DOCTORAL THESIS

Extracellular Vesicles and Endocrine-Disrupting Chemicals as Modulators of Ovarian Follicle Physiology

Inge Varik

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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.

Inge Varik

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Rakuvälised vesiikulid ja endokriinsüsteemi kahjustavad kemikaalid kui munasarjade füsioloogiat mõjutavad tegurid

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Contents

List of publications

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

I **Varik I**, Saretok KJ, Rosenberg K, Quintero I, Puhka M, Volkova N, Trošin A, Guazzi P, Velthut-Meikas A. Small and Large Extracellular Vesicles From Human Preovulatory Follicular Fluid Display Distinct ncRNA Cargo Profiles and Differential Effects on KGN Granulosa Cells. J Extracell Vesicles. 2025 Jul;14(7):e70119. DOI: 10.1002/jev2.70119.

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III **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. Environ Int. 2024 Sep;191:108960. DOI: 10.1016/j.envint.2024.108960.

*Contributed equally

Author's contribution to the publications

The author's contributions to the publications included in this thesis are as follows:

I The author performed all experimental procedures except ovarian puncture, transmission electron microscopy, single-particle interferometric reflectance imaging, and the sequencing runs; conducted all statistical and bioinformatic analyses; prepared all figures and tables; wrote the first draft and finalised the manuscript.

II The author supervised the experimental work; performed extracellular vesicle labelling and uptake assays; conducted the statistical and bioinformatic analyses; prepared part of Figure 1 and fully generated Figures 2–5 and the supplementary figures; wrote the first draft and finalised the manuscript.

III The author performed RNA-seq library preparation; conducted RNA-seq and small RNA-seq bioinformatic analyses, including cell-type deconvolution; prepared Figures 1–2 and the graphical abstract; drafted the Introduction and Discussion sections and contributed to the Methods section; and critically revised the final manuscript before submission.

Introduction

Female fertility relies on the successful growth and maturation of ovarian follicles, which support the development of the oocyte. For women who experience difficulty conceiving, fertility treatment—particularly assisted reproductive technologies (ART)—provides an alternative route to pregnancy. In treatments such as *in vitro* fertilisation (IVF), a key early step is controlled ovarian stimulation (COS), during which gonadotropins are administered to promote the simultaneous development of multiple follicles. Increasing the number of mature oocytes available for fertilisation enhances the likelihood of obtaining viable embryos and ultimately achieving pregnancy.

Women, however, differ widely in how their ovaries respond to gonadotropin stimulation. Ovarian sensitivity, defined as the number of oocytes produced for a given gonadotropin dose, can range from very low to excessively high. This variability makes COS outcomes difficult to predict. A low response may yield too few oocytes for a successful IVF cycle, whereas an excessive response increases the risk of ovarian hyperstimulation syndrome (OHSS), a potentially life-threatening complication. Understanding the molecular mechanisms underlying this variability is therefore essential for improving COS planning and enhancing the safety and effectiveness of ART.

Ovarian response to COS is likely shaped by processes occurring within the developing follicles. Follicular growth depends on coordinated communication among granulosa cells, theca cells, immune cells, and the oocyte. These interactions occur through direct cell-to-cell contacts and through signalling molecules present within the surrounding follicular fluid (FF). Given that FF contains the molecular cues exchanged among follicular cells, it provides a valuable window into how the local follicular environment may relate to differences in ovarian responsiveness.

One component of follicular communication that has gained increasing attention is the role of extracellular vesicles (EVs). These membrane-bound nanoparticles, released by virtually all cell types, transport proteins, lipids, and nucleic acids between cells. EVs are abundant in human FF, where they have been implicated in supporting granulosa cell function, oocyte competence, and overall follicular health. Despite this, their potential role in modulating ovarian sensitivity to gonadotropin stimulation remains unexplored.

Beyond intrinsic mechanisms, ovarian function is also influenced by external factors, including environmental exposures. Endocrine-disrupting chemicals (EDCs) such as phthalates can enter the follicular environment and interfere with molecular processes that support follicular development. Di(2-ethylhexyl) phthalate (DEHP) and its metabolites are among the most extensively studied phthalates in reproductive toxicity, yet the molecular mechanisms through which they alter granulosa cell function or contribute to variation in ovarian response to COS remain unclear.

This thesis investigates EVs in human FF as mediators of intercellular communication and explores their potential role in ovarian sensitivity to gonadotropin stimulation. Particular emphasis is placed on the RNA cargo of small and large FF EVs, their functional effects on granulosa cells, and differences in EV composition and activity among women with varying ovarian responses to COS. In parallel, the thesis explores the impact of DEHP exposure on FF microRNA profiles, somatic follicular cell transcriptomes, and steroid hormone production. Together, this doctoral thesis contributes to a deeper understanding of ovarian physiology and its modulation under gonadotropin stimulation, with implications for both natural reproductive processes and ART.

Abbreviations

3β/17β-HSD	3β/17β-hydroxysteroid dehydrogenase
AFC	Antral follicle count
AGO2	Argonaute 2
ALIX	Programmed cell death 6-interacting protein
AMH	Anti-Müllerian hormone
AR	Androgen receptor
ART	Assisted reproductive technologies
BMP	Bone morphogenetic protein
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
circRNA	Circular RNA
CL	*Corpus luteum*
COC	Cumulus-oocyte complex
COS	Controlled ovarian stimulation
CYP11A1	Cholesterol side-chain cleavage enzyme
CYP17A1	17α-hydroxylase/17,20-lyase
CYP19A1	Aromatase
DE	Differentially expressed
DEHP	Di(2-ethylhexyl) phthalate
DF	Dominant follicle
DHEA	Dehydroepiandrosterone
DOR	Diminished ovarian reserve
ECM	Extracellular matrix
EDC	Endocrine-disrupting chemical
EGF	Epidermal growth factor
ER	Oestrogen receptor
EV	Extracellular vesicle
FDR	False discovery rate
FF	Follicular fluid
FGF2	Fibroblast growth factor 2
FSH	Follicle-stimulating hormone
FSHR	Follicle-stimulating hormone receptor
GDF9	Growth-differentiation factor 9
GnRH	Gonadotropin-releasing hormone
hCG	Human chorionic gonadotropin
HGF	Hepatocyte growth factor
HPG	Hypothalamic-pituitary-gonadal axis
HR	Hyporesponder
ICSI	Intracytoplasmic sperm injection
IGF	Insulin-like growth factor

IGF2BP	IGF-2 mRNA-binding protein
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
KITL	KIT ligand
LEV	Large extracellular vesicle
LH	Luteinising hormone
LHR	Luteinising hormone receptor
lncRNA	Long non-coding RNA
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
miRNA	MicroRNA
MMP	Matrix metalloproteinase
mTORC1	Mechanistic target of rapamycin complex 1
MVB	Multivesicular body
ncRNA	Non-coding RNA
NR	Normoresponder
OHSS	Ovarian hyperstimulation syndrome
OSI	Ovarian sensitivity index
PCB	Polychlorinated biphenyls
PCOS	Polycystic ovarian syndrome
PFAS	Per- and polyfluoroalkyl substances
PGC	Primordial germ cell
PGR	Progesterone receptor
PI3K	Phosphoinositide 3-kinase
piRNA	Piwi-interacting RNA
POI	Premature ovarian insufficiency
POR	Poor ovarian response
PPAR	Peroxisome proliferator-activated receptor
PTEN	Phosphatase and tensin homologue
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
rFSH	Recombinant follicle-stimulating hormone
ROS	Reactive oxygen species
SEC	Size-exclusion chromatography
SEV	Small extracellular vesicle
snRNA	Small nuclear RNA
snoRNA	Small nucleolar RNA
STAR	Steroidogenic acute regulatory protein
TFF	Tangential flow filtration
TGF-β	Transforming growth factor-beta
TNF	Tumour necrosis factor

TSG101	Tumour susceptibility gene 101
TZP	Transzonal projection
UC	Ultracentrifugation
UF	Ultrafiltration
UTR	Untranslated region
ZP	*Zona pellucida*

1 Review of the literature

1.1 Folliculogenesis in humans

The human ovary is a dynamic, multi-compartmental organ with two principal functions: (1) production of developmentally competent, fertilisable oocytes and (2) synthesis of steroid hormones essential for the development and maintenance of female secondary sex characteristics, preparation of the reproductive tract for fertilisation, and support of early pregnancy. Both functions rely on the tightly regulated and highly coordinated process of follicular development, or folliculogenesis (Oktem & Oktay, 2008).

Folliculogenesis refers to the maturation of ovarian follicles, the fundamental structural and functional units of the ovary (Oktem & Oktay, 2008). In humans, this process begins during early foetal development when primordial germ cells (PGCs)—the embryonic precursors of gametes—migrate from the yolk sac to the genital ridge, where the gonads will form (Figure 1) (De Felici, 2013). During migration, PGCs proliferate extensively and colonise the developing ovary. Once settled, they differentiate into oogonia, which continue mitotic divisions while remaining interconnected through cytoplasmic bridges, forming germ cell nests—clusters of clonally related germ cells (Pepling & Spradling, 1998).

When proliferation ceases, oogonia enter meiosis and differentiate into primary oocytes. These cells progress through prophase I and arrest at diplotene, also referred to as the germinal vesicle stage, where they remain until puberty (Farini & De Felici, 2022; Pan & Li, 2019). At approximately 21 weeks of gestation, germ cell nests undergo programmed breakdown, during which surrounding pre-granulosa cells invade and encapsulate individual oocytes, giving rise to primordial follicles of roughly 40 µm in diameter (Bristol-Gould et al., 2006; Westergaard et al., 2007). These follicles localise to the ovarian cortex and are separated from the surrounding stroma by the follicular basal lamina, a specialised extracellular matrix (ECM) that provides structural support (Grosbois et al., 2023; Heeren et al., 2015).

The total pool of primordial follicles constitutes the ovarian reserve, a finite and non-renewable resource that determines the length of a woman's reproductive lifespan. Follicle numbers peak at mid-gestation and decline thereafter, continuing throughout life until depletion at menopause. By mid-gestation, the ovaries contain approximately 6–7 million oocytes, a number that falls to fewer than 1 million at birth, to ~500,000 by puberty, and to <1,000 by menopause (Fauser, 2000; Hansen et al., 2008). Notably, substantial inter-individual variation exists in both the size of the ovarian reserve and the rate at which it declines, influenced by genetic, epigenetic, and environmental factors (Pelosi et al., 2015). Moreover, recent single-follicle analyses have revealed marked inter-follicular heterogeneity within the same ovary, although the origins and functional significance of this variation remain unclear (Rooda et al., 2024).

Although most primordial follicles remain dormant, a small subset is continuously recruited into the growing follicle pool via initial recruitment, a process independent of gonadotropins (Hutt & Albertini, 2007). Each follicle faces one of three developmental fates: (1) remain quiescent, (2) undergo atresia, or (3) initiate growth. Of those that activate, only a minority—around 400 follicles over a woman's reproductive lifespan—reach ovulation, while the remainder are lost through atresia (Adhikari & Liu, 2009; Oktem & Oktay, 2008; Tingen et al., 2009). Follicular atresia occurs at all stages of folliculogenesis but is most prevalent at the early antral stage. In antral follicles, cell loss is predominantly apoptotic, whereas in preantral follicles it proceeds mainly via

autophagy (Meng et al., 2018). The resulting cellular debris is cleared by tissue-resident macrophages, helping to maintain ovarian homeostasis (Cacciottola et al., 2025). Importantly, atresia is not pathological but rather the default outcome for most follicles, functioning as a quality-control mechanism that ensures only developmentally competent follicles continue to progress (Celestino et al., 2018).

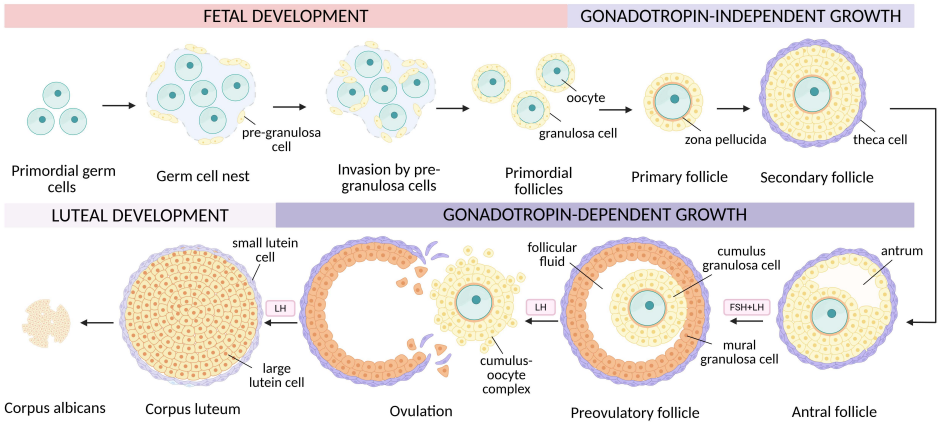

Figure 1. *Overview of human folliculogenesis. During embryonic development, primordial germ cells migrate into the ovary, proliferate, and form germ cell nests, which give rise to primordial follicles. Follicles then progress through gonadotropin-independent stages, during which the oocyte enlarges, granulosa cells proliferate, and theca cells differentiate to form the follicle's outer layers. With antrum formation, growth becomes dependent on follicle-stimulating hormone (FSH). After puberty, cohorts of antral follicles are cyclically recruited, although typically only one follicle achieves dominance and progresses to the preovulatory stage, where the granulosa cells differentiate into mural and cumulus populations. The luteinising hormone (LH) surge induces ovulation, releasing the mature oocyte within the cumulus-oocyte complex. The residual follicle luteinises to form the corpus luteum, which either regresses to a corpus albicans or is maintained in early pregnancy (adapted from Bristol-Gould et al., 2006 and Doherty et al., 2022; created with BioRender.com).*

The transition from primordial to primary follicle marks the onset of follicular growth and involves coordinated changes in both the oocyte and the surrounding somatic cells. The oocyte increases in size (Liu et al., 2006), initiates the synthesis of the *zona pellucida* (ZP)—a glycoprotein-rich ECM crucial for species-specific sperm recognition and for preventing polyspermy (Törmälä et al., 2008; Wassarman & Litscher, 2022)—and begins to accumulate maternal RNAs, proteins, and organelles such as mitochondria, which are essential for supporting early embryonic development (Clarke, 2018). At the same time, pre-granulosa cells shift from a flattened to a cuboidal morphology and enter a proliferative state (Clarke, 2018; Da Silva-Buttkus et al., 2008; Lintern-Moore & Moore, 1979).

Follicle activation is initiated primarily through the mechanistic target of rapamycin complex 1 (mTORC1) signalling within pre-granulosa cells, which promotes their differentiation into granulosa cells and induces the expression of KIT ligand (KITL). KITL subsequently binds to its receptor KIT on the oocyte surface, activating phosphoinositide 3-kinase (PI3K) signalling, thereby driving the oocyte's transition from dormancy to growth (Zhang et al., 2014). The essential role of this axis is underscored by studies showing that oocyte-specific deletion of phosphatase and tensin homologue (PTEN),

a negative regulator of PI3K, results in premature activation and depletion of the follicle pool (Reddy et al., 2008). Beyond the mTORC1-KITL-PI3K axis, follicle activation is modulated by additional signalling cascades: Hippo, anti-Müllerian hormone (AMH), and transforming growth factor-beta 1 (TGF-β1) help preserve quiescence, whereas mitogen-activated protein kinase (MAPK) and insulin-like growth factor 1 (IGF-1) promote growth (Kawamura et al., 2013; Knight & Glister, 2006; Zhao et al., 2021).

As granulosa cells proliferate and form multiple layers, the follicle progresses to the secondary follicle stage. Paracrine signals from both granulosa cells and the oocyte recruit adjacent stromal cells and drive their differentiation into the theca cell lineage (Guahmich et al., 2023; Young & McNeilly, 2010). The emerging thecal layer becomes vascularised and is infiltrated by immune cells, including macrophages, which contribute to follicle development by supporting angiogenesis, ECM remodelling, and the clearance of apoptotic cells (Wu et al., 2004; Young & McNeilly, 2010). The follicular basal lamina maintains a physical boundary between the theca and granulosa cell compartments (Rodgers et al., 2003). Importantly, follicular growth up to this stage remains gonadotropin-independent and is sustained by local intercellular communication (McGee & Hsueh, 2000).

Antrum formation marks the onset of the gonadotropin-dependent phase of folliculogenesis. This phase begins at puberty, when hypothalamic gonadotropin-releasing hormone (GnRH) stimulates the secretion of follicle-stimulating hormone (FSH) and luteinising hormone (LH) from the anterior pituitary. During each menstrual cycle, rising FSH levels initiate the recruitment of a cohort of small antral follicles (~2–5 mm in diameter) in a process termed cyclic recruitment (McGee & Hsueh, 2000; Stamatiades et al., 2019). Within this cohort, a single dominant follicle (DF) is selected, while the remaining follicles undergo atresia. The DF is characterised by elevated oestrogen and inhibin production, which exert negative feedback on the hypothalamus and pituitary (Figure 2), leading to a decline in circulating FSH levels, which in turn limits the growth of subordinate follicles (Webb et al., 2003). Follicular dominance is thought to arise from the mural granulosa cells of the leading follicle acquiring LH receptors (LHR), permitting continued growth even as systemic FSH levels fall (Nogueira et al., 2007; Webb et al., 2004). In parallel, intra-ovarian factors such as activins, growth differentiation factor-9 (GDF9), AMH, and bone morphogenetic proteins (BMPs) fine-tune DF selection by modulating FSH- and IGF-mediated signalling (Knight & Glister, 2006).

During antral development, the theca layer differentiates into two compartments: the theca interna, an endocrine layer producing androgens, and the theca externa, which provides vascularisation and structural support (Hatzirodos et al., 2014). At the same time, the antrum expands and fills with follicular fluid (FF), derived mainly from blood plasma but further enriched with granulosa cell secretions. This fluid contains hormones, growth factors, and signalling molecules that create a specialised microenvironment for oocyte maturation and granulosa-oocyte communication (Pan et al., 2024; Rodgers & Irving-Rodgers, 2010). As the follicle enlarges, granulosa cells differentiate into two functionally specialised subtypes: mural granulosa cells, which line the follicular wall, and cumulus granulosa cells, which surround the oocyte and provide metabolic and nutritional support (Clarke, 2018; Uhde et al., 2018).

In the preovulatory follicle, the oocyte reaches its full size (~100 µm in diameter) but remains arrested in meiosis I (Mehlmann, 2005). The preovulatory LH surge triggers germinal vesicle breakdown, completion of the first meiotic division, and extrusion of the first polar body. These events are facilitated in part by the closure of gap junctions

between granulosa cells and the oocyte, which reduces the transfer of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). The resulting decrease in cyclic nucleotides permits activation of maturation-promoting factor (MPF)—a complex of cyclin-dependent kinase 1 and cyclin B1—which drives meiotic resumption. Following meiosis I, the oocyte arrests at metaphase II, where it remains until fertilisation (Pei et al., 2023).

Ovulation represents the culmination of folliculogenesis. Approximately 34–36 hours after the LH surge, a coordinated sequence of events remodels the follicular wall, enabling release of the cumulus-oocyte complex (COC) (Reed & Carr, 2000). LH induces the expression of proteolytic enzymes, including matrix metalloproteinases (MMPs) and ADAMTS (a disintegrin and metalloproteinase with thrombospondin-like motifs) proteases, which degrade the ECM and weaken the follicular wall (Ohnishi et al., 2005). Concurrently, cumulus granulosa cells undergo expansion and synthesise a hyaluronan-rich ECM that facilitates oocyte release and capture by the oviduct (Russell & Robker, 2007). Ovulation resembles an inflammatory reaction, involving leukocyte infiltration, cytokine release, and prostaglandin synthesis, which together support cumulus expansion and the meiotic progression of the oocyte (Duffy et al., 2019; Niringiyumukiza et al., 2018).

Following ovulation, granulosa and theca cells undergo luteinisation, forming large and small luteal cells, respectively. Together, these cells constitute the *corpus luteum* (CL), a transient, highly vascularised endocrine gland that secretes progesterone to maintain endometrial receptivity and support early pregnancy (Abedel-Majed et al., 2019). CL function is supported by LH and, if fertilisation occurs, maintained by embryonic human chorionic gonadotropin (hCG) (Todorović & Fatemi, 2010). In the absence of fertilisation, luteolysis leads to CL regression and the formation of a fibrotic *corpus albicans*, thereby concluding the ovarian cycle (McCracken et al., 1999).

1.1.1 Hormonal regulation of folliculogenesis

The hormonal regulation of folliculogenesis relies on a finely tuned interplay between systemic endocrine signals and local intra-ovarian factors. At the systemic level, the hypothalamic–pituitary–gonadal (HPG) axis coordinates follicular growth with the cyclical rhythm of the menstrual cycle (Orisaka et al., 2021). Pulsatile release of GnRH from the hypothalamus drives pituitary gonadotropin secretion, with pulse frequency determining the relative balance of the two gonadotropins: low-frequency pulses favour FSH release, while high-frequency pulses stimulate LH secretion (Figure 2) (Dalkin et al., 1989; Kaiser et al., 1997). Loss of pulsatility—as occurs under continuous GnRH exposure—suppresses gonadotropin secretion, underscoring the critical role of rhythmic hypothalamic signalling for follicle growth, steroidogenesis, and ovulation (Conn & Crowley, 1994).

From the secondary follicle stage onwards, FSH becomes the principal driver of follicle development (McGee & Hsueh, 2000). Acting via FSH receptors (FSHR), FSH promotes granulosa cell proliferation and survival, enabling progression beyond the preantral stage (McGee & Hsueh, 2000). A central function of FSH is the induction of aromatase (CYP19A1), allowing granulosa cells to convert theca-derived androgens into oestrogens (Gore-Langton & Dorrington, 1981). Androgens can further reinforce follicle growth by acting through the androgen receptor (AR) to stimulate granulosa cell proliferation and upregulate *FSHR* expression, thereby amplifying responsiveness to FSH (Franks & Hardy, 2018). Intra-ovarian factors further modulate FSH responsiveness: AMH decreases

granulosa cell sensitivity to FSH, whereas IGF signalling enhances it by supporting FSH-induced *CYP19A1* expression and cell proliferation (Baumgarten et al., 2015; Pellatt et al., 2011).

A key mechanism by which gonadotropins regulate follicular development is through the control of ovarian steroidogenesis, described by the two-cell, two-gonadotropin model (Hillier et al., 1994). Steroidogenesis begins with cholesterol transport from the outer to the inner mitochondrial membrane by steroidogenic acute regulatory protein (STAR), where cholesterol is converted by the cholesterol side-chain cleavage enzyme (CYP11A1) into pregnenolone (Figure 3) (Miller & Auchus, 2011).

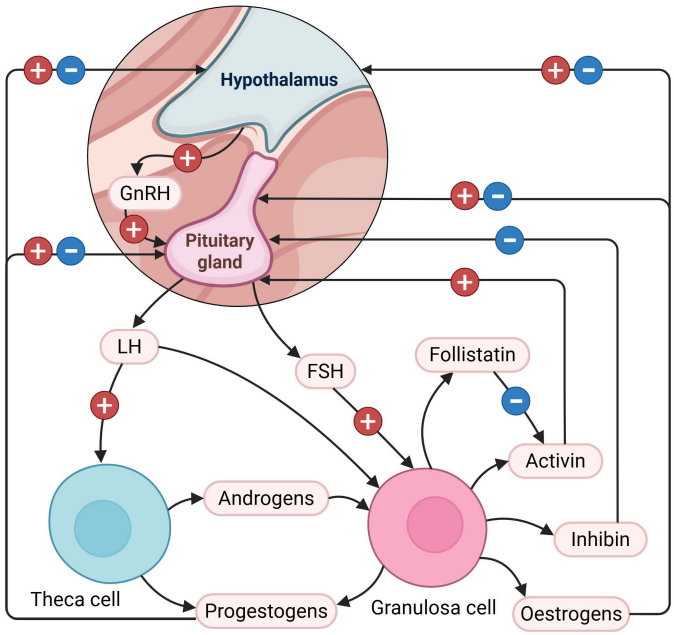

Figure 2. *The hypothalamic–pituitary–gonadal (HPG) axis. Pulsatile gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus stimulates pituitary release of follicle-stimulating hormone (FSH) and luteinising hormone (LH), which act on granulosa and theca cells, respectively, with granulosa cells acquiring LH receptors in later stages of follicular maturation. This axis is regulated by ovarian feedback signals, including oestrogens, progestogens, activin, inhibin, and follistatin, which modulate hypothalamic GnRH and pituitary gonadotropin release (created with BioRender.com).*

From this precursor, two principal pathways operate in the human ovary. In the Δ5 pathway, pregnenolone is hydroxylated by 17α-hydroxylase/17,20-lyase (CYP17A1) in theca cells to form 17α-hydroxypregnenolone, which is subsequently cleaved to dehydroepiandrosterone (DHEA), an androgen precursor (Andersen & Ezcurra, 2014; Wickenheisser et al., 2006). Because granulosa cells lack CYP17A1, these intermediates must diffuse from the theca to the granulosa compartment. In granulosa cells, 3β-hydroxysteroid dehydrogenase (3β-HSD) converts DHEA to androstenedione, which can then be converted via two converging routes to oestrogens. In the first, CYP19A1 converts androstenedione to oestrone, which is then reduced to oestradiol by 17β-hydroxysteroid dehydrogenase (17β-HSD). In the second route, androstenedione is reduced to testosterone by 17β-HSD, and testosterone is subsequently aromatised to

oestradiol by CYP19A1 (Hillier et al., 1994; Jamnongjit & Hammes, 2006). The Δ5 pathway, therefore, requires cooperation between theca and granulosa compartments to generate oestrogens (Hillier et al., 1994).

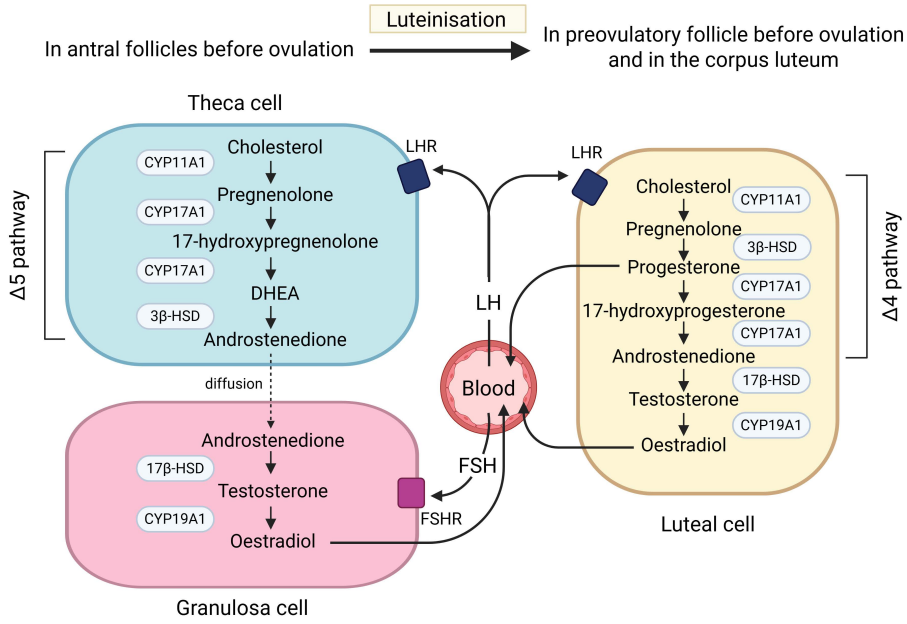

Figure 3. *Steroidogenesis pathways in ovarian cells before and after luteinisation. Cholesterol is converted to steroid hormones via the Δ5 or Δ4 pathway. In antral follicles, theca cells initiate androgen synthesis through the Δ5 pathway, producing dehydroepiandrosterone (DHEA) and androstenedione. These androgens diffuse into granulosa cells, where they are converted to oestradiol. After the LH surge, granulosa cells luteinise and shift toward progesterone production via the Δ4 pathway. In humans, this route contributes little to androgen synthesis because the conversion of progesterone to androstenedione is inefficient (adapted from Jones & Lopez, 2014; created with BioRender.com).*

In the Δ4 pathway, pregnenolone is converted to progesterone by 3β-HSD. Although progesterone may be further hydroxylated to 17-hydroxyprogesterone by CYP17A1, this reaction proceeds inefficiently in humans and therefore contributes little to classical androgen synthesis; however, the 17-hydroxyprogesterone that is produced can enter the backdoor pathway, which provides an alternative route for androgen formation (Miller & Auchus, 2011; Vazakidou et al., 2024). As a result, the Δ4 pathway serves mainly as a source of progesterone, particularly following ovulation when progesterone synthesis is upregulated in large lutein cells of the CL (Hurwitz et al., 1989; Taraborrelli, 2015). Together, the Δ5, Δ4, and backdoor pathways support ovarian steroidogenesis across the menstrual cycle, with oestrogen production predominating during follicular growth and progesterone becoming the dominant steroid after ovulation, while oestrogens and androgens continue to be produced at lower levels (Vazakidou et al., 2024).

Oestradiol produced within granulosa cells exerts both local and systemic effects. Within the ovary, its actions are mainly mediated through oestrogen receptor β (ERβ), the predominant isoform expressed in granulosa cells. In contrast, systemic feedback at

the HPG axis is largely mediated by ERα, which is expressed in both hypothalamic and pituitary cells and regulates gonadotropin synthesis and release (Lindzey et al., 2006; Nalvarte & Antonson, 2023). During the growth of small to medium antral follicles, when circulating oestradiol levels remain relatively low, oestradiol exerts negative feedback on the HPG axis, reducing FSH secretion and thereby limiting the number of follicles that continue to grow towards dominance (Messinis et al., 2014). These systemic effects are further fine-tuned by inhibins, activins, and follistatin, which act at the pituitary to modulate FSH secretion (de Kretser et al., 2002). Inhibin B, secreted by small antral follicles, acts in concert with oestradiol to suppress FSH secretion, becoming the predominant signal as follicle growth progresses (Andersen et al., 2010; Welt et al., 2003). Conversely, activins stimulate FSH synthesis and release (Gregory & Kaiser, 2004), an effect tightly restrained by follistatin, another granulosa-derived factor that binds activins with high affinity and prevents their signalling (Findlay et al., 2002).

Once a DF is established and oestradiol secretion increases markedly, feedback at the HPG axis switches from negative to positive, promoting GnRH release and triggering the preovulatory LH surge (Kauffman, 2022; Reed & Carr, 2000). By this stage, mural granulosa cells of the DF have acquired LHRs, enabling them to respond directly to LH (Richards & Ascoli, 2018). The LH surge induces a steroidogenic shift in granulosa cells, characterised by declining CYP19A1 activity and the onset of progesterone synthesis as the predominant output (Carletti & Christenson, 2009; Duffy et al., 2019; Fitzpatrick et al., 1997). In parallel, LH upregulates progesterone receptor (*PGR*) expression in granulosa cells (Akison & Robker, 2012; Park & Mayo, 1991). Once activated, PGR initiates the transcriptional programme required for follicle rupture and oocyte release (Akison & Robker, 2012). To sustain progesterone production, granulosa cells accumulate cholesterol-rich lipid droplets, which provide a readily available substrate (Duffy et al., 2019). Collectively, these changes ensure the transition from follicle growth, primarily driven by FSH, to ovulation and luteinisation, which are governed by LH.

1.1.2 Intercellular communication in the ovarian follicle

From the primordial stage until ovulation, the oocyte and surrounding somatic cells engage in continuous bidirectional communication, a process essential for proper follicular growth and oocyte maturation. This dialogue also occurs among somatic cells and is mediated through multiple mechanisms, including gap junctions, transzonal projections (TZPs), paracrine signalling, and extracellular vesicles (EVs) (Figure 4) (Andrade et al., 2019).

Gap junctions are specialised intercellular membrane channels composed of connexins that allow the passage of small molecules (<1 kDa), such as ions, amino acids, glucose metabolites, and nucleotides (Kidder & Mhawi, 2002). Within the ovarian follicle, connexin 37 localises to the oocyte-granulosa cell interface, while connexin 43 is mainly expressed between granulosa cells (Kidder & Mhawi, 2002; Veitch et al., 2004). The functional significance of these junctions has been demonstrated in knockout models: mice deficient in connexin 37 fail to form preovulatory follicles and are unable to ovulate (Simon et al., 1997), whereas connexin 43-deficient ovaries exhibit arrested follicular development with impaired granulosa cell proliferation and oocyte maturation (Ackert et al., 2001). *In vitro* studies further support the requirement for granulosa cell contact in oocyte growth and maturation: isolated immature oocytes fail to develop, whereas those retaining gap junctions with granulosa cells progress normally (McGee & Hsueh, 2000). In addition to enabling direct contact, gap junctions support metabolic cooperation by mediating the transfer of second messengers (e.g., cAMP) and key

metabolites such as pyruvate, lactate, and cholesterol intermediates (Fontana et al., 2019; Turathum et al., 2021). Their assembly is hormonally regulated, with FSH enhancing connexin expression in granulosa cells and the oocyte to maintain efficient intracellular communication (El-Hayek & Clarke, 2015).

Complementing gap junctions, TZPs are cytoplasmic extensions that originate from cumulus granulosa cells and traverse the ZP to establish direct physical contact with the oocyte membrane. Individual cumulus cells extend multiple TZPs, some of which terminate in bulbous structures at the oocyte surface (Baena & Terasaki, 2019; Makabe et al., 2006). At their tips, TZPs form both gap junctions and adherens junctions (Li & Albertini, 2013), facilitating the transfer of metabolites, mRNAs, long non-coding RNAs (lncRNAs), and small organelles (Andrade et al., 2019; Macaulay et al., 2014). TZPs display structural diversity, with most containing an actin backbone (Macaulay et al., 2014) and a smaller subset incorporating microtubules (Li & Albertini, 2013). Although traditionally viewed as mediators of oocyte-cumulus communication, TZPs also interconnect granulosa cells, suggesting a broader role in coordinating granulosa-granulosa signalling (Baena & Terasaki, 2019).

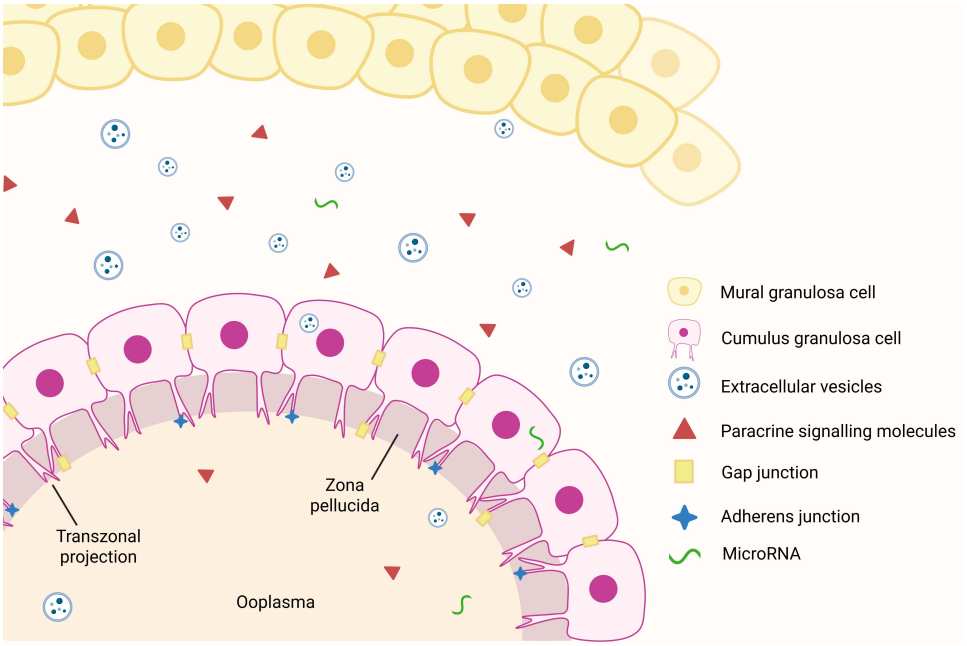

Figure 4. *Modes of intercellular communication within the ovarian follicle. Both germ cells and somatic cells exchange signals through several coordinated mechanisms. These include the secretion of locally acting paracrine factors, direct exchange of molecules through transzonal projections (TZPs) that establish gap or adherens junctions with the oocyte, and the movement of extracellular vesicles either along TZPs or freely in the follicular fluid (adapted from Andrade et al., 2019; created with BioRender.com).*

In addition to direct physical contacts, follicular cells also communicate through paracrine signals carried in FF. This is a biochemically rich environment containing hormones, growth factors, cytokines, metabolites, and nucleic acids (Martinez et al., 2018; Revelli et al., 2009). Key mediators of oocyte-cumulus communication include oocyte-derived TGF-β family members, particularly GDF9 and BMP15, which regulate

granulosa cell function and promote follicular growth (Gilchrist et al., 2004). GDF9, for instance, stimulates TZP formation, thereby reinforcing oocyte-cumulus cell contacts (El-Hayek et al., 2018). Conversely, granulosa cells secrete KITL, which activates its receptor KIT on the oocyte to promote oocyte growth and survival, exemplifying the reciprocal nature of oocyte-cumulus communication (Packer et al., 1994; Thomas & Vanderhyden, 2006).

Beyond oocyte-granulosa interactions, communication between granulosa cell subpopulations plays a pivotal role in coordinating follicle development. A well-characterised example is the epidermal growth factor (EGF) signalling cascade, which transmits the LH signal from mural to cumulus granulosa cells, thereby inducing cumulus expansion, ECM production, and oocyte meiotic resumption via EGF-like ligands such as amphiregulin and epiregulin (Conti et al., 2006; Park et al., 2004). Recent transcriptomic analyses have expanded this view, revealing that mural-cumulus granulosa cell communication involves additional signalling routes. These include ephrin ligand–receptor interactions linked to steroidogenesis, Wnt–LRP6 (low-density lipoprotein receptor-related protein 6) signalling associated with lipid transport, and TGF-β–TGFBR1 (TGF-β receptor 1) signalling implicated in hyaluronan synthesis (Shirafuta et al., 2024).

Granulosa and theca cells also engage in reciprocal paracrine communication. Granulosa-derived factors such as Desert hedgehog (*DHH*) and Indian hedgehog (*IHH*), whose expression is stimulated by oocyte-derived GDF9, regulate theca cell differentiation and proliferation (Liu et al., 2015). In turn, theca cells produce growth factors such as hepatocyte growth factor (HGF), keratinocyte growth factor, EGF and BMP7, which promote granulosa cell proliferation and inhibit apoptosis (Orisaka et al., 2021). Notably, HGF and KITL participate in a positive feedback loop: HGF stimulates *KITL* expression in granulosa cells, and KITL, in turn, enhances HGF production in theca cells (Parrott & Skinner, 1998). Co-culture studies further confirm that theca-granulosa cell communication enhances proliferation and steroidogenesis in both cell types (Kotsuji & Tominaga, 1994).

More recently, EVs—small vesicles delimited by a lipid bilayer—have been recognised as an additional mode of paracrine signalling within the follicle (da Silveira et al., 2012). Through the transfer of selectively packaged molecular cargo, EVs can influence transcriptional programmes and cellular behaviour in neighbouring cells (Abels & Breakefield, 2016). Together with contact-mediated and soluble paracrine cues, they contribute to an integrated signalling network that synchronises oocyte maturation with somatic cell support.

1.2 Extracellular vesicles (EVs)

EVs were first reported in incidental observations and were not immediately recognised as an intercellular communication system. Early reports of platelet-derived “dust” (Wolf, 1967) were followed by the discovery that intraluminal vesicles form within multivesicular bodies (MVBs) and are secreted as exosomes (Harding et al., 1983). For years, EV release was mainly interpreted as a cellular waste-disposal mechanism, particularly during reticulocyte maturation (Johnstone et al., 1987, 1991). The discovery that human B lymphocytes secrete antigen-presenting EVs prompted recognition of their potential physiological roles (Raposo et al., 1996). This view was solidified in the mid-2000s by evidence that EVs transfer functional proteins and nucleic acids between cells, establishing them as active mediators of intercellular communication (Ratajczak et al., 2006; Valadi et al., 2007).

EV-mediated communication is evolutionarily conserved across bacteria, archaea and eukaryotes (Gill et al., 2019). In humans, EVs have been detected in most biofluids, including blood, saliva, urine, breast milk, and tears, as well as cerebrospinal, synovial, seminal, amniotic, uterine, oviductal fluid, and FF (del Rivero et al., 2022; Ditchfield et al., 2025; Fasoli et al., 2025; Kanei et al., 2025; Kumar et al., 2024). In physiological settings, EVs contribute to maintaining homeostasis—for example, neutrophil-derived EVs suppress bacterial growth (Timár et al., 2013), and circulating EVs can modulate vascular tone and blood pressure (Good et al., 2020). At the same time, EVs have gained attention for their role in the pathogenesis and progression of major disorders—including cardiovascular disease (Reiss et al., 2023), diabetes (Xiao et al., 2019), cancer (Kalluri & McAndrews, 2023), and neurodegeneration (Sigdel et al., 2023)—making them attractive biomarker candidates.

1.2.1 EV biogenesis, uptake and molecular composition

Based on their biogenesis, EVs are commonly classified into three broad categories: exosomes (30–150 nm), microvesicles (50–1000 nm), and apoptotic bodies (50–5000 nm) (Colombo et al., 2014; Crescitelli et al., 2013; Kakarla et al., 2020). Exosomes form as intraluminal vesicles within MVBs and are released when an MVB fuses with the plasma membrane (Figure 4). Although some MVBs are delivered to lysosomes for degradation, a subset is transported along microtubules to the cell surface for exosome release (van Niel et al., 2018). By contrast, microvesicles form by outward budding and scission of the plasma membrane, whereas apoptotic bodies are produced during membrane blebbing and fragmentation in apoptosis (Dixson et al., 2023). Additional EV subtypes have been described, including migrasomes generated from retraction fibres of migrating cells (Zhang et al., 2023), exophers that contain damaged organelles or protein aggregates (Melentijevic et al., 2017), and tumour-derived large oncosomes (Minciacchi et al., 2015).

Following their release, EVs may act locally—via uptake by the secreting cell or neighbouring cells—or enter the circulation to mediate effects at distant sites (Salomon et al., 2022). Circulating EVs are not uniformly derived from all tissues; in fact, it has been estimated that only ~0.2% of plasma EVs originate from solid tissues, with adipose tissue contributing the largest share, whereas most circulating EVs arise from haematopoietic cells (Li et al., 2020b). The specificity of EV uptake remains an area of ongoing investigation. Some studies report broad internalisation of fluorescently labelled EVs across a wide range of cell types, suggesting limited selectivity (Svensson et al., 2013). In contrast, other studies indicate preferential uptake by cells of the same origin. For example, HEK293T-derived EVs are internalised more efficiently by HEK293T cells compared to other cell lines, such as HUVEC and C3A (Jurgielewicz et al., 2020). Similarly, breast cancer cell-derived EVs have been reported to exhibit preferential uptake by their parent cell lines, with reduced uptake observed in allogenic and non-cancerous cells (Alagundagi et al., 2026).

Upon reaching a recipient cell, EVs can engage in one of several entry mechanisms: (1) trigger signalling cascades via receptor-ligand interactions at the cell surface, (2) be internalised through phagocytosis, macropinocytosis, or clathrin-/caveolin-mediated endocytosis, or (3) fuse with the plasma membrane to deliver their cargo directly into the cytoplasm (Mulcahy et al., 2014) (Figure 5). EVs have also been proposed to undergo sequential uptake and re-release, enabling them to traverse multiple cell layers (Record et al., 2011).

Following internalisation, EVs are trafficked through the endosomal system, first entering early endosomes that mature into late endosomes, which may eventually fuse with lysosomes, leading to EV degradation (Ribovski et al., 2023). Therefore, endosomal escape is required for the functional delivery of EV contents. This typically occurs via back-fusion of EVs with the limiting membrane of endosomes, allowing cargo release into the cytosol without disrupting endosomal integrity—a process estimated to occur for ~30% of internalised EVs (Bonsergent et al., 2021). Alternatively, MVBs or late endosomes may fuse with the plasma membrane, resulting in the re-secretion of EVs (O'Brien et al., 2022).

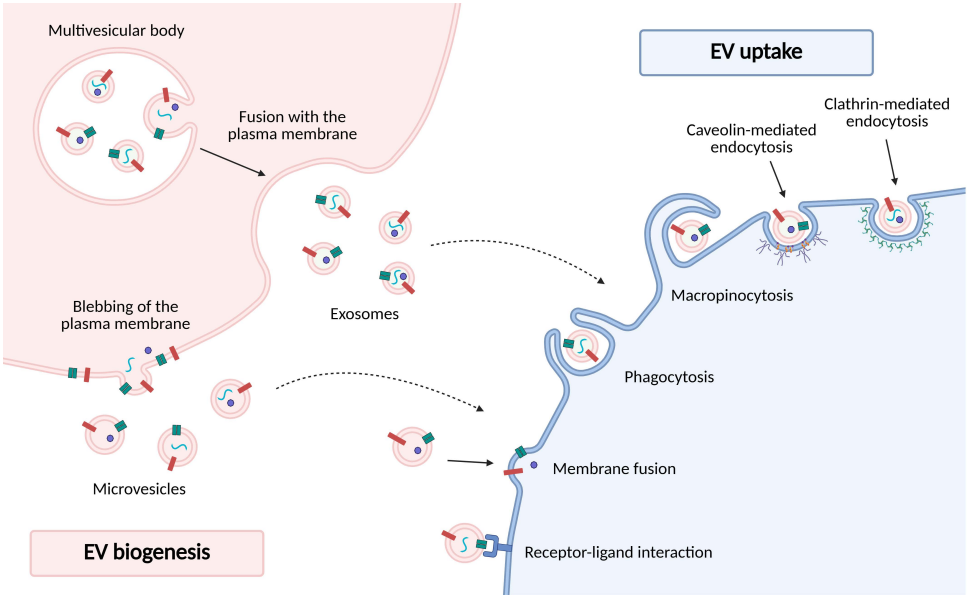

Figure 5. *Mechanisms of extracellular vesicle (EV) biogenesis and uptake. Cells release EVs via two main routes: (1) exosomes form when multivesicular bodies fuse with the plasma membrane and release their intraluminal vesicles, and (2) microvesicles arise by outward budding and scission of the plasma membrane. Recipient cells take up EVs through clathrin- or caveolin-mediated endocytosis, macropinocytosis, phagocytosis, or via direct membrane fusion. EVs may also trigger surface signalling through receptor–ligand interactions (adapted from Thompson & Papoutsakis, 2023; created with BioRender.com).*

The molecular composition of EVs reflects the identity and state of the source cell (van Niel et al., 2018) and includes lipids, proteins, metabolites, and nucleic acids (Pathan et al., 2019). EV membranes are enriched in cholesterol, sphingomyelin, and ceramides and commonly display phosphatidylserine on the outer leaflet (Gurung et al., 2021; Théry et al., 2009). Typical protein cargo includes tetraspanins (CD9, CD63, CD81; CD, cluster of differentiation), biogenesis and trafficking machinery (TSG101, tumour susceptibility gene 101; ALIX, programmed cell death 6-interacting protein), heat-shock proteins (HSP70, HSP90), metabolic enzymes, nuclear and cytosolic proteins (e.g., histones, ubiquitin), integrins and other adhesion receptors, and antigen-presentation molecules (MHC-I and -II; MHC, major histocompatibility complex) (Abels & Breakefield, 2016; Record et al., 2011).

In biofluids, EVs acquire a biomolecular corona—an adsorbed layer of proteins, lipids, glycans, and nucleic acids from the extracellular milieu (Buzas, 2022; Palviainen et al., 2020). Emerging evidence indicates that corona formation can augment the functional

effects of EVs and promote their uptake (Esmaeili et al., 2025). The biomolecular corona can modulate docking to recipient cells, interactions with the ECM, and activation of signalling pathways in recipient cells (Hallal et al., 2022).

Unlike freely soluble signalling molecules, EVs protect their cargo from enzymatic degradation and support targeted delivery, providing a stable and efficient means of communication (Kwok et al., 2021; Mulcahy et al., 2014). EVs encapsulate a diverse repertoire of nucleic acids, including DNA, mRNAs, and multiple classes of non-coding RNAs (ncRNAs), which are often detected in fragmented forms. These ncRNAs include rRNAs, tRNAs, and various regulatory RNA species (Batagov & Kurochkin, 2013; Huang et al., 2013). EVs are particularly enriched in small ncRNAs (<200 nucleotides in length), including microRNAs (miRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), PIWI-interacting RNAs (piRNAs), vault RNAs, and Y RNAs (Batagov & Kurochkin, 2013; Huang et al., 2013; Li et al., 2013; Nolte-'t Hoen et al., 2012). In addition, EVs can contain longer ncRNAs such as circular RNAs (circRNAs) and long non-coding RNAs (lncRNAs), which exceed 200 nucleotides in length (Huang et al., 2013; Li et al., 2015).

miRNAs are evolutionarily conserved small ncRNAs widely present in animals and plants, with confirmed occurrence in a limited number of additional eukaryotic lineages (Tarver et al., 2012). They are among the most intensively studied EV-associated ncRNAs, typically ~22 nucleotides in length. They regulate gene expression post-transcriptionally by binding complementary sequences, most often within the 3' untranslated regions (UTRs) of mRNAs, resulting in mRNA degradation and/or translational repression (O'Brien et al., 2018). In vertebrates, a single miRNA can modulate the expression of hundreds of transcripts (Krek et al., 2005). The miRNA content of EVs is selectively packaged rather than representing a random sample of the cellular pool, with RNA-binding proteins contributing to miRNA sorting (Abdelgawad et al., 2025; Garcia-Martin et al., 2022). Despite their biological significance, miRNAs are present at low absolute copy numbers in EVs—often at fewer than one molecule per ~100 EVs for the most abundant species (Chevillet et al., 2014). Not all extracellular miRNAs are EV-associated; a substantial fraction circulates bound to Argonaute 2 (AGO2) or lipoproteins (Arroyo et al., 2011; Vickers et al., 2011).

piRNAs are small ncRNAs found in animals that are generally 24-30 nucleotides in length, and associate with PIWI proteins, a specialised subfamily of Argonaute proteins (Klattenhoff & Theurkauf, 2008). Together, they form piRNA-induced silencing complexes that translocate to the nucleus, where they mediate transcriptional silencing of transposable elements, thereby preserving genomic integrity (Sato & Siomi, 2020). In addition to their canonical role in transposon repression, piRNAs can also regulate gene expression post-transcriptionally in a manner analogous to miRNAs. This regulation can occur through base-pairing with transposon-derived sequences in mRNAs, typically within 3'-UTRs, which leads to mRNA degradation or translational repression (Watanabe & Lin, 2014). Although originally considered germline-specific (Klattenhoff & Theurkauf, 2008), components of the piRNA pathway are also present in somatic ovarian cells: PIWI proteins are expressed in human granulosa cells of primordial and preantral follicles (Lim et al., 2013), and piRNAs have been identified in human cumulus granulosa cells (Chen et al., 2017). The relative abundance of piRNAs among EV-associated small RNAs varies widely across body fluids, ranging from less than 1% in urine-derived EVs to over 40% in plasma-derived EVs (Goh et al., 2022). Given their presence in both oocytes and granulosa cells, EV-associated piRNAs may contribute to intercellular communication within the follicular microenvironment.

1.2.2 Methods for EV isolation

Numerous techniques are available for EV isolation, including ultracentrifugation (UC), ultrafiltration (UF), density gradient centrifugation, size-exclusion chromatography (SEC), tangential flow filtration (TFF), precipitation, and immunoaffinity capture (Liu et al., 2025). While efforts have been made to harmonise EV isolation approaches, methodological standardisation remains challenging due to variation in both research objectives and sample types. In practice, this often leads researchers to combine multiple techniques to balance purity and yield. Each method has specific limitations (Table 1), and high recovery often comes at the expense of purity (Clos-Sansalvador et al., 2022).

UC remains one of the most widely adopted approaches for EV isolation. Standard workflows begin with low-speed centrifugation (up to 2,000 × g) to remove cells and large cellular debris, followed by optional removal of apoptotic bodies and large EVs at 10,000 × g, and finally pelleting small EVs at ≥100,000 × g (Monguió-Tortajada et al., 2019; Shami-Shah et al., 2023). As a refinement of this method, density gradient centrifugation separates EVs based on their buoyant density: stepwise or continuous sucrose/iodixanol gradients are layered, and during centrifugation, EVs migrate to their equilibrium density, enabling separation from protein aggregates and lipoproteins (Onódi et al., 2018).

Precipitation is among the simplest isolation methods, in which volume-excluding polymers—typically polyethylene glycol (PEG)—are used to co-aggregate EVs with other macromolecules into a pellet. While suitable for small sample volumes or clinical workflows, this method may be less appropriate for functional studies due to residual polymer and co-precipitated proteins/lipoproteins that may confound results (Monguió-Tortajada et al., 2019). UF is another user-friendly technique that concentrates EVs via pressure-driven flow across membranes with defined molecular weight cut-offs, retaining EVs while allowing smaller molecules to pass through. However, pore blockage and vesicle adsorption to the membrane can reduce recovery and alter size distributions (Ljungström & Oltra, 2025). TFF, an advanced form of UF, directs sample flow parallel to the membrane surface, minimising membrane clogging and enabling scalable EV concentration and buffer exchange (Busatto et al., 2018; Kawai-Harada et al., 2024).

SEC separates particles by hydrodynamic size using columns packed with porous resin. Larger particles, such as EVs, are excluded from the pores and elute early, while proteins enter the pores and elute later. The main advantage of this method is that, while user-friendly, it yields EV preparations of substantially higher purity than UC, UF, or precipitation, though overall vesicle recovery may be lower or comparable (Clos-Sansalvador et al., 2022). Immunoaffinity capture achieves very high purity by using antibody-conjugated magnetic beads to bind EV surface proteins. Due to the absence of universal EV markers, this approach selectively enriches specific EV subpopulations and therefore may introduce selection bias in downstream analyses (Bellotti et al., 2021).

Table 1. *Advantages and disadvantages of commonly used EV isolation methods.*

Isolation method	Advantages	Disadvantages	References
Ultracentrifugation (UC)	Widely used protocols, moderate yield, scalable for medium volumes	Time-consuming, low to moderate purity, requires expensive equipment, potential EV aggregation or deformation, sensitive to rotor type and protocol variability	Brennan et al., 2020; Coughlan et al., 2020; Gardiner et al., 2016; Monguió-Tortajada et al., 2019
Ultrafiltration (UF)	Inexpensive equipment, fast extraction, moderately scalable, high yield	Low purity, pore blockage, EV loss by membrane adsorption, potential EV deformation	Clos-Sansalvador et al., 2022; Kim et al., 2025; Ljungström & Oltra, 2025
Density gradient centrifugation	Higher purity compared to UC, preserves EV morphology	Time-consuming, expensive equipment, low yield, complex preparation, limited throughput and scalability	Duong et al., 2019; Van Deun et al., 2014; Webber & Clayton, 2013
Size-exclusion chromatography (SEC)	High purity, low to moderate yield, preserves EV morphology, fast extraction, inexpensive equipment	Sample is diluted, manual fraction collection may introduce variability, cannot entirely remove lipoproteins of similar size	Gardiner et al., 2016; Lobb et al., 2015; Sidhom et al., 2020; Stranska et al., 2018
Tangential flow filtration (TFF)	High yield, higher purity compared to UC, fast extraction, can isolate from large volumes, preserves EV morphology	Protein-rich samples can cause membrane clogging, potential EV aggregation during concentration, requires specialised equipment	Busatto et al., 2018; Kawai-Harada et al., 2024; Wadenpohl et al., 2024
Precipitation	Low cost, fast extraction, very high yield, preserves EV morphology	Very low purity, polymer carryover, not recommended for functional studies	Coughlan et al., 2020; Lobb et al., 2015; Sidhom et al., 2020
Immunoaffinity capture	Very high purity, enables enrichment of EV subpopulations	Time-consuming, low yield, lack of specific markers for isolation, selection bias, possible nonspecific binding, expensive reagents, suitable for only small volumes, harsh elution conditions may alter EV integrity	Clos-Sansalvador et al., 2022; Ljungström & Oltra, 2025; Sidhom et al., 2020; Zhu et al., 2020

The choice of isolation method remains a significant bottleneck in EV research, mainly due to the complex composition of biofluids and the overlapping physical properties of EVs and non-vesicular particles, such as lipoproteins, exomeres and supermeres (Jeppesen et al., 2024). These contaminants, which closely resemble EVs in size and density, make selective isolation inherently difficult; for example, very-low-density lipoproteins (VLDL) and chylomicrons often co-isolate with EVs when using SEC (Wei et al., 2020). Further complicating matters, some co-isolating molecules can adsorb onto EV surfaces and form part of a biomolecular corona. This raises a critical trade-off: pursuing maximal purity through aggressive isolation may strip corona components and reduce EV functionality, while insufficient purification risks exaggerating apparent effects due to residual co-isolates. As such, the optimal degree of purification is context-dependent and remains an active subject of debate. (Manno et al., 2025). Methodological diversity adds another layer of complexity, as differences in isolation workflows introduce variability in EV concentration, size distribution, and purity, limiting cross-study comparability (Tang et al., 2017).

Attributing biogenetic origin to EVs post-isolation remains a significant challenge. Biogenesis pathways operate in parallel, and EV subtypes overlap in size, making it unreliable to assign origin based solely on physical characteristics (van Niel et al., 2018). Moreover, no universal markers exist for all EVs or for distinguishing specific subtypes, such as exosomes and microvesicles (Welsh et al., 2024). Although these subtypes are defined by their biogenesis mechanisms, they are difficult to resolve experimentally; therefore, most EV isolates represent heterogeneous mixtures of vesicles derived from multiple pathways (Willms et al., 2016). To address these nomenclature-related challenges, the International Society for Extracellular Vesicles (ISEV) issued the Minimal Information for Studies of EVs (MISEV) guidelines, which advocate the use of operational terms (e.g., small EVs, large EVs) when biogenesis cannot be confidently assigned. The guidelines also emphasise the importance of transparently reporting pre-analytical variables and isolation parameters, and characterising EVs using multiple, orthogonal markers to improve reproducibility and comparability across studies (Welsh et al., 2024).

1.2.3 EVs in ovarian biology

EVs have been consistently detected in FF across multiple species, including human, bovine, equine, and porcine, indicating that their presence in the follicular environment is evolutionarily conserved (da Silveira et al., 2012; Martinez et al., 2018; Rooda et al., 2020; Sohel et al., 2013). In human FF, mural and cumulus granulosa cells are considered the principal sources of EVs, while theca cells and the oocyte may also contribute (Machtinger et al., 2021). Most studies report FF EV sizes ranging from 30 to 300 nm (da Silveira et al., 2017; Hung et al., 2015; Rooda et al., 2020; Soares et al., 2023); however, this likely underrepresents the true size range because UC, the predominant isolation method across studies, typically includes a pre-clearing step that removes larger vesicles (Monguió-Tortajada et al., 2019). Canonical EV markers, such as CD9, CD63, CD81, TSG101, and ALIX have been identified in FF EVs (da Silveira et al., 2017; Rooda et al., 2020; Santonocito et al., 2014; Soares et al., 2023), and cryo-electron microscopy has revealed considerable heterogeneity in FF EV morphology (Neyroud et al., 2022).

Both granulosa cells and oocytes have been reported to internalise FF EVs (da Silveira et al., 2012; Gabryś et al., 2022; Hung et al., 2017; Lipinska et al., 2025; Uzbekova et al., 2020). Within COCs, EVs traverse the ZP via TZPs and accumulate in the perivitelline space and ooplasm (Gabryś et al., 2022; Lipinska et al., 2025; Uzbekova et al., 2020).

In granulosa cells, uptake appears to occur predominantly via clathrin-independent pathways, including caveolin-mediated endocytosis, macropinocytosis, and phagocytosis (Wang et al., 2024). Uptake efficiency varies with follicle stage, with EVs from smaller follicles entering granulosa cells more readily (Hung et al., 2017).

Multiple studies demonstrate that FF EVs influence granulosa cell behaviour and steroidogenic output in culture conditions. In equine, bovine, and porcine models, they promote cumulus cell expansion (Gabryś et al., 2024; Hung et al., 2015; Kim et al., 2023), although one porcine study reported no effect under its specific conditions (Matsuno et al., 2017). FF EVs also stimulate granulosa cell proliferation and reduce apoptosis, suggesting a broader role in supporting cell survival (Couty et al., 2024; Hung et al., 2017; Wang et al., 2021). They can also increase progesterone, oestradiol and testosterone secretion, suggesting modulation of steroidogenic enzyme activity (Couty et al., 2024). Beyond somatic cells, FF EVs can influence male gametes by promoting sperm viability and inducing key fertilisation processes such as capacitation and the acrosome reaction (Hasan et al., 2021). FF EVs also exert protective effects during early embryonic development: in bovine COCs subjected to heat shock, supplementation with FF EVs during oocyte maturation improved cleavage rates and blastocyst formation, suggesting that FF EVs may mitigate stress-induced damage during oocyte maturation (Rodrigues et al., 2019).

FF EVs carry a diverse RNA cargo, predominantly composed of ncRNAs. In human cohorts, FF EVs are enriched in miRNAs but also contain mRNA fragments and a broad range of other ncRNAs, including tRNAs, rRNAs, piRNAs, snRNAs, snoRNAs, lncRNAs, and circRNAs (Navakanitworakul et al., 2016; Santonocito et al., 2014; Wyse et al., 2023; Yu et al., 2023). This RNA composition is dynamic: the miRNA repertoire shifts with follicular stage and hormonal cues, suggesting that EV-mediated signalling adapts to the physiological needs of the follicle (de Ávila et al., 2020; Gebremedhn et al., 2023). Comparative analyses reveal substantial overlap in miRNA profiles between FF EVs and granulosa cells (Andrade et al., 2017), as well as between FF EVs and granulosa cell-derived EVs, with shared networks predicted to regulate key folliculogenesis pathways (Zhou et al., 2024).

EV cargo profiles undergo notable changes in ovarian pathologies and may participate in or reflect disease-associated processes. In polycystic ovary syndrome (PCOS), FF EV concentrations are elevated, and ncRNA profiles are altered, with adiposity identified as a major contributor to these transcriptomic changes (Rooda et al., 2020; Wyse et al., 2023). Proteomic analyses of FF EVs from PCOS patients reveal changes associated with inflammation, reactive oxygen species (ROS), and cell migration (Li et al., 2020). In ovarian hyperstimulation syndrome (OHSS), miR-27-3p is significantly downregulated in FF EVs, and functional assays implicate it in ROS production and granulosa cell apoptosis (Liu et al., 2021). In diminished ovarian reserve (DOR), FF EV abundance is reduced, and miRNA profiles are altered, with differentially expressed (DE) miRNAs linked to PI3K/AKT signalling and apoptotic regulation (Xie et al., 2024). These findings suggest that EV cargo alterations may reflect underlying pathology and potentially influence granulosa cell function in disease states.

Small RNAs in FF, whether associated with EVs or stabilised by AGO2 or lipoprotein complexes, are gaining recognition as promising biomarkers for predicting *in vitro* fertilisation (IVF) outcomes. Some studies have profiled total FF miRNAs in relation to fertilisation and embryo quality; for example, Machtinger et al. (2017) identified miRNAs that differed between follicles yielding fertilised oocytes or top-quality embryos versus

those with poorer developmental potential. Other investigations have explicitly focused on EV-associated miRNAs. Martinez et al. (2018) reported three FF EV miRNAs that distinguished top- from low-quality day 3 embryos, while Zhang et al. (2021) reported 47 FF EV miRNAs that varied between superior and poor-quality oocyte groups. Despite these promising associations, the requirement to isolate FF from individual follicles to link EV cargo with specific oocyte outcomes remains a major obstacle to clinical translation.

Across species, supplementation with FF EVs during oocyte *in vitro* maturation (IVM) or embryonic development has been shown to improve outcomes in assisted reproductive technologies (ART). In horses, FF EVs added to IVM media significantly improved oocyte maturation rates (Gabryś et al., 2022). In cattle, FF EV supplementation increased blastocyst yield (da Silveira et al., 2017), reduced vitrification-induced oocyte cryodamage (Diaz-Muñoz et al., 2025), and rescued oocyte competence under metabolic stress (Lipinska et al., 2025). These effects appear to be dose- and context-dependent: for instance, low-dose FF EV supplementation in individually cultured bovine COCs enhanced developmental outcomes, whereas higher doses or group culture settings yielded no benefit or even adverse effects (Azari-Dolatabad et al., 2025). Preliminary human studies have begun to explore FF EV supplementation in IVM, but current reports assess only oocyte maturation rates or molecular endpoints, without evaluating fertilisation, embryo development, or pregnancy outcomes (Makieva et al., 2025; Zingerenko et al., 2025). Comprehensive studies including functional endpoints and rigorous EV characterisation are therefore needed to assess the safety and efficacy of FF EV supplementation in human ART.

1.3 Clinical aspects and treatment of infertility

Infertility, defined as the inability to conceive after 12 months of regular unprotected intercourse, affects an estimated 8–12% of reproductive-age couples worldwide (Vander Borght & Wyns, 2018). The global burden of infertility is rising, driven by a complex interplay of factors, including delayed childbearing, increasing obesity rates, exposure to environmental pollutants, psychological stress, and lifestyle shifts such as poor diet and reduced physical activity (Aitken, 2024).

Infertility is commonly categorised into female, male, combined, and unexplained origins. In women, frequent aetiologies include ovulatory dysfunction, fallopian tube obstruction, pelvic adhesions, endometriosis, and DOR, which may result from age-related decline, genetic abnormalities, ovarian surgery, or chemotherapy (Carson & Kallen, 2021). Primary ovarian insufficiency (POI), defined as severe ovarian failure before age 40, is another important cause of infertility and is characterised by reduced ovarian reserve, menstrual irregularities, hypoestrogenism, and early menopause, indicative of near-complete follicle depletion (Chon et al., 2021; Pastore et al., 2018; The ESHRE Guideline Group on POI et al., 2016). Complete absence of ovulation can eliminate any chance of natural conception, whereas age-related decline in ovarian reserve tends to exert a more gradual, though clinically significant, impact on reproductive potential. Female fertility remains relatively stable until around age 31, after which it progressively declines. By age 38, the monthly probability of conception leading to live birth is approximately 25% of that in women under 30. This decline reflects a gradual depletion of ovarian reserve and an increase in meiotic errors that result in chromosomal aneuploidy, thereby compromising oocyte quality (te Velde & Pearson, 2002). For couples affected by infertility, ART offer a viable route to conception when natural attempts prove unsuccessful.

1.3.1 Assisted reproductive technologies (ART)

ART comprise a set of procedures involving *in vitro* handling of oocytes, sperm, or embryos, used to treat infertility (Zegers-Hochschild et al., 2017). Since the birth of the first IVF baby in 1978 (Steptoe & Edwards, 1978), ART has become central to reproductive medicine, with millions of children born worldwide through these techniques (European IVF Monitoring Consortium (EIM) et al., 2022). Core ART procedures include IVF and intracytoplasmic sperm injection (ICSI). Intrauterine insemination, though widely used in infertility treatment, is generally classified as medically assisted reproduction rather than ART (Zegers-Hochschild et al., 2017).

Intrauterine insemination involves placing processed sperm directly into the uterine cavity around the time of ovulation, bypassing potential cervical barriers and reducing the distance sperm must travel to reach the oocyte. It is often indicated in cases of mild male factor, cervical factor, or unexplained infertility, and may be performed with or without ovarian stimulation or ovulation induction to improve success rates (Practice Committee of the American Society for Reproductive Medicine, 2020). IVF represents a more advanced intervention, involving the retrieval of oocytes, their fertilisation with sperm *in vitro*, and subsequent embryo culture and transfer. ICSI is a refinement of IVF, in which a single sperm is injected directly into the cytoplasm of a mature oocyte. Originally developed to address severe male-factor infertility, ICSI is now also used in cases of low oocyte yield (Huang & Rosenwaks, 2012; Palermo et al., 2017).

For IVF and ICSI, treatment typically begins with controlled ovarian stimulation (COS), in which exogenous gonadotropins are administered to promote the development of multiple preovulatory follicles. GnRH agonists or antagonists are used alongside recombinant FSH (rFSH) to suppress premature LH surges, which could otherwise trigger untimely ovulation. COS is a critical step in fertility treatment, as it increases the number of oocytes available for fertilisation and thereby improves the likelihood of obtaining viable embryos (Farquhar et al., 2017). Follicular growth is monitored via ultrasound, and final oocyte maturation is triggered with hCG or, in women at increased risk of OHSS, with a GnRH agonist (The ESHRE Guideline Group on Ovarian Stimulation et al., 2020). Oocytes are retrieved transvaginally, fertilised, and embryos are cultured to cleavage or blastocyst stages before transfer (Glujovsky et al., 2022). Luteal phase support, typically with progesterone, may be provided to prepare the endometrium for implantation (The ESHRE Guideline Group on Ovarian Stimulation et al., 2020), and surplus embryos can be cryopreserved for future use. Despite decades of refinement, the live birth rate per IVF cycle remains modest, with large cohort analyses reporting a live birth rate of ~29.5% in the first IVF cycle across all age groups, higher rates in women under 40 (32.3%) and markedly lower success after the age of 40 (12.3%) (Smith et al., 2015). This limited success often necessitates multiple treatment cycles, highlighting the need for continued research into the biological, clinical, and environmental factors that influence ART outcomes.

1.3.2 Ovarian response to controlled ovarian stimulation (COS)

Ovarian response to COS varies considerably among individuals, and its accurate prediction remains a clinical challenge (Lebovitz et al., 2022). Based on their response to exogenous gonadotropins, ART patients are typically categorised as hyporesponders (HR), normoresponders (NR), or hyperresponders (Drakopoulos et al., 2023). HR patients, also referred to as poor responders, show limited follicular recruitment and generally yield three or fewer oocytes, often resulting in cycle cancellation (Ferraretti et al., 2011;

Romito et al., 2020). NR patients typically produce 10–15 oocytes, a range considered clinically optimal and associated with favourable outcomes. Hyperresponders display excessive follicular development, yielding more than 15 oocytes, and are at increased risk of developing OHSS (Drakopoulos et al., 2023). OHSS is a complication characterised by abdominal pain, nausea, ascites, and electrolyte imbalance, which in severe cases can progress to thromboembolic events or renal failure (Kumar et al., 2011).

Given this spectrum of responses, early identification of ovarian response profiles is critical for optimising COS. Tailored stimulation strategies can improve outcomes at both extremes: HR patients may require intensified or prolonged gonadotropin regimens, whereas hyperresponders may benefit from dose adjustments based on hormonal and ultrasound monitoring, or alternative ovulation trigger protocols to minimise the risk of OHSS (Drakopoulos et al., 2023; Lebovitz et al., 2022). Accurate patient stratification, therefore, supports safer, more personalised care and may improve ART success rates (La Marca & Sunkara, 2014).

Efforts to standardise the definition of poor ovarian response (POR) have evolved over time. The Bologna criteria, introduced in 2011, represented the first formal attempt to standardise POR classification using a combination of age, prior oocyte yield, and ovarian reserve markers such as AMH and antral follicle count (AFC) (Ferraretti et al., 2011). However, the Bologna criteria have been criticised for grouping heterogeneous patients with differing prognoses. In response, the POSEIDON (Patient-Oriented Strategies Encompassing Individualised Oocyte Number) classification, introduced in 2016, addressed this limitation by distinguishing expected from unexpected poor responders and stratifying patients into four prognostic groups, thereby supporting more individualised clinical decision-making. (Alviggi et al., 2016).

Traditionally, ovarian response has been predicted using ovarian reserve markers, most notably AMH and AFC. These indicators reflect the pool of recruitable follicles and allow early assessment before COS begins. However, ovarian reserve tests reflect follicle quantity rather than follicular performance, and women with similar AMH or AFC values may exhibit markedly different responses to stimulation (Zhuang et al., 2019). This discrepancy highlights that ovarian reserve and ovarian response to COS represent related but distinct dimensions of ovarian function. Such variability has also contributed to inconsistent use of several clinical terms related to ovarian biology.

Although POR and DOR are sometimes used interchangeably in the literature, they represent clinically distinct conditions. POR describes a functional outcome observed during COS—poor follicular recruitment and low oocyte yield despite adequate stimulation. DOR, in contrast, refers to a quantitative reduction in the pool of recruitable follicles, typically assessed using AMH and AFC, and does not inherently predict poor response to COS. In other words, DOR describes a reserve-based condition, whereas POR reflects an impairment in how follicles respond to stimulation—an aspect of ovarian physiology that cannot be inferred from static reserve markers.

This distinction has motivated the development of dynamic measures that quantify ovarian response to COS. One such measure is the ovarian sensitivity index (OSI), which relates the number of retrieved oocytes to the total dose of exogenous rFSH administered (Biasoni et al., 2011). OSI provides a more integrative measure of ovarian responsiveness by accounting for both stimulation intensity and outcome. It has been shown to correlate with embryo quality, clinical pregnancy and live birth rates, making it a valuable metric for individualised treatment planning (Weghofer et al., 2020). However, OSI is calculated inconsistently across studies—either as oocytes per rFSH dose

or the inverse—meaning that a low OSI value may indicate hyporesponse or hyperresponse depending on the formula applied (Biasoni et al., 2011; Revelli et al., 2009). Clear reporting of the calculation method is therefore essential for meaningful comparison between studies and for accurate clinical interpretation.

The mechanisms underlying varied ovarian response, particularly hyporesponse, remain incompletely understood. Both genetic and environmental factors appear to shape individual outcomes following COS. Among genetic contributors, polymorphisms affecting gonadotropins and their receptors, most notably variants in the *FSHR* gene, have been linked to altered ovarian sensitivity to FSH stimulation (Mayorga et al., 2000). Environmental influences, including exposure to endocrine-disrupting chemicals (EDCs), are also increasingly recognised as contributors to impaired ovarian response (Bellavia et al., 2023), underscoring the multifactorial nature of ovarian responsiveness.

1.4 Environmental influences on female reproduction

Since the mid-20th century, widespread industrialisation and chemical innovation have introduced tens of thousands of synthetic compounds into the environment, with over 800 now recognised or suspected to act as EDCs (Encarnação et al., 2019). These substances, both natural and synthetic, can interfere with hormonal regulation and compromise reproductive health in both males and females (Sifakis et al., 2017). EDCs are exogenous compounds that mimic, block, or otherwise alter the activity of endogenous hormones. They exert their effects through multiple mechanisms, including binding to hormone receptors, altering hormone synthesis, metabolism, or transport, and interfering with intracellular signalling pathways (La Merrill et al., 2020). Given that reproductive processes rely on precise hormonal coordination, even minor disruptions can result in significant physiological consequences.

EDCs span a broad spectrum of chemical classes and applications, with widespread use in manufacturing, agriculture, household goods, and personal care products. Notable examples include dioxins, pesticides, phthalates, bisphenols, polychlorinated biphenyls (PCBs), parabens, heavy metals, as well as per- and polyfluoroalkyl substances (PFAS) (Yilmaz et al., 2020). These compounds are typically classified based on their persistence in biological systems. Persistent EDCs, such as PCBs, dioxins, PFAS, and certain heavy metals, resist degradation and accumulate in tissues, often remaining in the body for years or even decades. This persistence allows them to exert continuous biological effects long after initial exposure. In contrast, non-persistent EDCs, including bisphenols, phthalates, and parabens, are more readily metabolised and excreted, yet frequent or chronic exposure can still result in sustained endocrine disruption (Kumar et al., 2020).

Although EDCs are often associated with synthetic compounds, certain naturally occurring substances also exhibit potent endocrine activity. Phytoestrogens, found in soy, green tea, flaxseed, and other plant-based foods, can mimic endogenous oestrogens and bind to hormone receptors with affinities that, in some cases, exceed those of synthetic EDCs. Despite their natural origin, these compounds may exert significant biological effects, particularly when exposure occurs during hormonally sensitive life stages or in conjunction with other environmental stressors (Autrup et al., 2020; Virtuoso et al., 2024).

Due to their widespread use in everyday products, including plastics, food packaging, cosmetics, and household items, humans are exposed to EDCs daily through ingestion, inhalation, and dermal contact. The biological impact of this exposure is not uniform; it varies significantly depending on the timing, duration, and dose (Yilmaz et al., 2020).

Notably, women may experience higher cumulative exposure due to more frequent use of personal care products, cosmetics, and cleaning products that contain EDCs (Kim et al., 2023b). Developmental periods such as fetal life, infancy, and puberty are particularly vulnerable due to ongoing hormonal programming and tissue differentiation. Disruption during these time windows can lead to long-lasting alterations in reproductive function, with effects that may not manifest until later in life (Lopez-Rodriguez et al., 2021).

In females, EDCs can interfere with reproductive physiology at multiple levels, targeting the HPG axis, ovarian folliculogenesis, steroidogenesis, and uterine function (Craig et al., 2011; Pan et al., 2024b). These disruptions may manifest as altered menstrual cyclicity, anovulation, reduced oocyte quality, and impaired endometrial receptivity, ultimately leading to diminished fertility (Cho et al., 2019; Land et al., 2022; Lavogina et al., 2022). Epidemiological and experimental studies have linked EDC exposure to a range of reproductive disorders, including uterine fibroids, endometriosis, PCOS, and POI (Bariani et al., 2020; Evangelinakis et al., 2024; Kandaraki et al., 2011; Stephens et al., 2022). Additionally, EDCs may reduce the efficacy of ART by compromising oocyte competence and causing implantation failure (Ehrlich et al., 2012; Fujimoto et al., 2011; Mahalingaiah et al., 2012). When exposure occurs during pregnancy, EDCs have been associated with increased risk of fetal growth retardation, as well as disruptions in neurobehavioral development, including impairments in cognition, motor skills and language acquisition (Lauritzen et al., 2017; Yang et al., 2024). While direct evidence of transgenerational effects in humans remains limited, studies have shown that EDCs can alter the abundance of epigenetic regulators and their cofactors, leading to changes in DNA methylation, histone modifications, and ncRNA expression (Alavian-Ghavanini & Rüegg, 2018). Animal models further demonstrate that EDC-induced epigenetic modifications may persist across generations, potentially affecting offspring not directly exposed to EDCs (Rattan & Flaws, 2019).

Despite growing evidence of the harmful effects of EDCs, significant gaps remain in our understanding of their mechanisms, interactions, and long-term consequences (Tzouma et al., 2025). Many studies rely on animal models or *in vitro* systems, which, while informative, may not fully capture the complexity of human endocrine physiology (Patisaul et al., 2018). Additionally, substantial variability in study design, exposure assessment, and outcome measures makes it difficult to compare findings across studies or establish causal relationships (Tzouma et al., 2025). The latency between exposure and disease manifestation, often spanning years or even decades, further complicates efforts to link EDCs to specific reproductive outcomes (Patisaul et al., 2018).

Given the ubiquity of EDCs in consumer products, mitigating exposure has become a pressing public health priority. Regulatory efforts began gaining momentum in the early 2000s, with the European Union taking a leading role through the REACH regulation (Registration, Evaluation, Authorisation and Restriction of Chemicals), enacted in 2006 (Kassotis et al., 2020). REACH has been central to chemical safety governance, resulting in restrictions or bans on several EDCs, including bisphenol A in thermal paper and baby bottles, multiple phthalates in toys, childcare articles, and other plasticised materials, and nonylphenol ethoxylates in textiles (European Commission, 2011, 2016b, 2016a, 2018). Regulatory stringency varies across world regions, with differing levels of precaution and implementation deadlines (Kassotis et al., 2020).

To support chemical regulation, the Organisation for Economic Co-operation and Development (OECD) has developed internationally standardised test guidelines to identify substances with endocrine-disrupting properties. These guidelines comprise a

range of *in vitro* and *in vivo* methods for detecting hormone-mediated effects. Most validated assays focus on disruption of oestrogenic and androgenic signalling and associated hormone-dependent responses in animal models, commonly evaluated through changes in reproductive organ weights, sexual development, and fertility outcomes (OECD, 2018). Although these approaches provide a consistent basis for regulatory decision-making, they have important limitations. The assays are optimised mainly to detect effects mediated by endogenous hormones acting through classical receptor-mediated mechanisms, which may limit sensitivity to low-potency chemicals or substances acting via non-classical endocrine pathways. In addition, disruption of the thyroid axis is comparatively poorly addressed relative to oestrogen and androgen pathways, while other hormonally regulated systems, including metabolic and glucocorticoid signalling, are not covered by the current guidelines (Kassotis et al., 2020).

1.4.1 Phthalates and their impact on ovarian biology

Phthalates, or phthalic acid esters, are a major class of non-persistent EDCs widely used as plasticisers to enhance the flexibility and durability of polymers such as polyvinyl chloride (PVC) (Basso et al., 2022). Because they are not covalently bound to the polymer matrix, phthalates can readily leach into the surrounding environment, contaminating air, dust, water, and food (Wang & Qian, 2021). Following human exposure, the parent compounds are rapidly metabolised in the gastrointestinal tract and liver to monoester metabolites, which often exhibit greater biological activity and toxicity than their precursors. Although phthalates are rapidly excreted, continuous low-level exposure is maintained through daily contact with contaminated food, water, and consumer products (Frederiksen et al., 2007). Biomonitoring, typically based on single-sample urine analysis, captures only recent exposure and fails to capture cumulative burden, complicating efforts to associate phthalate levels with long-term health outcomes (Johns et al., 2015; Koch & Calafat, 2009; Wang et al., 2019).

Among phthalates, di(2-ethylhexyl) phthalate (DEHP) is the most extensively used, being incorporated into numerous medical, industrial, and consumer products, including intravenous bags, medical tubing, catheters, furniture upholstery, wall coverings, flooring, food packaging, and personal care items (Rowdhwal & Chen, 2018; Wang et al., 2019). DEHP undergoes rapid biotransformation in the human body (Figure 6). Its primary metabolite is mono(2-ethylhexyl) phthalate (MEHP), which is subsequently metabolised by oxidation and hydroxylation reactions to secondary metabolites, including mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono(2-ethyl-5-carboxypentyl) phthalate (MECPP), and mono(2-carboxymethylhexyl) phthalate (MCMHP) (Silva et al., 2006). These metabolites have been consistently detected in biological fluids relevant to female reproduction, such as amniotic fluid, breast milk, and FF (Bellavia et al., 2023; Bräuner et al., 2022; Hung et al., 2021). These findings indicate that exposure begins early in development and extends into adulthood, raising concerns about both developmental programming and ovarian health.

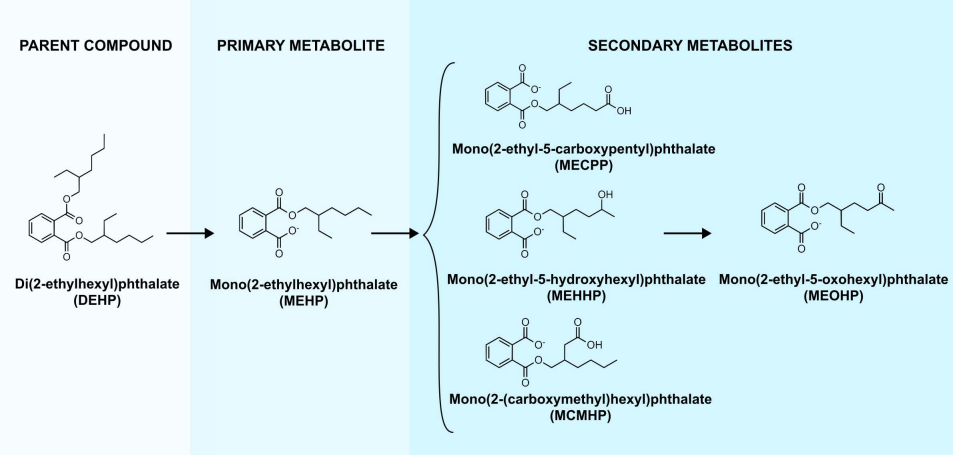

Figure 6. *Metabolism of di(2-ethylhexyl) phthalate (DEHP). Adapted from Lorber et al., 2010 and created using ChemDraw.*

While the reproductive toxicity of phthalates in males is well documented, their effects on female reproductive health remain comparatively less characterised. Epidemiological studies have linked elevated urinary DEHP metabolite levels to impaired ovarian function, including lower oocyte yield, reduced fertilisation rates, and diminished embryo quality (Machtinger et al., 2018). Higher DEHP exposure has also been associated with reduced AFC in women undergoing infertility treatment, indicating a negative impact on ovarian reserve (Messerlian et al., 2016). Extending these observations to the follicular microenvironment, elevated concentrations of DEHP metabolites in FF have been inversely associated with the OSI, reflecting reduced ovarian responsiveness to gonadotropin stimulation in the context of phthalate exposure (Bellavia et al., 2023). Together, these impairments may help explain observed associations between DEHP and adverse fertility outcomes, including reduced probabilities of embryo implantation, clinical pregnancy, and live birth, as well as increased risks of pregnancy loss and preterm birth (Ferguson et al., 2014; Hauser et al., 2015; Mínguez-Alarcón et al., 2019; Toft et al., 2012). In addition, phthalate exposure has been linked to reproductive pathologies, with elevated DEHP levels reported in FF of women with PCOS (Jin et al., 2019), and increased serum concentrations of monobutyl phthalate (MBP) observed in women with POI (Özel et al., 2019).

Mechanistically, phthalates and their metabolites have been reported to interact with multiple components of the endocrine system. For instance, MEHP has been consistently shown to activate peroxisome proliferator-activated receptors (PPARs), particularly PPARα and PPARγ, thereby altering transcriptional programmes related to lipid metabolism, steroidogenesis, and cellular differentiation (Engel et al., 2017; Feige et al., 2007; Hurst & Waxman, 2003; Komar, 2005). In addition to PPAR signalling, *in vitro* reporter gene assays have shown that several phthalates can weakly modulate sex steroid receptor activity, including stimulation or inhibition of oestrogen receptors ERα and ERβ and inhibition of AR signalling (Engel et al., 2017). MEHP has also been reported to activate the aryl hydrocarbon receptor (AHR) (Neff et al., 2024), a transcription factor increasingly recognised as a regulator of female reproductive physiology (Hernández-Ochoa et al., 2009). Beyond receptor-mediated effects, *in silico* studies suggest that phthalates may interfere with hormone transport by binding to sex hormone-binding globulin (SHBG),

potentially altering the bioavailability of circulating oestrogens and androgens (Sheikh et al., 2016).

The downstream consequences of these primary interactions have been characterised across multiple ovarian cell and tissue models. In human primary granulosa cells, physiologically relevant mixtures of commonly used phthalates impair ovulatory function by reducing progesterone production and downregulating *PGR* expression (Hannon et al., 2023). DEHP also suppresses oestradiol synthesis and reduces expression of *CYP19A1* and *FSHR* in the KGN granulosa-like tumour cell line, but only in the presence of FSH, indicating interference with FSH-dependent signalling pathways (Ernst et al., 2014). This is further supported by findings in human cumulus granulosa cells, where DEHP has been shown to impair two major FSH-activated pathways, cAMP and ERK1/2 (extracellular signal-regulated kinase 1/2), leading to reduced *CYP19A1* expression and diminished production of both oestradiol and progesterone (Tesic et al., 2023). Beyond effects on granulosa cells, animal studies indicate that DEHP may exert direct effects on the oocyte. In fetal mouse oocytes cultured *in vitro*, DEHP exposure impairs meiotic progression and disrupts DNA damage repair, indicating potential interference with oocyte maturation (Liu et al., 2017). At the tissue level, MEHP has been shown to compromise the survival and growth of human ovarian follicles. *In vitro* exposure of adult human ovarian tissue to epidemiologically relevant MEHP concentrations reduces follicular growth, increases degeneration, and disrupts cytoskeletal organisation and Hippo signalling (Panagiotou et al., 2024). Collectively, previous findings demonstrate that DEHP and its metabolites can impair ovarian function by targeting steroidogenesis, oocyte maturation, and follicle viability.

Despite the growing body of evidence linking phthalates to adverse effects on ovarian physiology, the molecular events by which DEHP and its metabolites disrupt human follicle development, particularly through effects on granulosa cell function, remain incompletely defined. Resolving these mechanisms is critical for establishing causal links between exposure and reproductive outcomes and for developing more predictive toxicity testing frameworks that incorporate female-specific endpoints.

2 Aims of the study

The aims of the thesis are as follows:

- Characterise and compare the molecular profiles of small and large EVs in human preovulatory FF, with a focus on surface tetraspanin expression and small RNA cargo.
- Examine the functional effects of small and large FF EVs on granulosa cells, using the KGN cell line to assess changes in steroid hormone production and alterations in mRNA and small RNA expression.
- Characterise FF EV and cell-free FF miRNA profiles across patients with varying OSI and identify regulatory signatures associated with differences in ovarian responsiveness to gonadotropin stimulation.
- Identify gene expression changes in KGN granulosa cells treated with patient-derived EVs that correlate with OSI, and determine the biological pathways linked to these OSI-associated genes.
- Investigate the molecular consequences of elevated DEHP metabolite levels in human FF, focusing on alterations in FF miRNA expression, FF steroid hormone levels, and transcriptomic profiles of primary granulosa cells.

3 Materials and methods

The following methods, described in detail in the respective publications, were used in this dissertation:

- Collection of FF – Publications I, II and III
- Cell culture – Publications I, II
- Isolation of FF EVs by SEC – Publications I, II
- Isolation of FF EV subpopulations by TFF – Publication I
- Nanoparticle tracking analysis – Publications I, II
- Total protein quantification – Publications I, II
- Western blot – Publications I, II
- Transmission electron microscopy – Publications I, II
- Single particle interferometric reflectance imaging – Publication I
- EV labelling and uptake assay – Publications I, II
- Fluorescence microscopy – Publications I, II
- Cytotoxicity assay – Publication I
- Enzyme-linked immunosorbent assay – Publications I, II
- Liquid chromatography with tandem mass spectrometry – Publication III
- RNA extraction – Publications I, II and III
- Library preparation and bulk RNA-seq – Publications I, II and III
- Library preparation and small RNA-seq – Publications I, II and III
- Differential gene expression analysis – Publications I, II and III
- Pathway enrichment analysis of DE genes – Publications I, III
- Over-representation analysis of DE genes – Publications I, III
- Gene set enrichment analysis – Publication II
- Cell-type deconvolution analysis – Publication III
- Bioinformatic prediction of miRNA targets – Publications I, III
- Statistical data analysis – Publications I, II and III

4 Results and discussion

4.1 Molecular divergence of FF EV subtypes (Publication I)

Despite growing recognition of EVs as key mediators of intercellular communication within the ovarian follicle (Machtinger et al., 2021), fundamental questions remain regarding the heterogeneity and functional specificity of distinct EV subtypes. Human FF contains a complex and dynamic mixture of EV populations (Neyroud et al., 2022), yet the majority of studies have approached these vesicles as a largely homogenous entity. As a result, potential size-dependent differences in EV abundance, molecular composition, and biological function remain insufficiently explored. This apparent conceptual simplification reflects the practical constraints of the field. Persistent challenges—including the absence of standardised approaches for isolating EV subpopulations, the limited availability of subtype-specific molecular markers, and an evolving EV nomenclature (Welsh et al., 2024)—have made it difficult to investigate EV heterogeneity within complex biofluids such as FF routinely.

Given the central role of granulosa cells in coordinating follicular development, steroidogenesis, and oocyte maturation (Gilchrist et al., 2004), resolving whether distinct FF EV subtypes carry unique molecular signatures with the capacity to influence granulosa cell behaviour is essential for understanding intrafollicular communication. In Publication I, FF EVs were isolated using SEC and stratified into small EVs (SEVs; <200 nm) and large EVs (LEVs; >200 nm) using TFF, enabling a comparative assessment of their relative abundance, surface marker composition, and RNA cargo.

Clear size-dependent distinctions were observed between the two EV subpopulations. SEVs exhibited a mean diameter of 93 nm, whereas LEVs averaged 299 nm in diameter (Publication I, Figure 2B). SEVs were also substantially more abundant, with particle concentrations exceeding those of LEVs by more than an order of magnitude ($p < 0.01$) (Publication I, Figure 2A). This predominance of SEVs in FF is consistent with observations from other biofluids, in which vesicles smaller than 200 nm typically constitute the major EV fraction (Gámez-Valero et al., 2016). Consequently, studies analysing bulk FF EV isolates are likely to be dominated by SEV-derived molecular signals, potentially masking functional contributions from less abundant LEV populations.

Despite shared expression of canonical EV markers, SEVs and LEVs displayed distinct surface tetraspanin profiles. SEVs contained a higher proportion of triple-positive particles, whereas LEVs were enriched in CD9/CD81- and CD63/CD81-positive particles (Publication I, Figure 3A–C). This pattern aligns with previous observations indicating that EVs enriched in CD9 and CD81, but relatively low in CD63, are more frequently associated with vesicles originating from the plasma membrane rather than the endosomal pathway, as reported in HeLa and HEK293 cells (Han et al., 2023; Mathieu et al., 2021). Accordingly, the CD9/CD81-enriched LEVs observed here exhibit surface marker profiles resembling those reported for microvesicles. However, it is important to note that surface marker expression alone cannot definitively determine EV biogenesis.

Statistical comparison of the observed tetraspanin distributions with simulated random patterns demonstrated that the detected colocalisation profiles were non-random (Publication I, Figure 3D-E), supporting the notion that specific tetraspanin combinations are preferentially incorporated into distinct EV subtypes. Such compositional bias likely reflects differences in EV formation pathways, including endosomal versus plasma membrane origin, and may have functional implications for downstream interactions

with recipient cells, such as cellular targeting and uptake efficiency. Moreover, as EVs derived from distinct cell types are known to display characteristic tetraspanin signatures (Breitwieser et al., 2022; Mizenko et al., 2021), it is plausible that SEVs and LEVs not only differ in their biogenesis routes but may also originate from distinct ovarian cell populations. While differences in biogenesis likely provide the primary explanation for the observed tetraspanin patterns, cell type-specific expression of tetraspanins may further contribute to the molecular diversity distinguishing SEV and LEV populations.

Following the characterisation of EV surface tetraspanin profiles, the molecular cargo of SEVs and LEVs was examined to assess size-dependent differences in RNA content. Small RNA sequencing identified a total of 148 miRNAs and 223 piRNAs across all samples (Publication I, Table S4), of which 48 miRNAs and 50 piRNAs were DE between SEVs and LEVs at a false discovery rate (FDR) < 0.05 (Publication I, Figure 6B; Table S5). The vast majority of DE miRNAs (45/48; 93.4%) were upregulated in SEVs, whereas most DE piRNAs (37/50; 74%) were upregulated in LEVs. This preferential enrichment of miRNAs in SEVs and piRNAs in LEVs aligns with emerging evidence from other biological fluids, supporting the concept that EVs of different sizes carry distinct RNA signatures. For instance, in human serum and plasma, SEVs are enriched in Y RNAs and miRNAs, whereas LEVs contain a higher proportion of tRNAs (Xie et al., 2025). Similarly, in porcine seminal plasma, most DE miRNAs were more abundant in SEVs, while DE piRNAs were evenly distributed across EV subtypes (Barranco et al., 2024). Collectively, these findings support the hypothesis that EV size and/or biogenetic origin influence the selective packaging of specific RNA classes into distinct EV subpopulations.

To explore the potential functional relevance of the DE miRNAs, pathway enrichment analysis was performed. The most significantly enriched Reactome terms were associated with broad regulatory processes, including transcriptional regulation, cellular senescence, cellular responses to external stimuli, cell cycle regulation, and tRNA processing (Publication I, Figure 6C; Table S6). Such categories are commonly identified in miRNA-based enrichment analyses and reflect the capacity of miRNAs to influence fundamental cellular regulatory networks rather than acting on isolated signalling pathways. Within this broader regulatory context, several signalling pathways with well-established roles in ovarian biology—including TGF-β, WNT, Ca^{2+}, tumour necrosis factor (TNF), and ER signalling—were also significantly represented. These pathways govern key aspects of follicular development and function, encompassing granulosa cell proliferation and differentiation (TGF-β), follicle maturation and steroidogenesis (WNT), oocyte maturation (ER and Ca^{2+}), and follicle rupture during ovulation (TNF) (Chen et al., 2024; Gifford, 2015; Knight & Glister, 2006; Liu et al., 2017; Yamamoto et al., 2014). Importantly, evidence from equine, porcine, and human FF studies further supports the relevance of these pathways in EV-mediated communication. Across species, FF EV miRNAs have consistently been reported to target genes involved in cell cycle regulation, TGF-β, WNT, TNF, Ca^{2+}, and oestrogen signalling (da Silveira et al., 2012; Gad et al., 2022; Martinez et al., 2018; Santonocito et al., 2014), indicating a conserved regulatory signature with potential roles in follicle development, oocyte maturation, and ovulation. In contrast, LEVs were characterised primarily by enrichment of piRNAs, for which reliable and widely accepted tools for pathway-level functional annotation are currently lacking. This limitation highlights a current gap in understanding the potential regulatory roles of LEV-associated RNA cargo within the ovarian follicle.

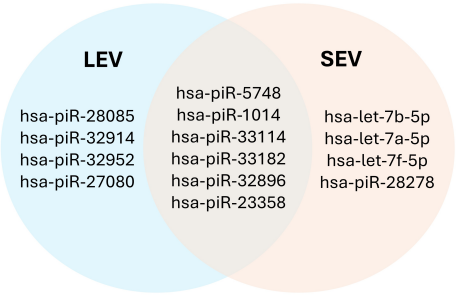

Figure 7. *Top ten most abundant miRNAs and piRNAs within small (SEV) and large (LEV) FF EVs. Data derived from Publication I, Table S4.*

Across both SEV and LEV populations, the most abundant small RNA was hsa-piR-5748 (Figure 7). Although piRNAs are traditionally associated with transposon silencing (Sato & Siomi, 2020), increasing evidence indicates that they can also regulate gene expression at the post-transcriptional level (Watanabe & Lin, 2014). In mesenchymal stem cells, hsa-piR-5748 has been shown to downregulate multiple target genes, including fibroblast growth factor 2 (*FGF2*) (Bogusławska et al., 2025). FGF2 is a well-established regulator of ovarian biology, known to promote granulosa cell proliferation while limiting differentiation and steroidogenesis (Price, 2016). Consistent with the idea that piRNAs may have broader regulatory roles in the follicle, previous studies have detected piRNAs in human FF EVs and reported variations in their abundance under different physiological conditions and pathological conditions, including pregnancy and PCOS (Hu et al., 2020; Muraoka et al., 2024). Notably, hsa-piR-5748 is downregulated in FF EVs from PCOS patients (Hu et al., 2020), suggesting that altered levels of this piRNA may have functionally relevant consequences for follicle development.

Among the miRNAs, the most abundant species in SEVs belonged to the let-7 family, including hsa-let-7a-5p, hsa-let-7b-5p, and hsa-let-7f-5p (Figure 7). Although these miRNAs were also detected in LEVs, their abundance was two- to four-fold lower (Publication I, Table S4), highlighting their preferential enrichment in SEVs. Substantial evidence supports the functional relevance of the let-7 family in ovarian biology. In mice, let-7b regulates CL angiogenesis by modulating the anti-angiogenic factor TIMP1, thereby promoting luteal vascularisation (Otsuka et al., 2008). In porcine ovaries, let-7a and let-7b are significantly downregulated during follicular atresia (Cao et al., 2015), implicating this family in the regulation of follicle fate. In humans, consistent downregulation of let-7 family miRNAs in developmentally arrested oocytes compared to normal oocytes (Wei et al., 2022) suggests that adequate levels of these miRNAs are required to sustain oocyte competence. Taken together, these findings suggest that EV-enriched let-7 miRNAs may contribute to the regulation of angiogenesis, follicle survival, and oocyte competence. Although their precise mechanisms of action remain unresolved, the consistent enrichment of let-7 miRNAs in FF EVs provides a strong rationale for future studies investigating their transfer to recipient cells and their functional roles within the ovarian follicle.

In summary, the molecular profiling performed in Publication I demonstrates that FF EVs are not a homogeneous population but rather comprise functionally distinct SEV and LEV subtypes with distinct molecular signatures that may mediate different aspects of intrafollicular communication. This molecular characterisation establishes a foundation for subsequent investigation of their functional effects on granulosa cells.

4.2 Impact of FF EVs and their subpopulations on KGN granulosa cells (Publications I and II)

Following the molecular characterisation of FF EV subpopulations, their functional effects on granulosa cell gene expression were investigated using complementary experimental designs in Publications I and II. Publication I focused on subtype-specific effects by comparing size-resolved FF EV populations derived from pooled FF samples, whereas Publication II addressed patient-specific variation by analysing total FF EVs isolated from clinically characterised individuals stratified by ovarian sensitivity to gonadotropin stimulation. Together, these approaches enabled the assessment of both EV subtype-dependent and patient-dependent influences on granulosa cell function.

To first establish the overall impact of FF EVs on granulosa cells, KGN cells were exposed to patient-specific FF EVs or to a vehicle control for 24 hours. The EV concentrations used in these experiments reflected both the maximal particle yields achievable with the isolation workflow and the physiological predominance of SEVs within FF. EV treatment induced extensive transcriptional remodelling relative to vehicle-treated cells (Publication II, Figure 3A), with 667 genes upregulated and 328 genes downregulated at FDR < 0.05 and $|\log_2 FC| > 0.6$ (Publication II, Figure 3B; Table S2). These transcriptional responses were highly consistent across EVs derived from both NR and HR patients, indicating that FF EVs broadly modulate granulosa cell gene expression independent of ovarian response classification. Pathway enrichment analysis revealed upregulation of ECM-related pathways, including collagen biosynthesis and fibril assembly, whereas downregulated pathways included those involved in cell cycle regulation, cholesterol biosynthesis, and steroid metabolism (Figure 8). Together, these patterns suggest that FF EV exposure promotes transcriptional programmes associated with ECM remodelling while attenuating pathways linked to steroidogenic activity.

Although cell cycle-related pathways were among the most strongly downregulated transcriptional programmes in EV-treated granulosa cells ($\log_2 FC$: -0.63 to -0.08; median - 0.2), this did not translate into measurable changes in cell proliferation within the 48-hour observation period (Publication I, Figure 5B). Importantly, transcriptional changes in cell cycle-associated genes do not necessarily result in immediate alterations in proliferation rates, as cell cycle progression is regulated at multiple levels beyond transcription. Such changes may instead reflect modulation of cell cycle timing, checkpoint activity, or cellular readiness to proliferate rather than net cell division. The absence of detectable effects on proliferation may also relate to the experimental timeframe and the use of the KGN granulosa-like tumour cell line, which may buffer transcriptional perturbations that would have more pronounced effects in primary granulosa cells. Together, these considerations suggest that EV-induced transcriptional changes in cell cycle-associated genes likely represent early regulatory events, the functional consequences of which may require prolonged exposure or more sensitive assays to detect.

The enrichment of ECM-related pathways following FF EV exposure highlights a potential role for EV-mediated signalling in shaping the follicular microenvironment. Follicular growth requires continuous ECM expansion and remodelling to accommodate increasing follicular size (Woodruff & Shea, 2007). Disruptions in these processes, including reduced ECM gene expression in cumulus granulosa cells, have been described in PCOS during the peri-ovulatory period (Hassani et al., 2019). Supporting a direct role

for EVs in ECM regulation, proteomic analyses have detected ECM components, including laminin, collagen, and fibronectin, in porcine FF EVs (Grzesiak et al., 2020). Together, these findings suggest that FF EVs may help maintain ECM integrity and structural adaptability during follicle development.

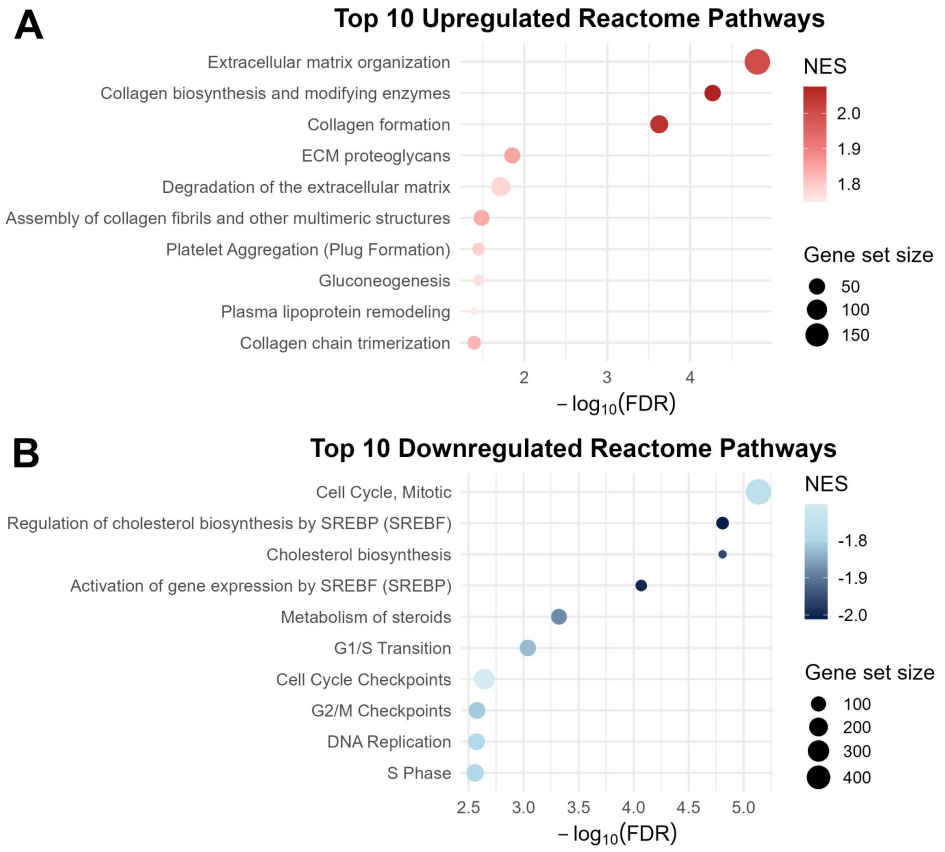

Figure 8. *Top 10 (**A**) upregulated and (**B**) downregulated Reactome pathways in FF EV-treated KGN cells. Pathways are ranked by normalised enrichment score (NES), with significance defined as FDR < 0.05. Dot size corresponds to gene set size, and colour indicates the direction and magnitude of NES (adapted and modified from Publication II, Figure 3C).*

In parallel with ECM-related changes, FF EV exposure was associated with downregulation of cholesterol and steroid biosynthesis pathways, suggesting reprogramming of granulosa cell metabolic activity. Cholesterol, the essential precursor for all ovarian steroids, can be derived from *de novo* synthesis, mobilisation of intracellular lipid stores, or uptake from extracellular lipoproteins such as high-density lipoprotein (HDL) and low-density lipoprotein (LDL) present in FF (Azhar et al., 1998; Grummer & Carroll, 1988). Reduced expression of cholesterol biosynthesis genes in EV-treated cells may therefore reflect a physiologically economical strategy that favours the utilisation of stored or extracellular cholesterol rather than the energetically costly *de novo* synthesis. At the same time, suppression of cholesterol biosynthetic pathways has been linked to DOR and impaired steroidogenic capacity (Yang et al., 2022), raising the possibility that EV-mediated transcriptional changes reflect underlying patient-specific reproductive physiology.

To determine whether these transcriptional changes translated into functional alterations in steroid output, oestradiol, progesterone, and testosterone concentrations were measured in the culture medium of EV-treated KGN cells. No changes in oestradiol or progesterone levels were detected 24 hours after treatment. Notably, however, treatment with LEVs and with combined SEV+LEV preparations increased testosterone production at specific concentrations (Publication I, Figures 5C-E), despite the absence of transcriptional changes in steroidogenesis-related genes following LEV treatment (Publication I, Figure 7B). This dissociation between gene expression and hormone output suggests that LEVs may influence androgen production through mechanisms other than transcriptional regulation, such as the delivery of steroidogenic enzymes, modulation of enzymatic activity, or direct transfer of androgens.

Given these functional outcomes, FF EV subtype-specific effects on granulosa cells were examined in greater detail. Treatment of KGN cells with SEVs or LEVs for 24 hours revealed a marked contrast in transcriptional impact. SEVs induced extensive gene expression changes relative to control (692 genes upregulated, 904 genes downregulated) (Publication I, Figure 7C), with strong induction of ECM-related pathways and suppression of cholesterol biosynthesis genes (Publication I, Figure 7D-E), closely mirroring the signatures observed following exposure to total FF EVs. In contrast, LEVs altered the expression of only five genes (Publication I, Figure 7B; Table S7-S8) despite efficient cellular uptake within six hours (Publication I, Figure 4), indicating a limited effect on gene expression within this timeframe.

Despite their minimal impact on mRNA expression, LEVs substantially altered the small RNA landscape of KGN cells. Exposure to LEVs resulted in upregulation of 46 miRNAs and downregulation of 42 miRNAs relative to SEV-treated cells, with many of the upregulated miRNAs detected within LEVs themselves (Publication I, Figure 8E), supporting their direct delivery to granulosa cells. The absence of widespread transcriptomic changes following LEV treatment suggests that LEV-associated miRNAs may primarily act at the translational level or that prolonged exposure is required for downstream effects on mRNA abundance to become apparent.

Following the observation that LEVs alter the small RNA landscape, over-representation analysis was performed to identify pathways potentially influenced by these miRNAs. miRNAs upregulated following LEV treatment were linked to pathways involving IGF-2 mRNA-binding proteins (IGF2BPs) and PTEN signalling, both of which are highly relevant to granulosa cell physiology. IGF2BP2, which is upregulated in PCOS, has been reported to promote glycolysis and proliferation in KGN cells, whereas its knockdown suppresses these processes and improves reproductive outcomes in mice (Han et al., 2026). PTEN likewise plays a critical role in regulating granulosa cell survival and lifespan (Fan et al., 2008).

In contrast, miRNAs upregulated in SEV-treated cells were predominantly associated with transcriptional regulation and TGF-β/SMAD signalling (Publication I, Figure 8C). Integrative analysis across four SEV-related datasets consistently highlighted RNA polymerase II-associated pathways, along with recurrent enrichment of TGF-β, cytokine, and receptor tyrosine kinase signalling, as well as integrin-mediated interactions (Figure 9). Collectively, these findings identify SEVs as dominant modulators of granulosa cell gene expression, whereas LEVs appear to exert more subtle regulatory effects, primarily through small RNAs. Future studies incorporating higher LEV doses and extended treatment durations will be important to determine whether LEVs can elicit broader transcriptomic changes and to further define their functional roles within the follicular environment.

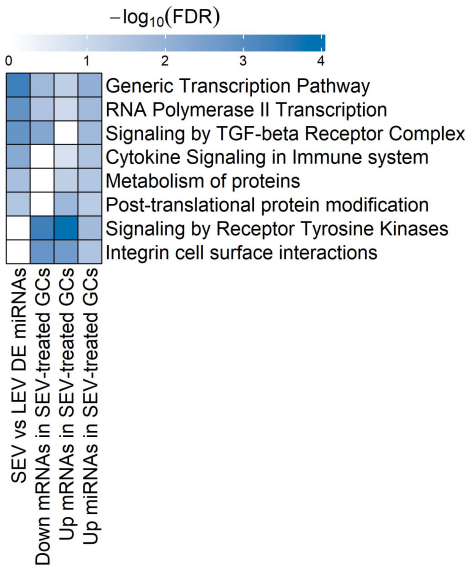

Figure 9. *Heatmap of statistically significant Reactome pathways associated with SEVs: SEV vs LEV DE miRNAs (45 out of 48 up in SEVs), downregulated mRNAs in SEV-treated KGN cells, upregulated mRNAs in SEV-treated KGN cells, and upregulated miRNAs in SEV-treated KGN cells. Colour intensities represent the -log$_{10}$(FDR) values for each pathway, and only pathways detected in more than three datasets are shown (adapted and modified from Publication I, Figure 9).*

4.3 Small RNA profiles of FF EVs and cell-free FF across patients with varying sensitivity to COS (Publication II)

Ovarian sensitivity to COS varies considerably among patients undergoing assisted reproduction (Huber et al., 2013) and is clinically relevant, as reduced sensitivity has been associated with fewer high-quality embryos and lower live birth rates (Weghofer et al., 2020). In Publication II, ovarian sensitivity was quantified using OSI, which relates the total dose of exogenous rFSH to the number of retrieved oocytes, with lower OSI values corresponding to NR patients and higher OSI values to HR patients.

In earlier work, our research group identified distinct transcriptomic profiles in somatic cells from preovulatory follicles of NR and HR patients, highlighting differences in ECM organisation, cholesterol synthesis and steroid metabolism pathways (Roos et al., 2022). Together with our observation that FF EVs, particularly SEVs, modulate similar pathways in granulosa cells, these findings led us to hypothesise that EV-mediated regulation may contribute to interindividual differences in sensitivity to COS. Prior to this study, the potential involvement of FF EVs in ovarian sensitivity to COS had not been investigated.

To address this knowledge gap, Publication II analysed small RNA profiles in FF EVs and cell-free FF samples from women spanning a range of OSI values (Publication II, Table 1). As an initial step, we first compared the global small RNA composition of matched FF EV and cell-free FF samples. This analysis revealed clear compartment-specific differences, with FF EVs predominantly enriched in piRNAs and cell-free FF enriched in miRNAs (Publication II, Figure 2B-D). To our knowledge, this compartment-specific distribution of

small RNA classes has not been previously described and suggests selective partitioning of small RNAs within the follicular milieu.

Having established these compartmental differences, we next examined whether specific small RNAs correlated with ovarian sensitivity across the OSI spectrum. Several small RNAs showed significant correlations with OSI, with distinct profiles observed between FF EVs and cell-free FF (Figure 10). In FF EVs, four small RNAs (three miRNAs and one piRNA) were correlated with OSI ($|\rho| \geq 0.48$, FDR < 0.05), whereas nine OSI-associated small RNAs (two miRNAs and seven piRNAs) were identified in cell-free FF ($|\rho| \geq 0.56$, FDR < 0.05). Overlap between compartments was minimal, with hsa-let-7f-5p exhibiting a positive correlation with OSI in both FF EVs and cell-free FF, while hsa-let-7a-5p and hsa-miR-26-5p correlated with OSI exclusively in FF EVs.

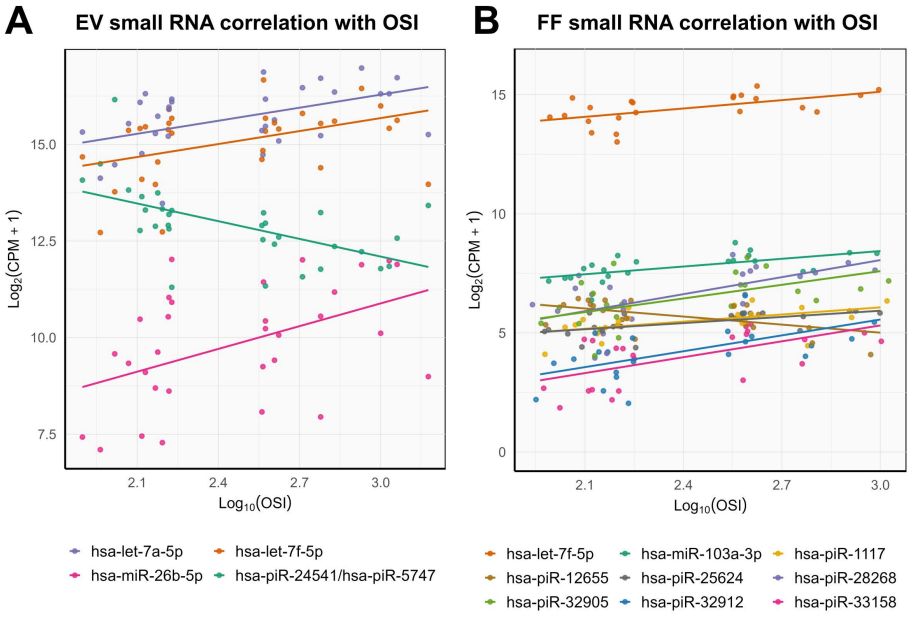

Figure 10. *Correlation between small RNA expression and ovarian sensitivity index (OSI) in (**A**) EV and (**B**) FF samples. Log_2-transformed counts per million (CPM) for the top small RNAs are plotted against log_{10}(OSI), with Spearman correlations adjusted for patient age. In both panels, each colour represents a different small RNA. Points correspond to individual samples, and lines show linear fits derived from correlation models. The collapsed piRNA feature hsa-piR-24541/hsa-piR-5747 represents two highly similar piRNAs. Because the alignment pipeline allows up to 2 mismatches, individual detection is uncertain; therefore, their counts were averaged for plotting and analysis (adapted and modified from Publication II, Figure 4).*

The biological relevance of these OSI-associated small RNAs is supported by previous studies linking them to granulosa cell function and follicular health. Elevated levels of hsa-let-7a-5p and hsa-let-7f-5p have been reported in FF from women with ovarian endometrioma, a condition that often presents with DOR. Functional experiments in that study demonstrated that let-7 agonists increase ROS production and reduce cellular ATP levels in rat granulosa cells (Shi et al., 2025), suggesting that elevated let-7 expression may compromise granulosa cell metabolism and, in turn, ovarian sensitivity to stimulation, although species differences should be considered.

Similarly, members of the miR-26 family have been implicated in follicular atresia and granulosa cell apoptosis. In porcine ovaries, miR-26 is upregulated in atretic follicles and promotes granulosa cell apoptosis *in vitro* (Lin et al., 2012). Consistent with this, increased has-miR-26b-5p levels have been reported in the FF of POR patients aged 40 or older (Zhang et al., 2017). Together, these observations support the hypothesis that elevated miR-26b-5p within FF EVs may reflect or contribute to a granulosa cell environment characterised by increased cellular stress or susceptibility to apoptosis in women with reduced sensitivity to COS.

Within cell-free FF, a distinct set of small RNAs showed associations with ovarian sensitivity. In the present study, hsa-miR-103a-3p correlated positively with OSI, a finding that aligns with evidence linking elevated FF levels of this miRNA to impaired oocyte maturation and poor embryo quality in women undergoing IVF (Zhang et al., 2021b). Additionally, the piRNA hsa-piR-25624 displayed increased abundance with higher OSI values. Although this piRNA has been detected exclusively in POI patients of a small cohort (Ding et al., 2025), its presence across the OSI spectrum in the current study—at higher levels in HR patients—suggests that it may reflect a broader continuum of diminished ovarian responsiveness rather than a discrete pathological state.

In summary, FF EVs and cell-free FF harbour distinct small RNA repertoires, subsets of which correlate with reduced ovarian sensitivity to COS. The compartment-specific associations identified in this study support the concept that altered small RNA signalling within the follicular environment is linked to interindividual variability in ovarian responsiveness. Future work should prioritise functional validation of these candidate small RNAs in granulosa cells and COC cultures and explore their potential utility as minimally invasive biomarkers of reduced sensitivity to rFSH stimulation.

4.4 EV-driven transcriptional changes in granulosa cells associated with diminished response to COS (Publication II)

Our next objective was to investigate whether EVs derived from patients with differing OSI values elicit distinct transcriptional responses in granulosa cells. To explore this, KGN cells were treated with patient-specific FF EVs for 24 hours, followed by RNA sequencing. As shown earlier, EV treatment elicited widespread gene expression changes (Figure 8); however, these responses did not clearly separate between the NR and HR groups. Instead, EV-induced transcriptional responses varied progressively with OSI, supporting a graded relationship between EV-mediated signalling and ovarian sensitivity.

Correlation analysis identified 116 OSI-associated transcripts, including 78 negatively and 38 positively correlated genes (Publication II, Figure 5A). Notably, the observed expression differences were modest, indicating that EVs from HR patients do not strongly up- or downregulate individual genes but instead induce subtle, coordinated shifts across multiple pathways. Such low-amplitude changes are consistent with the clinical phenotype of ovarian hyporesponse, which is characterised by functional attenuation rather than overt cellular dysfunction.

To place the OSI-associated genes in a broader biological context, gene set enrichment analysis (GSEA) was performed on all tested genes ranked by their correlation with OSI. This analysis revealed positive associations with pathways related to cell cycle regulation and replication stress, alongside negative associations with interferon signalling and iron uptake/transport pathways (Figure 11). The enrichment of cell cycle pathways is particularly noteworthy given that granulosa cells normally exit the cell cycle and initiate

luteinisation after the preovulatory LH surge (Richards et al., 1998). Rather than indicating increased proliferative activity, these signatures likely reflect subtle differences in the regulation of cell cycle exit during this transition. This interpretation aligns with observations by Woo et al. (2018), who reported that granulosa cells from DOR patients fail to show the expected LH-induced reduction in proliferation.

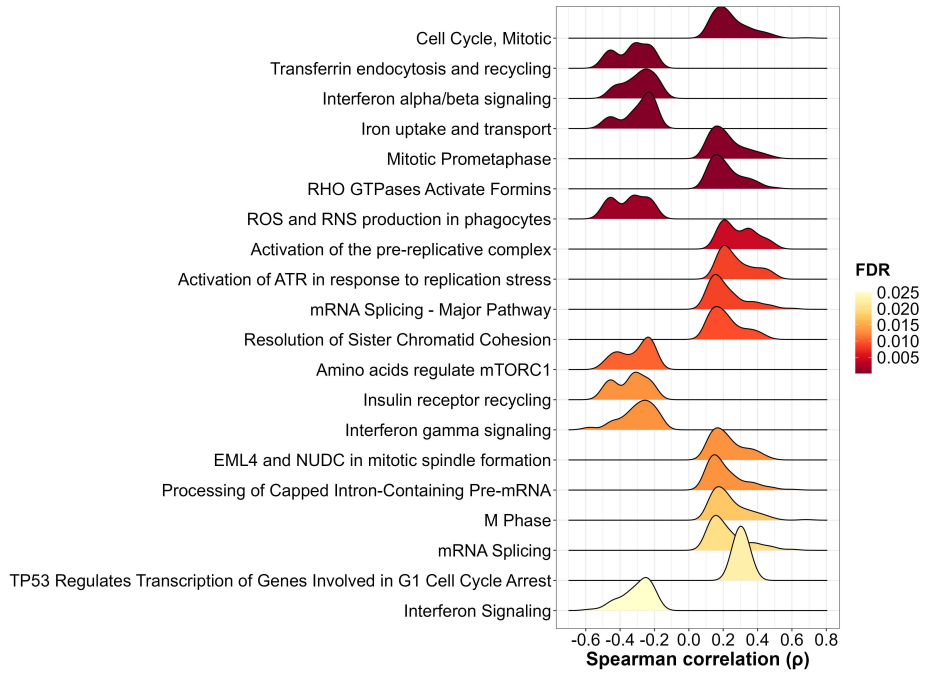

Figure 11. *Ridgeplot of the top 20 Reactome pathways enriched among genes correlating with OSI. The y-axis lists pathway names, and the x-axis represents Spearman correlation coefficients used to rank the genes. Ridge shapes depict the distribution of gene correlation values across the ranked list, while the fill colour indicates pathway significance (FDR) (adapted and modified from Publication II, Figure 5D-E).*

Consistent with this pathway-level interpretation, the two genes showing the strongest correlations with OSI—*PPP2R2D* (PP2A subunit B isoform delta; positively correlated) and *RERG* (RAS-like oestrogen-regulated growth inhibitor; negatively correlated) (Publication II, Figure 5B-C)—provide gene-level support for altered cell cycle control. *PPP2R2D* encodes a regulatory subunit of PP2A (protein phosphatase 2A) that modulates mitotic entry and exit (Chen et al., 2012), whereas *RERG* encodes a Ras-related GTPase that suppresses cellular proliferation (Finlin et al., 2001). Together, these correlations suggest that EVs from HR patients may influence checkpoint regulation during the shift from a proliferative to a luteinised granulosa cell state.

Among the negatively OSI-correlated genes, *CYP19A1*, which encodes aromatase and is essential for oestrogen biosynthesis in granulosa cells, was of particular interest. *CYP19A1* is a validated target of hsa-let-7f-5p, a miRNA that showed positive correlation with OSI in FF EVs (Publication II, Figure 5F), suggesting a plausible miRNA-mediated regulatory mechanism. To assess whether these transcriptional differences translate into altered steroidogenic output, we examined hormone production in EV-treated KGN cells.

EVs from HR patients did not significantly alter oestradiol production in KGN cells following 24 hours of exposure (Publication II, Figure 6A). This lack of effect may reflect subtle transcriptional changes below the threshold of functional impact, limited assay sensitivity, or insufficient incubation time for hormonal shifts to manifest.

In contrast, EVs from HR patients led to a significant reduction in progesterone production in KGN cells compared with vehicle-treated controls (Publication II, Figure 6B). This finding is notable given that FF was collected after hCG administration—a stage at which granulosa cells normally luteinise and transition from oestrogen to progesterone production (Jensen et al., 2025). The reduced progesterone output complements the cell cycle findings, both pointing toward alterations in the processes governing granulosa cell luteinisation. Notably, this decrease in progesterone production was not accompanied by significant OSI-associated changes in the expression of genes encoding core enzymes involved in its biosynthesis, suggesting that EV-mediated modulation may occur through non-transcriptional mechanisms. Integration of proteomic and metabolomic profiling of FF EVs would therefore be a valuable next step in elucidating how EVs from patients with reduced ovarian sensitivity alter steroidogenic output. Similar patterns have been reported in POR patients, whose granulosa cells display impaired expression of steroidogenic enzymes, including *CYP19A1*, and reduced progesterone production (Bildik et al., 2022). Taken together, these EV-mediated effects point to subtle alterations in granulosa cell physiology that resemble features observed in poor responders.

In addition to luteinisation-related changes, OSI-associated transcripts also pointed to alterations in cellular metabolism, particularly iron handling. Reduced expression of iron uptake and transport genes in granulosa cells treated with HR EVs may indicate disrupted iron homeostasis in patients with reduced ovarian sensitivity to COS. While iron overload can suppress granulosa cell proliferation and induce oxidative stress (Chen et al., 2017b), insufficient iron may likewise impair granulosa cell function. In a mouse model, iron deficiency disrupted follicular development by reducing ovarian ATP levels and downregulating key granulosa cell markers such as *FSHR* and *CYP19A1* (Tonai et al., 2020). Notably, ferroportin—an iron export protein—has been reported to be downregulated in granulosa cells of infertile women, further supporting the link between impaired iron handling and reproductive dysfunction (Moreno-Navarrete et al., 2017). Whether the OSI-associated enrichment of iron pathways reflects an adaptive response to limit iron-driven oxidative stress or contributes to granulosa cell dysfunction remains unclear.

These interpretations should be viewed in the light of several limitations. First, all functional assays were performed using the tumour-derived KGN granulosa cell line. Functional validation in primary human granulosa cells will therefore be required to establish the physiological relevance of the observed EV-mediated effects. Second, markers of ovarian reserve, such as AMH and AFC, were unavailable for this cohort, limiting our ability to distinguish the contribution of ovarian sensitivity from underlying ovarian reserve. Third, the patient cohort did not include the extremes of ovarian responsiveness, as COS cycles with very low OSI (risk of OHSS) or very high OSI (no follicular development) are typically cancelled, thereby limiting the availability of FF samples from individuals at these extremes. As a result, the OSI-associated molecular signatures identified here likely represent moderate phenotypes rather than extreme cases.

4.5 Molecular alterations in the follicular environment associated with DEHP exposure (Publication III)

Ovarian responsiveness to COS is shaped by a complex interplay between intrinsic follicular regulatory mechanisms and exogenous influences within the follicular microenvironment. A substantial body of epidemiological and experimental work has established EDCs, including phthalates, as detrimental modifiers of ovarian physiology, with documented effects on folliculogenesis and steroidogenesis (Basso et al., 2022). Despite extensive evidence for their reproductive toxicity, less is known about how phthalate exposure is reflected in the regulatory molecular landscape of the human preovulatory follicle. Based on our previous finding that higher follicular DEHP exposure is associated with reduced ovarian sensitivity to gonadotropin stimulation (Bellavia et al., 2023), Publication III was designed to characterise the molecular alterations accompanying elevated DEHP levels within the human preovulatory follicle, with a focus on integrating small RNA profiles and gene expression changes relevant to granulosa cell function.

For this, a subset of patients from the Bellavia et al. (2023) cohort was stratified according to follicular ΣDEHP concentrations, defined as the summed levels of the major DEHP metabolites (MEHP, MECPP, MEHHP, and MEOHP) measured in FF (Publication III, Table 1). Ovarian responsiveness to COS was assessed using an alternative OSI formulation compared with Publication II, with lower OSI values indicating reduced responsiveness to gonadotropin treatment. Importantly, molecular profiling was performed on matched samples from the same follicles, integrating FF small RNA sequencing with transcriptomic analysis of FF-derived somatic cells.

Publication III revealed that elevated ΣDEHP exposure was associated with distinct alterations in the small RNA landscape of the preovulatory follicle, with 16 DE FF miRNAs between exposure groups (FDR < 0.1) (Publication III, Figure 1B). Among these, miR-203a-3p stood out as the only small RNA that also correlated with ovarian sensitivity to COS, showing higher abundance in high-exposure follicles and a negative association with OSI ($R = -0.28$, $p < 0.05$) (Publication III, Figure 1D). Notably, increased miR-203a-3p expression has previously been reported in the context of diminished follicular competence, including advanced maternal age and PCOS, where it has been linked to granulosa cell dysfunction and impaired oocyte quality (Battaglia et al., 2016; Rooda et al., 2020). Although these associations do not establish causality, the consistent upregulation of miR-203a-3p across diverse conditions of compromised ovarian function supports its potential role as a marker of follicular stress or diminished developmental capacity in the context of DEHP exposure.

Several additional DEHP-associated FF miRNAs were predicted to target transcripts involved in pathways critical for granulosa cell function. In particular, six DE miRNAs were predicted to target components of the AR signalling pathway (FDR < 0.05) (Publication III, Figure 1C). AR activity in granulosa cells plays a central role in early follicular development by promoting cellular proliferation, enhancing *FSHR* expression, and thereby amplifying FSH responsiveness (Franks & Hardy, 2018). Disruption of AR signalling has been linked to impaired folliculogenesis, defective luteinisation, and mitochondrial dysfunction in granulosa cells, underscoring its importance for normal ovarian function (Wang et al., 2015). Although computational modelling has suggested that DEHP metabolites may interact with the AR (Beg & Sheikh, 2020), experimental evidence for direct receptor binding remains limited (Kim et al., 2019). Nonetheless,

phthalates are well recognised for their anti-androgenic properties (Borch et al., 2006), raising the possibility that DEHP exposure may modulate androgen-dependent processes indirectly, for example, through miRNA-mediated targeting of key AR pathway components. In addition to AR signalling, pathway enrichment analysis of DE FF miRNAs identified other implicated pathways, including RAC1/PAK1/p38/MMP2 signalling, adipogenesis, and AGE/RAGE signalling (FDR < 0.05) (Publication III, Figure 1C).

To determine whether DEHP-associated changes in the FF miRNA landscape were accompanied by transcriptional alterations in follicular cells, we next examined the gene expression profiles of FF-derived somatic cells from the same preovulatory follicles. Elevated ΣDEHP exposure led to widespread transcriptional differences, with 2454 DE genes between high- and low-exposure groups (FDR < 0.05) (Publication III, Figure 2A). Pathway-level analyses revealed prominent enrichment of inflammatory and immune-related signatures, alongside suppression of genes involved in lipid, cholesterol and steroid biosynthesis (FDR < 0.05) (Publication III, Figure 2B-C). These transcriptional patterns suggest that elevated DEHP exposure is associated with coordinated reprogramming of the follicular somatic cell transcriptome, affecting pathways central to follicular homeostasis and endocrine function.

Because transcriptional profiles derived from FF somatic cells reflect contributions from multiple cell types, we next considered whether the ΣDEHP-associated gene expression patterns were accompanied by shifts in the cellular composition of the follicular environment. Cell type deconvolution indicated that follicles with elevated ΣDEHP levels are enriched in immune cell signatures, including markers associated with T cells, macrophages, and neutrophils, together with a reduced representation of granulosa cell-associated transcripts ($p < 0.05$) (Publication III, Figure 2D). This immune-enriched signature is consistent with recent experimental and human studies reporting activation of inflammatory pathways, including NF-κB signalling and pyroptosis-related markers due to DEHP exposure (Sun et al., 2023), as well as positive associations between phthalate metabolite and inflammatory cytokine levels, such as TNF-α, in FF (Wang et al., 2023). In this context, the reduced representation of granulosa cell-associated transcripts observed here may reflect, at least in part, inflammation-associated granulosa cell loss driven by pyroptosis or related inflammatory cell death processes, rather than solely immune cell enrichment.

A prominent feature of the ΣDEHP-associated transcriptional response was the coordinated downregulation of genes involved in cholesterol and lipid biosynthesis within FF somatic cells (Publication III, Figure 3A). Several of these genes were also predicted targets of DEHP-associated FF miRNAs (Figure 12), indicating post-transcriptional regulation. This suppression aligns with prior evidence that DEHP disrupts lipid metabolism systematically. For example, in vivo rat models show elevated serum cholesterol levels and impaired hepatic cholesterol metabolism following chronic DEHP exposure (Li et al., 2022), and DEHP-treated mice exhibit increased triglyceride and total cholesterol concentrations, indicative of systemic lipid dysregulation (Huang et al., 2022). In the ovarian context, such disruptions can be particularly consequential, as cholesterol availability constrains steroidogenic capacity. In line with this interpretation, exposure of mouse granulosa cells to phthalate metabolite mixtures has resulted in downregulation of cholesterol and lipid metabolic pathway genes, together with reduced *CYP11A1* expression (Alahmadi et al., 2024), closely paralleling the molecular patterns observed in Publication III. Notably, downregulation of cholesterol metabolism genes such as *IDI1* (isopentenyl-diphosphate delta isomerase 1), *FDFT1*, *CYP51A1* (lanosterol

14α-demethylase) and *STAR* has also been reported in granulosa cells from DOR patients (Yang et al., 2022), suggesting that DEHP-associated transcriptional changes may recapitulate molecular features of clinically impaired ovarian function.

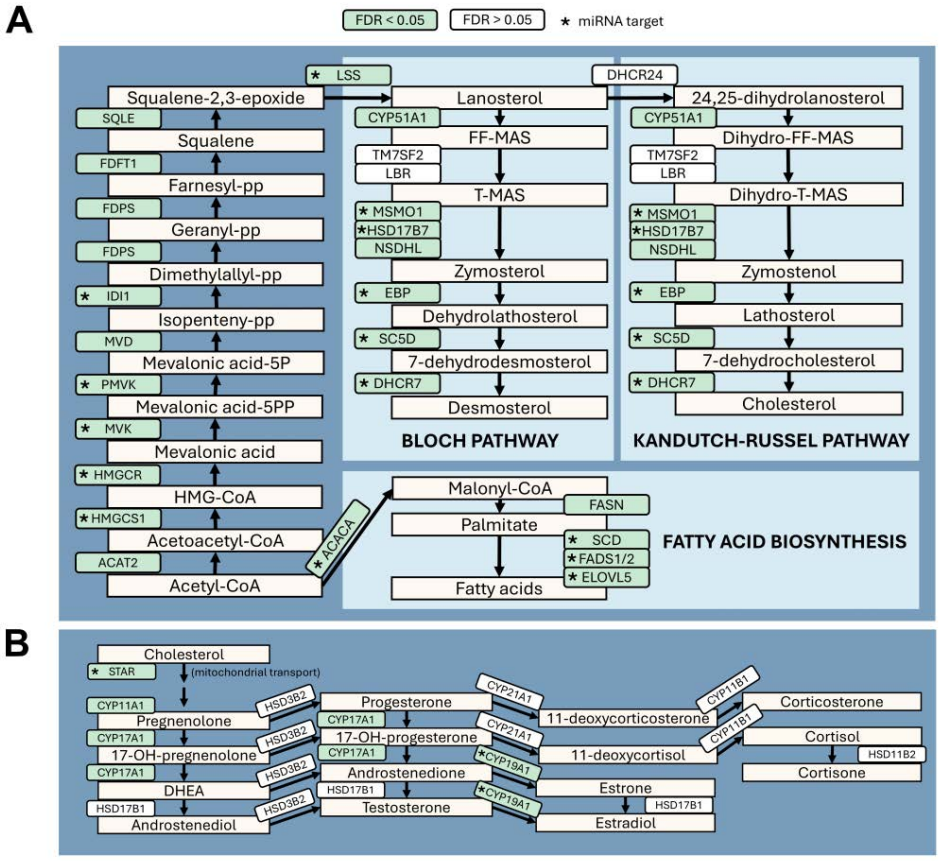

Figure 12. *Differentially expressed genes within (**A**) cholesterol and fatty acid biosynthesis pathways and (**B**) steroidogenesis pathway in FF somatic cells from high versus low ΣDEHP exposure groups. Genes shaded in green showed significant downregulation in high ΣDEHP follicles (FDR < 0.05). Asterisks indicate predicted targets of DEHP-associated FF miRNAs (adapted and modified from Publication III, Figure 3).*

Although ΣDEHP exposure was associated with coordinated transcriptional suppression of cholesterol synthesis and steroidogenesis-related pathways (Figure 12), FF concentrations of major steroid hormones did not differ significantly between exposure groups ($p > 0.05$) (Publication III, Figure 4). This observation aligns with several recent studies using human-relevant exposure levels, which similarly report preserved steroid hormone output following phthalate exposure in human ovarian tissue and granulosa cell models (Panagiotou et al., 2024; Tarvainen et al., 2023). Comparable findings have also been reported in mouse granulosa cells, where exposure to phthalate metabolite mixtures did not alter pregnenolone or progesterone levels despite significant downregulation of *CYP11A1* and upstream cholesterol biosynthesis genes (Alahmadi et al., 2024). In contrast, studies employing substantially higher phthalate

concentrations more frequently report suppression of steroid hormone production (Davis et al., 1994; Li et al., 2012; Neff et al., 2024), underscoring the dose-dependent nature of phthalate effects on ovarian steroidogenesis.

Despite the preservation of absolute hormone concentrations, we observed a reduced 17-hydroxyprogesterone-to-progesterone ratio in high ΣDEHP follicles (Publication III, Figure 4B), indicating a subtle alteration in steroidogenic flux. This finding is consistent with transcriptional downregulation of *CYP17A1* and suggests that DEHP exposure may preferentially affect specific enzymatic steps within the steroidogenic pathway. Such context-dependent sensitivity is further supported by epidemiological studies reporting associations between phthalate metabolite levels and circulating steroid hormones predominantly in postmenopausal women, with weaker or absent associations in premenopausal populations (Long et al., 2021), implying that phthalate-related steroidogenic disruptions may become more apparent under an altered hormonal milieu.

To conclude, these findings indicate that elevated DEHP exposure is associated with coordinated molecular alterations in the preovulatory follicular environment that modify steroidogenic pathway regulation without overt disruption of steroid hormone output.

4.6 Future perspectives and translational potential

The work presented in this thesis establishes FF-derived EVs as heterogeneous mediators of intrafollicular communication, carrying molecular signatures that reflect both EV subtype and patient-specific ovarian function. These findings point toward multiple translational trajectories: FF EV profiling may support improved patient stratification and personalisation of COS, while targeted manipulation of EV composition or function could provide novel avenues to support ovarian performance. Progress along these paths, however, will depend on addressing several methodological and practical challenges that currently limit clinical translation.

One of the primary reasons FF and FF-derived EVs have not yet been incorporated into routine IVF decision-making is logistical. FF is collected only at oocyte retrieval, after completion of COS, meaning that FF-derived molecular information cannot influence treatment decisions within the same cycle. Moreover, FF composition varies substantially between follicles, even those that appear to be at similar developmental stages (Wu et al., 2022), complicating the establishment of universal thresholds or reference ranges for biomarkers. Standardisation is further hindered by differences in aspiration technique, dilution with flushing media, and the absence of consensus regarding FF processing for molecular analyses. As a result, most FF-based biomarkers remain at the discovery or early validation stage, with limited reproducibility across cohorts and treatment protocols. Although a growing body of literature has proposed numerous FF-derived candidate biomarkers—including hormones, cytokines, metabolites, and small RNAs—that correlate with oocyte quality, fertilisation, or pregnancy outcomes, none have yet demonstrated sufficient robustness or reproducibility for routine clinical use. Meaningful clinical adoption will therefore require multi-centre validation using standardised protocols.

Despite these constraints, FF profiling may still hold considerable translational value. Rather than serving as a real-time decision tool, FF-derived molecular signatures could be used for patient stratification, particularly to inform protocol selection, dosing strategies, or risk assessment in subsequent COS cycles. Moving toward this goal will require multi-omics FF and FF EV profiling approaches that integrate small RNA analysis with proteomic and metabolomic information, thereby offering a more comprehensive

characterisation of the follicular environment than RNA-centric analyses alone. Although such multi-layered approaches are currently costly and not scalable for routine IVF, they provide a critical framework for identifying the most informative molecular features that could be translated into feasible and clinically applicable biomarker panels.

An important next step toward translational relevance is validating EV-mediated effects in physiologically relevant models. While the KGN granulosa cell line used in this thesis provided a controlled and reproducible system for mechanistic investigation, primary human granulosa cells exhibit greater heterogeneity, steroidogenic capacity, and gonadotropin responsiveness. Validation of EV effects in primary granulosa cells—and ideally in *ex vivo* follicle culture systems—will be essential to determine whether the transcriptional and functional responses observed here reflect *in vivo* ovarian physiology. Single-cell transcriptomic approaches could further refine this understanding by resolving how distinct follicular cell populations respond to EV signals, providing a more nuanced view of EV-mediated communication within the follicle.

In addition to their diagnostic and prognostic potential, EVs may also serve as tools to modulate ovarian function. This concept has gained increasing attention, with preclinical studies reporting beneficial effects of EV-based interventions on ovarian physiology and fertility outcomes. In a PCOS mouse model, treatment with mesenchymal stem cell-derived EVs restored ovarian function and rescued fertility, resulting in successful pregnancies and healthy offspring (Park et al., 2023). Similarly, in a chemotherapy-induced POI mouse model, EVs derived from human embryonic stem cells improved ovarian morphology, increased follicle numbers, and normalised oestrous cycling (Liu et al., 2020). EV-mediated support of ovarian function has also been proposed in the context of reproductive ageing, as exposure of preantral follicles from aged mice to human umbilical cord stem cell-derived EVs enhanced follicle growth and reduced oocyte spindle abnormalities (Zhang et al., 2023). Translational interest in EV-based approaches is further underscored by a recent human pilot study using autologous platelet-derived EVs, which reported an increase in the number of mature (MII) oocytes following treatment (Navarro et al., 2025). Although these findings provide proof-of-concept evidence, EV-based interventions remain highly experimental.

A major barrier to therapeutic translation is the intrinsic heterogeneity of EV preparations, which comprise diverse EV subtypes and co-isolated components whose composition depends on cell source, culture conditions, and isolation protocols. As a result, defining the exact composition of a therapeutic EV formulation beyond broad metrics, such as particle number or enrichment of presumed active cargo, remains challenging, raising concerns about reproducibility and potential unintended biological effects. These limitations highlight the need for future studies to move beyond empirical administration of heterogeneous EV preparations and toward the identification of specific bioactive components responsible for therapeutic effects.

In this context, engineered EVs represent a promising strategy to overcome some of the limitations associated with naturally derived EV populations. Engineered EVs can be designed to carry defined and well-characterised cargo, offering greater control over composition, reproducibility, and mechanism of action. Future studies could explore whether targeted delivery of selected miRNAs can be used to correct or compensate for molecular features associated with reduced ovarian responsiveness or environmental exposure. In parallel, engineering EV surface properties may enhance delivery to specific follicular cell populations, increasing therapeutic precision while reducing off-target effects.

The potential applications of EVs also extend to ART laboratory procedures. Oocyte cryopreservation and IVM remain limited by suboptimal survival and reduced developmental competence. Emerging evidence suggests that EV supplementation at different stages of *in vitro* handling may help mitigate these challenges. In a bovine model, supplementation of IVM media with EVs derived from large follicles enhanced nuclear and cytoplasmic maturation and improved mitochondrial function in oocytes (Diaz-Muñoz et al., 2025). In the same study, EV exposure was also associated with improved post-vitrification survival, supporting a protective role for EVs during cryopreservation (Diaz-Muñoz et al., 2025). Complementing these findings, a study in the domestic cat demonstrated that the addition of FF EVs to vitrification and/or thawing media improved the ability of frozen-thawed oocytes to resume meiosis, despite no difference in survival (de Almeida Monteiro Melo Ferraz et al., 2020). Beyond effects on oocytes, EVs may also influence early embryonic development: in a bovine model, supplementation of embryo culture media with FF EVs has been shown to increase blastocyst rates (da Silveira et al., 2017). Together, these studies indicate that FF EVs can support oocyte and embryo quality at multiple stages of ART; however, these findings are currently based on animal models, and further work will be required to define optimal EV sources, doses, timing, minimal bioactive components, and their relevance in human ART settings.

Finally, although this thesis did not directly examine how DEHP or other EDCs alter EV biology, emerging evidence indicates that environmental toxicants can influence EV-mediated signalling. Studies by Martinez et al. (2019) and Barnett-Itzhaki et al. (2021) have shown that the abundance of specific EV-associated miRNAs varies with phthalate metabolite levels, suggesting that EDC exposure modulates EV cargo composition and function. Systematic investigation of how EDCs affect EV release, their molecular cargo, and downstream effects on ovarian cells, therefore, represents an important future direction. Such work may not only clarify mechanisms linking environmental exposure to ovarian dysfunction but also support the development of EV-based biomarkers that capture toxicant burden in women of reproductive age.

5 Conclusions

The main conclusions of the thesis are as follows:

- SEVs and LEVs in FF are molecularly distinct, differing in abundance, tetraspanin colocalisation patterns, and non-coding RNA cargo.
- SEVs are enriched in miRNAs, whereas LEVs contain higher levels of piRNAs, indicating subtype-specific RNA sorting.
- In KGN granulosa cells, SEVs induce broad transcriptomic remodelling, affecting transcriptional regulation, TGF-β signalling, ECM remodelling, cholesterol synthesis, and cell cycle pathways.
- LEVs induce minimal changes in KGN cell gene expression but substantially alter cellular small RNA profiles.
- LEV and combined SEV+LEV treatments increase testosterone production in KGN cells at specific concentrations, suggesting that FF EVs can modulate steroidogenic output of granulosa cells.
- FF EVs and cell-free FF harbour distinct small RNAs, with EVs enriched in piRNAs and cell-free FF enriched in miRNAs.
- Specific small RNAs in FF EVs and cell-free FF, including hsa-let-7f-5p, hsa-let-7a-5p, hsa-miR-26b-5p, hsa-miR-103a-3p, and piR-25624, correlate with OSI and may serve as candidate biomarkers of ovarian responsiveness to COS.
- FF EVs derived from patients with reduced sensitivity to COS (hyporesponders) elicit transcriptional responses in KGN cells that correlate with OSI, affecting pathways related to cell cycle regulation, replication stress, iron homeostasis, and interferon signalling.
- FF EVs derived from hyporesponders significantly reduce progesterone production in KGN cells.
- DEHP exposure is associated with differential miRNA profiles in FF, which are linked to pathways such as AR signalling, RAC1/PAK1/p38/MMP2 signalling, adipogenesis, and AGE/RAGE signalling.
- High follicular DEHP exposure is associated with downregulation of genes involved in cholesterol and steroid hormone biosynthesis in FF somatic cells, and upregulation of immune-related genes.
- Cell-type deconvolution of bulk transcriptomes suggests increased representation of macrophage, neutrophil, and T-cell signatures, and reduced representation of granulosa cells in follicles with high DEHP levels.
- Although steroid hormone concentrations did not differ significantly with DEHP exposure, a reduced 17-hydroxyprogesterone-to-progesterone ratio was detected in high-exposure follicles, consistent with *CYP17A1* downregulation, suggesting a mild impairment of steroidogenesis.

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Abstract

Extracellular Vesicles and Endocrine-Disrupting Chemicals as Modulators of Ovarian Follicle Physiology

With fertility rates declining and the demand for assisted reproductive technologies (ART) rising, there is a growing need to better understand the biological processes that determine ovarian function and treatment success. A central step in in vitro fertilisation (IVF) is controlled ovarian stimulation (COS), during which gonadotropin hormones are administered to stimulate the concurrent growth of multiple ovarian follicles, enabling the retrieval of several mature oocytes within a single treatment cycle. However, women respond very differently to ovarian stimulation, and the biological reasons underlying this variability remain poorly understood.

The ovarian follicle is a tightly regulated microenvironment composed of the developing oocyte and the surrounding somatic cells. These cells communicate not only through endocrine and paracrine signalling but also via extracellular vesicles (EVs)—nanoparticles enclosed by a lipid bilayer that mediate the transfer of proteins, lipids, and nucleic acids between cells. EVs produced within the follicle accumulate in follicular fluid (FF), which fills the antral cavity during the final stages of follicular maturation. Although FF EVs are known to reflect the physiological state of the follicle, their molecular diversity, functional roles, and relationship to ovarian sensitivity to COS have not yet been fully characterised. Because FF also serves as a reservoir for compounds entering the follicle from the circulation, it may contain endocrine-disrupting chemicals (EDCs), such as the plasticiser di(2-ethylhexyl) phthalate (DEHP), which can interfere with pathways regulating follicle development and steroid hormone production.

This doctoral thesis investigates the molecular environment of the human preovulatory ovarian follicle, focusing on EV-mediated intercellular communication, differences associated with ovarian responsiveness to COS, and the impact of DEHP exposure. Using FF samples from women undergoing IVF treatment, this work integrates small RNA profiling of FF and FF EVs with functional experiments in granulosa cells—the somatic cells that support oocyte development—to characterise regulatory processes within the follicular microenvironment.

The findings of this thesis demonstrate that FF contains distinct EV subtypes with different molecular composition and regulatory effects on granulosa cell gene expression. Importantly, both the composition of FF EVs and their impact on granulosa cell gene expression differed between women with normal and reduced ovarian responsiveness to COS, indicating altered EV-mediated intercellular communication in women with reduced ovarian sensitivity to COS. Additionally, this work shows that higher follicular exposure to DEHP is associated with increased levels of inflammatory markers and coordinated suppression of pathways involved in cholesterol handling and steroid hormone biosynthesis.

In conclusion, this thesis advances understanding of how intercellular communication and exposure to environmental chemicals influence the molecular features associated with ovarian responsiveness to gonadotropin stimulation in the preovulatory follicle. The identification of ovarian sensitivity-associated RNA profiles may, in the future, enable improved prediction of stimulation response. The findings also underscore the importance of assessing the reproductive toxicity of environmental chemicals before widespread use. Overall, this work provides a foundation for the optimisation of infertility treatment strategies, including future personalisation of ovarian stimulation.

Lühikokkuvõte

Rakuvälised vesiikulid ja endokriinsüsteemi kahjustavad kemikaalid kui munasarjade füsioloogiat mõjutavad tegurid

Seoses sündimuse languse ja viljatusravi tehnoloogiate aina kasvava rakendamisega on üha olulisem paremini mõista bioloogilisi protsesse, mis määravad munasarjade funktsionaalsust ja viljatusravi edukust. Kunstliku viljastamise ehk IVF ravi keskseks etapiks on kontrollitud munasarjade stimulatsioon (COS), mille käigus manustatakse patsiendile gonadotroopseid hormoone, et soodustada mitme munasarja folliikuli samaaegset kasvu, võimaldades seeläbi ühe ravitsükli jooksul mitme küpse munaraku kogumist. Naised reageerivad munasarjade stimulatsioonile siiski väga erinevalt ning nende erinevuste bioloogilised põhjused ei ole tänaseks piisavalt kirjeldatud.

Munasarja folliikul on rangelt reguleeritud mikrokeskkond, mis koosneb küpsevast munarakust ja seda ümbritsevatest somaatilistest rakkudest. Need rakud suhtlevad omavahel nii endokriinsete kui parakriinsete signaalide vahendusel, aga ka rakuväliste vesiikulite (EV) kaudu – need on kahekihilise lipiidse membraaniga piiritletud nanoosakesed, mis vahendavad valkude, lipiidide ja nukleiinhapete transporti rakkude vahel. Folliikulis toodetavad EV-d akumuleeruvad follikulaarvedelikus (FF), mis täidab folliikuli antraalse õõnsuse folliikulite küpsemise viimastes etappides. Kuigi FF-is leiduvad EV-d peegeldavad folliikuli füsioloogilist seisundit, ei ole nende molekulaarset mitmekesisust, funktsionaalseid rolle ega seost munasarjade stimulatsiooni tulemuslikkusega veel täielikult iseloomustatud. Kuna FF-is kogunevad ka vereringest folliikulisse jõudvad ühendid, võib see sisaldada endokriinsüsteemi kahjustavaid kemikaale (EDC-sid), näiteks plastifikaatorit di(2-etüülheksüül)ftalaati (DEHP), mis võivad mõjutada folliikulite arengut ja steroidhormoonide sünteesi reguleerivaid molekulaarseid signaaliradu.

Käesolev doktoritöö uurib inimese ovulatsioonieelse munasarjafolliikuli molekulaarset keskkonda, keskendudes EV-de poolt vahendatud rakkudevahelisele suhtlusele, munasarjade stimulatsiooni tulemuslikkust määravatele teguritele ning DEHP-iga kokkupuute mõjule. Antud töö ühendab IVF ravi läbivatelt naistelt kogutud FF proovide ja nendes sisalduvate EV-de lühikeste RNA-de analüüsi funktsionaalsete katsetega granuloosarakkudes, st somaatilistes rakkudes, mis toetavad munaraku arengut, eesmärgiga iseloomustada folliikuli mikrokeskkonnas toimuvaid regulatoorseid protsesse.

Doktoritöö tulemused näitavad, et FF sisaldab erinevaid EV-de alamtüüpe, millel on erinev molekulaarne koostis ja mõju granuloosarakkude geenide avaldumisele. Nii FF EV-de koostis kui ka nende mõju granuloosarakkude ekspressioonile erinesid oluliselt naiste vahel, kelle munasarjade stimulatsiooni tulemuslikkus oli vähenenud, võrreldes normaalse stimulatsioonivastusega patsientidega. See viitab EV-de poolt vahendatud rakkudevahelise suhtluse häirumisele madalama stimulatsioonivastusega naistel. Lisaks seostus kõrgem DEHP-iga kokkupuude folliikuli tasandil suurenenud põletikumarkerite ning kolesterooli käitlemist ja steroidhormoonide sünteesi eest vastutavate molekulaarsete radade allasurumisega.

Kokkuvõttes süvendab käesolev doktoritöö arusaama ovulatsioonieelses folliikulis toimuvatest molekulaarsetest mehhanismidest, mille kaudu rakkudevaheline suhtlus ja kokkupuude keskkonnakemikaalidega võivad mõjutada munasarjade vastust gonadotropiinidega stimuleerimisele. Munasarjade stimulatsiooni tulemuslikkusega

seotud RNA profiilide tuvastamine võib tulevikus avada võimalusi stimulatsioonivastuse paremaks ennustamiseks. Saadud tulemused rõhutavad ühtlasi keskkonnakemikaalide reprodutiivtoksilisuse hindamise olulisust enne nende laialdasemat kasutuselevõttu. Käesolev töö loob aluse viljatusravi strateegiate edasiseks optimeerimiseks, sealhulgas munasarjade stimulatsiooni personaliseerimiseks tulevikus.

Appendix 1

Publication I

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RESEARCH ARTICLE OPEN ACCESS

Small and Large Extracellular Vesicles From Human Preovulatory Follicular Fluid Display Distinct ncRNA Cargo Profiles and Differential Effects on KGN Granulosa Cells

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Keywords: extracellular vesicles | follicular fluid | granulosa cells | miRNA | ncRNA | ovary

ABSTRACT

Follicular fluid extracellular vesicles (FF EVs) facilitate communication between oocytes and somatic cells within the ovarian follicle, playing a pivotal role in follicular development. This study highlights the molecular and functional distinctions between small (SEV) and large (LEV) FF EV subpopulations, revealing their specialised regulatory roles in granulosa cell (GC) biology and their consequential impact on ovarian function. Single-EV profiling uncovered distinct tetraspanin distributions, with LEVs containing a lower proportion of CD9/CD63/CD81-positive particles compared to SEVs. Fluorescent labelling confirmed uptake of both SEVs and LEVs by GCs, supporting their capacity to impact cellular behaviour. Functionally, LEVs increased testosterone production by GCs, whilst SEVs had no effect on steroid hormone secretion, suggesting a specific role for LEVs in androgen biosynthesis. Transcriptomic analysis revealed extensive SEV-induced changes in GC gene expression, affecting pathways involved in transcription, TGF-β signalling, extracellular matrix (ECM) remodelling and cell cycle regulation. In contrast, LEVs elicited minimal transcriptional changes, primarily modulating genes associated with immune regulation and oxidative stress defence. Small RNA sequencing further revealed distinct non-coding RNA (ncRNA) profiles, with SEVs enriched in miRNAs targeting pathways critical for GC differentiation, whilst LEVs carried higher levels of piRNAs implicated in maintaining genomic stability. These findings advance our understanding of FF EV-mediated intercellular communication and underscore the importance of investigating EV subpopulations independently.

1 | Introduction

The primary function of the human ovary is to produce fertilisation-competent oocytes and steroid hormones, such as oestrogens, progesterone and testosterone. Its functional unit, the ovarian follicle, comprises an oocyte surrounded by granulosa cells (GCs) and theca cells (Edson et al. 2009). Follicular development progresses through multiple stages, starting with primordial follicles and culminating in the formation of a pre-ovulatory follicle, characterised by a fluid-filled antrum (Malo et al. 2024). This antrum, which delineates GCs into mural and cumulus subtypes (Khamsi and Roberge 2001), contains follicular

fluid (FF) derived from blood plasma and GC/oocyte secretions (Hennet and Combelles 2012). FF contains various biologically active molecules, including hormones, growth factors, proteins, peptides, amino acids and metabolites, which collectively support folliculogenesis (Revelli et al. 2009).

Effective follicle development depends on intercellular communication between somatic and germ cells (Matzuk et al. 2002). These interactions, vital for oocyte growth, maturation and eventual release, occur via paracrine and endocrine signalling, gap junctions and transzonal projections (Dumesic et al. 2015; Marchais et al. 2022). Previous studies have identified extracellular vesicles (EVs) in FF across multiple species, including equine, bovine, porcine and human (da Silveira et al. 2012; Hung et al. 2015; Matsuno et al. 2017; Rodrigues et al. 2019; Rooda et al. 2020; Santonocito et al. 2014; Sohel et al. 2013) as important mediators of this communication (Marchais et al. 2022).

EVs are a heterogeneous group of lipid bilayer-enclosed particles secreted by most cell types (Willms et al. 2018), serving as messengers in intercellular communication by transferring their cargo, comprising proteins, lipids and nucleic acids, from donor to recipient cells (Valadi et al. 2007; van Niel et al. 2018). Among their nucleic acid cargo, small non-coding RNAs (ncRNAs), such as microRNAs (miRNAs) and piwi-interacting RNAs (piRNAs), have attracted particular interest due to their roles in gene regulation (Abramowicz and Story 2020; Liu et al. 2019). EVs are categorised, for example, into exosomes, microvesicles and apoptotic bodies based on their biogenesis and size. Exosomes (30–150 nm), formed within multivesicular bodies (MVBs), are released when MVBs fuse with the plasma membrane (Simpson et al. 2009; van Niel et al. 2018), whilst microvesicles (50–1000 nm) bud directly from the plasma membrane (Muralidharan-Chari et al. 2010; van Niel et al. 2018), and apoptotic bodies (200–5000 nm) are shed from cells undergoing programmed cell death (Willms et al. 2018). EVs are often characterised by specific membrane-associated proteins, including tetraspanins CD9, CD63 and CD81 (Jankovičová et al. 2020); however, no universal EV markers are known yet (Welsh et al. 2024).

FF EVs have been implicated in supporting GC function and oocyte development. For instance, bovine FF EVs have been shown to promote GC proliferation (Hung et al. 2017), induce cumulus GC expansion (Hung et al. 2015) and protect cumulus-oocyte complexes from apoptosis and heat shock damage (Rodrigues et al. 2019). In humans, FF EV miRNAs have been linked to pathways that regulate follicle growth and oocyte maturation (Santonocito et al. 2014). Our research group has previously demonstrated that miRNA profiles in FF EVs differ significantly from those of the somatic follicular cells and bulk FF of the same follicle (Rooda et al. 2020), suggesting that EV miRNAs serve as specific means of molecular communication within the follicle. Furthermore, differences in EV miRNA cargo between patients with polycystic ovarian syndrome (PCOS) and healthy individuals (Rooda et al. 2020) highlight their potential as diagnostic biomarkers.

Despite their recognised importance, most studies have examined the collective pool of FF EVs, overlooking the diversity among EV subpopulations. Recent research has identified at least ten distinct FF EV subpopulations (Neyroud et al. 2022), each potentially fulfilling specialised roles in the ovarian follicle. For instance, small FF EV subpopulations have been shown to exhibit heterogeneous miRNA profiles (Wang et al. 2021), emphasising their unique molecular characteristics. These findings underscore the need for in-depth investigations into the specific functions of FF EV subpopulations, given their potential to serve distinct functions in ovarian physiology.

However, overlapping size and density ranges between EV subpopulations, such as exosomes and microvesicles, pose challenges for their complete isolation (Brennan et al. 2020). Moreover, there are currently no molecular markers that can definitively distinguish between these two subtypes (Welsh et al. 2024). In this study, we used size exclusion chromatography (SEC) and tangential flow filtration (TFF) with a 200 nm cut-off to separate small EVs (SEVs) and large EVs (LEVs) from human FF. We hypothesise that this size separation produced SEVs that are enriched in exosomes and LEV preparations enriched in microvesicles, though some degree of cross-contamination between the subpopulations was likely.

We characterised the molecular composition, biophysical properties and ncRNA cargo of FF EV subpopulations. To assess their functional effects on GCs, we used the human granulosa-like tumour cell line KGN, a well-established model that retains key GC features, including steroidogenic activity (Nishi et al. 2001). Although KGN cells do not fully replicate the physiological characteristics of primary GCs, they serve as a valuable model for studying GC responses. By integrating molecular and functional analyses, our study provides new insights into the specialised roles of FF EV subpopulations in ovarian physiology and contributes to a deeper understanding of EV-mediated intercellular communication within the ovarian follicle.

2 | Materials and Methods

2.1 | Collection of FF

FF samples were donated by patients undergoing assisted reproductive technology (ART) at Nova Vita Clinic and East Tallinn Central Hospital, both located in Tallinn, Estonia. All procedures in this study adhered to the Declaration of Helsinki and were approved by the Research Ethics Committee of the University of Tartu, Estonia (approval no. 356/M-4). Prior to participation, patients received both written and oral information about the study. Informed written consent was obtained from all subjects for the use of FF samples collected during ART treatment. To ensure confidentiality, all donated samples were anonymised before further procedures. No physiological nor clinical metadata about the patients was collected.

At Nova Vita Clinic, a gonadotropin-releasing hormone (GnRH) antagonist (Cetrotide, Merck, Darmstadt, Germany) protocol was used, along with the administration of recombinant follicle-stimulating hormone (Gonal-F, Merck; or Bemfola, Gedeon Richter Plc., Budapest, Hungary) for ovarian stimulation.

At East Tallinn Central Hospital, two ovarian stimulation protocols were employed. In long protocols, a GnRH agonist (Diphereline, Ipsen Pharma Biotech, France, Paris) was admin-

istered along with Gonal-F (Merck), Bemfola (Gedeon Richter Plc.), Ovaleap (Theramex, United Kingdom, London), Rekovelle (Ferring Pharmaceuticals, Switzerland, Saint-Prex), Pergoveris (Merck Serono) or Luveris (Merck Serono). In short protocols, a GnRH antagonist (Cetrotide, Merck) was administered, together with Gonal-F, Bemfola, Pergoveris, Ovaleap or Rekovelle.

At both clinics, ovarian puncture was carried out transvaginally under ultrasound guidance 36 h after human chorionic gonadotropin administration (Ovitrelle, Merck) if at least two follicles were ≥18 mm in diameter. The FF was collected from one or more mature follicles (diameter ≥18 mm) with minimal visible blood contamination and transported to the laboratory in a thermal flask. The samples were processed by centrifugation at 400 × *g* for 10 min to pellet intact somatic cells. The supernatant was subsequently centrifuged at 2000 × *g* for 10 min to remove any residual cellular debris. Finally, the cell-free FF samples were aliquoted to avoid repeated freeze-thaw cycles and stored at −80°C until further analysis.

2.2 | Isolation of FF EVs by SEC

EV isolation and characterisation were performed in adherence to the MISEV2023 guidelines (Welsh et al. 2024). A maxiPURE-EVs column (HansaBioMed Life Sciences, Tallinn, Estonia) was used to isolate vesicles by SEC. Before use, the column was washed three times with 1X Dulbecco's phosphate-buffered saline (DPBS; Corning, Massachusetts, USA). FF samples were thawed, and pooled FF samples were prepared by combining equal volumes of FF from seven patients per pool to average out patient-to-patient variability and focus on general trends rather than individual differences between patients. In total, 42 patient samples were used to generate six pooled samples for analysis. A 20 mL aliquot of pooled FF was loaded onto the maxiPURE-EVs column and allowed to pass through by gravity flow. Fractions of 1 mL were collected sequentially, and a total of 40 fractions was obtained, using DPBS as the elution buffer. The column was washed three times with 1X DPBS between uses and reused up to five times.

2.3 | Isolation of FF EV Subpopulations by TFF

Nanoparticle tracking analysis (NTA) and total protein quantification (described in detail below) indicated that fractions 15–31 were enriched in EVs and exhibited minimal protein contamination (Figure S1). These fractions were pooled and subjected to ultrafiltration with a TFF system. A TFF-MV cartridge (HansaBioMed Life Sciences) with 200 nm pore size was employed, following the manufacturer's instructions. Shortly, the sample was introduced into the cartridge with a clean 25 mL syringe attached to one of the TFF nozzles (syringe A). Another empty 25 mL syringe (syringe B) was connected to the second nozzle, and a collection tube was placed under the permeate nozzle. The syringes were alternatively pushed until emptied, after which syringe A was filled with 5 mL of 1X DPBS and the process was repeated. As a result, permeate containing SEVs (<200 nm) was collected. To collect the retained LEVs (>200 nm), the permeate nozzle was closed, syringe B was filled with 5 mL of 1X DPBS, re-attached to the nozzle and both syringes were alternatively pushed 15 times. The retentate containing LEVs was collected in one of the syringes. The TFF cartridge was thoroughly washed with Milli-Q water at least three times, air-dried and stored at room temperature (RT).

2.4 | Concentration of FF EV Samples

22 mL of SEV and 5 mL of LEV samples were concentrated using 10 kDa Amicon Ultra-15 centrifugal filters (Merck Millipore, Massachusetts, USA). Samples were centrifuged at 3200 × *g* at RT until a final volume between 300 and 700 μL was reached. Concentrated EVs were used immediately for experiments or aliquoted and stored in premium surface tubes (04-232-3500, Nerbe Plus, Germany, Winsen) at −80°C. The EVs were not subjected to more than one freeze-thaw cycle and were used within 18 months after storage. Upon use, EVs were thawed at RT.

2.5 | NTA

Size distribution and concentration of isolated EVs were measured with the ZetaView PMX 110 (Particle Metrix, Germany) instrument using its corresponding software (ZetaView 8.05.12 SP1). Before measurements, the instrument was calibrated with a 100 nm polystyrene nanoparticle standard (Applied Microspheres BV, Leusden, Netherlands) prepared in distilled water, following the manufacturer's recommendations. Automated quality control measurements, including cell quality checks and instrument alignment, were performed prior to measurements.

One millilitre of sample, diluted in 1X DPBS, was introduced into the cell, ensuring the detection of 50–200 particles per frame. Particle size and concentration were measured at 11 distinct positions, capturing 30 frames per position across three independent readings with a shutter speed of 100. The sensitivity for small particle measurements was set at 85, whilst for large particle measurements, it was set at 65. The cell temperature was maintained at 25°C for all measurements, and the cell was washed with 1X DPBS between each measurement.

2.6 | Total Protein Quantification

Protein abundance was determined with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Massachusetts, USA), according to the manufacturer's instructions, with 10 μL of undiluted intact EVs and bovine serum albumin (BSA) as a standard. The absorbance at 570 nm was measured with a TECAN GENios Pro plate reader (TECAN Group Ltd, Männedorf, Switzerland). EV purity was calculated by dividing the particle concentration (particles/mL) determined by NTA with the protein concentration (μg/mL) measured by bicinchoninic acid assay (BCA).

2.7 | Western Blot

SEV and LEV samples were obtained by TFF, and fractions 32–40 (containing FF proteins) were obtained by SEC as described earlier. To precipitate proteins from 300 μL of FF, 100 μL of Milli-

Q water, 400 µL of methanol (Sigma-Aldrich, Missouri, USA) and 100 µL of chloroform (Lach-Ner, Neratovice, Czech Republic) were added to the concentrated sample. The mixture was then centrifuged for 5 min at 14,000 × g. The top layer was removed, and the protein layer was washed with 400 µL of methanol. The sample was centrifuged again as before, the protein pellet was dried and resuspended in 0.25% SDS (SERVA Electrophoresis GmbH, Heidelberg, Germany) containing cOmplete Mini Protease Inhibitor Cocktail (Roche, Basel, Switzerland). For cell lysis, KGN cells were treated with RIPA buffer (50 mM Tris pH 7.4, 1% Triton X-100, 1 mM EDTA, 150 mM NaCl, 0.1% SDS) supplemented with cOmplete Mini Protease Inhibitor Cocktail. The lysate was maintained on ice for 30 min, vortexed every 10 min and then centrifuged at 10,000 × g for 10 min. Following centrifugation, the supernatant was transferred to a new tube. Lyophilised small EVs from the HCT116 cell line (HansaBioMed Life Sciences) were reconstituted in 100 µL of Milli-Q water.

10 µg of protein was mixed with either reducing 4X Laemmli buffer (250 mM Tris-HCl, 8% SDS, 40% glycerol, 0.05% bromophenol blue, 10% β-mercaptoethanol) or 4X non-reducing Laemmli buffer (250 mM Tris-HCl, 8% SDS, 40% glycerol, 0.05% bromophenol blue); the latter was used for the detection of CD9 and CD81 proteins. Samples were denatured for 5 min at 95°C and separated by 10% SDS-PAGE at 90 V for 30 min, followed by 150 V for 1 h using the Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad Laboratories, California, USA). Proteins were transferred onto the Immobilon-P polyvinylidene fluoride membrane (Merck Millipore) in Towbin buffer (25 mM Tris, 192 mM glycine, 10% ethanol) at 370 mA for 1 h with the Mini Trans-Blot cell (Bio-Rad Laboratories). The membranes were incubated in 5% non-fat dry milk in 0.1% PBST buffer (0.1% of Tween 20 in 1X DPBS) for 1 h at RT.

Immunoblotting was carried out with the following primary antibodies diluted in 2.5% non-fat dry milk in 0.1% PBST overnight at +4°C: mouse anti-CD9 antibody (HBM-CD9-100, 1:500, HansaBioMed Life Sciences), mouse anti-CD81 antibody (HBM-CD81-EM4-100, 1:500, HansaBioMed Life Sciences), rabbit anti-HSP70 antibody (10995-1-AP-20, 1:500, Proteintech), rabbit anti-albumin antibody (16475-1-AP, 1:10,000, Proteintech, Illinois, USA), mouse anti-ApoA1 antibody (sc-376818, 1:1000, Santa Cruz Biotechnology, Texas, USA) and rabbit anti-calnexin antibody (AB2301, 1:10,000, Merck Millipore). The membranes were washed 3 × 5 min with 0.1% PBST and then incubated with either HRP-conjugated goat anti-rabbit IgG (115-035-003, 1:10,000, Jackson Immunoresearch, Pennsylvania, USA) or goat anti-mouse IgG (G-21040, 1:20,000, Thermo Fisher Scientific) diluted in 2.5% non-fat dry milk in 0.1% PBST buffer for 1 h at RT. After further washing (6 × 5 min) with 0.1% PBST and incubation with SuperSignal West ECL Substrate (Thermo Fisher Scientific) at RT, the images were acquired using the ImageQuant LAS 4000 instrument (GE HealthCare Technologies, Inc., Illinois, USA).

2.8 | Transmission Electron Microscopy (TEM)

LEV (but not SEV) samples were concentrated using an Amicon Ultra-0.5 Centrifugal Filter (Merck Millipore) with a 10 kDa cutoff. All EV samples were prepared for TEM as in Puhka et al. 2017. Briefly, samples with particle concentrations between 10^{10}/mL and 10^{11}/mL were loaded directly onto carbon-coated and glow-discharged 200 mesh copper grids with formvar or pioloform support membrane. The loaded EVs were fixed with 2% paraformaldehyde in sodium phosphate buffer (pH 7.0), washed with deionised water (PURELAB Chorus 1 Water Purification System, ELGA LabWater) and then stained with 2% neutral uranyl acetate. Finally, the samples were embedded and further stained in 1.8% methyl cellulose (25 ctp)/0.4% uranyl acetate in deionised water on ice. The EVs were visualised by TEM using a JEM-1400 electron microscope (Jeol Ltd., Tokyo, Japan) operating at 80 kV. The images were acquired with a Gatan Orius SC 1000B CCD camera (Gatan Inc., USA) with a resolution of 4008 × 2672 pixels image size and no binning.

2.9 | Single Particle Interferometric Reflectance Imaging (SP-IRIS)

FF of individual patients, including SEV and LEV samples isolated from these FF samples, were analysed with SP-IRIS using the ExoView Tetraspanin kit and an ExoView R100 scanner (NanoView Biosciences, USA) according to the manufacturer's instructions. Samples were diluted using the incubation buffer provided in the kit in an optimised manner based on NTA measurements (ZetaView PMX-120, Particle Metrix). FF samples were diluted to a particle concentration of 10^{11}/mL, SEVs to 10^{8}/mL and LEVs to 5×10^{9}/mL (all determined by NTA). The samples were added directly to ExoView R100 chips coated with antibodies against CD9, CD63 and CD81, respectively, and incubated at RT for 16 h. The chips were then stained using fluorescently labelled antibodies (against CD9, CD63, CD81, provided in the kit), washed, dried and scanned. The data obtained was analysed using the NanoViewer analysis software (NanoView Biosciences) version 3.0 with sizing thresholds set to 50–200 nm diameter.

The strategy for assessing the randomness of tetraspanin colocalisation was based on a study by Breitwieser et al. (2022) and is illustrated in Figure S2. In brief, the probabilities for individual events were calculated using experimental colocalisation data. Individual event probabilities were then used to compute theoretical tetraspanin colocalisation ratios, which were subsequently compared with experimental event ratios using a Chi-square test.

2.10 | Cell Culture

Human granulosa tumour-derived cell line KGN (RIKEN BioResource Research Centre, Tsukuba, Japan) was used as a model for cell line experiments (Nishi et al. 2001). Cells were thawed and passaged 3–10 times before being used in the experiments. Cells were seeded at a density of 2×10^{4} –- 3×10^{4} cells per cm^2 and cultured in DMEM/F12 medium containing 4.5 g/L glucose, L-glutamine and sodium pyruvate (10-013-CV, Corning) supplemented with 10% foetal bovine serum (35-089-CV, Corning) and 1% penicillin-streptomycin (Gibco, Massachusetts, USA). Cells were cultured at 37°C in a humidified incubator with 5% CO_2. Cells were routinely passaged after a brief exposure to 0.05% trypsin and 0.53 mM EDTA (25-051-CI, Corning).

For steroidogenesis experiments, 50,000 KGN cells were seeded on a 24-well plate in a phenol red free growth medium con-

taining DMEM (DMEM-HXRXA, Capricorn Scientific, Hesse, Germany), 4 mM L-glutamine (GLN-B, Capricorn Scientific), 1 mM sodium pyruvate (NPY-B, Capricorn Scientific), 10% exosome-depleted foetal bovine serum (FBS, Thermo Fisher Scientific), 1% penicillin-streptomycin (Thermo Fisher Scientific), 10 μM androstenedione (LGC Standards, London, UK) and 20 ng/mL follicle stimulating hormone (Gonal-F, Merck Europe B.V., Amsterdam, Netherlands). Cells were allowed to adhere to the cell culture plate for 48 h, and were subsequently incubated with either SEVs (10^9/mL, 10^8/mL or 10^7/mL), LEVs (10^8/mL, 10^7/mL or 10^6/mL), combination of SEVs and LEVs (10^9/mL + 10^8/mL, 10^8/mL + 10^7/mL or 10^7/mL + 10^6/mL) or 1X DPBS (matching the highest EV sample volume) for 24 h. 500 μL of cell culture medium was collected from each well, centrifuged for 5 min at 1000 × g to remove cell debris. The supernatant was transferred to a new tube and stored at −80°C until further experiments.

For transcriptomics experiments, 50,000 KGN cells were seeded on a 24-well plate in a growth medium containing DMEM/F12 (10-013-CV, Corning), 10% exosome-depleted FBS (Thermo Fisher Scientific) and 1% penicillin-streptomycin (Thermo Fisher Scientific). The cells were allowed to adhere to the cell culture plate for 48 h, then incubated with 10^9/mL SEVs, 10^8/mL LEVs or 1X DPBS (equal to the largest EV sample volume) in 500 μL of culture medium for 24 h. Cell culture medium was removed, cells were washed with 1X DPBS and incubated with 700 μL of QIAzol Lysis Reagent (QIAGEN, Hilden, Germany) for 5 min. The lysates were then transferred to tubes, vortexed for 1 min and stored at −80°C until RNA extraction.

2.11 | EV Labelling and Uptake Assay

SEV and LEV samples were labelled using the ExoGlow-Membrane EV Labeling Kit (System Biosciences, California, USA) according to the manufacturer's instructions. Briefly, the labelling dye was mixed with the provided buffer, then centrifuged at 17,000 × *g* for 5 min to remove dye aggregates. The resulting supernatant was used to incubate 50 μg of EVs for 30 min at RT with shaking. Excess dye was removed by passing samples through miniPURE-EVs spin columns (HansaBioMed Life Sciences) twice via centrifugation at 200 × *g* for 3 min. As a negative control, DPBS was used at a volume equivalent to the lowest EV sample volume, labelled with dye and processed identically to the EV samples. Labelled EV concentrations were determined by NTA as described above.

For the uptake assay, 70,000 KGN cells were seeded onto 13 mm coverslips placed in 4-well plates (Thermo Fisher Scientific) and cultured for 24 h. Cells were then incubated with 10^8 SEVs, 10^8 LEVs, 10^7 LEVs or a negative control for 6 h. After incubation, cells were washed 3 × 5 min with DPBS and fixed with 4% paraformaldehyde (Santa Cruz Biotechnology) for 10 min. Following another set of 3 × 5 min DPBS washes, cells were permeabilised with 0.5% Triton X-100 (Scharlab, Barcelona, Spain) in DPBS for 5 min. After an additional wash, cells were blocked with 5% BSA (Sigma-Aldrich) in DPBS for 1 h. Cells were then incubated with mouse anti-tubulin antibody (2315513, 1:500, DSHB, Iowa, USA) overnight at +4°C. After washing 3 × 5 min with DPBS, DyLight 405 AffiniPure Donkey Anti-Mouse IgG (715-475-150, 1:300, Jackson ImmunoResearch) was added for 1 h at RT. After 3 × 5 min washes with DPBS, the coverslips were mounted with ProLong Gold Antifade Mountant (Thermo Fisher Scientific).

Fluorescence imaging was performed using an LSM 900 confocal microscope (Zeiss, Oberkochen, Germany) with a Plan-Apochromat 63x/1.4 oil objective. DyLight 405 and ExoGlow dyes were excited using 405 and 488 nm lasers, respectively. Both single-plane and serial z-sections were captured using bidirectional scanning at a scan speed of 6, with line averaging set to 4.

2.12 | Cytotoxicity Assay

Cytotoxicity analysis was performed using the CytoTox-Glo Cytotoxicity Assay (Promega Corporation, Wisconsin, USA) according to the manufacturer's instructions. This assay can quantify the extracellular activity of an intracellular protease, allowing the calculation of cell viability by measuring luminescence before and after cell lysis. In addition to assessing viability, the total luminescence signal measured after cell lysis was used as an indirect measure of cell proliferation, reflecting the total number of cells per well.

10,000 KGN cells were plated into each well of a white, clear-bottom 96-well plate (SPL Life Sciences, South Korea). Twenty-four hours after plating, cells were treated with either 10^6/mL, 10^7/mL or 10^8/mL of LEVs, 10^7/mL, 10^8/mL or 10^9/mL of SEVs or with 1X DPBS (equal to the largest EV sample volume) for 48 h. All samples were measured in triplicate with a TECAN GENios Pro plate reader (TECAN Group Ltd., Männedorf, Switzerland). For viability measurement, luminescence was recorded from intact cell cultures and for proliferation assessment, luminescence was measured after complete cell lysis. The mean values of three independent experiments were used in data analysis.

2.13 | Enzyme-Linked Immunosorbent Assay (ELISA)

Steroid hormone levels were measured from cell culture media with commercial oestradiol (DRG International, New Jersey, USA), testosterone (DRG International) and progesterone (Abcam, Cambridge, UK) ELISA kits according to the manufacturer's instructions. The limit of detection (LOD) values for the kits were 10.6 pg/mL, 0.083 and 0.05 ng/mL, respectively. Samples were diluted five-fold for oestradiol and progesterone assays, and 10-fold for testosterone assays. The Multiskan FC Microplate Photometer (Thermo Fisher Scientific) was used to quantify the absorbance at a wavelength of 450 nm. The mean absorbance values for two technical replicates were calculated. A four-parameter logistic regression model was fitted to the values of measured standards, which was then used to calculate the sample concentrations. All hormone levels were normalised against the corresponding values measured in DPBS-treated control samples.

2.14 | RNA Extraction

RNA extraction from isolated EVs was carried out using the miRNeasy Micro kit (QIAGEN, Hilden, Germany) following the user manual, with the modification of adding 5 µg of glycogen (R0561, Thermo Fisher Scientific) at the same time as chloroform. RNA isolation from cell lysates included the separation of a small RNA-enriched fraction from long RNAs (>200 nt), using the miRNeasy Micro kit and the RNeasy MinElute Cleanup Kit (QIAGEN) by strictly following the guidelines provided in the user manual.

RNA concentrations were measured using a Qubit 4 Fluorometer (Thermo Fisher Scientific) with either the Qubit RNA High Sensitivity Assay or the Qubit microRNA Assay (Thermo Fisher Scientific). The quality of the long RNA fraction was evaluated on an Agilent 2100 Bioanalyzer (Agilent Technologies, California, USA) with the RNA 6000 Pico kit (Agilent Technologies).

2.15 | Polyadenylated RNA Sequencing and Data Analysis

RNA samples with RNA integrity number (RIN) values >9.0 were used for RNA sequencing. Polyadenylated RNA was isolated from 500 ng of the long RNA fraction using the NEXTFLEX Poly(A) Beads 2.0 kit (Revvity, Massachusetts, USA), which was subsequently used to prepare strand-specific RNA libraries with the NEXTFLEX Rapid Directional RNA-Seq Kit 2.0 (Revvity) according to the manufacturer's instructions. Library size was estimated with Agilent DNA High Sensitivity chips on the Agilent 2100 Bioanalyzer instrument (Agilent Technologies), and library concentrations were determined with the Qubit 4.0 instrument using the Qubit High Sensitivity DNA Assay (Thermo Fisher Scientific). The libraries were pooled in equimolar amounts and 150 bp paired-end sequencing was performed on the NovaSeq X platform (Illumina, California, USA). On average, 38.9 million raw reads were produced per sample (Table S1).

Raw FASTQ reads were first assessed for their quality using FastQC version 0.11.8 (Andrews 2010). The reads were then passed through Trimmomatic version 0.39 (Bolger et al. 2014) for quality trimming and adapter sequence removal with the parameters *LEADING:3 SLIDINGWINDOW:3:20 MINLEN:36*. The remaining read pairs were mapped to the human reference genome GRCh38 (GENCODE annotation) using STAR version 2.7.1 (Dobin et al. 2012) with default parameters. Uniquely mapped reads were counted with HTSeq-count version 0.11.2 (Anders et al. 2014), using the primary assembly annotation GTF file (version 111) obtained from Ensembl. To annotate reads that overlap with multiple features, the option *intersection-nonempty* was used with HTSeq. Differential gene expression (DGE) analysis was performed with DESeq2 version 1.40.2 (Love et al. 2014) in R version 4.4.1 (R Core Team 2018). Genes with low counts were excluded from the analysis: the cut-off was set at ≥10 normalised counts in ≥50% of samples. The statistical significance cut-off for differentially expressed (DE) genes was set at Benjamini-Hochberg (BH) false discovery rate (FDR) < 0.05 with no cut-off for the $\log_2$(fold change). The PCA plot was generated with the plotPCA function in the DESeq2 package, using the top 500 most variable genes. Pathway enrichment analysis was performed for DE genes using the ShinyGO version 0.8 application tool (Ge et al. 2020) together with the Reactome database (Croft et al. 2011). Pathways with FDR <0.05 were considered statistically significant and reported. Statistically significant Reactome terms across multiple datasets were visualised as a heatmap using the R package ComplexHeatmap (Gu et al. 2016).

2.16 | Small RNA Sequencing and Data Analysis

Small RNA sequencing libraries were prepared from 5 ng of RNA with the QIAseq miRNA Library Kit (QIAGEN), following the manufacturer's manual. A total of 20 PCR cycles were used for library amplification. Library size was estimated with Agilent DNA High Sensitivity chips on the Agilent 2100 Bioanalyzer instrument (Agilent Technologies). Library concentrations were determined with the Qubit High Sensitivity DNA Assay (Thermo Fisher Scientific). The libraries were pooled in equimolar amounts, and single-end sequencing of 72 bp length with 10 bp dual indexing was performed on the NovaSeq X platform (Illumina). On average, approximately 30.2 million raw reads were produced per sample (Table S2).

The raw data analysis was conducted in the QIAGEN RNA-seq Analysis Portal 5.0 using the QIAseq miRNA Library Kit analysis workflow (QIAGEN). The list of unique molecular counts of miRNAs and piRNAs was used as input for DGE with DESeq2 (version 1.40.2) in R (version 4.4.1). miRNAs and piRNAs expressed at low levels were excluded by retaining those with a sum of ≥6 unique molecular identifier (UMI) reads across SEV or LEV samples. UMI counts per million (CPM) values were calculated with the cpm function of the EdgeR package (version 4.2.2) (Robinson et al. 2010). The statistical significance cut-off for DE miRNAs and piRNAs was set at FDR < 0.05 with no cut-off for the $\log_2$(fold change).

The list of DE miRNAs was used as an input for the miRNA Enrichment Analysis and Annotation Tool (miEAA) (Kern et al. 2020) for over-representation analysis (ORA) of Reactome pathways via miRPathDB (Backes et al. 2017). Pathways targeted by ≥2 miRNAs with FDR < 0.05 are reported. mRNA targets of the DE miRNAs were predicted with the miRWalk prediction tool (Sticht et al. 2018). Only targets that intersected among miRTarBase, miRDB and TargetScan, had target sites in their 3'-UTR region, and possessed a binding score of 1 were selected for further analysis. Cytoscape version 3.10.2 (Shannon et al. 2003) was used for plotting the network of DE miRNAs and their target genes.

2.17 | Statistical Analysis

R version 4.4.1 was used to generate graphs and to perform statistical analyses. Data were analysed using either a two-tailed Student's *t*-test, Mann-Whitney *U* test, analysis of variance (ANOVA) or Chi-square test. The specific statistical methods used in this study are described in each figure legend. Differences were considered statistically significant at $^{\#}p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

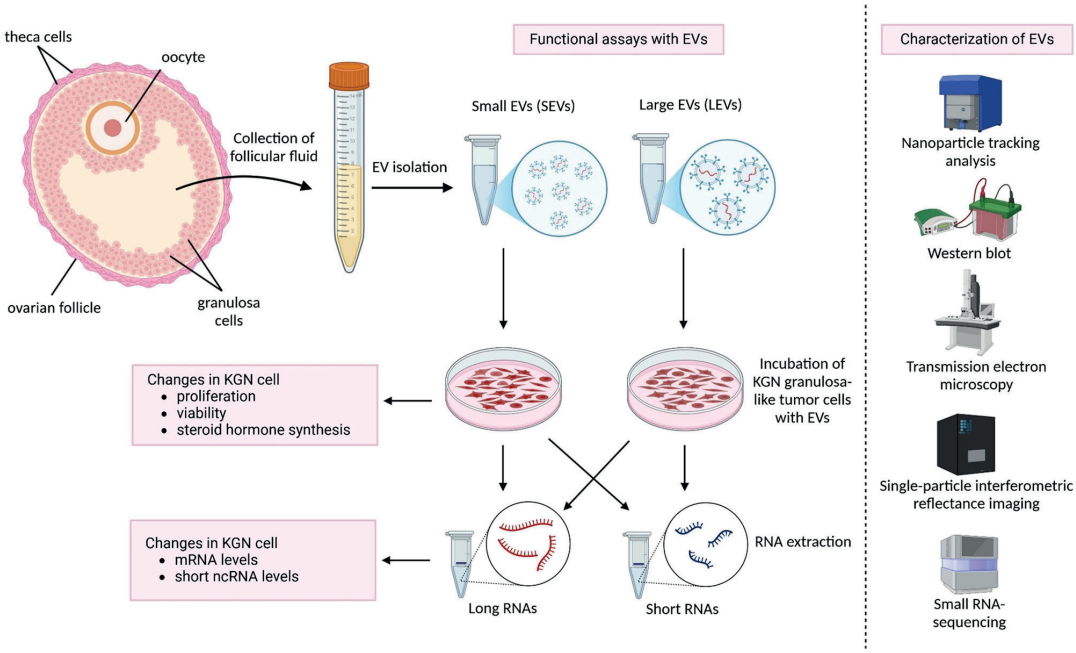

FIGURE 1 | Study design. Extracellular vesicles were isolated from human ovarian follicles and separated based on size. Small and large EVs were characterised with multiple methods. Functional studies were carried out on the KGN cell line serving as a homogenous granulosa cell model. Created in BioRender. Varik, I. (2025) https://BioRender.com/p12h544.

2.18 | Sequencing Data Availability

The raw data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB83132 (https://www.ebi.ac.uk/ena/browser/view/PRJEB83132).

3 | Results

3.1 | Isolation and Characterisation of Human FF EV Subpopulations

To investigate the functional differences between FF EV subpopulations and their potential roles in ovarian health, we first separated EVs from FF proteins using SEC, followed by TFF (Figure 1). We found that SEVs contained approximately 25 times more nanoparticles than LEVs, with concentrations of $1.36 \times 10^{11} \pm 4.18 \times 10^{10}$ particles/mL (mean ± SEM) for SEVs and $5.43 \times 10^{9} \pm 1.05 \times 10^{9}$ particles/mL for LEVs (Figure 2A). NTA measurement of particle diameters confirmed significant differences between the subpopulations, with SEVs averaging 93 ± 2 nm in diameter and LEVs 299 ± 9 nm (Figure 2B). Our isolation procedure yielded high-purity EV subpopulations, with mean particle/protein ratios of $7.93 \times 10^{7} \pm 1.7 \times 10^{7}$ for SEVs and $9.08 \times 10^{7} \pm 1.7 \times 10^{7}$ for LEVs (Figure 2C, Table S3).

To further assess EV purity, we performed Western blot analysis to detect albumin and apolipoprotein 1 (APOA1) (Figure 2D), which are abundant in FF (Kurdi et al. 2023) and often co-isolated with EVs (Simonsen 2017). Although albumin was detected both in SEVs and LEVs, and APOA1 in SEVs, their levels were substantially reduced compared to the crude FF and the protein fractions (FR 32–40) obtained during SEC. Calnexin, an endoplasmic reticulum (ER) protein, was expectedly not detected in any EV samples but was present in the KGN cell lysate (Figure 2D).

To confirm EV enrichment in our samples, we analysed well-established EV markers, CD9, CD81 and HSP70. Western blot revealed that CD9, CD81 and HSP70 were present in both SEV and LEV samples, whilst crude FF and protein fractions (FR 32–40) exhibited lower levels of these markers. Additionally, these markers were detectable in commercially produced EVs purified from the HCT116 cell line (HCT116 EV), which served as a positive control for EV marker detection (Figure 2D). TEM analysis further confirmed the presence of EV-like nanoparticles with spherical or cup-shaped morphology in both SEV and LEV samples (Figure 2E).

3.2 | Comparison of Tetraspanin Profiles Between FF EV Subpopulations

We further characterised the FF EV subpopulations using SP-IRIS to investigate the potential differences in tetraspanin colocalisation between SEV, LEV and FF samples. This method captures EVs using antibodies that target specific EV proteins, followed by incubation with fluorescently labelled antibodies that bind either the same or different EV proteins. In this study, we captured and probed EVs, using antibodies against CD9, CD63 and CD81.

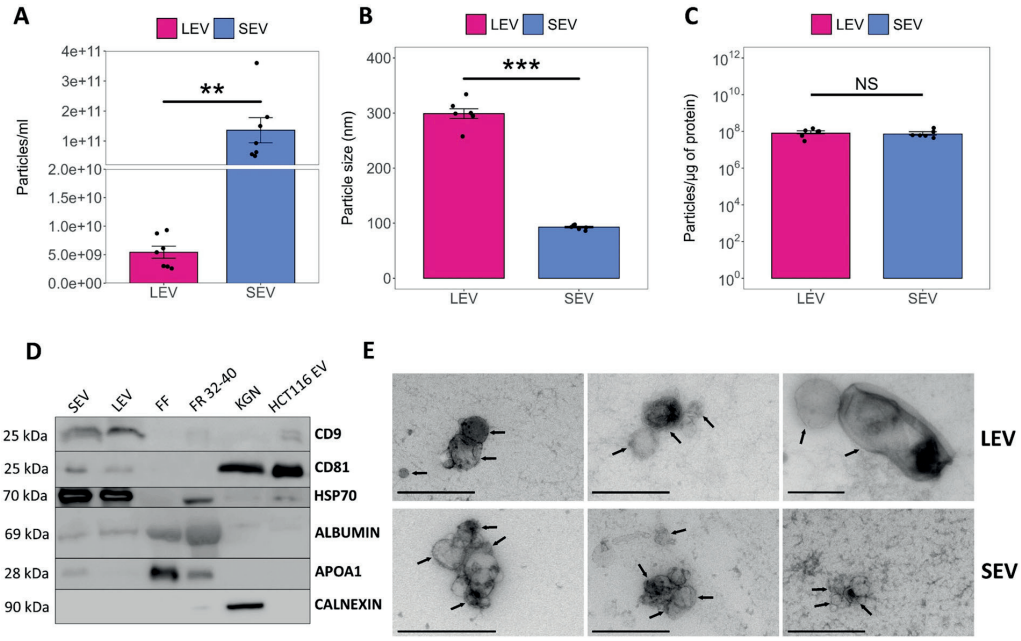

FIGURE 2 | Characterisation of small (SEV) and large (LEV) extracellular vesicles purified from human FF ($n = 6$). Mean particle concentrations (A), diameters (B), and purity (C) of SEV and LEV samples. (D) Western blot analysis showing EV (CD9, CD81 and HSP70) and non-EV proteins (albumin, APOA1 and calnexin). FF and protein fractions (FR 32–40) served as a positive control for the detection of albumin and APOA1. KGN cell lysate was used as a positive control for detecting calnexin. Commercial EVs isolated from the HCT116 cell line served as a positive control for EV protein detection. (E) Transmission electron microscopy images of SEV and LEV samples. EVs are indicated by arrows, scale bar = 500 nm. All results are shown as mean ± SEM. Statistical differences between groups were determined by Student's *t*-test and are indicated with asterisks (**$p < 0.01$, ***$p < 0.001$). NS –= not significant.

Our results show that both SEV and LEV samples contain all three tetraspanins—CD9, CD63 and CD81 (Figure 3). LEVs exhibit a lower percentage of CD9/CD63/CD81 colocalisation compared to SEV and FF samples on the CD9-capture spot (Figure 3A), compared to FF samples on the CD63-capture spot (Figure 3B), and compared to SEV samples on the CD81-capture spot (Figure 3C). These findings suggest that SEVs are more likely to contain all three tetraspanins compared to LEVs. Conversely, LEVs were enriched in CD9-positive particles (Figure 3A) and CD9/CD81-positive particles (Figure 3C) compared to FF samples.

Next, we assessed whether tetraspanin combinations occurred randomly or if specific tetraspanin combinations were preferentially formed in FF EVs. LEVs captured by the CD81 and CD63 antibodies displayed a statistically significant under-representation of CD9/CD63/CD81-positive particles (Figure 3D), whereas SEVs captured by the CD9 antibody showed a statistically significant over-representation of CD9/CD63/CD81-positive particles (Figure 3E) compared with simulated distributions. These results suggest that the tetraspanin profiles of SEVs and LEVs differ and that the distribution of these tetraspanins is non-random, potentially reflecting distinct characteristics of EV subpopulations.

3.3 | FF EVs Are Internalised by KGN Cells

To verify the internalisation of FF-derived EVs by GCs, we labelled SEVs and LEVs with ExoGlow membrane dye and purified the preparations to remove excess dye. After dye removal, KGN cells were treated with 10^8 SEVs, 10^8 LEVs or 10^7 LEVs for 6 h, with dye-containing DPBS as a negative control. Confocal microscopy analysis revealed the accumulation of fluorescently labelled EVs in the cytoplasm of KGN cells (Figure 4A,B), whilst minimal fluorescence was observed in the control condition. These findings confirm that both SEVs and LEVs are efficiently internalised by KGN cells, indicating their capability to deliver bioactive molecules and potentially influence cellular functions.

3.4 | The Impact of FF EVs on KGN Cell Proliferation, Viability and Steroid Hormone Production

In order to investigate the impact of different FF EV subpopulations on GC function, we treated the KGN cell line with varying concentrations of SEVs (10^7, 10^8 or 10^9 particles/mL), LEVs (10^6, 10^7 or 10^8 particles/mL) or DPBS as a control for 48 hours. The rationale for using lower concentrations of LEVs compared to

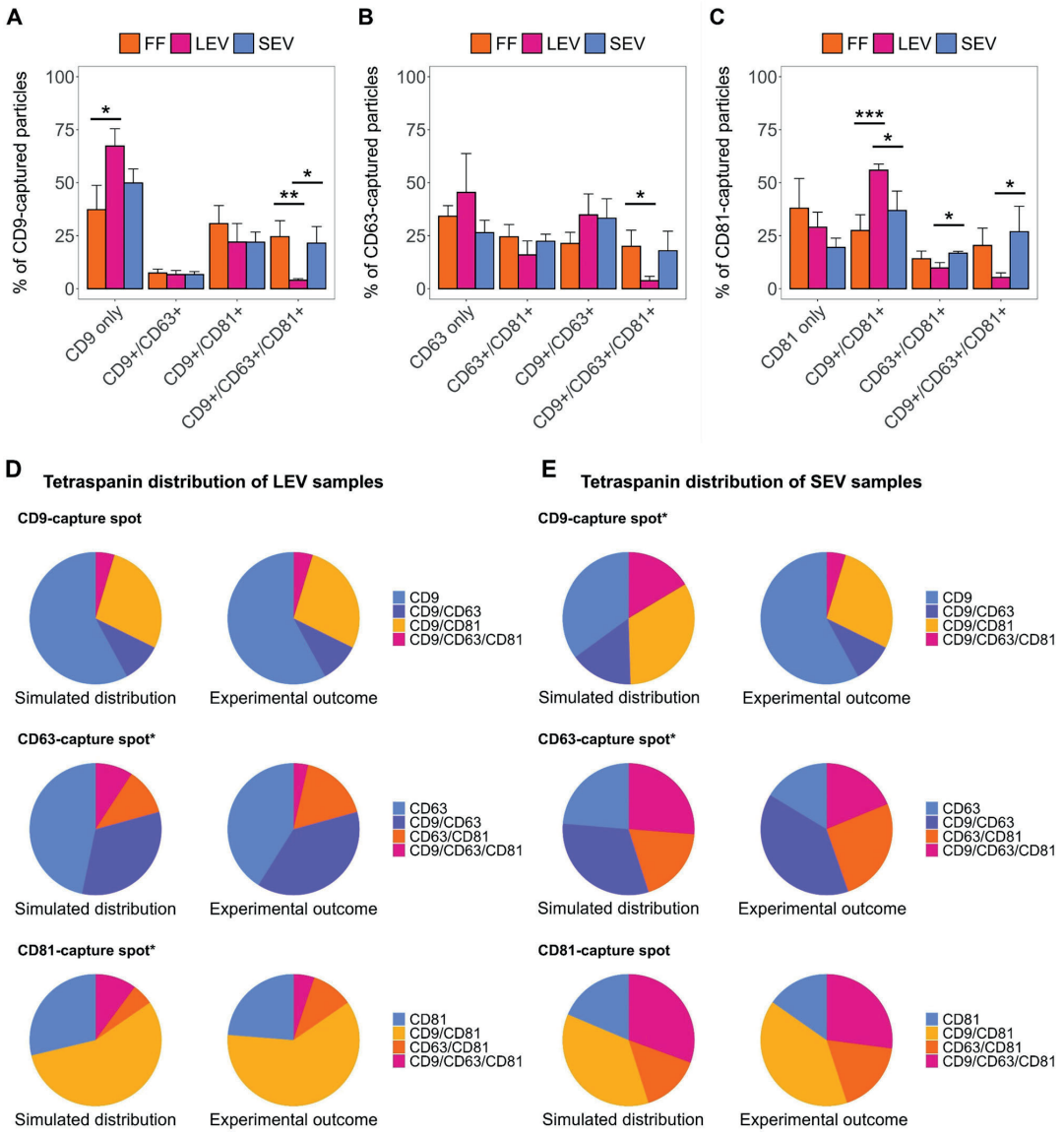

FIGURE 3 | Comparison of tetraspanin profiles of SEVs, LEVs and crude FF. SEVs ($n = 4$), LEVs ($n = 3$) and FF ($n = 6$) were loaded onto the ExoView Tetraspanin chips and analysed with the ExoView R100 scanner. Tetraspanin colocalisation fractions (mean percentage of all detected EVs ± SEM) are shown for the CD9-capture spot (A), the CD63-capture spot, (B) and the CD81-capture spot (C). Statistical significance was determined using one-way ANOVA, followed by Tukey's post-hoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (D) Comparison of tetraspanin distribution between the simulated and experimental values for LEV samples and (E) SEV samples. Capture spots for which Chi-square test between simulated and experimental colocalisation ratios resulted in $p < 0.05$ (indicating deviation from random colocalisation), are shown with asterisks (*).

SEVs in functional studies stems from their biological levels in FF being approximately a magnitude lower than those of SEVs (Figure S3).

Cytotoxicity assays showed that neither SEVs nor LEVs significantly affected KGN cell viability (Figure 5A) or proliferation (Figure 5B) at any of the tested concentrations. Next, we assessed whether FF EV subpopulations could influence steroid hormone production in KGN cells. For this, KGN cells were treated with SEVs (10^7, 10^8 or 10^9 particles/mL), LEVs (10^6, 10^7 or 10^8 particles/mL), SEVs and LEVs in combination (10^9/mL SEV + 10^8/mL LEV, 10^8/mL SEV + 10^7/mL LEV and 10^7/mL SEV + 10^6/mL LEV), or DPBS for 24 h, after which oestradiol, progesterone and testosterone levels were measured from the cell media by ELISA.

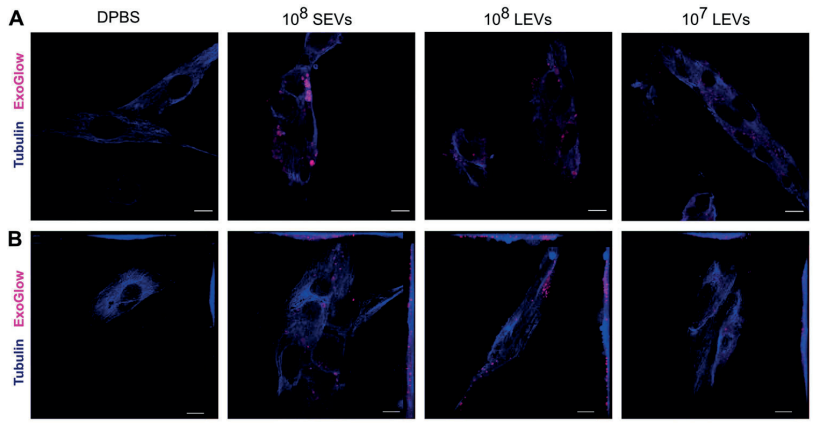

FIGURE 4 | Internalisation of FF-derived EVs by KGN cells. KGN cells were treated with 10^8 SEVs, 10^8 LEVs, or 10^7 LEVs for 6 h after labelling EVs with ExoGlow membrane dye (red), followed by a purification step to remove excess dye. As a negative control, cells were incubated with dye-containing DPBS that underwent the same purification procedure. Tubulin staining (blue) was used to visualise cell structure. (A) Confocal images of a single plane. (B) Maximum intensity projections of the z-stack optical sections. Scale bar = 10 μm.

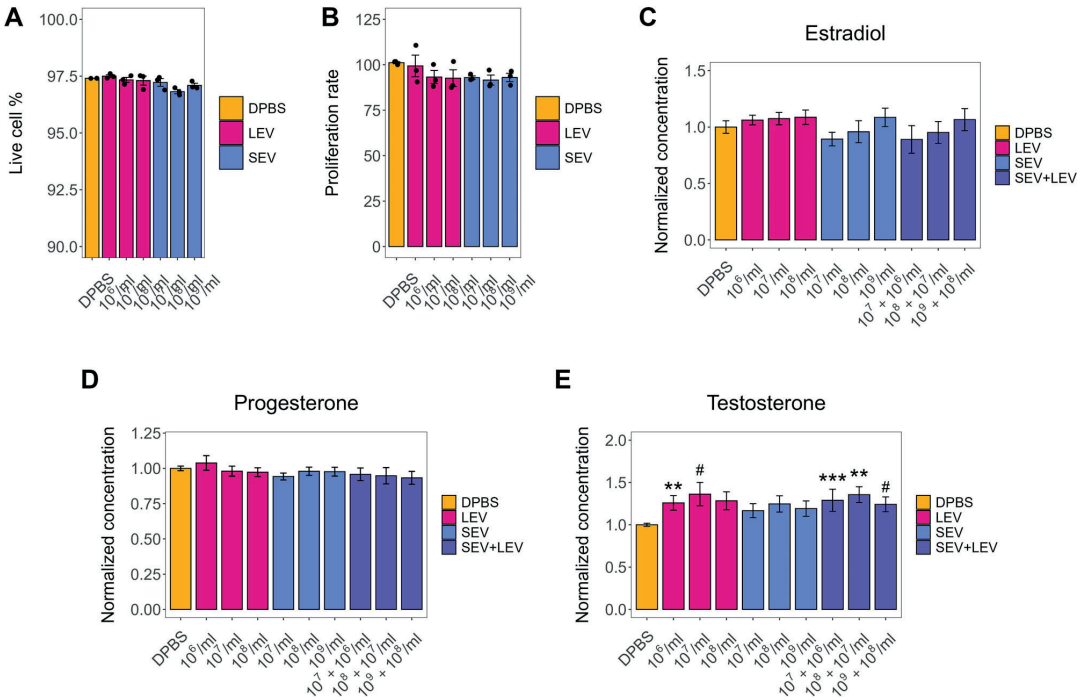

FIGURE 5 | Effects of SEVs and LEVs on KGN cell viability, proliferation and steroid hormone synthesis. For steroid hormone measurements, KGN cells were treated with increasing concentrations of SEVs ($n = 12$), LEVs ($n = 12$), a combination of SEVs and LEVs ($n = 6$) or DPBS ($n = 12$) for 24 h. For viability and proliferation measurements, cells were treated with SEVs ($n = 3$), LEVs ($n = 3$) or DPBS ($n = 3$) for 48 h. After the treatment, cell viability (A), proliferation (B), and the synthesis of oestradiol (C), progesterone (D), and testosterone (E) were assessed. All results are shown as mean ± SEM. Statistical significance was determined using the Mann-Whitney *U*-test (#$p < 0.1$, **$p < 0.01$, ***$p < 0.001$).

No significant changes in oestradiol (Figure 5C) or progesterone (Figure 5D) levels were observed in response to any of the treatments. However, treatment of KGN cells with LEVs alone, as well as with a combination of SEVs and LEVs, resulted in a significant increase in testosterone production (Figure 5E), suggesting that SEVs and LEVs vary in their ability to modulate steroidogenesis

3.5 | DE of Small ncRNAs in FF EV Subpopulations

Aiming to better understand the role of FF EV subpopulations in ovarian intercellular communication, we analysed their small ncRNA content through small RNA sequencing. Our analysis identified 148 miRNAs and 223 piRNAs across the analysed samples (Table S4).

Principal component analysis (PCA) revealed distinct expression profiles between SEV and LEV samples, with 31% of variance explained by PC1 (Figure 6A). We identified 48 differentially expressed (DE) miRNAs between SEVs and LEVs, of which 45 were upregulated in SEVs and 3 in LEVs (Figure 5B, Table S5). Additionally, 50 DE piRNAs were identified, with 13 upregulated in SEVs and 37 in LEVs (Figure 6B, Table S5). These results suggest that miRNAs are preferentially packaged into SEVs, whilst piRNAs are predominantly incorporated into LEVs by the follicular cells.

To explore the biological processes potentially influenced by DE miRNAs, we performed ORA with miEAA to identify enriched Reactome terms. ORA highlighted pathways related to generic transcription, cellular senescence, insulin-like growth factor-2 mRNA binding proteins (IGF2BPs), the cell cycle, cellular responses to external stimuli and tRNA processing (Figure 6C). Additionally, pathways associated with signalling by the TGF-β receptor complex, oestrogen receptor (ESR), WNT signalling, TP53 regulation of metabolic genes, Ca^{2+} signalling and TNF signalling were identified (Table S6). The upregulation of miRNAs associated with these pathways in SEVs suggests that small FF EVs may influence critical cellular processes in target cells.

3.6 | SEVs Trigger Significant Changes in KGN Cell Gene Expression

Our next objective was to determine whether FF EV subpopulations differentially influence the transcriptome of KGN cells. To address this, KGN cells were treated with the highest achievable concentration of SEVs (10^9 particles/mL), LEVs (10^8 particles/mL) or DPBS as a control for 24 h, followed by RNA-sequencing. PCA of gene expression data revealed that LEV-treated cells clustered closely with DPBS-treated cells, whilst SEV-treated cells clustered more distinctly (Figure 7A).

LEV treatment induced changes in the expression of five genes in KGN cells: KIRREL3 and CCBE1 were downregulated, whilst VCAM1, C1S and SOD2 were upregulated (Figure 7B, Table S7). In contrast, SEV treatment caused extensive transcriptomic changes, with 692 genes upregulated and 904 genes downregulated (Figure 7C, Table S8). Enrichment analysis for Reactome pathways revealed that genes downregulated by SEV treatment were enriched in pathways related to cholesterol biosynthesis, cell-ECM interactions, homologous recombination repair (HRR) and Rho GTPase signalling (Figure 7D, Table S9). In contrast, genes upregulated by SEV treatment were primarily associated with extracellular matrix (ECM) remodelling, including laminin and syndecan interactions, collagen biosynthesis, non-integrin membrane-ECM interactions, degradation of ECM, ECM proteoglycans, as well as MET-activated PTK2 signalling (Figure 7E, Table S10).

To investigate SEV miRNA and KGN mRNA interactions upon incubation with SEVs, we predicted target genes of miRNAs upregulated in SEVs using miRWalk. Since miRNAs primarily mediate gene silencing (Quévillon Huberdeau and Simard 2019), we compared the predicted miRNA targets with genes downregulated in SEV-treated cells and identified 74 miRNA-mRNA interactions (Figure S4, Table S11). The largest interaction network involved hsa-let-7b-5p and its target genes, which regulate cell cycle progression (CCND1, PDGFB) and cytoskeletal dynamics (FARP1, UTRN, FIGN and NAP1L1).

3.7 | SEV and LEV Treatments Alter Small ncRNA Levels in KGN Cells

To further characterise the impact of FF EVs on the KGN cell transcriptome, we performed small RNA sequencing of KGN cells treated with the highest achievable concentration of SEVs (10^9 particles/mL), LEVs (10^8 particles/mL) or DPBS as a control for 24 h. PCA revealed distinct clustering for all treatments (Figure 8A), highlighting the differential effects of SEVs and LEVs on small ncRNA levels in KGN cells.

DE analysis between SEV- and LEV-treated samples identified 159 DE ncRNAs, including 88 DE miRNAs and 71 piRNAs (Figure 8B, Table S12). Of the DE miRNAs, 42 were upregulated and 46 downregulated in SEV-treated cells; whilst of the DE piRNAs, 27 were upregulated and 44 downregulated in SEV-treated cells (Table S12).

ORA using the Reactome database revealed distinct functional associations for miRNAs upregulated in SEV- and LEV-treated cells. miRNAs upregulated upon LEV treatment were enriched in pathways related to IGF2BPs (FDR = 6.09×10^{-3}) and PTEN regulation (FDR = 0.0176), whereas miRNAs upregulated upon SEV treatment were associated with pathways related to generic transcription, receptor tyrosine kinase signalling, ER to Golgi transport, TGF-β signalling and Smad2/Smad3/Smad4 transcriptional activity (Figure 8C, Table S13).

We examined whether miRNAs present in FF EVs were upregulated in KGN cells, suggesting their potential delivery via EVs. Among the 42 miRNAs upregulated upon SEV treatment in KGN cells, 11 were present in SEVs (Figure 8D), and among the 46 miRNAs upregulated upon LEV treatment, 26 were detected in LEVs (Figure 8E). Notably, there was no overlap between miRNAs potentially delivered by SEVs and LEVs. For piRNAs, 20 of the 27 piRNAs upregulated in SEV-treated cells were present in SEVs (Figure S5), whilst 28 of the 44 upregulated piRNAs in LEV-treated cells were present in LEVs (Figure S6).

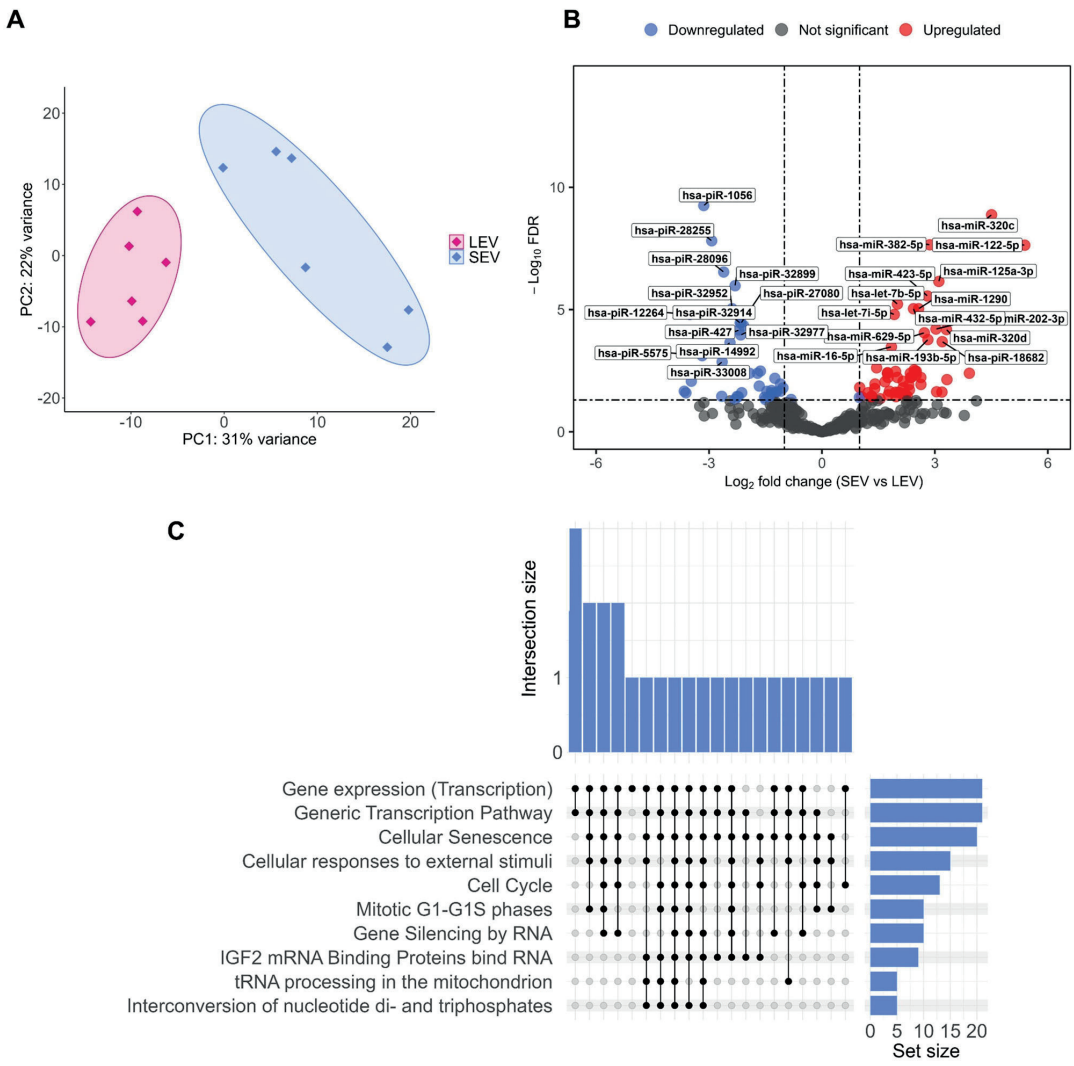

FIGURE 6 | Identification of differentially expressed (DE) small noncoding RNAs between FF EV subpopulations. (A) PCA based on all miRNAs and piRNAs detected in SEV ($n = 6$) and LEV samples ($n = 6$). (B) DE miRNAs and piRNAs between SEV and LEV samples. RNAs that are more abundant ($\log_2$ fold change >1) in SEVs are indicated by red dots, whereas those more abundant ($\log_2$ fold change <−1) in LEVs are indicated by blue dots. (C) Top 10 enriched pathways for DE miRNAs identified via miEAA over-representation analysis using the Reactome database. Interaction size represents the number of shared miRNAs among pathways. Set size represents the number of DE miRNAs annotated to a specific pathway.

3.8 | Integration of Gene Ontology Datasets Reveals Central Pathways Modulated by SEVs

To identify key pathways influenced by SEVs, we integrated pathway enrichment results from four independent analyses: DE miRNAs between SEVs and LEVs (45 of 48 DE miRNAs were upregulated in SEVs), downregulated mRNAs in SEV-treated GCs, upregulated mRNAs in SEV-treated GCs and upregulated miRNAs in SEV-treated GCs. This systems-level approach identified overlapping pathways, highlighting their robustness and biological significance.

The analysis identified transcriptional regulation as a central target of SEVs, with both the generic transcription pathway and RNA polymerase transcription consistently enriched across all four datasets (Figure 9). Notably, SEV treatment led to the upregulation of POLR2A (Table S8), the largest subunit of RNA polymerase II and GTF2F1, a component of the RNA polymerase II preinitiation complex, suggesting that SEVs may enhance transcriptional efficiency or activity in GCs.

In addition to transcriptional regulation, six pathways were consistently enriched across three datasets, underscoring their importance in SEV-mediated processes. Among these, signalling

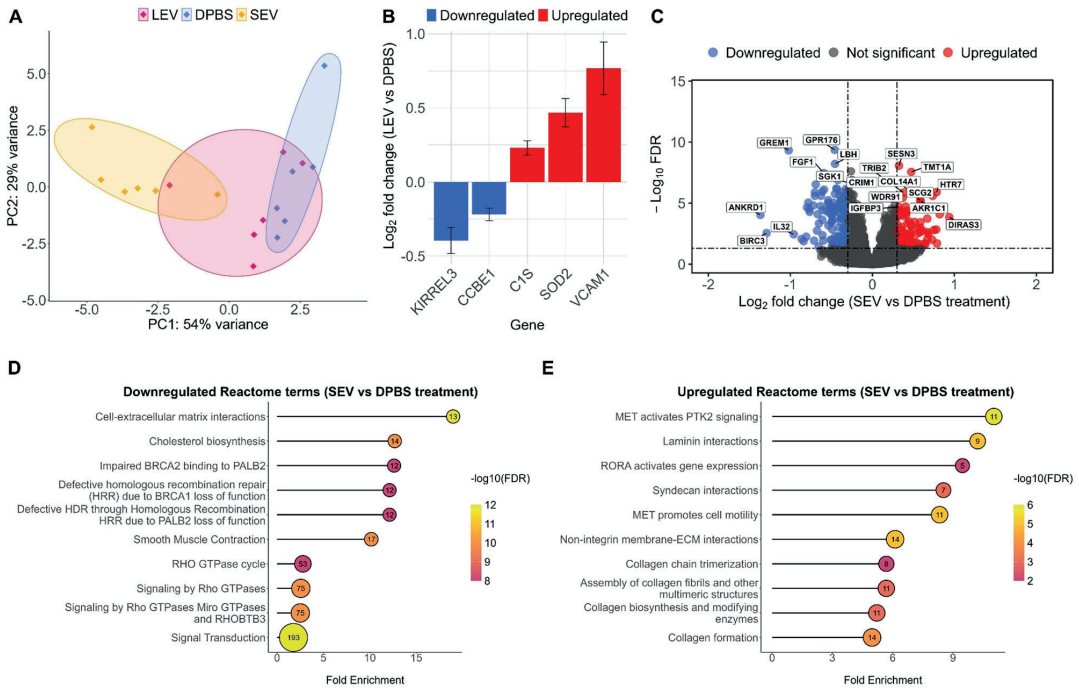

FIGURE 7 | Identification of differentially expressed genes (DEGs) in KGN cells treated with SEVs ($n = 6$), LEVs ($n = 6$) or DPBS ($n = 6$). (A) PCA of mRNA expression data from KGN cells treated with SEVs, LEVs or DPBS. (B) Log_2 fold change values for statistically significant (FDR <0.05) DEGs between LEV and DPBS treatments (mean ± SEM). (C) Volcano plot showing DEGs upon SEV treatment, compared to DPBS-treatment. Red dots represent upregulated DEGs (log_2 fold change >0.3), and blue dots represent downregulated DEGs (log_2 fold change <−0.3) with FDR <0.05. (D) Top 10 Reactome terms (FDR <0.05) enriched with downregulated genes between SEV and DPBS treatments. (E) Top 10 Reactome terms (FDR <0.05) enriched with upregulated genes between SEV and DPBS treatments. The number of DEGs observed by comparing SEV versus DPBS in each pathway is noted within circles in D and E.

by the TGF-β receptor complex, which is known for its roles in follicular development, GC proliferation and oocyte maturation (Knight and Glister 2006), emerged as a key pathway. The enrichment of the cytokine signalling pathway reflects the role of SEVs in immune regulation within the follicle, whilst pathways related to protein metabolism and post-translational protein modifications emphasise the potential role of SEVs in fine-tuning protein activity, turnover and stability in GCs. The consistent enrichment of receptor tyrosine kinase signalling points to the regulation of cell survival and differentiation, and integrin cell surface interactions link SEVs to ECM remodelling and cell-ECM communication.

Pathways enriched across two datasets further reinforce the multifunctional roles of SEVs, particularly in cellular stress responses and cell cycle regulation. Cellular stress pathways, including oxidative stress-induced senescence and cellular responses to stress, indicate SEV-driven mechanisms that may help protect GCs from cellular damage and maintain their viability. Taken together, these findings offer a systems-level understanding of SEV-mediated intercellular communication and suggest that SEVs play a multifaceted role in reprogramming GC functions, particularly through the modulation of transcriptional activity and cellular signalling.

4 | Discussion

FF-derived EVs play a crucial role in facilitating intercellular communication between oocytes and somatic cells of the ovarian follicle. To better understand their distinct contributions to the follicular microenvironment, small and large EVs were studied separately, as they may differ in molecular composition and biological activities. By isolating and characterising these EV subpopulations, we aimed to gain a clearer understanding of the GC function, including viability, proliferation, steroidogenesis and gene expression.

Our results demonstrated that SEVs are significantly more abundant than LEVs in FF, consistent with earlier reports by Neyroud et al. (2022) and Franz et al. (2016), who similarly observed higher abundance of SEVs among FF EVs. Single-EV tetraspanin profiling revealed a non-random distribution of tetraspanins, with LEVs containing a lower proportion of CD9/CD63/CD81-positive particles compared to SEVs and crude FF. These findings suggest that distinct tetraspanin combinations may characterise each EV subpopulation.

The non-random distribution of tetraspanins could be explained by several factors. First, SEVs and LEVs likely originate from

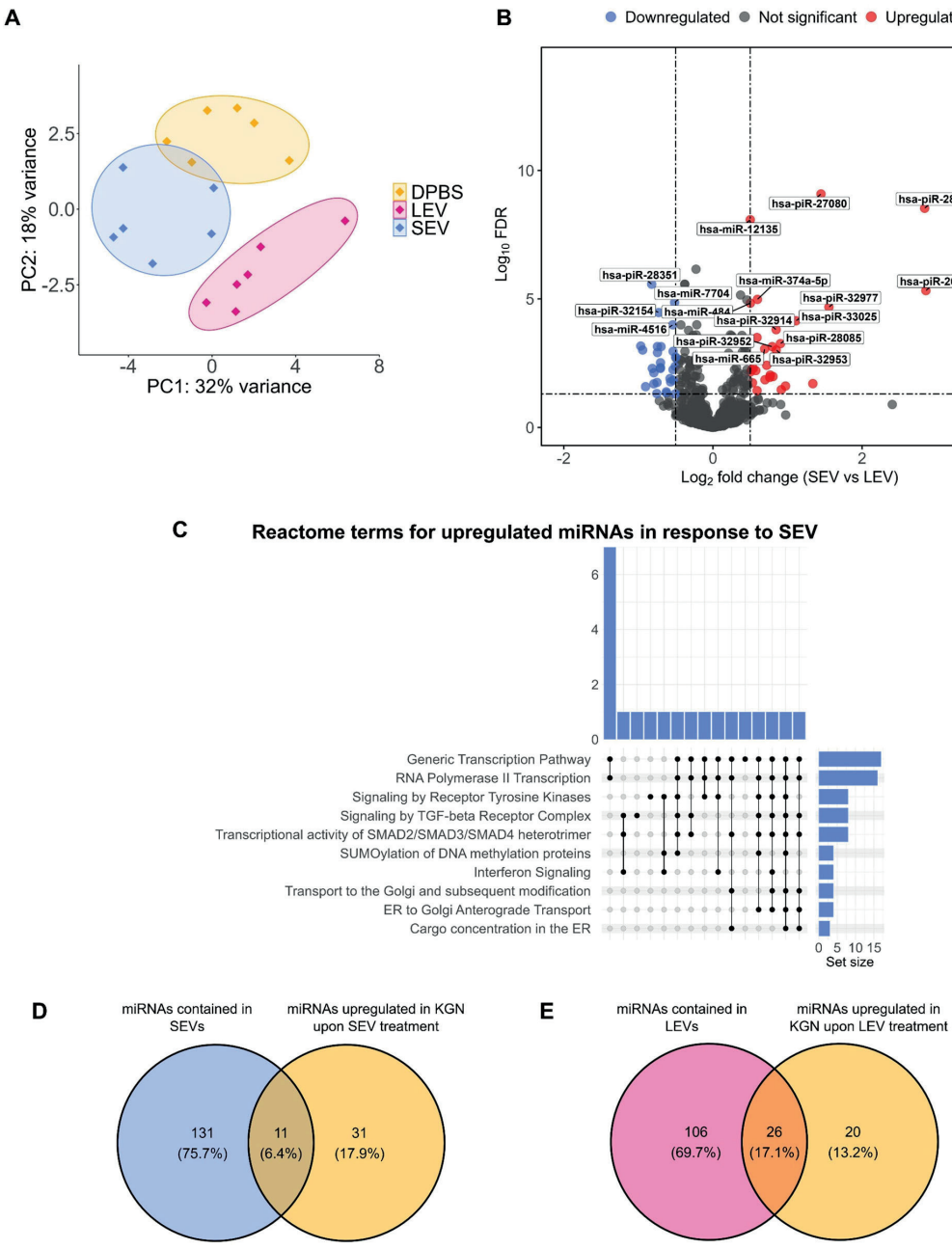

FIGURE 8 | Identification of DE ncRNAs in KGN cells treated with SEVs ($n = 6$), LEVs ($n = 6$) or DPBS ($n = 6$). (A) PCA based on piRNA and miRNA expression levels of KGN cells treated with SEVs, LEVs or DPBS. (B) Volcano plot showing DE ncRNAs between SEV and LEV treatments. Red dots represent upregulated ncRNAs in SEVs ($\log_2$ fold change >0.3), and blue dots represent upregulated ncRNAs in LEVs ($\log_2$ fold change <−0.3) with FDR <0.05. (C) Top 10 Reactome terms for upregulated miRNAs in response to SEV treatment. Reactome terms were identified via miEAA over-representation analysis. Interaction size represents the number of shared miRNAs. Set size represents the number of miRNAs annotated to a specific pathway. (D) Overlap between miRNAs upregulated in KGN cells upon SEV treatment and miRNAs contained in SEVs. (E) Overlap between miRNAs upregulated in KGN cells upon LEV treatment and miRNAs contained in LEVs.

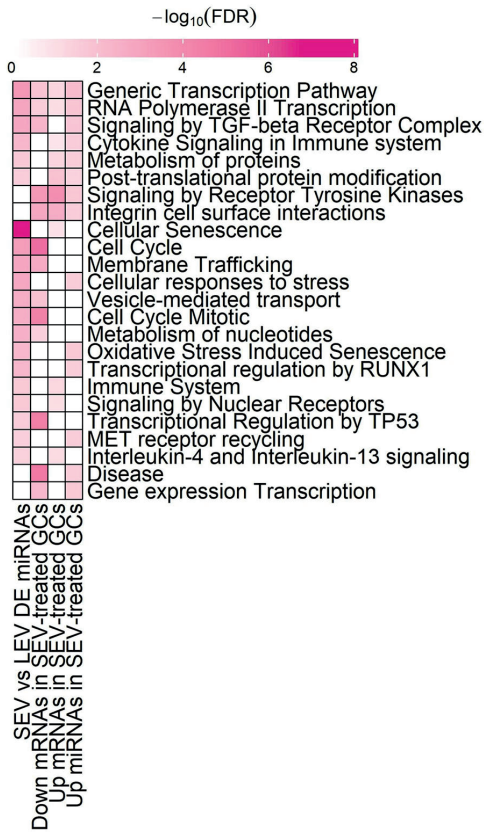

FIGURE 9 | Heatmap of statistically enriched Reactome terms associated with SEVs: DE miRNAs predominantly upregulated in SEVs compared to LEVs, downregulated mRNAs in SEV-treated GCs, upregulated mRNAs in SEV-treated GCs and upregulated miRNAs in SEV-treated GCs. Colour intensities represent the $-\log_{10}$(FDR) values for each Reactome term.

different biogenesis pathways, which may influence the incorporation of specific tetraspanins into these EV subpopulations (Han et al. 2023). Second, the cellular origins of FF EVs may shape their tetraspanin profiles. For example, SEVs isolated from various biofluids and brain tissue have been shown to exhibit tetraspanin heterogeneity, with distinct patterns and predominant tetraspanins specific to each sample type (Okada-Tsuchioka et al. 2022). Given that fourteen somatic cell populations have been identified in FF (Roos et al. 2022), the heterogeneous tetraspanin profiles observed in FF EVs likely reflect their diverse cellular origins within the follicular microenvironment. Third, tetraspanin incorporation may be regulated by cellular signalling or external stimuli during EV biogenesis (Breitwieser et al. 2022; Duke et al. 2025). Further investigation is needed to elucidate the molecular mechanisms governing EV sorting in follicular cells and to determine how variations in tetraspanin composition influence EV-mediated intercellular communication.

Although previous studies in bovine and human models reported that FF EVs promote GC proliferation (Couty et al. 2024; Hung et al. 2017; Ying et al. 2023), our findings showed no such effect in SEV- or LEV-treated KGN cells. This discrepancy may arise from differences in EV isolation methods, biological variations between species and/or experimental conditions. Importantly, we employed size-exclusion chromatography, a method known to reduce FF protein contamination compared to ultracentrifugation (UC) (Soares et al. 2023), which likely improved the purity of our EV preparations. This raises the possibility that proliferative effects reported in UC-based studies were partly influenced by residual FF proteins rather than EV internal cargo. Interestingly, whilst both our results and those of Couty et al. (2024) showed no effect under normal physiological conditions, Zhou et al. (2023) demonstrated that FF EVs reduced GC apoptosis under stress conditions in a PCOS-like model. This suggests that FF EVs may serve protective functions under stress conditions, whilst exhibiting minimal effects under normal physiological conditions.

Consistent with animal studies showing that FF EVs can modulate steroidogenesis (Ying et al. 2023; Yuan et al. 2021), we observed EV-type specific effects on hormone production in human GCs. Although oestradiol or progesterone levels remained unchanged after EV treatment, LEVs—either alone or in combination with SEVs—significantly increased testosterone production. Notably, this effect occurred without concurrent changes in the expression of steroidogenesis-related genes, suggesting that LEVs may influence testosterone synthesis through the delivery of regulatory RNAs or proteins that affect steroidogenesis at post-transcriptional or translational levels. Supporting this, both SEVs and LEVs were efficiently internalised by KGN cells, confirming that the observed functional responses were likely mediated by EV-derived cargo. Collectively, our findings highlight the potential role of FF EVs, particularly LEVs, in modulating the steroidogenic environment of the preovulatory follicle.

Small RNA sequencing revealed distinct ncRNA profiles in SEVs and LEVs, further highlighting their functional divergence. SEVs, enriched in miRNAs, were associated with pathways critical for GC proliferation and differentiation, including cell cycle regulation, IGF2BPs, TGF-β, WNT, TNF, TP53 and Ca^{2+} signalling. Several of these pathways have previously been identified as targets of FF EV miRNAs in both animal (da Silveira et al. 2012; Liu et al. 2024) and human (Martinez et al. 2018; Santonocito et al. 2014) studies, underscoring the conserved regulatory roles of FF EV miRNAs across species. In contrast, LEVs, enriched in piRNAs, appear to play a role in preserving genome stability, as piRNAs are essential for silencing transposable elements and maintaining genomic integrity in both germ and somatic cells (Wu et al. 2020).

These differences in ncRNA profiles were reflected in the distinct transcriptomic responses of KGN cells treated with SEVs or LEVs. LEV treatment resulted in minimal transcriptomic changes, with only five differentially expressed genes, primarily linked to immune regulation (VCAM, C1A) and oxidative stress defence (SOD2), suggesting that LEVs may play a role in maintaining cellular homeostasis. In contrast, SEV treatment induced extensive transcriptomic changes, with 1596 genes showing differential expression. SEVs upregulated genes involved in ECM remodelling and MET-activated PTK2 signalling, both of which are essential for follicular expansion and oocyte-GC communication

(McGinnis and Kinsey 2014; Vasse et al. 2024). ECM remodelling within the ovarian follicle involves the controlled synthesis and degradation of structural components like collagens and laminins (Irving-Rodgers and Rodgers 2005). PTK2, activated through MET signalling, regulates focal adhesions — specialised cellular structures that transmit mechanical forces and signalling cues between the ECM and interacting cells, essential for tissue remodelling. These findings underscore the role of SEVs in facilitating the structural reorganisation necessary for ovulation, aligning with previous studies highlighting FF EV roles in ECM formation (de Almeida Monteiro Melo Ferraz et al. 2020) and ECM-receptor interactions (Martinez et al. 2018).

SEV treatment led to the downregulation of genes associated with Rho GTPases, cell cycle regulation, cholesterol biosynthesis and HRR. The suppression of Rho GTPases, which regulate cytoskeletal organisation and mitosis (Chircop 2014), likely reflects necessary cytoskeletal and cell cycle adaptations for GC differentiation during luteinisation, a process characterised by cell cycle exit (Robker and Richards 1998). Furthermore, the downregulation of cholesterol biosynthesis genes may represent a metabolic shift favouring cholesterol uptake rather than synthesis to meet the demands of hormone production. Alternatively, it may reflect underlying fertility challenges, as reduced cholesterol biosynthesis is linked to diminished ovarian reserve (Yang et al. 2022). Similarly, suppression of HRR genes, such as BRCA2 and RAD51, indicates potential genomic instability, which is a recognised contributor to infertility (Dunce and Davies 2024). Collectively, these findings suggest SEVs influence both the structural and metabolic states of GCs, supporting their transition during luteinisation.

Analysis of ncRNA profiles in FF EV-treated GCs further distinguished the effects of SEVs and LEVs. LEV-treated GCs exhibited upregulated miRNAs linked to IGF2BPs and PTEN regulation, pathways associated with GC survival. Elevated levels of PTEN, a negative regulator of the PI3K-AKT pathway, have been implicated in impaired follicular development and increased GC apoptosis (Yao et al. 2021). Although LEV supplementation itself did not alter PTEN mRNA levels after 24 h of incubation, miRNAs targeting PTEN could repress its translation, suggesting that LEVs may contribute to GC survival and cellular homeostasis. Conversely, SEV-treated GCs exhibited upregulated miRNAs associated with transcription, TGF-β signalling and Smad transcriptional activity. These pathways align with the functional roles of SEV-enriched miRNAs, suggesting SEVs may impact GCs through direct miRNA delivery, but also through secondary transcriptional changes.

The integration of pathway enrichment analyses further strengthens these findings by revealing recurring pathways influenced by SEVs. Modulation of transcriptional pathways by SEVs is particularly noteworthy, as GCs must undergo extensive transcriptional changes to adapt to the dynamic physiological demands of follicular development and luteinisation. Another key pathway impacted by SEVs is TGF-β signalling, which was significantly downregulated in SEV-treated GCs. TGF-β is known to suppress oocyte maturation (Coskun and Lin 1994) and maintain meiotic arrest (Yang et al. 2019) during early follicular development, and its suppression after the LH surge is essential for meiotic resumption. SEV-mediated downregulation of TGF-β signalling may also support GC luteinisation by alleviating its inhibitory effects on *STAR* expression and progesterone synthesis (Fang et al. 2014; Zheng et al. 2009). Furthermore, TGF-β regulates ECM remodelling and GC apoptosis during the periovulatory period (Wang et al. 2019). By regulating this pathway, SEVs may facilitate ECM reorganisation and inhibit apoptosis to ensure GC survival during luteinisation.

Similarly, the enrichment of the integrin cell surface interactions pathway highlights the role of SEVs in ECM remodelling. Integrins, which mediate cell-ECM adhesion, are critical for cellular communication and structural organisation (Anderson et al. 2013). Among the upregulated genes in this pathway, fibronectin (FN1) stands out as a key ECM glycoprotein that binds integrins and supports GC adhesion. The SEV-induced upregulation of fibronectin is particularly significant, as it has been shown to promote GC luteinisation and cumulus expansion (Kitasaka et al. 2018). The concurrent downregulation of genes encoding collagens and specific integrin subunits suggests a regulated loosening of cell-ECM adhesion, enabling the dynamic remodelling required during follicular maturation.

Finally, cytokine signalling emerged as another pathway influenced by SEVs. Cytokines, particularly interleukins (ILs), are well-known for their roles in immune regulation and inflammation. However, the ovarian follicle itself is a site of inflammatory-like processes, where ovarian cells both produce and respond to cytokines. Among these, IL-1 has been shown to regulate ovulation-associated events, including the production and activation of proteolytic enzymes, such as prostaglandins, which mediate follicle rupture (Gérard et al. 2004; Peterson et al. 1993). The upregulation of IL1R1, the receptor for IL-1, in SEV-treated GCs suggests that SEVs may enhance IL-1 signalling necessary for follicle wall breakdown and oocyte release.

Despite the significant findings, this study has several limitations. First, the inclusion of patients undergoing ovarian stimulation may have influenced the FF EV profile. Ovarian stimulation is known to alter FF miRNA profiles (Noferesti et al. 2015), potentially introducing variability into our findings. Additionally, the variation in stimulation protocols between patients may further complicate the interpretation of our findings, as different treatment regimens can have distinct effects on FF EV composition. Future studies utilising patient material collected during natural cycles would provide a clearer understanding of how ovarian stimulation affects EV composition. However, such samples are not available from the majority of infertility clinics. Secondly, whilst the presented research focuses on the differences between SEV and LEV properties and functions, the aspect of various aetiologies of infertility was left out of the scope of the study. The presented results were derived from six pools of seven FF samples (42 patients in total) and do not present inter-patient variability. The recruited patients possess variable confounding factors that we aimed to even out by using the pooling strategy. Thirdly, whilst the KGN cell line is advantageous for reducing variability across experiments, it is a tumour-derived GC-like model that may not fully replicate the behaviour of healthy GCs *in vivo*. Further research involving primary human GCs would provide more physiologically relevant insights into FF EV-mediated mechanisms. Furthermore, the SEV and LEV populations were not exclusively separated based on the TEM

visualisation. However, a complete separation would be very difficult to achieve. Finally, results considering the pathways affected by EVs should be considered preliminary, and further studies are needed to confirm the functional relevance of these pathways.

Nevertheless, this study has notable strengths. By employing rigorous EV isolation and characterisation protocols, including single-EV profiling and small RNA sequencing, we ensured high purity and size-specific enrichment of SEVs and LEVs. Additionally, the integration of transcriptomic and ncRNA analyses enabled us to uncover nuanced functional and molecular distinctions between FF EV subpopulations, addressing gaps in prior research that often examined pooled FF EVs.

Taken together, this study highlights the functional and molecular differences between SEVs and LEVs in human FF, emphasising their distinct contributions to ovarian function. SEVs, more abundant and enriched in miRNAs, were found to regulate pathways related to transcription, TGF-β signalling, ECM remodelling and cell cycle, suggesting their role in driving structural and functional changes during follicular development. LEVs, less abundant and enriched in piRNAs, were linked to IGF2BPs and PTEN regulation, emphasising their role in maintaining genomic stability and cellular homeostasis. Importantly, LEVs also demonstrated the capacity to increase testosterone production in GCs, highlighting their functional role in modulating steroidogenesis. These findings advance our understanding of intercellular communication within the ovarian follicle and underscore the importance of considering EV heterogeneity in ovarian biology studies.

Author Contributions

Inge Varik: Data curation (lead), formal analysis (lead), investigation (equal), methodology (lead), visualization (lead), writing–original draft (lead). **Katariina Johanna Saretok**: Methodology (supporting), writing–review and editing (equal). **Kristine Rosenberg**: Resources (equal), writing–review and editing (equal). **Ileana Quintero**: Methodology (supporting), writing–review and editing (equal). **Maija Puhka**: Methodology (supporting), writing–review and editing (equal). **Nataliia Volkova**: Methodology (supporting), writing–review and editing (equal). **Aleksander Trošin**: Resources (equal), writing–review and editing (equal). **Paolo Guazzi**: Conceptualization (equal), supervision (equal), writing–review and editing (equal). **Agne Velthut-Meikas**: Conceptualization (equal), data curation (supporting), funding acquisition (lead), investigation (equal), project administration (lead), supervision (equal), writing–review and editing (equal).

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Conflicts of Interest

Paolo Guazzi is the Chief Operating Officer at HansaBioMed Life Sciences. All other authors declare no conflicts of interest.

Data Availability Statement

The raw data for this study have been deposited in the ENA at EMBL-EBI under accession number PRJEB83132 (https://www.ebi.ac.uk/ena/browser/view/PRJEB83132).

Declaration of Generative AI and AI-Assisted Technologies in the Writing Process

During the preparation of this work, the authors used ChatGPT-4o in order to improve sentence structure and correct grammar. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting Fig.1: Particle concentrations and protein concentrations for each individual fraction collected during SEC. **Supporting Fig.2**: Strategy for the calculation of tetraspanin colocalization randomness. **Suppporting Fig.3**: Particle concentrations and diameters of small EVs (SEVs) and large EVs (LEVs) after isolation with TFF. **Supporting Fig.4**: miRNA-mRNA network illustrating upregulated miRNAs in SEVs that are predicted to downregulate several mRNAs in KGN cells. **Supporting Fig.5**: The overlap between piRNAs upregulated in KGN cells upon SEV treatment and piRNAs contained in SEVs. **Supporting Fig.6**: The overlap between piRNAs upregulated in KGN cells upon LEV treatment and piRNAs contained in LEVs. **Supporting Table 1**: Read counts for RNA-seq analysis of EV-treated KGN cells. **Supporting Table 2**: Read counts for ncRNA-seq analysis of SEVs, LEVs, and EV-treated KGN cells. **Supporting Table 3**: Particle and protein concentrations of SEV and LEV samples. **Supporting Table 4**: Average UMI counts per million (CPM) and standard deviation (SD) for ncRNAs detected at ≥6 normalized UMI counts in FF EVs. **Supporting Table 5**: Differentially expressed (DE) short ncRNAs between SEV and LEV samples. **Supporting Table 6:** Over-representation analysis of DE miRNAs (SEV vs LEV) with miEAA (using Reactome database). **Supporting Table 7**: DE genes between LEV- and DPBS-treated KGN cells. **Supporting Table 8**: DE genes between SEV- and DPBS-treated KGN cells. **Supporting Table 9**: Reactome terms for downregulated genes in KGN cells upon SEV treatment (SEV vs DPBS). **Supporting Table 10**: Reactome terms for upregulated genes in KGN cells upon SEV treatment (SEV vs DPBS). **Supporting Table 11**: List of upregulated SEV miRNAs and their predicted targets which were experimentally shown to be downregulated in KGN cells upon SEV treatment. **Supporting Table 12**: DE short ncRNAs between SEV- and LEV-treated KGN cells. **Supporting Table 13**: Over-representation analysis of miRNAs upregulated in SEV-treated KGN cells with miEAA (using Reactome database).

Appendix 2

Publication II
(**Manuscript**) Saretok KJ*, **Varik I***, Rosenberg K, Trošin A, Quintero I, Puhka M, Guazzi P, Velthut-Meikas A. Follicular fluid extracellular vesicle small RNA profiles reflect ovarian sensitivity to controlled ovarian stimulation.

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To comply with publisher guidelines for pending manuscripts, **Publication II** is excluded from the public version of this dissertation. A copy will be made available to the official committee members and opponents to facilitate a comprehensive evaluation of the thesis.

Appendix 3

Publication III

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. Environ Int. 2024 Sep;191:108960. doi: 10.1016/j.envint.2024.108960.

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Full length article

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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Reproductive health
Inflammation

ABSTRACT

The plasticizer di(2-ethylhexyl) phthalate (DEHP) is known to have endocrine-disrupting properties mediated by its many metabolites that form upon exposure in biological systems. In a previous study, we reported an inverse association between DEHP metabolites in the human ovarian follicular fluid (FF) and the responsiveness of the follicles to controlled ovarian stimulation during *in vitro* fertilization (IVF) treatments. Here, we explored this association further through molecular analysis of the ovarian FF samples.

Ninety-six IVF patients from Swedish (N = 48) and Estonian (N = 48) infertility clinics were selected from the previous cohort (N = 333) based on the molar sum of DEHP metabolites in their FF samples to arrive at "high" (mean 7.7 ± SD 2.3 nM, N = 48) and "low" (0.8 ± 0.4 nM, N = 48) exposure groups. Extracellular miRNA levels and concentrations of 15 steroid hormones were measured across FF samples. In addition, FF somatic cells, available for the Estonian patients, were used for RNA sequencing.

Differential expression (DE) and interactions between miRNA and mRNA networks revealed that the expression levels of genes in the cholesterol biosynthesis and steroidogenesis pathways were significantly decreased in the high compared to the low DEHP group. In addition, the DE miRNAs were predicted to target key enzymes within these pathways (FDR < 0.05). A decreased 17-OH-progesterone to progesterone ratio was observed in the FF of the high DEHP group ($p < 0.05$). Additionally, the expression levels of genes associated

Abbreviations: OSI, ovarian sensitivity index; IVF, *in vitro* fertilization; DEHP, di(2-ethylhexyl) phthalate; ASD, androstenedione; TES, testosterone; PRO, progesterone; HPR, 17-OH-hydroxyprogesterone; RNA-seq, RNA sequencing; FF, follicular fluid.

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with inflammatory processes were elevated in the FF somatic cells, and a computational cell-type deconvolution analysis suggested an increased immune cell infiltration into the high DEHP follicles ($p < 0.05$).

In conclusion, elevated DEHP levels in FF were associated with a significantly altered follicular milieu within human ovaries, involving a pro-inflammatory environment and reduced cholesterol metabolism, including steroid synthesis. These results contribute to our understanding of the molecular mechanisms of female reprotoxic effects of DEHP.

1. Introduction

Endocrine disrupting chemicals (EDCs) can pose a risk to human health by interfering with the endocrine system by, for instance, disrupting hormone synthesis, transport, secretion, activity, and elimination (Schug et al., 2011). Among the many classes of chemicals harboring endocrine disrupting activities are phthalates, commonly used as plasticizers or additives in personal care products and cosmetics (David et al., 2012). Di(2-ethylhexyl) phthalate (DEHP), one of the most commonly used phthalates, has long been a component of various plastic products, including but not limited to food and beverage packaging, medical devices, toys, flooring, cosmetics, and personal care products (Koniecki et al., 2011). DEHP is known to leach from plastics due to its non-covalent association with polymers, and can enter the human body through ingestion, inhalation, or dermal absorption (Andersen et al., 2018; Schettler, 2006). Once in the body, DEHP undergoes rapid metabolism, resulting in the formation of metabolites such as mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), and mono(2-ethyl-5-carboxypentyl) phthalate (MECPP). These metabolites are subsequently eliminated primarily through the urine (Koch et al., 2006). Despite being classified as a non-persistent chemical, the widespread presence of DEHP in everyday products with long half-lives causes continuous exposure to humans. Consequently, DEHP metabolites have been routinely detected in different human matrices, including urine, serum, breast milk, amniotic fluid, and follicular fluid (FF) (Bellavia et al., 2023; Frederiksen et al., 2010; Högberg et al., 2008; Katsikantami et al., 2020; Paoli et al., 2020).

The widespread use of, and exposure to, phthalates has raised concerns over potential adverse health effects, particularly on reproduction. For example, DEHP has been classified as an endocrine disruptor and toxic to reproduction by the European Chemicals Agency based primarily on data from males (European Chemicals Agency, 2024). There is, however, emerging evidence also suggesting effects on female reproductive health (Basso et al., 2022). For instance, DEHP can impact adult mouse ovarian health by inhibiting antral follicle growth, inducing follicle atresia, and disturbing steroidogenesis and granulosa cell proliferation (Hannon et al., 2023; Liu et al., 2021). Moreover, recent findings also suggests DEHP reducing the maturation and fertilization capacity of mouse oocytes through DNA damage and oxidative stress (Lu et al., 2019; Machtinger et al., 2018).

A limited number of human studies suggest an association between elevated DEHP levels and disrupted folliculogenesis, irrespective of exposure taking place during development or adulthood. The primary metabolite MEHP can induce the expression of genes controlling cholesterol and lipid synthesis in human fetal ovaries *in vitro*, potentially impacting ovarian development (Muczynski et al., 2012). Also, several epidemiological studies have demonstrated that high levels of DEHP metabolites in urine are linked to a significant decrease in antral follicle count, a lower number of mature and fertilized oocytes, and reduced quantity of top-quality embryos, suggesting that DEHP may impair *in vitro* fertilization (IVF) outcomes (Hauser et al., 2016; Messerlian et al., 2016). Coupled with our recent findings that increased concentrations of DEHP metabolites in FF are inversely correlated with the ovarian sensitivity index (OSI) – a measure of ovarian competence that links the dosage of recombinant follicle stimulating hormone (rFSH) to the number of retrieved oocytes in IVF treatment (Bellavia et al., 2023) – heightens the concern over the potential for DEHP to negatively impact human ovarian function. OSI is a major predictor of IVF treatment success and, although it also correlates with known ovarian reserve markers like anti-Müllerian hormone (AMH), it more accurately predicts live birth rates. This is likely because OSI provides a more functional measure of follicles than AMH levels (Revelli et al., 2020; Vaegter et al., 2019; Weghofer et al., 2020). Thus, our earlier findings imply that the functionality of the follicles may be modified by phthalates in the FF, potentially leading to a lower number of oocytes retrieved during IVF treatments.

Exactly how DEHP or its metabolites affect ovarian follicle growth in humans at the molecular level remains largely unknown. However, such evidence is needed to provide a causal link between initial chemical perturbation and adverse health outcomes. Such knowledge will also aid with developing better alternative test methods to assess female reproductive toxicity. To address this knowledge gap, we aimed to elucidate the molecular pathways by which DEHP metabolites may alter ovarian sensitivity. For this purpose, we generated RNA sequencing datasets from cell-free FF and the corresponding follicular somatic cells collected from IVF patients at infertility clinics in Sweden and Estonia. The patients were stratified into high and low DEHP groups based on previously measured DEHP metabolite levels in their FF. In addition, we assessed a panel of 15 steroid hormones from the same FF samples. By assessing the molecular signatures that differ between FF samples with high and low DEHP, a deeper understanding of how DEHP exposure may influence female fertility is gained.

2. Materials and methods

2.1. Ethical statement

The study was conducted in accordance with the Declaration of Helsinki. Both oral and written information was provided to the study participants after which they signed an informed consent form. 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, the European General Data Protection Regulation, and the Estonian Data Protection Act). The Swedish study was approved by the Swedish Ethical Review Authority (original license No. 2015/798–31/2, amendments 2016/360–32 and 2016/1523–32). The Estonian study was approved by the Research Ethics Committee of the University of Tartu (approval No. 356/M-4).

2.2. The patient cohort and previously measured variables

This study is based on the previously described merged cohort of 333 women recruited from Carl von Linnékliniken in Sweden and Nova Vita Clinic in Estonia (Bellavia et al., 2023). All patients underwent controlled ovarian stimulation (COST) and ovum pick-up procedure as described previously (Bellavia et al., 2023). Shortly, Swedish patients were stimulated according to the gonadotropin-releasing hormone (GnRH) agonist protocol (77 % of patients) using Suprecur (Cheplapharm Arzneimittel GmbH, Greifswald, Germany) or Synarela (Pfizer, New York City, New York, USA) where the pituitary was desensitized starting the luteal phase, or GnRH antagonist protocol (23 % of patients) where GnRH antagonist Orgalutran (N.V. Organon, Oss, The Netherlands) was given on day 6 of menses. rFSH (Gonal-F or Fostimon,

Bemfola, Gedeon Richter Plc., Budapest, Hungary) alone or with human menopausal gonadotropin (Menopur, Ferring Pharmaceuticals Ltd, Saint-Prex, Switzerland) were administered from day 3 of menses. Once ≥3 follicles with a diameter of ≥17 mm were detected, human chorionic gonadotropin (hCG) was given. After 36–37 h, oocytes were retrieved through the transvaginal ultrasound-guided ovarian puncture. For the Estonian patients, COST was conducted according to the GnRH antagonist (Cetrotide, Merck, Darmstadt, Germany) protocol with the administration of rFSH (Gonal-F®, Merck; Bemfola, Gedeon Richter Plc). All patients underwent oocyte retrieval 36 h after hCG injection (Ovitrelle®, Merck) if at least two follicles were observed with a diameter of ≥18 mm.

Once all cumulus-oocytes-complexes had been collected for the IVF procedure of the patient, the remaining FF was donated for research. Samples collected in Sweden (N = 48) contained FF from multiple follicles and were pooled for each patient, thereafter centrifugation was performed at 500 × g for 15 min. FF samples from the Estonian clinic (N = 48) were collected from a single leading follicle. The somatic cells from the follicular aspirate were pelleted by centrifugation at 300 × g for 10 min. Cell debris was removed from FF by an additional centrifugation at 2000 × g for 10 min. The cell-free FF samples were aliquoted and stored at −80 °C until further analysis. In addition, somatic cells were collected from the Estonian patients. Follicular cell pellets from the first round of centrifugation were lysed in 700 μL of QIAzol Lysis Reagent (QIAGEN, Hilden, Germany) and stored at −80 °C.

As described in our previous study, the concentrations of 59 EDCs were quantified in the FF samples using isotope dilution liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Bellavia et al., 2023), and OSI was calculated for each patient according to the previously published formula(Huber et al., 2013): OSI = ln((number of oocytes retrieved)/(total rFSH dose (IU)) × 1000).

2.3. Selection of high and low DEHP samples for the present study

According to the power analyses described in the Supplementary Method, at least 90 participants are needed to achieve adequate statistical power for this study. Therefore, a sub cohort of 96 women from the original merged cohort of 333 women was selected based on the previously obtained concentrations of DEHP metabolites in the FF samples (Bellavia et al., 2023). Data on four DEHP metabolites, MEHP, MECPP, MEHHP, and MEOHP, were used. The concentrations for each compound were divided by their molar weight and summed to a variable referred to as ΣDEHP hereafter. Women were ranked by their ΣDEHP value, and samples were chosen from the top and bottom quartile with the following criteria: there should be equally many patients from Sweden and Estonia in both 1st and 4th ΣDEHP quartiles. To ensure comparability of two groups, we also considered the distribution of typical confounding factors in fertility studies: age, body-mass index (BMI), OSI, parity, and existence of experience with previous IVF cycles. The mean ΣDEHP concentration in the high and low groups (4th and 1st quartile, respectively) were 8 ± 2 nM (SD) and 0.9 ± 0.6 nM, respectively. The study design is depicted in Supplementary Fig. 1.

2.4. Quantification of steroids from follicular fluid

Steroid hormone levels were measured in selected FF samples by LC-MS/MS as previously described for plasma samples, with minor modifications (Draskau et al., 2019). All samples from Sweden (N = 48) were analyzed, while 23 and 17 samples from the low and high ΣDEHP groups, respectively, were available for analysis from the Estonian patients due to the more limited sample volume (Supplementary Fig. 1). A 100 μL of FF sample was added to a microcentrifuge tube containing 300 μL 2 % formic acid in acetonitrile with 3.33 ng/mL internal standard (deoxycortisol-d8 and cortisol-d4 from Cerilliant Corporation, Round Rock, TX, USA; methyltestosterone-d3, beta-testosterone-d2, beta-estradiol-d3 and progesterone-c2 from RIKILT, Wageningen, Netherlands), and then vortexed for 5 s. The vial was then placed in a freezer (−18 °C) for 20 min and centrifuged at 10,000 g for 7 min at 4 °C. The supernatant was subsequently transferred to a tube containing approximately 50 mg Supel™ QuE Z-Sep powder (Supelco, Bellefonte, PA, USA, #55418-U) and shaken for 60 s, before centrifugation for 3 min at 3,500 g. The supernatant was transferred to a centrifuge glass and dried using a nitrogen evaporator at 50 °C after which it was reconstituted in 50 μl acetonitrile, vortexed briefly, and mixed with 450 μl of MilliQ water.

Steroid hormones were separated, detected, and quantified using online-SPE LC-MS/MS using an Oasis HLB column (2.1 × 20 mm, 15 μm). For 17β-estradiol and estrone analysis, a Kinetex C18 column (2.1 × 100 mm, 2.6 μm) was used with an injection volume of 100 μl, measuring in negative ESI-mode using methanol, and 0.2 mM ammonium fluoride in water as the mobile phases (gradient flow rate was 0.4 mL/min). For the other hormones, an Ascentis Express C8 column (2.1 × 100 mm, 2.7 μm) was used with an injection volume of 100 μl, measuring in positive and negative ESI-mode with acetonitrile and 0.1 % formic acid in water as the mobile phases (gradient flow rate was 0.25 mL/min).

Fifteen hormones were assessed: aldosterone, testosterone (TES), epitestosterone, androstenedione (ASD), dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT), corticosterone, cortisol, hydroxycortisol, deoxycortisol, pregnenolone, progesterone (PRO), 17a-OH-progesterone (HPR), beta-estradiol, and estrone. All hormones were detected, except for aldosterone, DHEA and DHT.

Limit of quantification (LOQ) was estimated as the concentration corresponding to 10 times the signal to noise ratio of FF spiked with analyte. LOQs were estimated to be 0.05 ng/mL for hydroxycortisol, 0.1 ng/mL for ASD, cortisol, PRO, TES, beta-estradiol and estrone, 0.2 ng/mL for deoxycortisol and epitestosterone, 0.3 ng/mL for corticosterone, HPR and aldosterone, 1.0 ng/mL for DHEA and DHT, and 2.0 ng/mL for pregnenolone. For quantification, external calibration standards were run before and after the samples at concentrations of 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 ng/mL, with 2.0 ng/mL internal standard. Blank FF were spiked with an analyte at three concentration levels: 0.0 (blank sample), 0.5, and 2.0 ng/mL and run for quality control. The mass spectrometer was an EVOQ Elite Triple Quadrupole Instrument from Bruker (Bremen, Germany) and the UPLC system was an Ultimate 3000 system with a DGP-3600RS dual-gradient pump. Data handling was performed with MS Workstation v. 8.2.1 software.

2.5. RNA extraction

The extraction of cell-free RNA from FF was performed with miRNeasy Micro kit (QIAGEN) with some modifications to the user manual. Shortly, 5 volumes of QIAzol Lysis Reagent (QIAGEN) was added to 500 μL of FF sample. After incubation, 500 μL chloroform and 5 μg of glycogen (Thermo Scientific) were added to the tube, followed by steps according to the miRNeasy Micro kit (QIAGEN) user manual. RNA concentration was measured using the Qubit small RNA assay kit on the Qubit 4.0 instrument (Thermo Fisher Scientific). All samples, 48 for low and 48 for high ΣDEHP group, were used (Supplementary Fig. 1).

RNA from FF somatic cells was extracted with miRNeasy Micro kit (QIAGEN) according to the kit protocol. The quality of RNA was assessed with the Bioanalyzer RNA 6000 Pico kit on Agilent 2100 Bioanalyzer instrument (Agilent Technologies, Waldbronn, Germany). RNA integrity number (RIN) > 7 was the accepted quality threshold for downstream analyses and 24 samples passed the threshold (13 and 11 samples in the high and low ΣDEHP groups, respectively) (Supplementary Fig. 1). RNA concentration was measured with the Qubit 4.0 instrument and Qubit RNA HS assay kit (Thermo Fisher Scientific).

2.6. Small RNA library preparation and sequencing

Small RNA libraries were prepared with QIAseq miRNA Library Kit (QIAGEN) according to the manufacturer's protocol. Libraries were

prepared from 5 μl of RNA extracted from 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 small RNA libraries were crushed with pellet pestles (Fisher Scientific). Then, 300 μL of RNase free water (Thermo Fisher Scientific) was added to the gel debris and the tubes were rotated for 2 h at room temperature to elute miRNA libraries. Eluate and gel debris were transferred to the Spin X centrifuge tube filter (Merck, Darmstadt, Germany) and centrifuged for 2 min at 16,000 g. Thereafter 10 μg of glycogen (Thermo Fisher 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 the 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 DNA Assay kit (Thermo Fisher Scientific) before pooling in equimolar amounts. Single-end sequencing of 76 bp length was performed on NextSeq 500 platform with NextSeq 500/550 High Output Kit v2.5 (Illumina, San Diego, CA, USA) with 32 libraries per flow cell.

2.7. mRNA library preparation and sequencing

RNA with sufficient quality and concentration was available for 24 Estonian samples (N = 13 in the high ΣDEHP and N = 11 in the low ΣDEHP group) (Supplementary Fig. 1). Libraries of RNA extracted from FF somatic cells were prepared according to the Smart-seq2 protocol (Picelli et al., 2014) with small modifications. Ten ng of total RNA was used for cDNA synthesis and ten cycles of PCR for pre-amplification, instead of using single cells as described by the original Smart-seq2 protocol. KAPA HiFi DNA polymerase was replaced with Phusion High-Fidelity DNA Polymerase (Thermo Scientific) compatible with the original protocol. The protocol selects for polyadenylated RNA molecules and provides full coverage. The size and concentration of the final libraries were measured as described above for small RNA sequencing. Single-end sequencing of 122 bp length was performed on NextSeq 2000 platform on the P2 flow cell with NextSeq2000 P2 100 cycles kit (Illumina, San Diego, CA, USA).

2.8. Small RNA-seq data analysis

The raw data analysis was performed in the My QIAGEN GeneGlobe environment using the QIAseq miRNA Library Kit analysis workflow (QIAGEN). Briefly, the raw reads were trimmed of adapter sequences, quality-filtered and sequences with identical unique molecular identifiers were collapsed. The remaining reads of 15–55 nucleotides in length were annotated according to the miRBase v22 (Kozomara et al., 2019). The list of unique molecular counts of each miRNA was used as input for differential expression (DE) analysis in DESeq2 v1.40.2 (Love et al., 2014) in R v4.3.1 (R Core Team, 2023). MiRNAs expressed at low levels, determined as below 2 counts per million (cpm) in <25 % of samples were removed from the analysis.

Principal component analysis (PCA) was performed within the DESeq2 package for all detected miRNAs (N = 465). Variance stabilizing transformation was performed with option blind = FALSE for the normalized count table before drawing the PCA plot. Because PCA detected 3 outliers, 93 samples were ultimately included in the miRNA analysis. The country of origin (i.e. Sweden or Estonia) was used as a covariate in all DE analyses. The statistical significance cut-off for differentially expressed (DE) miRNAs in DESeq2 analysis was set at Benjamini-Hochberg (BH) false discovery rate (FDR) <0.1.

For predicting mRNA targets for the DE miRNAs, miRWalk (Sticht et al., 2018) prediction tool was used. Only genes with target sites in their 3́UTR mRNA region with a binding score of 1 are reported.

In addition, the list of statistically significant DE miRNAs was used as input to miRNA Enrichment Analysis and Annotation Tool (miEAA), which performs miRNA target prediction and over-representation analysis of gene ontology terms simultaneously by combining linked external databases (Aparicio-Puerta et al., 2023). Over-representation analysis was performed for WikiPathways (Agrawal et al., 2024) via miRPathDB (Kehl et al., 2020). Pathways targeted by ≥3 miRNAs with BH FDR < 0.05 are reported. The network between miRNAs and their targeted pathways was visualized in Cytoscape v3.9.1 (Shannon et al., 2003).

2.9. mRNA-seq data analysis

Raw FASTQ files were quality filtered using Trimmomatic v0.39 with the options LEADING:3 SLIDINGWINDOW:3:20 (Bolger et al., 2014). Reads below 36 nucleotides in length were discarded and all adapter sequences provided by Illumina were removed from the reads. The remaining reads were aligned to the primary assembly of human genome GRCh38 retrieved from GENCODE using STAR aligner v2.7.1 with standard settings (Dobin et al., 2013). Annotation of reads to genes was performed with htseq-count algorithm (Anders et al., 2015) (HTSeq v 0.11.2, Python v3.6.4) by using the primary assembly annotation GTF file v104 from Ensembl. The intersection nonempty option was used in htseq-count for annotating reads that overlapped with multiple features.

The count file produced by htseq-counts was used as input for DeSeq2 for DE analysis between high and low ΣDEHP groups with standard options. Genes expressed at low levels (<10 cpm across 50 % of samples) were removed from the analysis. The statistical significance cut-off for DE mRNAs in DESeq2 analysis was set at BH FDR <0.05. Volcano plot was created with the R package EnhancedVolcano v1.22.0.

The enrichment of DE genes in Reactome pathways was analyzed by g:Profiler (Kolberg et al., 2023) and results with BH FDR <0.05 are reported. The enriched pathways were visualized using publicly available web application ShinyGO (Ge et al., 2020).

2.10. Cell-type deconvolution analysis

CIBERSORTx (Newman et al., 2019) was used to estimate the proportion of different cell types in the FF somatic cell pool used in RNA-seq. To predict the cell types, we used our previously published single cell RNA-seq dataset describing cell types in human ovarian follicles as a reference (Roos et al., 2022). Importantly, the stimulation and sample collection protocols for the single-cell RNA-seq study (Roos et al., 2022) matched those of the current study as the patients were recruited from the same infertility clinic. A signature single-cell expression matrix of each cell type was generated with S-batch correction. The permutation value was set to 100. The relative abundance of cell type fractions was calculated as an average of three runs. Cell type proportion differences between the high and low ΣDEHP groups were compared using the Student's *t*-test.

2.11. Statistical analysis

Mann-Whitney *U* test was used to analyze the differences in patient baseline characteristics for continuous variables and chi-squared test was used for categorical variables. Spearman correlation was used to determine associations between ΣDEHP and other EDCs with $p < 0.05$ suggesting statistical significance. In case of Mann-Whitney U tests and correlation analyses, the statistical significance is noted in figures as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. For all genome-wide sequencing analyses, the statistical significance of the results has been corrected for multiple testing by using the BH FDR and the cut-off (FDR < 0.1 or FDR < 0.05) has been specified for each analysis.

3. Results

3.1. Description of patients and ΣDEHP groups

IVF patients from Sweden and Estonia were recruited to study the links between FF EDCs and the outcomes of infertility treatment. The analysis of 59 EDCs in FF and their associations with OSI in the merged cohort of 333 women were reported previously (Bellavia et al., 2023). Here, a sub cohort of these women was analyzed further to reveal molecular alterations underlying the negative association between ΣDEHP and ovarian function.

The FF samples were selected for the molecular analysis based on their concentrations of ΣDEHP while keeping confounders similar. The low ΣDEHP group was composed of samples from the 1st and high ΣDEHP group from the 4th quartile of measured concentrations, without any overlap, resulting in a high statistical significance of the concentration differences ($p < 0.001$, Table 1 and Supplementary Fig. 2A). Patients from both countries were equally represented (N = 48 + 48). Attention was given to maintaining comparable average OSI across the patient groups, ensuring the ability to draw conclusions about the impact of ΣDEHP on the ovarian microenvironment, uninfluenced by ovarian sensitivity (Table 1 and Supplementary Fig. 2B). In addition, general patient characteristics, were evenly distributed between two groups (Table 1), including typical confounding factors in fertility studies (BMI, age, parity, and previous IVF treatments).

Exposure to mixtures of EDCs is widespread, and the exposure levels may vary among individuals due to factors like the local use of chemicals, lifestyle, diet, age, and parity. Consequently, the concentrations of other EDCs, apart from ΣDEHP, may also differ between the patients in our study. Therefore, we analyzed correlations between ΣDEHP and the other previously measured EDCs (Bellavia et al., 2023). We found country-specific differences in correlations between chemicals: namely, ΣDEHP concentrations demonstrated a statistically significant positive correlation ($p < 0.05$) with methylparaben and PFHxS only in the Estonian women, while MEP and cxMiNP correlated with ΣDEHP in the combined cohort (Table 2). As these chemicals may possess independent molecular effects on ovarian function, we have normalized all subsequent analyses to the country of sample origin.

3.2. ΣDEHP levels correlate with altered FF miRNA profile

We aimed to analyze miRNAs in the FF, the liquid that makes up most of the volume of the preovulatory follicle, because of the suggested role of miRNAs in intercellular signaling (Valadi et al., 2007). Among the 465 miRNAs present in ≥25 % of the samples with expression level of ≥2 cpm, the country of origin explained most of the variance of miRNA expression ($p = 1.768*10^{-11}$ of the difference between PCA scores), while the ΣDEHP group separated the samples across the second principal component ($p = 0.0077$, Fig. 1A and Supplementary Fig. 3). This result again justified the use of the country of origin as a covariate in downstream DE analyses.

In total, 16 miRNAs were differentially expressed between the high and low ΣDEHP groups (FDR < 0.1) with the most robust differences observed for hsa-miR-203a-3p (FDR = 0.0001), hsa-miR-150-5p (FDR = 0.0382) and hsa-miR-28-3p (FDR = 0.0410) that were all more abundantly expressed in the high ΣDEHP group (Fig. 1B and Supplementary Table 1). To understand their potential function in the ovary, the target genes for the DE miRNAs were predicted and their interactions with signaling pathways were modeled. We identified 51,697 regions in total in the human transcriptome targeted by the 16 DE miRNAs (Supplementary Table 2). This high number is not surprising as multiple mRNA isoforms may be transcribed from one gene, and each transcript isoform may have multiple predicted miRNA target sites. The identified targets represented 10,998 unique genes. We aimed to understand which signaling pathways are potentially co-targeted by multiple DE miRNAs, and carried out a gene enrichment analysis together with miRNA target prediction. Adipogenesis, RAC1/PAK1/p38/MMP2, androgen receptor (AR) signaling, and AGE/RAGE pathways were each targeted by ≥ 5 DE miRNAs (FDR < 0.05, Fig. 1C). Three different pathways involving the Bone Morphogenetic Protein (BMP) signaling are targeted by 3 miRNAs each (FDR < 0.05, Supplementary Table 3).

Next, we examined if any of the DE miRNAs correlate with OSI that may explain the negative association between ΣDEHP concentration and OSI reported in our previous study[10]. Indeed, the most up-regulated miRNA in the high ΣDEHP group, hsa-miR-203a-3p, correlated negatively with OSI (age-adjusted $R = -0.27$, $p < 0.05$, Fig. 1D). None of the other DE miRNA showed statistically significant correlations.

3.3. Gene expression changes in follicular fluid cells indicate ΣDEHP-linked disturbances in the immune system and steroidogenesis pathways

The FF somatic cells consist mainly of mural granulosa cells (Roos et al., 2022) responsible for producing steroid hormones necessary for the feedback within the hypothalamus-pituitary-ovarian axis and endometrial maturation. Our samples were collected after the hCG trigger, which initiates the luteinization of the cells and switches the steroid production from estradiol to PRO. While extracellular miRNAs may be involved in modulating gene expression in the cells, ΣDEHP in FF could also directly influence the functionality and differentiation of the somatic cells.

To understand the overall effect of ΣDEHP to the cellular gene expression in the preovulatory follicle, we carried out RNA-seq from the follicular somatic cells corresponding to the same FF samples analyzed for ΣDEHP concentration and miRNA levels. Such cellular samples were available for a subset of the Estonian patients (Supplementary Fig. 1).

We detected 14,820 genes expressed at ≥ 10 cpm in ≥ 50 % of samples, out of which 2,454 were DE between the high and low ΣDEHP groups (FDR < 0.05, Fig. 2A, Supplementary Table 4). We carried out signaling pathway enrichment analyses to understand what processes were upregulated in the presence of high or low ΣDEHP, and found striking differences. Follicles with high ΣDEHP showed enrichment in processes related to immune system pathways (Fig. 2B, Supplementary Table 5). In contrast, follicles with low ΣDEHP demonstrated enrichment of different metabolic processes: for instance, cholesterol biosynthesis as well as lipid and steroid hormone metabolism were among the top 10 enriched Reactome pathways. In addition, follicles with low ΣDEHP showed enrichment in pathways involving mitochondria: tricarboxylic acid (TCA) cycle, gluconeogenesis, respiratory electron transport, and mitochondrial translation (Fig. 2C, Supplementary Table 6), meaning that these crucial metabolic pathways were downregulated in follicles containing high ΣDEHP.

Cytokine signaling is an important mechanism of cellular communication in the ovarian follicle. However, the upregulated immune system pathways in the high ΣDEHP group may also indicate a disproportionate number of leukocytes among the somatic cell pool. To study this further, we performed a cell population deconvolution

Table 1
Descriptive statistics of study groups.

Characteristic	Low ΣDEHP (N = 48)	High ΣDEHP (N = 48)	p-value
Age (years)	34.8 (4.2)	33.6 (4.7)	0.19
BMI (kg/m2)	22.4 (3.2)	23.8 (3.4)	0.05
OSI	0.6 (0.4)	0.7 (0.4)	0.51
∑DEHP (nM)	0.9 (0.6)	8 (2)	<0.001
Parity (≥1)	24 (50 %)	20 (42 %)	0.54
Previous IVF treatment (Yes)	20 (42 %)	21 (44 %)	0.84

Statistics are presented in mean (SD) or number (percentage); *t*-test, Mann–Whitney *U* test, or chi-square test was performed for between-group comparison. Abbreviations: ΣDEHP, sum of di(2-ethylhexyl) phthalate metabolites; BMI, body mass index; OSI, ovarian sensitivity index; IVF, *in vitro* fertilization.

Table 2
Spearman correlation between the molar concentration of ΣDEHP and concentrations of other endocrine-disrupting chemicals detected in >90 % of subjects (N = 96).

Cohort		MEP	cxMiNP	MOHiBP	MePAR	PFHxS	PFOA	PFOS	PFNA	PFDA	PFunDA	Sum[#]
All	ΣDEHP	0.22*	0.40*	0.15	0.04	0.15	0.18	0.12	0.16	0.16	0.09	0.13
Swedish		0.31*	0.21	0.10	−0.19	−0.11	0.19	−0.09	0.12	0.10	0.11	−0.01
Estonian		0.07	0.62*	0.19	0.48*	0.36*	0.17	0.24	0.11	0.16	0.001	0.51*

* $p < 0.05$.
[#] Sum refers to the sum of molar concentrations of all chemicals except ΣDEHP. Abbreviations: MEP, monoethyl phthalate; cxMiNP, mono-(4-methyl-7-carboxyheptyl)phthalate; MOHiBP, mono(2-hydroxyisobutyl)phthalate; MePAR, methyl paraben; PFHxS, perfluoro-1-hexanesulfonate; PFOA, perfluoro-n-octanoic acid; PFOS, perfluoro-octanesulfonate (sum); PFNA, perfluoro-n-nonanoic acid; PFDA, perfluoro-n-decanoic acid; PFunDA, perfluoro-n-undecanoic acid.

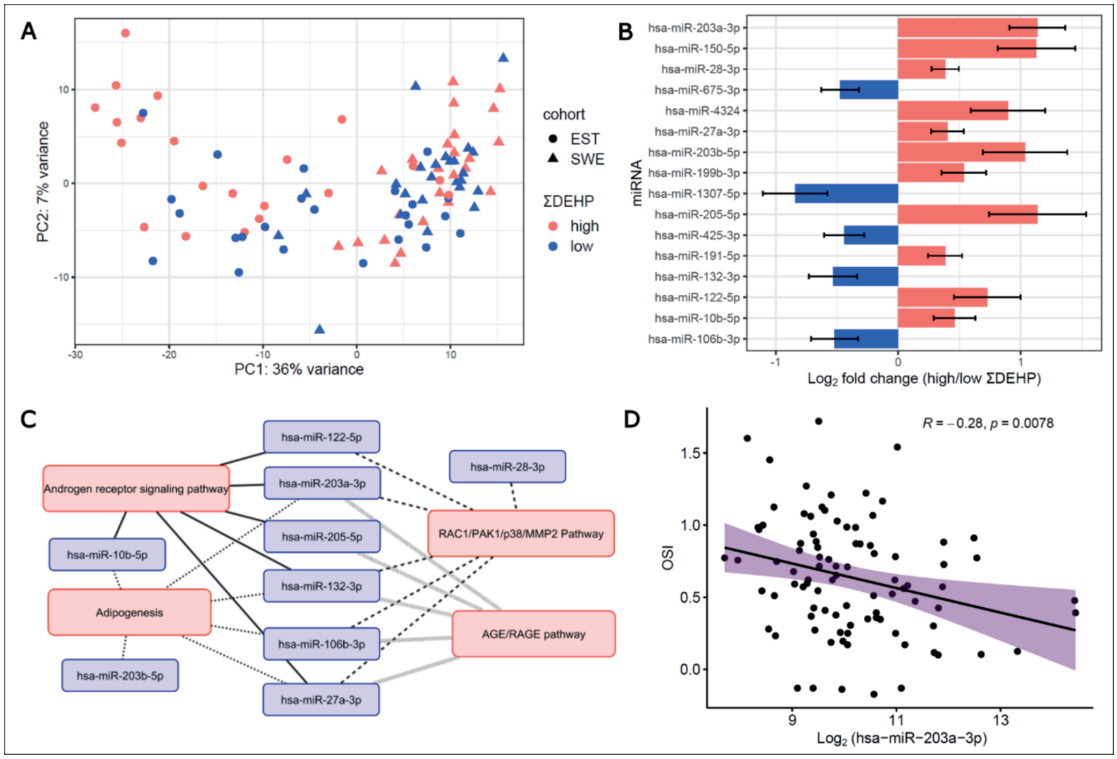

Fig. 1. Differential expression of miRNAs between high vs low ΣDEHP groups. A: Principal component (PC) analysis segregating the analyzed samples according to the expression of 465 detected miRNAs. B: Differentially expressed (DE) miRNAs between high vs low ΣDEHP group (FDR < 0.1). C: Interaction map of DE miRNAs and their targeted molecular pathways. Only pathways targeted by ≥ 5 DE miRNAs (FDR < 0.05) are presented. Each line type links miRNAs to a specific pathway. D: Negative correlation between the expression of miRNA hsa-miR-203a-3p and ovarian sensitivity index (OSI).

analysis by merging the current bulk mRNA sequencing data with our previous single-cell RNA-seq data on FF cells from fertile women with normal ovarian response to COST (Roos et al., 2022). Interesting findings were observed for the leukocyte proportions: the marker genes for T-cells, M1 and M2 macrophages as well as neutrophils were all expressed at increased levels in the high ΣDEHP group suggesting higher proportions of these cell types in the follicle ($p < 0.05$ for neutrophils and $p < 0.01$ for other leukocytes, Fig. 2D). At the same time, the analysis results suggested a reduced proportion of granulosa cells in the samples ($p < 0.001$) (Fig. 2D).

3.4. Coding and non-coding gene expression differences associated with ΣDEHP concentrations interfere with the cholesterol metabolism and steroidogenesis pathways

As the genes involved in cholesterol biosynthesis and steroidogenesis pathways were the most robustly down-regulated in the high ΣDEHP group (Supplementary Table 6), we aimed to further focus on the miRNA:mRNA interactions in these pathways.

Cholesterol is the precursor molecule for the steroid hormones produced by the somatic cell populations in the follicle. Its synthesis starts with multiple steps converting Acetyl-CoA to lanosterol. Thereafter two pathways exist: the Bloch pathway and Kandutsch-Russell pathway both produce cholesterol. Multiple genes from both pathways were expressed at lower levels in the FF cells derived from the high ΣDEHP group (Table 3A). Additionally, the low-density lipoprotein receptor (LDLR), essential for the import of cholesterol from circulating lipoproteins into cells (Miller and Bose, 2011), was downregulated in the high ΣDEHP group ($log_2FC = -1.65$, FDR = 0.013), suggesting a potential disruption in cholesterol acquisition (Supplementary Table 4). Multiple DE miRNAs target the mRNAs of genes involved in both pathways as well as in the fatty acid biosynthesis pathway that uses cholesterol levels as a sensor

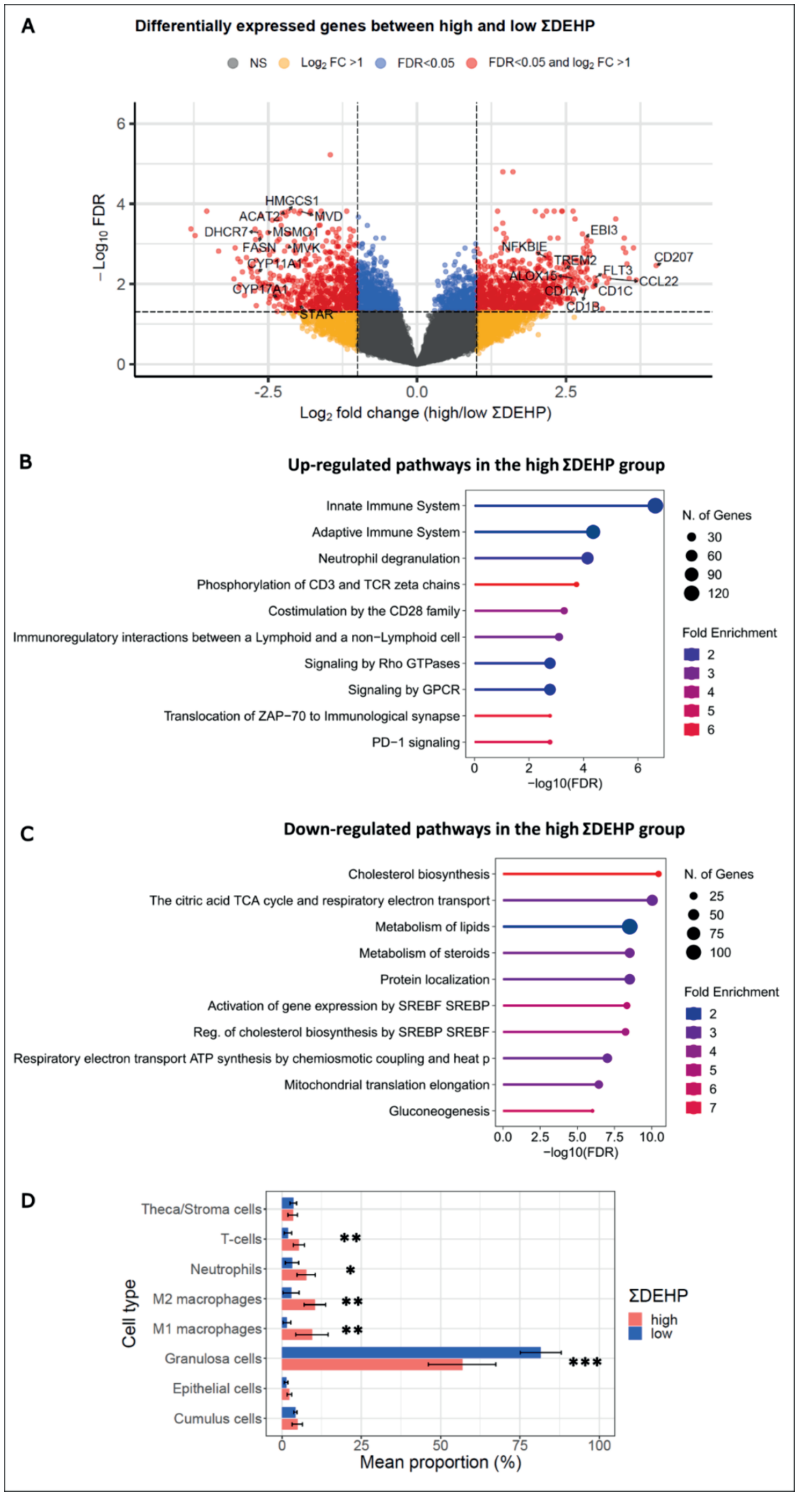

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Fig. 2. Gene expression changes in the follicular fluid somatic cells in the high ΣDEHP group. A: Volcano plot depicting the distribution of differentially expressed genes in the high ΣDEHP group compared to low ΣDEHP group. B: Enriched Reactome pathways associated with upregulated genes in the high ΣDEHP group. C: Enriched Reactome pathways associated with downregulated genes in the high ΣDEHP group. D: Computationally predicted cell type proportions in the high and low ΣDEHP groups. Cell types according to Roos et al 2022. * $p < 0.05$, **$p < 0.01$, *** $p < 0.001$. NS – not significant, FC – fold change, FDR – false discovery rate.

Table 3
miRNA:mRNA interactions in the cholesterol metabolism (A) and steroidogenesis (B) pathways. Differentially expressed (DE) genes between high vs low ΣDEHP group (FDR < 0.05) and the corresponding DE miRNAs predicted to target them according to miRWalk are displayed. Text in brackets indicates the number of binding sites of the miRNA to the corresponding mRNA.

A Cholesterol metabolism			
Gene symbol	**log_2FoldChange**	**FDR**	**Targeting DE miRNA**
MVD	−1.96	1.52E-04	none
HMGCS1	−2.16	1.52E-04	hsa-miR-150-5p, hsa-miR-205-5p (2x), hsa-miR28-3p (2x), hsa-miR-425-3p, hsa-miR-675-3p (2x)
ACAT2	−2.22	1.62E-04	none
FDPS	−2.27	1.72E-04	none
IDI1	−1.61	2.14E-04	hsa-miR-106b-3p, hsa-miR-122-5p, hsa-miR-203b-5p
FDFT1	−1.42	4.30E-04	none
MSMO1	−2.50	5.23E-04	hsa-miR-1307-5p (2x)
DHCR7	−2.65	5.23E-04	hsa-miR-199b-3p (2x), hsa-miR-425-3p
FASN	−2.63	6.43E-04	none
FADS1	−1.77	7.06E-04	hsa-miR-205-5p (2x), hsa-miR-4324
LSS	−2.32	7.65E-04	hsa-miR-106b-3p (3x), hsamiR-10b-5p (3x), hsa-miR-132-3p (3x)
SCD	−2.71	7.77E-04	none
MVK	−2.17	0.0011	hsa-miR-10b-5p (2x), hsa-miR-675-3p (2x)
SQLE	−2.00	0.0011	none
SC5D	−1.23	0.0015	hsa-miR-106b-3p
FADS2	−2.23	0.0015	hsa-miR-4324 (3x), hsa-miR-675-3p (3x)
CYP51A1	−2.04	0.0017	none
NSDHL	−1.87	0.0024	none
CYP27A1	2.66	0.0025	none
EBP	−1.95	0.0026	hsa-miR-122-5p
HMGCR	−1.45	0.0032	hsa-miR-4324 (4x)
HSD17B7	−1.13	0.0054	hsa-miR-675-3p
ELOVL5	−1.73	0.0063	hsa-miR-150-5p, hsa-miR-191-5p, hsa-miR-203b-5p, hsa-miR-28-3p (3x), hsa-miR-425-3p, hsa-miR-675-3p (2x)
SREBF1	−1.22	0.0085	hsa-miR-132-3p (3x), hsa-miR-199b-3p
PMVK	−0.83	0.0086	hsa-miR-205-5p
ABCG1	2.33	0.0110	hsa-miR-27a-3p, hsa-miR-4324 (2x), hsa-miR-106b-3p, hsa-miR-122-5p, hsa-miR-1307-5p, hsa-miR-191-5p
B Steroidogenesis			
Gene symbol	**log_2FoldChange**	**FDR**	**Targeting DE miRNA**
CYP11A1/P450scc	−2.68	0.0049	none
CYB5A/cytochrome b5	−2.24	0.0087	hsa-miR-106-3p (2x), hsa-miR-132-3p, hsa-miR-27a-3p (2x), hsa-miR-675-3p
CYP17A1/P450c17	−2.34	0.0217	none
POR	−0.85	0.0342	none
STAR	−1.99	0.0348	hsa-miR-28-3p, hsa-miR-675-3p
CYP19A1/P450Aro	−1.62	0.0407	hsa-miR-132-3p (x2), hsa-miR-150-5p (x2), hsa-miR-199b-3p (x2), hsa-miR-205-5p (x3), hsa-miR-28-3p (x2)

(Ye and DeBose-Boyd, 2011) (Fig. 3A, Table 3A).

In the steroidogenesis pathway, the expression of *STAR* mRNAs, a transporter of cholesterol from the mitochondrial outer to the inner membrane (Miller, 2007) was lower in the high ΣDEHP group ($log_2FC = -1.99$, FDR = 0.035) (Table 3B). Additionally, the key steroidogenic enzymes *CYP11A1, CYP17A1* and *CYP19A1* as well as steroidogenesis regulators *POR* and *CYB5A* were all downregulated in the high ΣDEHP group (FDR < 0.05). Furthermore, we observed that *STAR*, *CYB5A* and *CYP19A1* are potential targets for the DE miRNAs present in the FF of the same follicles (Fig. 3B, Table 3B).

It must be emphasized that most miRNA-regulated mRNAs in Fig. 3 were predicted to be targeted by either multiple DE miRNAs or by miRNAs with multiple target sites on a particular mRNA sequence (Tables 3A and B) indicating strong targeting evidence (Sætrom et al., 2007).

3.5. Differences in the follicular fluid steroid levels between high and low ΣDEHP groups

Lastly, the concentrations of 12 steroid hormones were analyzed in the same FF samples with available ΣDEHP measurements and were compared between the high and low ΣDEHP groups adjusting for the country of origin. Although ASD and TES levels were lower in the high ΣDEHP group, the difference was not statistically significant ($p > 0.05$, Fig. 4A). To account for the individual variations in the FF steroid levels, we next considered the ratios between steroids as indicators of the associated enzyme activities. The analysis of the product-to-precursor ratios revealed that PRO to HPR ratio was lower in the high ΣDEHP group ($p = 0.046$, Fig. 4B). As mentioned above, the expression level of the mRNA of the enzyme in P450c17 (encoded by the *CYP17A1* gene) performing the conversion of PRO to HPR was 5 times lower in the follicular cells of the high ΣDEHP group ($log_2FC = -2.33$, FDR = 0.022, Fig. 3B, Supplementary Table 4).

4. Discussion

DEHP metabolites have been detected in human FF and linked to altered ovarian function: women with higher levels of 4 main DEHP metabolites (ΣDEHP) in their FF demonstrated diminished response to rFSH in COST (Bellavia et al., 2023). However, the underlying mechanisms for this association remain poorly understood. In this study, we sought to characterize the impact of ΣDEHP on the miRNA profile and

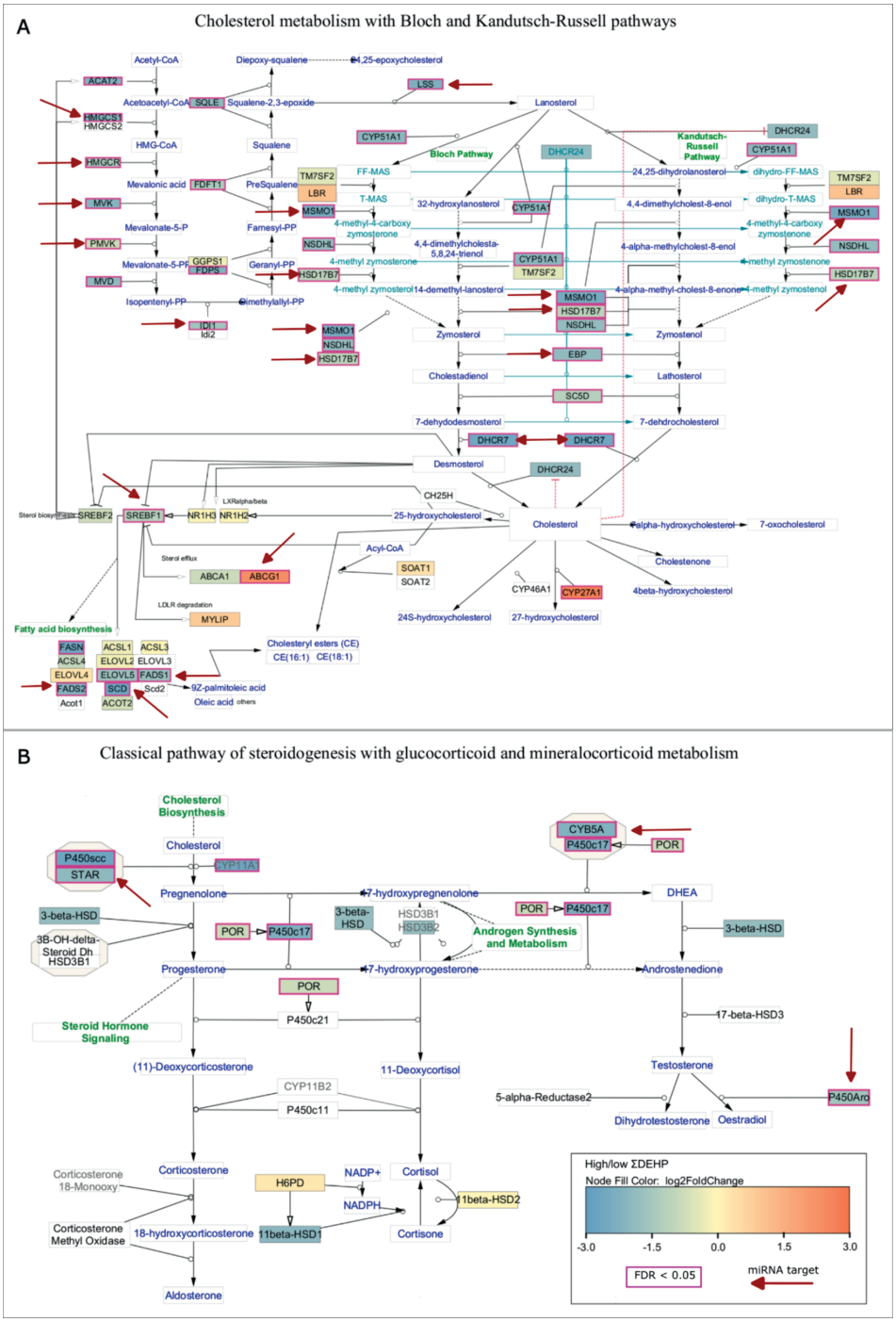

Fig. 3. Down-regulation of lipid and steroidogenesis synthesis pathways in the high ΣDEHP group. A: Cholesterol metabolism with Bloch and Kandutsch-Russell pathways; B: Classical pathway of steroidogenesis with glucocorticoid and mineralocorticoid metabolism. Gene expression fold differences are color-coded as depicted in the legend: blue colors refer to lower expression in the high ΣDEHP group. Differentially expressed (DE) genes between high and low ΣDEHP groups (FDR < 0.05) are highlighted with violet boxes. Genes predicted to be targeted by DE miRNAs are noted by red arrows. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

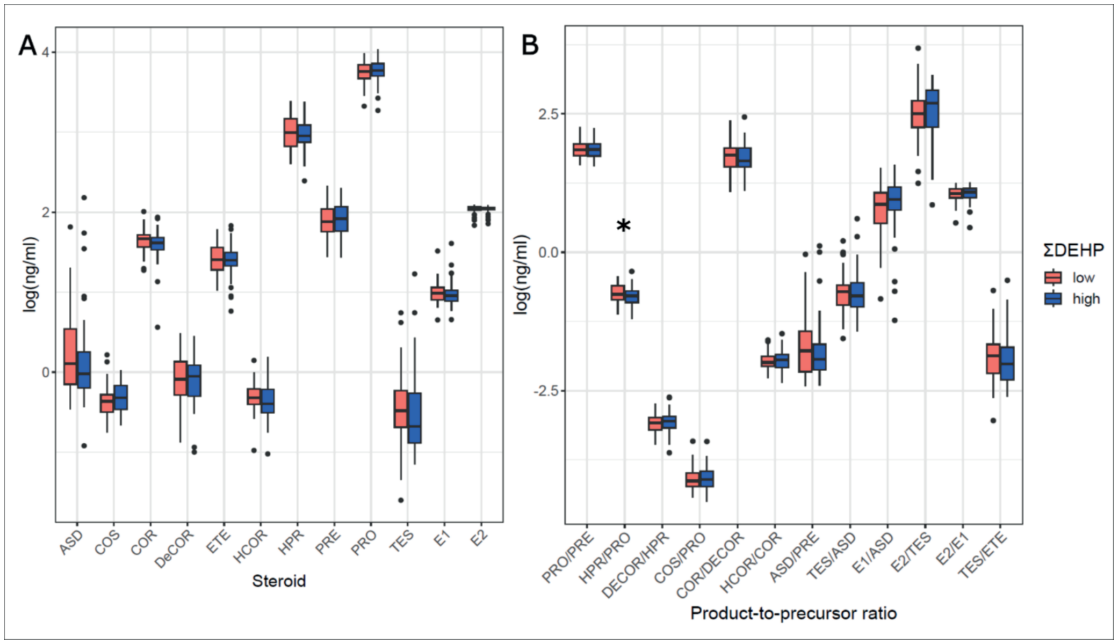

Fig. 4. Differences in the steroid hormone levels in the follicular fluid of women from low and high ΣDEHP groups. A: Steroid hormone levels; B: Product-to-precursor ratios of detected steroids. * p = 0.046, Welch's *t*-test adjusted for the country. ASD – androstenedione, COS – corticosterone, COR, cortisol, DeCOR – deoxycortisol, ETE – epi-testosterone, HCOR – hydroxycortisol, HPR – 17a-OH-progesterone, PRE – pregnenolone, PRO – progesterone, TES – testosterone, E1 – estrone, E2 – 17-β-estradiol.

the levels of steroid hormones of FF, as well as on mRNA expression in granulosa cells. To our knowledge, this is the first human cohort-based study to investigate the impact of the presence of ΣDEHP in follicles on human granulosa cell transcriptome and steroidogenesis, and to link the results with extracellular miRNA levels in the FF. Importantly, all measurements were performed from the same follicles, which allowed us to better scrutinize potential associations between different layers of information.

Our study presents compelling evidence for ΣDEHP-associated alterations in extracellular miRNA levels within FF. A total of 465 miRNAs were identified, 16 of which exhibited differential expression between high and low ΣDEHP groups. Changes in ovarian miRNA expression due to DEHP exposure have been observed in several animal studies (Liu et al., 2018; Patiño-García et al., 2020; Zhang et al., 2019). Culturing neonatal rodent ovaries with DEHP upregulates miR-28a-3p (Zhang et al., 2019), which suggests a causal connection between DEHP exposure and miRNA expression. Similarly, we observed an upregulation of miR-28a-3p in human FF in the presence of high ΣDEHP. Such an analogous response could suggest that miR-28a-3p modulation occurs across various organisms in response to DEHP, thus indicating a common molecular mechanism. While studies exploring the changes in miRNA profiles related to increased DEHP levels in human ovaries are limited, a negative correlation between urinary MEHP levels and miR-106b expression in FF extracellular vesicles (EV) has been reported (Martinez et al., 2019). In line with this, we observed a downregulation of miR-106b-3p in the FF of the high ΣDEHP group. However, another study exploring the association between FF phthalate concentration and EV miRNA expression did not find overlapping miRNAs with our study (Barnett-Itzhaki et al., 2021). This can be explained by partly distinct profiles between the cell-free and EV-packed miRNA sequences (Rooda et al., 2020). Secondly, the measured phthalate metabolites were different from those of our study. In addition, the absence of common miRNAs may be attributed to differences in patient cohorts, inclusion criteria, material collection and stimulation protocols as well as geographical differences in EDC exposure.

Our study revealed DE miRNAs associated with various reproductive conditions. Specifically, the upregulation of miR-27a-3p, miR-28a-3p, miR-122-5p and miR-199b-3p, and downregulation of miR-1307 in the high ΣDEHP group align with the expression patterns observed in patients with diminished ovarian reserve (Rapani et al., 2021; Xie et al., 2024). Additionally, the expression patterns of miR-122-5p and miR-132-3p in ovarian exosomes of mice with primary ovarian insufficiency was mirrored in our observations from the FF of the high ΣDEHP group (Zhang et al., 2022). A downregulation of miR-132-3p in FF has been linked with the generation of low-quality IVF embryos (Feng et al., 2015; Machtinger et al., 2017). The upregulation of miR-122-5p and miR-10b-5p, known to induce human granulosa cell apoptosis (Jiajie et al., 2017; Zhang et al., 2022), indicates that high ΣDEHP levels may contribute to altered granulosa cell dynamics. The upregulation of miR-203a-3p, previously observed in MII oocytes in older women and considered an indication for ovarian aging (Battaglia et al., 2016), is striking. Our results not only confirm the upregulation of miR-203a-3p in the high ΣDEHP group but also reveal a negative correlation with the OSI. This is noteworthy as decreased ovarian sensitivity is widely recognized as an indication of ovarian aging, and as a predictor for worse outcome in IVF treatments (Weghofer et al., 2020). Our findings thus suggest the possibility that ΣDEHP, by altering miR-203a-3p levels, may accelerate ovarian aging, contributing to the observed decline in ovarian sensitivity.

One of the mechanisms by which ΣDEHP may increase ovarian aging is by affecting AR signaling. Computational structural binding analysis suggests that DEHP metabolites have the potential to disrupt the AR function, as these metabolites closely resemble TES (Beg and Sheikh, 2020). However, direct binding of the DEHP metabolite MEHP has not

been confirmed in either yeast AR assays (Kim et al., 2019) nor in the human AR *in vitro* binding tests, even at supraphysiological concentrations (Parks et al., 2000). Our data shows that 6 of the DE miRNAs between the high and low ΣDEHP group target the AR signaling pathway that, in addition to the receptor, involves a plethora of AR-dependent transcription co-factors. The importance for AR signaling in the ovary has been established in model organisms and monogenic disorders (Panagiotou et al., 2022). The granulosa cell-specific AR knock-out mouse exhibits the signs of premature ovarian failure leading to subfertility (Sen and Hammes, 2010). Women suffering from androgen insensitivity syndrome-like monogenic disorders also exhibit poor ovarian response to gonadotrophins (Lissaman et al., 2023). Therefore, the potential disruption of AR signaling by ΣDEHP via miRNAs could have adverse consequences for the follicular development and function.

Molecular pathway enrichment analysis of DE miRNAs in FF revealed their involvement in adipogenesis, AGE/RAGE and RAC1/PAK1/p38/MMP2 pathways. Notably, rodent studies have demonstrated that DEHP acts as an obesogen, causing a substantial increase in body weight, food intake, and visceral adipose tissue compared to controls (Schmidt et al., 2012). These findings have been further supported by human studies, establishing an association between DEHP metabolites and obesity in adult females (Buser et al., 2014). The impact of DEHP and its metabolites on adipogenesis primarily occurs through the activation of peroxisome proliferator-activated receptors (PPARs) (Feige et al., 2007). Despite the absence of adipocytes in the ovarian follicle, the dysregulation of miRNAs targeting adipogenesis may signify potential alterations in fatty acid metabolism. Indeed, our results from RNA-seq of granulosa cells revealed the enrichment of downregulated genes in lipid and fatty acid metabolism pathways. Therefore, we hypothesize that differentially regulated miRNAs in the ovaries with high ΣDEHP levels could contribute to disturbances in fatty acid metabolism by targeting several genes, such as *FADS1, FADS2* and *SC5D*, within this pathway.

Advanced glycation end products (AGEs) are highly reactive molecules formed through lipid and protein glycation which exert their effects via receptor RAGEs. The accumulation of AGEs into the ovarian follicle has been proposed to trigger premature ovarian aging by promoting pro-inflammatory conditions (Tatone et al., 2008). Treatment of human cumulus cells with glycated albumin (a precursor for AGEs) has been shown to induce the expression of key steroidogenic genes (*CYP11A1, 3-βHSD, STAR, CYP17A1* and *LHR*), highlighting the pivotal role of AGEs in regulating steroidogenesis (Merhi, 2014). We hypothesize that dysregulation of the AGE/RAGE pathway through miRNAs in granulosa cells could be one of the mechanisms by which ΣDEHP alters the expression of steroidogenic enzymes and induces a pro-inflammatory environment within the follicle.

While the involvement of the RAC1/PAK1/p38/MMP2 pathway in normal ovarian function is not well-established, its implication in ovarian cancer is noteworthy (Gonzalez-Villasana et al., 2015). Matrix metalloproteinases, including MMP2, play crucial roles in follicle formation and rupture (Vos et al., 2014). Therefore, any disruptions in this signaling pathway that lead to MMP2 dysregulation could prove detrimental to these essential ovarian processes.

We identified 2,454 DE genes in granulosa cells between high and low ΣDEHP groups. Particularly interesting is the observation that the upregulated genes in high ΣDEHP follicles predominantly belong to immune system pathways. This finding is further substantiated by cellular deconvolution of our RNA-seq data, which revealed an increased presence of T-cells, neutrophils and macrophage markers within the ovarian follicle. This provides a potential explanation for the observed immune system related gene expression changes. These results also align with previous findings demonstrating the upregulation of immune response-associated genes in the ovaries of prepubertal mice exposed to DEHP (Lai et al., 2017). Moreover, phthalate metabolites or their mixtures have been shown to alter inflammatory cytokine levels in human FF (Wang et al., 2023). The enhanced presence of immune cells may indicate increased inflammation within the ovarian follicles, potentially compromising the microenvironment essential for successful ovulation. While further research is needed to clarify the immune mechanisms integral to ovarian function, our findings suggest that higher ΣDEHP levels may influence these processes.

Regulation of ovarian steroidogenesis is vital for the normal development and function of the ovarian follicles. In our study, higher concentration of ΣDEHP was associated with the reduced mRNA levels of genes encoding essential components of the steroidogenic pathway, including *CYP11A1, CYP17A1, CYP19A1* and *STAR*. Similar results have been observed in rodent studies, where *CYP17A1* and *STAR* were downregulated in rodent ovaries, and *CYP11A1, CYP19A1* and *STAR* expression levels were inhibited in rat granulosa cells upon DEHP exposure, accompanied by lower levels of PRO and estradiol in both studies (Lai et al., 2017; Tripathi et al., 2019). Our results also agree with experiments performed using primary human granulosa cells (Jin et al., 2019), cumulus cells (Tesic et al., 2023) and the granulosa-like tumor cell-line KGN (Jin et al., 2019). In these studies, DEHP decreased the expression of the same key steroidogenic enzymes (Guerra et al., 2016) accompanied by reduced PRO and estradiol production (Guerra et al., 2016; Tesic et al., 2023). Additionally, DEHP has been associated with reduced PRO levels in human FF (Du et al., 2019). Although we did not observe significant changes in FF steroid hormone levels between high and low ΣDEHP groups, we observed an altered ratio between PRO and HPR, which could be attributed to lower *CYP17A1* mRNA levels in the ovarian somatic cells of the high ΣDEHP group. Considering the predominantly luteinized state of the studied somatic cells, alterations in PRO conversion could negatively affect the developing corpus luteum and the maintenance of pregnancy. HPR serves as a precursor for ASD and cortisol production (Chakraborty et al., 2021; Honour, 2014), suggesting a potential influence on the synthesis of these hormones. Although a trend toward lower ASD and TES levels was observed between the study groups, we cannot confirm that the main mechanism of ΣDEHP action in the adult ovary is the inhibition of androgen synthesis as has been described for the testis (Desdoits-Lethimonier et al., 2012; Parks et al., 2000). Instead, our findings propose that *CYP19A1* and *STAR* mRNAs are targeted by multiple DE FF miRNAs, and the expression of *CYP17A1* is significantly reduced in the ovaries with high ΣDEHP. These results are indicative of decreased PRO metabolism.

Our transcriptional analyses revealed downregulation of 17 key genes involved in cholesterol, lipid and fatty acid metabolism pathways. mRNAs of 11 of these genes are potentially targeted by the DE FF miRNAs. Furthermore, considering that granulosa cells predominantly rely on cholesterol import from circulating lipoproteins (Miller and Bose, 2011), the downregulation of *LDLR* implies a potential imbalance in cellular cholesterol homeostasis. Given that cholesterol serves as a crucial precursor for the synthesis of steroid hormones, this downregulation could explain the observed decrease in steroidogenesis-related genes. Notably, the reduction in the expression of cholesterol metabolism genes *IDI1, FDFT1, CYP51A1* and *STARD1* has been implicated in granulosa cells of individuals with diminished ovarian reserve (Yang et al., 2022). Downregulation of genes involved in lipid and fatty acid metabolism could be detrimental for the maintenance of energy supplies in the granulosa cells (Zhang et al., 2023). Reduced utilization of fatty acids can lead to their accumulation in granulosa cells which can induce ovarian inflammation (Lai et al., 2022). This collective evidence strengthens the growing body of evidence connecting ΣDEHP to disruptions in ovarian function.

As a limitation of the current study we acknowledge that using samples from two heterogeneous populations might lead to fewer statistically significant results. Population heterogeneity can be attributed to differences not only in individual lifestyles, potentially influencing EDC levels, but also in stimulation protocols and sample collection routines between the clinics. However, this aspect effectively demonstrates the complexity of human studies and explains the frequent

discordances between different papers. As a strength, the current study stresses the robustness of the presented results as these have been validated in two patient sub cohorts covering the expected variability between lifestyle and fertility treatment standards between IVF patients from different countries.

5. Conclusions

In summary, our comprehensive analysis - spanning cell-free miRNA, cellular mRNA expression and steroid hormone measurements - reveals that high ΣDEHP level in human FF is linked with disrupted cholesterol and steroid hormone biosynthesis, altered PRO to HPR ratio, as well as with a pro-inflammatory environment within the ovarian follicle. Notably, some of these perturbations can be attributed to interrupted inter-cellular molecular communication via extracellular miRNAs. Our study suggests different mechanisms of action for ΣDEHP disruption in the adult ovaries to what has been previously described in the testis. This further demonstrates that EDCs such as ΣDEHP may have multiple mechanisms of action contributing to adverse outcomes, including in the ovaries. Overall, many of the changes reported here are associated with ovarian aging in other studies, indicating that EDCs like DEHP may accelerate reproductive senescence. There are still unresolved issues that warrant further scrutiny based on our findings, not least investigating in more detail how lipid metabolism, steroidogenesis, and inflammation is affected by ΣDEHP exposure in ovaries. However, our findings help explain the impact of EDC exposure on increasing female fertility problems.

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used Microsoft Copilot in order to improve wording and correct grammar. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

CRediT authorship contribution statement

Inge Varik: Writing – original draft, Visualization, Formal analysis. **Runyu Zou:** Writing – original draft, Visualization, Methodology, Formal analysis. **Andrea Bellavia:** Writing – review & editing, Methodology. **Kristine Rosenberg:** Writing – review & editing, Resources, Data curation. **Ylva Sjunnesson:** Writing – review & editing, Resources. **Ida Hallberg:** Writing – review & editing, Resources. **Jan Holte:** Resources. **Virissa Lenters:** Writing – review & editing, Methodology. **Majorie Van Duursen:** Writing – review & editing, Funding acquisition, Conceptualization. **Mikael Pedersen:** Methodology, Investigation, Formal analysis. **Terje Svingen:** Writing – review & editing. **Roel Vermeulen:** Writing – review & editing. **Andres Salumets:** Writing – review & editing, Funding acquisition. **Pauliina Damdimopoulou:** Writing – original draft, Supervision, Project administration, Conceptualization. **Agne Velthut-Meikas:** Writing – original draft, Visualization, Supervision, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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

The raw sequencing data files have been published in the European Nucleotide Archive under project number PRJEB74279.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2024.108960.

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Curriculum vitae

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Education

2021–2026	Tallinn University of Technology, PhD student in Chemistry and Biotechnology
2018–2020	University of Tartu, MSc in Biomedicine, diploma *cum laude*
2015–2018	University of Tartu, BSc in Gene Technology, diploma *cum laude*
2012–2015	Võru Kreutzwaldi High School

Language competence

English	Fluent
Estonian	Native
Russian	Basic
Finnish	Basic

Professional employment

2021	Estonian Health Board, Laboratory Specialist
2020–2021	Celvia CC AS, Laboratory Specialist

Scholarships & awards

2025	TalTech School of Science award for best popular science article of the year
2025	Võru City Youth of the Year
2024	Estonian Academy of Sciences “Science in 3 minutes” laureate
2024	Special prize at the competition “Science in Vikipeedia”
2024	2nd prize for a poster presentation at the Estonian Doctoral School’s career conference
2024	Kristjan Jaak Scholarship
2024	Vikipeedia competition “Terminikratt” scholarship
2024	The best Young Scientist award at the annual conference of the Estonian Society of Human Genetics
2023	Best flash talk at the TalTech Department of Chemistry and Biotechnology symposium
2023	Nominated among the 5 best posters in the field of Reproductive Endocrinology, 39th Annual Meeting of the European Society of Human Reproduction and Embryology

2019	Lydia and Felix Krabi scholarship
2017	Rotalia Foundation scholarship

Conferences

2025	Karl Ernst von Baer seminar, Tartu, Estonia, **poster presentation**
2025	European Society of Human Genetics (ESHG) Annual Conference, Milan, Italy, **poster presentation**
2025	International Society of Extracellular Vesicles (ISEV) Annual Meeting, Vienna, Austria, **poster presentation**
2025	Estonian Doctoral School's Gene Technology Winter Seminar, Jäneda, Estonia, **oral presentation**
2024	Society for the Study of Reproduction (SSR) Annual Meeting, Dublin, Ireland, **poster presentation**
2024	Estonian Doctoral School's Career Conference, Tallinn, Estonia, **poster presentation**
2024	International Society of Extracellular Vesicles (ISEV) Annual Meeting, Melbourne, Australia, **poster presentation**
2024	Estonian Society of Human Genetics Annual Conference, Viljandi, Estonia, **oral presentation**
2024	XVI Annual Conference of the School of Science, Tallinn, Estonia, **oral presentation**
2023	Tallinn University of Technology, Department of Chemistry and Biotechnology symposium, Tallinn, Estonia, **oral presentation**
2023	European Society of Human Reproduction and Embryology (ESHRE) Annual Meeting, Copenhagen, Denmark, **oral and poster presentations**
2023	Estonian Society of Human Genetics Annual Conference, Rakvere, Estonia, **poster presentation**

Supervised dissertations

2025	**Katariina Johanna Saretok**, MSc, (sup) Agne Velthut-Meikas & Inge Varik, "The effect of follicular fluid extracellular vesicles from patients with varying ovarian sensitivity on the KGN cell line"
2025	**Triin Sild**, BSc, (sup) Inge Varik & Agne Velthut-Meikas, "The impact of di(2-ethylhexyl) phthalate and its primary metabolite on the gene expression of KGN granulosa cells"
2024	**Thor Tristan Karafin**, BSc, (sup) Inge Varik & Agne Velthut-Meikas, "Effects of di(2-ethylhexyl) phthalate on KGN cell viability, proliferation, and miRNA expression"
2023	**Katariina Johanna Saretok**, BSc, (sup) Agne Velthut-Meikas & Inge Varik, "Isolation and characterisation of extracellular vesicle subpopulations in human follicular fluid"

Publications

Varik I, Saretok KJ, Rosenberg K, et al. Small and Large Extracellular Vesicles From Human Preovulatory Follicular Fluid Display Distinct ncRNA Cargo Profiles and Differential Effects on KGN Granulosa Cells. *J Extracell Vesicles*. 2025;14(7):e70119. doi:10.1002/jev2.70119.

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Elulookirjeldus

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2018–2020	Tartu Ülikool, magistrikraad biomeditsiinis, diplom *cum laude*
2015–2018	Tartu Ülikool, bakalaureusekraad geenitehnoloogias, diplom *cum laude*
2012–2015	Võru Kreutzwaldi gümnaasium

Keelteoskus

Inglise keel	Kõrgtase
Eesti keel	Emakeel
Vene keel	Algtase
Soome keel	Algtase

Teenistuskäik

2021	Eesti Terviseamet, laborispetsialist
2020–2021	Celvia CC AS, laborispetsialist

Tunnustused ja auhinnad

2025	TalTechi Loodusteaduskonna aasta parima populaarteadusliku artikli auhind
2025	Võru linna aasta noor
2024	Eesti Teaduste Akadeemia konkursi “Teadus 3 minutiga” laureaat
2024	Eripreemia konkursil “Teadus Vikipeediasse”
2024	Vikipeedia Terminikrati konkursi stipendium
2024	Posterettekande 2. preemia Eesti doktorikooli karjäärikonverentsil
2024	Kristjan Jaagu välislähetuse stipendium
2024	Parim noorteadlane Eesti Inimesegeneetika Ühingu aastakonverentsil
2023	Parim välkettekanne Tallinna Tehnikaülikooli keemia ja biotehnoloogia instituudi sümpoosiumil
2023	Viie parima teaduspostri seas reproduktiivbioloogia-endokrinoloogia valdkonnas, 39. Euroopa Inimese Reproduktsiooni ja Embrüoloogia Ühingu aastakonverents

2019	Lydia ja Felix Krabi stipendium
2017	Rotalia Foundation stipendium

Konverentsid

2025	Karl Ernst von Baeri seminar, Tartu, Eesti, **posterettekanne**
2025	Euroopa Inimesegeneetika Ühingu (ESHG) aastakonverents, Milano, Itaalia, **posterettekanne**
2025	Rahvusvahelise Rakuväliste Vesiikulite Ühingu (ISEV) aastakonverents, Viin, Austria, **posterettekanne**
2025	Eesti doktorikooli geenitehnoloogia talveseminar, Jäneda, Eesti, **suuline ettekanne**
2024	Reproduktiivuuringute Ühingu (SSR) aastakonverents, Dublin, Iirimaa, **posterettekanne**
2024	Eesti doktorikooli karjäärikonverents, Tallinn, Eesti, **posterettekanne**
2024	Rahvusvahelise Rakuväliste Vesiikulite Ühingu (ISEV) aastakonverents, Melbourne, Austraalia, **posterettekanne**
2024	Eesti Inimesegeneetika Ühingu aastakonverents, Viljandi, Eesti, **suuline ettekanne**
2024	Tallinna Tehnikaülikooli XVI loodusteaduskonna konverents, Tallinn, Eesti, **suuline ettekanne**
2023	Tallinna Tehnikaülikooli keemia ja biotehnoloogia instituudi sümpoosium, Tallinn, Eesti, **suuline ettekanne**
2023	Euroopa Inimese Reproduktsiooni ja Embrüoloogia Ühingu (ESHRE) aastakonverents, Kopenhaagen, Taani, **suuline ettekanne ja posterettekanne**
2023	Eesti Inimesegeneetika Ühingu aastakonverents, Rakvere, Eesti, **posterettekanne**

Lõputööde juhendamine

2025	**Katariina Johanna Saretok**, MSc, (juh) Agne Velthut-Meikas & Inge Varik, “Erineva munasarjade tundlikkusega patsientide follikulaarvedelikust eraldatud rakuväliste vesiikulite mõju KGN rakuliinile”
2025	**Triin Sild**, BSc, (juh) Inge Varik & Agne Velthut-Meikas, “Di(2-etüülheksüül)ftalaadi ja selle primaarse metaboliidi mõju KGN granuloosarakkude geeniekspressioonile”
2024	**Thor Tristan Karafin**, BSc, (juh) Inge Varik & Agne Velthut-Meikas, “Di(2-etüülheksüül)ftalaadi mõju KGN rakkude elulemusele, proliferatsioonile ja miRNA ekspressioonile”
2023	**Katariina Johanna Saretok**, BSc, (juh) Agne Velthut-Meikas & Inge Varik, “Inimese follikulaarvedelikust rakuväliste vesiikulite alampopulatsioonide eraldamine ja iseloomustamine”

Publikatsioonid

Varik I, Saretok KJ, Rosenberg K, et al. Small and Large Extracellular Vesicles From Human Preovulatory Follicular Fluid Display Distinct ncRNA Cargo Profiles and Differential Effects on KGN Granulosa Cells. *J Extracell Vesicles*. 2025;14(7):e70119. doi:10.1002/jev2.70119.

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