FF sample was concentrated to 150 μL using 10 kDa Amicon® Ultra centrifugal filter units (Merck Millipore Ltd., Tullagreen, Carrigtwohill, Ireland). Commercially available size exclusion chromatography (SEC) column (qEVsingle/70 nm by Izon Sciences, UK) was used for the isolation of EVs. The column was prewashed with 10 mL filtered (0.2 μm Minisart® syringe filters) Dulbecco's phosphate-buffered saline (DPBS, Sigma® Life Science, UK) and 150μl of the concentrated sample was added to the top of the column filter. After the sample had passed down, DPBS was added immediately on the top of the column filter and a total of 20 fractions, 200 μL each, were collected separately. The concentration of nanoparticles (NP) of each fraction was measured on ZetaView® nanoparticle tracking analyzer (NTA, PMX 120 by Particle Metrix GmbH, Inning am Ammersee, Germany). The protein concentration of each fraction was determined with the Quick Start™ Bradford Protein Assay (Bio-Rad, California, USA) according to the manufacturer's protocol. Based on these analyses, fraction 6-9 (800 μL) showed the presence of the highest number of particles and the least protein contamination Supplementary Figure S1). Fractions 6–9 were pooled together, concentrated with 10 kDa Amicon® Ultra 2 centrifugal filter units, and used for downstream experiments.

4.4. Nanoparticle Tracking Analysis

The size profile and concentration of NPs/EVs in the samples were carried out using the ZetaView® nanoparticle tracking analyzer. A standard of 100 nm particles (Applied Microspheres BV, Leusden, the Netherlands) was used for instrument calibration. During the analysis, standard manufacturer's procedure was followed for NPs/EVs size distribution and concentration measurement. The size profile and concentration of NPs/EVs were measured using the scatter mode under the following settings: sensitivity 85, shutter speed 70, frame rate 30 frames per second, and the number of cycles 3. All samples were measured in triplicates. In order to minimize the inter-sample contamination, the measurement cell of the instrument was washed thoroughly using Milli-Q® water, and the cell was filled with DPBS before the injection of the next sample.

4.5. Western Blot Analysis

Fractions 6–9 (contain follicular fluid EVs) and fractions 10–14 (contain FF proteins) were obtained by SEC as described earlier. Respective fractions were pooled and concentrated with 10 kDa Amicon® Ultra-15 centrifugal filter to 300 μL. To precipitate the proteins, 100 μL of water, 400 μL of methanol (Sigma-Aldrich, Schnelldorf, Germany), and 100μl of chloroform (Lach-Ner, Neratovice, Czech Republic) were added to the concentrated sample and centrifuged for 5 min at

14,000× *g*. The top layer was removed, and proteins in the interphase were washed with 400 µL of methanol. After centrifugation, the pellet was dried, resuspended in 0.25% SDS, and the protein concentration was measured with Bradford assay. Proteins from 50µl of FF were precipitated using the same protocol. For each sample, 10 µg of protein was mixed with either non-reducing Laemmli buffer or reducing Laemmli buffer, heated for 5 min at 95 °C and separated by 12% SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride membranes (Thermo Scientific, Rockford, IL, USA), and the membranes were incubated in blocking buffer (5% nonfat dry milk in PBS-Tween 0.05%) for 1 h at room temperature (RT). Subsequently, the membranes were incubated with the following primary antibodies overnight at 4 °C: mouse anti-human CD63 antibody (556019, 1:1000, BD Biosciences, San Jose, CA, USA), mouse anti-CD9 antibody (sc-59140, 1:250, Santa Cruz Biotechnology Inc., Dallas, TX, USA), mouse anti-apoA-I antibody (sc-376818, 1:1000, Santa Cruz Biotechnology Inc.), mouse anti-human CD81 antibody (555675, 1:1000, BD Biosciences), rabbit anti-Grp94 antibody (ADI-SPA-851-D, 1:1000, Enzo Life Sciences, Farmingdale, NY, USA) and rabbit anti-albumin antibody (16475-1-AP, 1:10 000, Proteintech, Chicago, IL, USA). Membranes were washed with PBS-Tween 0.05% and then incubated with either HRP-conjugated goat anti-rabbit IgG secondary antibody (G21234, 1:20 000, Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA) or goat anti-mouse IgG secondary antibody (G21040, 1:20 000, Invitrogen, Thermo Fisher Scientific) for 1 h at RT. After washing the membranes with PBS-Tween 0.05% and incubating in ECL Select Western Blotting Detection Reagent solution (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK), the protein bands were visualized using ImageQuant RT ECL Imager (GE Healthcare).

4.6. Transmission Electron Microscopy

Fractions 6-9 (800 µL) of isolated EVs on SEC were pooled and subsequently concentrated to 150µl using Amicon® Ultra 2 centrifugal filter units (10 kDa) (Merck Millipore Ltd.). A previously described method [39] was followed for *transmission electron microscopy* (TEM) analysis. A droplet from the purified EV samples was deposited on Formvar-carbon-coated 200 mesh copper grids (Agar Scientific, Essex, UK) and allowed to absorb for 20 min. The sample was fixed on a grid in 2% paraformaldehyde (Sigma-Aldrich) and 1% glutaraldehyde (Polysciences, Warrington, PA, USA), contrasted in uranyl oxalate (a mixture of 4% uranyl acetate (Polysciences) and 0.15 M oxalic acid (Sigma-Aldrich)) and embedded in a mixture of methylcellulose (Sigma-Aldrich) and uranyl acetate (Polysciences). Samples were observed with a JEM 1400 transmission electron microscope (JEOL Ltd. Tokyo, Japan) at 80 kV, and digital images were acquired with a numeric camera (Morada TEM CCD camera, Olympus, Germany).

4.7. RNA Extraction

miRNA extraction from isolated EVs was performed using miRNeasy Micro kit (QIAGEN) according to the user manual with the exception of 5 µg of glycogen (Thermo Scientific) added to chloroform.

Starting amount of miRNA extraction from FF was 500 µL. Extraction was performed with miRNeasy Micro kit (QIAGEN) with some modifications to the user manual [68]. Shortly, 500 µL of FF was transferred into a 15 mL tube and 5x volumes of QIAzol Lysis Reagent (QIAGEN) was added. After incubation 500 µL chloroform and 5 µg of glycogen (Thermo Scientific) were added to the tube. Following steps of RNA exactions were performed according to the miRNeasy Micro kit (QIAGEN) user manual.

Total RNA from cells was extracted with miRNeasy Mini kit (QIAGEN). In addition, small fraction RNA (≤200 nucleotides) was separated by RNeasy Mini Elute Cleanup Kit (QIAGEN). Both total and small RNA extraction were performed according to the user manual.

The quality and concentration of cellular RNA samples was evaluated on Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

4.8. Small RNA Library Preparation and Sequencing

Small RNA libraries were prepared with QIAseq miRNA Library Kit (QIAGEN) according to the manufacturer's protocol. Starting amount of RNA in library preparation was 10 ng from cellular small RNA fraction and 5 µL of RNA from EV and FF samples. Final libraries were separated and excised from 5% TBE gels (Bio-Rad Laboratories) after staining with 1X SYBR Gold stain (Thermo Fisher Scientific). Gel pieces containing the miRNA libraries were crushed with pellet pestles (Fisher Scientific). 300 µL RNase free water (Thermo Fisher Scientific) was added to the gel debris and rotated for 2 h at RT to elute miRNA libraries. Eluate and gel debris were transferred to the Spin X centrifuge tube filter (Merck, Darmstadt, Germany) and centrifuged 2 min at 16,000 g. Thereafter 2 µL glycogen (Thermo Scientific), 30 µL 3 M NaOAc (Thermo Fisher Scientific), 1 µL 0.1x Pellet Paint (Merck) and 975 µL of cold 100% ethanol (Naxo, Tartu, Estonia) were added to the eluate, and centrifuged for 20 min at 20,000× *g* at 4 °C. Pellet was washed with 500 µL of 70% ethanol and centrifuged for 2 min at 20,000× g. The final libraries were resuspended in 7 µL of resuspension buffer (PerkinElmer, Massachusetts, USA). The size of libraries was estimated with Agilent DNA High Sensitivity chips on the Agilent 2100 Bioanalyzer system (Agilent Technologies). Library concentrations were measured using Qubit High Sensitivity Assay kit (Thermo Fisher Scientific) before pooling in equimolar amounts. Single-end sequencing of 75 bp length was performed on NextSeq 500 platform with NextSeq 500/550 High Output Kit v2.5 (Illumina, San Diego, CA, USA).

4.9. cDNA Synthesis and RT-qPCR

For the validation of miRNA expression levels cDNA was synthesized using miRCURY LNA RT Kit (QIAGEN) from 30 ng of cellular small RNA fraction or 5 µL of extracted small RNA fraction from FF and EV samples.

The RT-qPCR analysis was carried out on LightCycler 480 instrument (Roche, Basel, Switzerland). For the detection of miRNA expression miRCURY LNA SYBR Green (QIAGEN) was used according to the user manual. The specificity of amplified PCR products was determined by melt curve analysis. miRCURY LNA miRNA PCR Assay primers were used in all reactions (QIAGEN).

4.10. Data Analysis and Statistics

4.10.1. miRNA Sequencing Analysis

Raw FASTQ files were quality-filtered with Trimmomatic v 0.39 [69] with the options of SLIDINGWINDOW:2:20. Adapter sequences (3'adapter AACTGTAGGCACCATCAAT and 5´ adapter GTTCAGAGTTCTACAGTCCGACGATC) were removed and reads below 17 nucleotides in length were discarded and the remaining filtered and trimmed reads were counted and mapped to the primary assembly of human genome GRCh38 using miRDeep2 with standard settings [70].

Count tables from individual samples were merged using edgeR package v.3.28.1 [71] and formed count matrix was used as input for DESeq2 v.1.26.0 [72] in R version 3.6.3 [73] for differential gene expression analysis between groups with standard options. miRNAs expressed at low level were removed from analysis: cut-off was set at ≥5 raw reads in 50% of samples. For visualization purposes, variance stabilizing transformation of data was performed with option blind = FALSE.

The statistical significance cut-off for differentially expressed miRNAs in DESeq2 analysis was set at false discovery rate (FDR) <0.05 in case of comparing three tissue types in oocyte donor samples. Cut-off for statistical significance was set at FDR <0.1 when comparing patient groups.

4.10.2. RT-qPCR Data Analysis

miRNA expression levels in cellular fraction were normalized for U6 snRNA and hsa-miR-132-3p. Endogenous control for FF and EV samples was hsa-miR-16-5p. All normalizations were performed according to the ΔΔCt method of relative quantification [74]. Statistical significance was calculated

by two-tailed Student's t-test in Microsoft Office Excel 2017. Statistical significance level was set at $p < 0.05$.

4.10.3. miRNA Target Prediction, Gene Ontology and Over-Representation Analysis

Novel miRNA targets were predicted with miRDB [75] custom prediction tool. Obtained miRNA targets list was an input for gene enrichment analysis with g:Profiler [76], using g:GOst functional profiling tool where significance threshold was set at FDR <0.05. Results were visualized with REVIGO [77].

For annotated miRNAs the lists of statistically significant differentially expressed miRNA lists were used as input to miRNA Enrichment Analysis and Annotation Tool (miEAA), that performs miRNA target prediction and over-representation analysis of gene ontology terms simultaneously by combining linked external databases [78]. Over-representation analysis was performed for Reactome Pathways via miRPathDB [79] for each up- and down-regulated miRNA list separately. Background list was created from all detected miRNAs in our small RNA sequencing dataset. Pathways targeted by >50% of miRNAs (minimum 3) in each list with Benjamini-Hochberg FDR < 0.05 are reported.

Similarly, miEAA was used to analyze Reactome Pathway over-representation for all miRNAs observed exclusively in FF or in EV samples.

4.10.4. Novel miRNA Candidate Filtering

Predicted novel miRNAs were filtered with a cut-off of miRDeep2 score >1. Remaining potential novel miRNA candidates were aligned against human transcriptome with NCBI nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), discarded if the sequences overlapped with a coding region of an annotated gene, demonstrated high similarity to other know miRNAs, were detected only in one sample or with the average occurrence in positive samples <10 raw counts. miRNA sequences with similar seed region to the potential novel miRNA were obtained from miRBase v22.1 and visualized in Jalview 2.11.1.0 [80].

4.10.5. EV Size Profile and Concentration

To test if the sample means of the EV size profiles are non-normally distributed 1000 samples of 1000 EVs were drawn from the NTA data of PCOS and donors' group, their means calculated and tested using Shapiro–Wilk test. Student's t-test was then used to test if the difference between the size profile means is statistically significant. The behavior of p-values with smaller sample size was further analyzed by drawing 1000 random samples of size 100, 1000, 2000, and 5000 EVs each and plotting the p-value histograms (Supplementary Figure S2). The difference in EV concentrations was tested using two-tailed Student's t-test and a p-value <0.05 was considered statistically significant.

4.10.6. Data Availability

The datasets generated for this study can be found in the Gene Expression Omnibus repository (GSE157037).

5. Conclusions

The current study proposes novel signaling pathways underlying the infertility of patients with PCOS. We demonstrate that the follicular environment is affected by the PCOS differently depending on the studied compartment, i.e., MGC, EV, and FF, indicating potential changes in intercellular communication in the ovaries of these patients. We predict that alterations in cellular miRNA expression levels lead to changes in estrogen receptor signaling and the dysregulation of transcription and apoptosis. EV-mediated miRNA signalization potentially affects IGF1R pathways in the recipient cells.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/24/9550/s1.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

AFC	Antral follicle count
CGC	Cumulus granulosa cells
COC	Cumulus oocyte complex
DE	Differential expression
ESR	Estrogen receptors
EV	Extracellular vesicles
FDR	False discovery rate
FF	Follicular fluid
MGC	Mural granulosa cells
NOR	Normal ovarian reserve
NP	Nanoparticles
NTA	Nanoparticle tracking analyzer
PCOM	Polycystic ovarian morphology
PCOS	Polycystic ovary syndrome
RBP	Ribo-protein complexes
SEC	Size exclusion chromatography
TEM	Transmission electron microscopy
WB	Western blot

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Curriculum vitae

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2011–2014	Tallinn University of Technology, Faculty of Science, Department of Gene Technology, Gene Technology, BSc
2008–2011	Tallinn Gustav Adolf High School

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Professional employment

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2017–2019	Nova Vita Clinic AS, embryologist/lab manager
2016–2017	BioEximi OÜ, andrology lab specialist
2012–2014	North Estonian Regional Hospital, lab technician

Scholarships & awards

2022	The best Young Scientist award at the annual conference of the Estonian Society of Human Genetics
2022	Nominated among the 5 best posters in the field of Reproduction-Endocrinology, 38th Annual Meeting of European Society of Human Reproduction and Embryology

Conferences

Sept 2022	Annual conference of the Estonian Society of Human Genetics; oral presentation
July 2022	Conference “38th Annual Meeting of European Society of Human Reproduction and Embryology”; oral and poster presentation “Heterogeneity of preovulatory follicle cell types between normo- and hyporesponders”
June 2022	FEBS3+ Conference of Estonian, Latvian and Lithuanian Biochemical Societies; poster presentation

Supervised dissertations

Robyn-Stefany Keif, Master’s Degree, 2021, (sup) Agne Velthut-Meikas; Kristine Roos, Cell types in human preovulatory follicle and their gene expression, Tallinn University of Technology, School of Science, Department of Chemistry and Biotechnology

Publications

1. Varik I, Zou R, Bellavia A, **Rosenberg K**, Sjunnesson Y, Hallberg I, Holte J, Lenters V, Van Duursen M, Pedersen M, Svingen T, Vermeulen R, Salumets A, Damdimopoulou P, Velthut-Meikas A.
   Reduced ovarian cholesterol and steroid biosynthesis along with increased inflammation are associated with high DEHP metabolite levels in human ovarian follicular fluids.
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   Epidemiologically relevant phthalates affect human endometrial cells in vitro through cell specific gene expression changes related to the cytoskeleton and mitochondria.
   Reprod Toxicol. 2024 Jul 9:128:108660. doi: 10.1016/j.reprotox.2024.108660. PMID: 38992643
3. Vazakidou P, Evangelista S, Li T, Lecante LL, **Rosenberg K**, Koekkoek J, Salumets A, Velthut-Meikas A, Damdimopoulou P, Mazaud-Guittot S, Fowler PA, Leonards PEG, van Duursen MBM.
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4. Tarvainen I, Soto DA, Laws MJ, Björvang RD, Damdimopoulos A, **Roos K**, Li T, Kramer S, Li Z, Lavogina D, Visser N, Kallak TK, Lager S, Gidlöf S, Edlund E, Papaikonomou K, Öberg M, Olovsson M, Salumets A, Velthut-Meikas A, Flaws JA, Damdimopoulou P.
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   Reproductive Toxicology, 119, 108393. DOI: 10.1016/j.reprotox.2023.108393. PMID: 37160244
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   Association between chemical mixtures and female fertility in women undergoing assisted reproduction in Sweden and Estonia.
   Environ Res. 2023 Jan 1;216(Pt 1):114447. doi: 10.1016/j.envres.2022.114447. PMID: 36181890
6. Tagoma A, Haller-Kikkatalo K, Oras A, **Roos K**, Kirss A, Uibo R. Plasma cytokines during pregnancy provide insight into the risk of diabetes in the gestational diabetes risk group. J Diabetes Investig. 2022 Sep;13(9):1596-1606. doi: 10.1111/jdi.13828. PMID: 35524472
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9. **Roos K.**
Gestational diabetes encompasses at least two types of conditions - autoimmune diabetes and insulin resistance-based diabetes. Meditsiin Fookuses, veebruar 2019, 10-14.

10. Tagoma A, Haller-Kikkatalo K, **Roos K**, Oras A, Kirss A, Ilonen J, Uibo R.
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11. **Roos K**, Tagoma A, Kirss A, Metsküla K, Uibo R, Haller-Kikkatalo K.
The serum vitamin D level in the second trimester of pregnancy affects pregnancy outcomes and maternal health. Eesti Arst, 96 (2), 81−91.

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Juuli 2022	ESHRE Aastakonverents, Euroopa Inimese Reproduktsiooni ja Embrüoloogia Ühing; suuline ja poster ettekanne teemal "Heterogeneity of preovulatory follicle cell types between normo- and hyporesponders"
Juuni 2022	FEBS3+ Eesti, Läti ja Leedu Biokeemia Seltsi konverents, poster

Juhendatud väitekirjad

Robyn-Stefany Keif, magistrikraad, 2021, (juh) Agne Velthut-Meikas; Kristine Roos Ovulatsioonieelse folliikuli rakutüübid ja nende geenikspressioon, Tallinna Tehnikaülikool, Loodusteaduskond, Keemia ja biotehnoloogia instituut

Publikatsioonid

1. Varik I, Zou R, Bellavia A, **Rosenberg K**, Sjunnesson Y, Hallberg I, Holte J, Lenters V, Van Duursen M, Pedersen M, Svingen T, Vermeulen R, Salumets A, Damdimopoulou P, Velthut-Meikas A.
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