

SPYRIDON PANAGIOTIS DELIGIANNIS

Multi-omics insights into
ovarian health preservation



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Multi-omics insights into
ovarian health preservation



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Institute of Clinical Medicine, University of Tartu, Estonia

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LIST OF ORIGINAL PUBLICATIONS

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- I. **Spyridon Panagiotis Deligiannis**, Tianyi Li, Elisabeth Moussaud-Lamodiere, Akos Végvári, Anastasios Damdimopoulos, Darja Lavogina, Kiriaki Papaikonomou, Roman Zubarev, Ganesh Acharya, Agne Velthut-Meikas, Pauliina Damdimopoulou, Andres Salumets, Valentina Di Nisio (2026). “Acute High-Dose Irradiation Disrupts Cell Adhesion and Silk-Ovarioid Formation in Human Primary Ovarian Cells”. *The Journal of Ovarian Research*, DOI: *10.1186/s13048-025-01932-8*
- II. **Spyridon P. Deligiannis**, Keiu Kask, Vijayachitra Modhukur, Nina Boskovic, Marilin Ivask, Ülle Jaakma, Pauliina Damdimopoulou, Timo Tuuri, Agne Velthut-Meikas & Andres Salumets (2024). “Investigating the impact of vitrification on bovine ovarian tissue morphology, follicle survival and transcriptomic signature” *Journal of Assisted Reproduction and Genetics*, DOI: *10.1007/s10815-024-03038-4*
- III. Alberto Sola-Leyva, Inmaculada Pérez-Prieto, Valentina Di Nisio, **Spyridon Panagiotis Deligiannis**, Lucia Blanco-Rodriguez, Ganesh Acharya, Merli Saare, Kiriaki Papaikonomou, Pauliina Damdimopoulou, Andres Salumets, Signe Altmäe (2025). “Assessing the ovarian microbiome: lack of a distinguishable microbial signature beyond contamination”. *Reproductive BioMedicine Online*, DOI: *10.1016/j.rbmo.2025.104988*

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Author's contribution to the original publication

- Study I: Participated in the study conception, formulating the study hypothesis, aims and the development of the experimental approach; performed the wet-lab experiments (ovarian tissue collection, ovarian cortex and medulla dissociation, irradiation of primary ovarian cells, performed ATP production and mitochondrial dehydrogenases assay, immunofluorescence staining, RNA extraction, Silk-ovarioids formation, H&E staining); collected the data, performed the statistical analysis of the data and interpreted the bioinformatics results; wrote the initial and final version of the manuscript.
- Study II: Participated in the study conception, formulating the study hypothesis, aims and the development of the experimental approach; performed the wet-lab experiments (ovarian tissue collection, separation of ovarian cortex from medulla, ovarian cortex vitrification/thawing, tissue embedding in paraffin, immunofluorescence staining, H&E staining, RNA extraction and library preparation for bulk RNA

sequencing, follicle isolation, live/dead test); collected the data, performed the statistical analysis of the data and interpreted the bio-informatics results; wrote the initial and final version of the manuscript.

Study III: Joined an ongoing project and performed wet-lab experiments (collecting ovarian tissue and data collection); participated in the writing of the final draft of the manuscript.

ABBREVIATIONS

2D	Two-Dimensional
3D	Three-Dimensional
ACN	Acetonitrile
Act A	Activin A
AMA	Advanced Maternal Age
AMH	Anti-Müllerian Hormone
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia-Telangiectasia and RAD3-Related
BAX	BCL2-associated X protein
BCL2	B-Cell Lymphoma 2
bFGF	Basic Fibroblast Growth Factor
BH	Benjamini & Hochberg's
BMP-4	Bone Morphogenic Protein-4
BSA	Bovine Serum Albumin
CDC25	Cell Division Cycle 25
CGCs	Cumulus Granulosa Cells
CHK1	Checkpoint Kinases 1
CHK2	Checkpoint Kinases 2
CL	Corpus Luteum
cl-Cas3	Cleaved Caspase-3
COC	Cumulus-Oocyte-Complex
cPOCs	Cortex-derived Primary Ovarian Cells
DAPI	4',6-Diamidino-2-Phenylindole Dihydrochloride
DAZL	Deleted in Azoospermia Like Protein
DEA	Differential Expression Analysis
DEGs	Differentially Expressed Genes
DEPs	Differentially Expressed Proteins
DF	Dominant Follicle
DMEM/F12	Dulbecco's Modified Eagle Medium F12
DPBS	Dulbecco's Phosphate Buffered Saline
DSB	Double-Strand Breaks
DSBs	Double-Strand Breaks
DTT	Dithiothreitol
E₂	Oestradiol
ECM	Extracellular Matrix
EthD-1	Ethidium Homodimer
EtOH	Ethanol
FA	Formaldehyde
FA	Formic Acid
FDR	False Discovery Rate
FOXL2	Forkhead Box Protein-L2
FP	Fertility Preservation

FSD	Focus-to-Skin Distance
FSH	Follicle-Stimulation Hormone
GCs	Granulosa Cells
GDPR	General Data Protection Regulation
GnRH	Gonadotropin-Releasing Hormone
GO	Gene Ontology
GSEA	Gene Set Enrichment Analysis
GV	Germinal Vesicle
Gy	Gray
H&E	Hematoxylin and Eosin
HCD	Higher-energy Collisional Dissociation
HI-FBS	Heat-Inactivated Foetal Bovine Serum
HSA	Human Serum Albumin
HVL	Half-Value Layer
ICSI	Intracytoplasmic Sperm Injection
ITS	Insulin–Transferrin–Selenium
IVM	<i>In Vitro</i> Maturation
KGF	Keratinocyte Growth Factor
KL	Kit Ligand
LC-MS	Liquid chromatography-tandem mass spectrometry
LH	Luteinizing Hormone
LIF	Leukaemia Inhibitory Factor
log₂FC	log ₂ Fold Change
MGCs	Mural Granulosa Cells
mPOCs	Medulla-derived Primary Ovarian Cells
NCE	Normalised Collision Energy
NDS	Normal Donkey Serum
NOTCH	Neurogenic Locus Notch Homolog Protein
NR	Neutral Red
OSE	Ovarian Surface Epithelium
OTC	Ovarian Tissue Cryopreservation
p21	CDKN1A, Cyclin-Dependent Kinase Inhibitor 1A
PBS -/-	Phosphate Buffered Saline without Ca ²⁺ and Mg ²⁺
PCA	Principal Component Analysis
p-Chk1	Phosphorylated Chk1
PCoA	Principal Coordinate Analysis
PCOS	Polycystic Ovarian Syndrome
PF	Primary Follicles
PFA	Paraformaldehyde
PGCs	Primordial Germ Cells
PMF	Primordial Follicles
POCs	Primary Ovarian Cells
POI	Premature Ovarian Insufficiency
p-p21	Phosphorylated p21
p-p53	Phosphorylated p53

PUMA	p53-Upregulated Modulator of Apoptosis
RA	Retinoic Acid
rAct	recombinant Activin A
rhFSH	recombinant human FSH
RIN	RNA Integrity Value
ROS	Reactive Oxygen Species
RSPO1	Roof Plate-Specific Spondin-1
RT	Radiotherapy
rTdT	recombinant Terminal Deoxynucleotidyl Transferase
scRNA-seq	Single-cell RNA sequencing
SF1	Steroidogenic Factor 1
SSBs	Single-Strand Breaks
SSC	Saline Sodium Citrate
STRA8	Stimulated by Retinoic Acid 8
TNFα	Tumour Necrosis Factor-alpha
VST	Variance Stabilising Transformation
WHO	World Health Organization
WNT4	Wingless-Type integration site family member 4
WT1	Wilms Tumour-1 Protein

1. INTRODUCTION

Fertility preservation has become an essential component of reproductive and oncological care, particularly as survival rates among cancer patients continue to improve. While advances in cancer therapies have significantly increased long-term survival, many chemotherapeutic and radiotherapy regimens have profound gonadotoxic effects on the ovary. The human ovary contains a finite pool of primordial follicles established during foetal development, making it particularly vulnerable to DNA damage, oxidative stress and microenvironmental disruption. This underscores the importance of developing strategies to safeguard reproductive potential.

Among available fertility preservation methods, ovarian tissue cryopreservation (OTC) represents the only viable option for prepubertal girls and for patients who require urgent oncological treatment. In addition to preserving fertility, OTC offers the potential to restore endocrine function following transplantation. Despite these advantages, several challenges remain, including tissue damage during cryopreservation and the risk of reintroducing malignant cells. Moreover, the slow-freezing method, currently the most widely used approach, requires specialised equipment and is associated with high costs, limiting its accessibility. In contrast, vitrification has emerged as a potential alternative cryopreservation strategy that may offer advantages in terms of simplicity, speed and cost-effectiveness.

Recent technological advances have enabled deeper insight into ovarian tissue biology and preservation. High-throughput approaches, including transcriptomics, proteomics and microbial DNA analysis, allow comprehensive tissue profiling. In parallel, the development of the 3D ovary-like organoid models, such as Silk-Ovarioids, provides a platform to study ovarian tissue organisation and function *in vitro*. Using these approaches, it is possible to investigate molecular changes in ovarian tissue induced by irradiation and vitrification, assess protein-level alterations, evaluate the capacity of primary ovarian cells to form Silk-Ovarioids following irradiation, and explore the presence of microbial DNA in fresh ovarian tissue. Together, these analyses may provide further information for the optimisation of cryopreservation protocols.

Collectively, these approaches provide complementary insights into the effects of irradiation on ovarian tissue and the potential of vitrification as an alternative to slow freezing. Ultimately, this knowledge may contribute to the refinement of fertility preservation strategies and improve clinical outcomes for patients undergoing gonadotoxic treatments.

2. LITERATURE REVIEW

The female reproductive system is a complex network of organs that function together to support reproduction, foetal development, and hormone production. It consists of the ovaries, fallopian tubes, uterus, cervix, and vagina. Among these, the ovaries are the primary reproductive organs responsible for producing mature oocytes as well as synthesising essential reproductive hormones (Magoffin *et al.*, 2005).

2.1. Ovarian biology

The ovaries are located bilaterally in the pelvis and are connected to the uterus via the fallopian tubes, ovarian ligaments, and broad ligaments (Oktem and Oktay, 2008). Each ovary consists of two structurally distinct compartments: the outer cortex (~1 mm thick), which contains primordial and growing follicles, and the inner medulla, a highly vascularised region housing larger developing follicles. These compartments differ in texture: the medulla is smoother, whereas the cortex is denser and more fibrous.

Recent single-cell RNA sequencing (scRNA-seq) studies have demonstrated that both the cortex and the medulla harbour diverse somatic cell populations, including stroma, immune, vascular, and granulosa cells (GCs). A key distinction lies in the presence of theca cells and broader diversity of GCs in the medulla due to multiple GC sub-populations derived from antral follicles (Fan *et al.*, 2019; Wagner *et al.*, 2020; Roos *et al.*, 2022).

The ovary is surrounded by a single layer of cells known as ovarian surface epithelium (OSE), which plays a role in rapid proliferation after ovulation, to efficiently repair the ovulatory wound (Auersperg *et al.*, 2001; Alzamil, Nikolakopoulou and Turco, 2021). Lastly, the ovarian hilum serves as the entry point for major blood vessels and nerves, supporting ovarian endocrine and reproductive functions.

2.1.1. Development and folliculogenesis

The ovarian reserve is derived from primordial germ cells (PGCs), the first germ cell population established during early development (Saitou and Yamaji, 2012). In humans, the induction of the PGC population begins around the second and third week of embryonic development and formed in the epiblast, from which they first migrate to yolk sac and from there to gonads (Ge *et al.*, 2015). These cells give rise to oogonia in females (and spermatogonia in males) and migrate from the yolk sac endoderm to the genital ridge (future gonads) by approximately the sixth gestational week (De Felici, 2016; Kojima *et al.*, 2017). During migration, PGCs undergo rapid mitotic proliferation, expanding the pool of future gametes (Jaszczak *et al.*, 2024).

Around gestational week 6, gonadal sex differentiation is initiated and it depends on the presence or absence of *SRY* and *SOX9*, which determine whether the gonad develops into a testis or an ovary (Figure 1) (Kashimada and Koopman,

2010). In the absence or dysfunction of *SRY* gene on the Y chromosome, the ovarian differentiation pathway is initiated. Key molecular regulators of sex differentiation, ovarian development, and subsequent follicle formation include Wingless-Type integration site family member 4 (*WNT4*)/ β -catenin pathway, Roof Plate-Specific Spondin-1 (*RSPO1*), Forkhead Box Protein-L2 (*FOXL2*), Wilms Tumour-1 Protein (*WT1*), Steroidogenic Factor 1 (*SFI*), Anti-Müllerian Hormone (*AMH*) and Deleted in Azoospermia Like Protein (*DAZL*) (Vainio *et al.*, 1999; Hughes, 2001; Yao *et al.*, 2004; Parma *et al.*, 2006; Ottolenghi *et al.*, 2007; Chang *et al.*, 2008; Tomizuka *et al.*, 2008; Katoh-Fukui *et al.*, 2012). Moreover, in some cases translocation of the *SRY* gene onto an X chromosome can result in a male phenotype (Margarit *et al.*, 2000). Conversely, *SRY*-negative cases may also occur, particularly when they involve alterations in *SOX9* or *DAX1*, and typically present with small, firm testis (Hanley *et al.*, 2000).

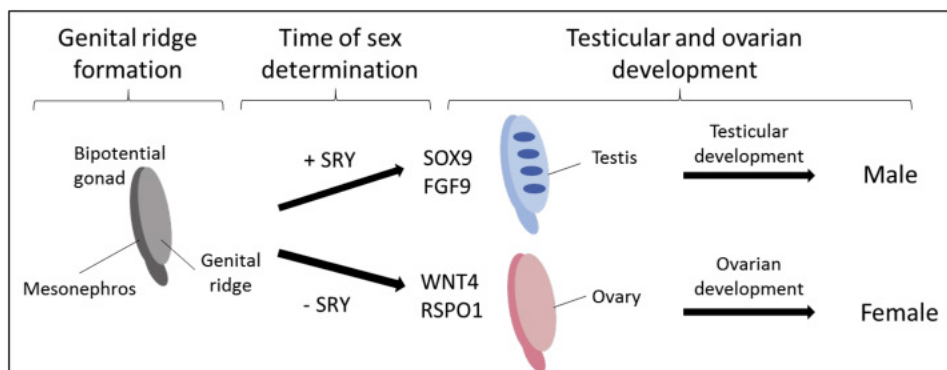


Figure 1: Sex determination pathways during foetal development. Sex determination during embryonic development is regulated by key genetic factors, with the *SRY* gene playing a central role. In XY embryos, *SRY* expression triggers upregulation of *SOX9*, leading to testis development. In the absence of *SRY*, or when its expression is delayed, *WNT4* and *RSPO1* signalling promote ovarian development. Figure reproduced by Kurtz and Petersen, 2019 (DOI: <https://doi.org/10.1016/j.theriogenology.2019.05.039>) under the terms of the Creative Commons CC-BY licence. Copyright 2019 by the Elsevier Inc. All rights reserved. License Number: 6200591146237.

Upon reaching the genital ridge, PGCs undergo mitosis with incomplete cytokinesis, forming germ cell cysts containing up to ~30 cells connected by intracellular bridges (De Felici, 2009; Wang, Zhou and Xia, 2017). In mice, retinoic acid (RA), activin A (Act A), *WNT4* and *RSPO1* secreted by the neighbouring mesonephros induce meiotic initiation in oogonia within the cysts, promoting their transition into primary oocytes (Bowles *et al.*, 2006; Koubova *et al.*, 2006; Le Bouffant *et al.*, 2010; Liu, Parker and Yao, 2010; Chassot *et al.*, 2011; Childs *et al.*, 2011; Mu *et al.*, 2013; Liang *et al.*, 2015).

RA induces the expression of Stimulated by Retinoic Acid 8 (*STR48*), essential for meiotic entry during prophase I (Xu *et al.*, 2005; Bowles *et al.*, 2006; Koubova *et al.*, 2006; Griswold *et al.*, 2012) and for the expression of synaptonemal complex proteins (Tedesco *et al.*, 2009, 2013). Oocyte numbers expand through proliferation, peaking at ~10 million by gestational week 20 (Lopes *et al.*, 2008; Edson, Nagaraja and Matzuk, 2009).

2.1.1.1. Formation of primordial follicles

The breakdown of germ cell cysts, which leads to the formation of primordial follicles (PMFs), is essential for establishing the ovarian reserve. This process requires coordinated development and communication between oocytes and pre-granulosa (pre-GCs). During cyst breakdown, a large proportion of oocytes undergo apoptosis, allowing the surviving oocytes to associate with pre-GCs to form individual PMFs (Skinner, 2005).

This process is regulated by oestradiol (E_2), which acts as inhibitory regulator by suppressing oocyte apoptosis and maintaining oocyte within germinal cysts, thereby delaying cyst breakdown and PMF formation, and by Neurogenic Locus Notch Homolog protein (NOTCH) signalling pathways, which promote pre-GCs differentiation and facilitate the encapsulation of individual oocytes, promoting as well cyst breakdown and PMF formation (Kurilo, 1981; Baker and Zuckerman, 1997; Reynaud *et al.*, 2004). In rats, progesterone inhibits cyst breakdown (Kezele and Skinner, 2003), while tumour necrosis factor-alpha ($TNF\alpha$) promotes this process by inducing oocyte apoptosis and PMF formation (Marcinkiewicz, Balchak and Morrison, 2002). The follicular development begins when PMFs are activated and recruited into the growing follicle pool.

2.1.1.2. Follicle recruitment: The initial recruitment

Follicle recruitment occurs in two phases: initial and cyclic. The initial phase is gonadotropin-independent and begins immediately after the formation of PMFs, continuing throughout life until menopause (Skinner, 2005). The cyclic phase begins at puberty and is gonadotropin-dependent (McGee and Hsueh, 2000).

During the initial phase, PMFs transition into primary follicles (PFs), a process characterised by the transformation of GCs from a squamous to a cuboidal shape and by oocyte growth (Figure 2) (Skinner, 2005). In humans and many other species, these changes are readily observed histologically. In bovines, PMFs often appear ellipsoidal and may already contain some cuboidal GCs (Rodgers and Irving-Rodgers, 2010).

The transition is regulated by several local intra-ovarian factors, including kit ligand (KL), basic fibroblast growth factor (bFGF), leukaemia inhibitory factor (LIF), keratinocyte growth factor (KGF), bone morphogenic protein-4 (BMP-4), FOXL2 and AMH (Skinner, 2005; Edson, Nagaraja and Matzuk, 2009). GCs produce KL, which stimulates oocyte growth, follicular development and the proliferation of theca cell precursors (Parrott and Skinner, 2000; Kezele and Skinner,

2003). Oocytes secrete bFGF, which promotes PMF activation (Kezele and Skinner, 2003), as does LIF (Nilsson, Kezele and Skinner, 2002). KGF, produced by theca precursor cells, also stimulates the PMF-to-PF transition (Kezele, Nilsson and Skinner, 2005), as does BMP-4 (Nilsson and Skinner, 2003). FOXL2 is essential for the transformation of squamous GCs into cuboidal GCs (Edson, Nagaraja and Matzuk, 2009), while AMH produced by growing follicles inhibits PMF activation, thereby limiting the size of the recruited follicle pool (Skinner, 2005).

2.1.1.3. Secondary and Antral follicle formation

PFs develop into secondary follicles in a process that remains independent from gonadotropins. Secondary follicles are larger (~0.1–0.2 mm) and contain a primary oocyte surrounded by multiple layers of proliferating cuboidal GCs, together with developing theca cells (Figure 2). At this stage, both GCs and theca cells are still considered precursor cells, as they are not yet fully differentiated and remain unresponsive to gonadotropins.

As follicles continue to grow through proliferation of GCs and increase in size (~0.2 – 0.4 mm), they begin to form a fluid-filled cavity termed the antrum. This marks the transition into the antral (tertiary) follicle stage, at which point the follicles become responsive and somewhat dependent to gonadotropins (Figure 2) (Craig *et al.*, 2007).

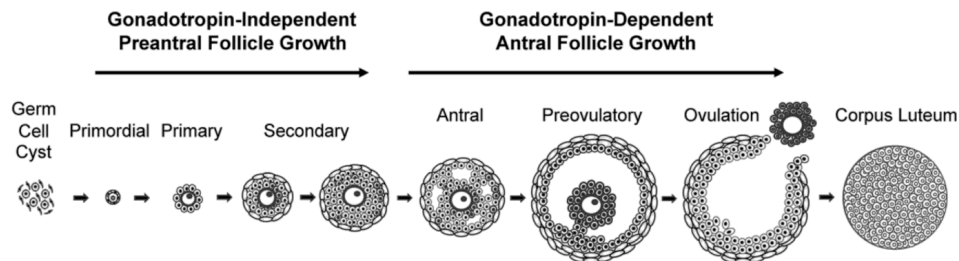


Figure 2: Schematic illustration of the main stages of folliculogenesis. Folliculogenesis begins with the activation of primordial follicles. Granulosa cells (GCs) undergo characteristic morphological changes, appearing as flattened cells in primordial follicles and forming a single layer of cuboidal GCs in primary follicles, followed by the development of multiple GC layers and the emergence of theca cells in secondary follicles. The transition from secondary to antral follicles marks the onset of FSH-dependent follicle growth. During this stage, continued GC proliferation and antrum formation divide GCs into two sub-populations: cumulus and mural GCs. A dominant follicle is then selected to progress towards ovulation, while the remaining follicles undergo atresia. Ovulation is triggered by the LH surge, resulting in the release of the cumulus-oocyte-complex (COCs). Following ovulation, the remaining theca cells and mural GCs luteinise to form the corpus luteum, which produces progesterone to support implantation and early pregnancy maintenance. Figure reproduced by Edson *et al.*, 2009 (DOI: <https://doi.org/10.1210/er.2009-0012>) under the terms of the Creative Commons CC-BY license. Copyright 2009 by The Endocrine Society. License Number: 6200581279027.

During antrum formation, GCs differentiate into two distinct populations: mural granulosa cells (MGCs) and cumulus granulosa cells (CGCs). CGCs surround the oocyte and, together with it, form the cumulus-oocyte-complex (COC), which is released at ovulation. MGCs align along the follicular basal membrane, forming a barrier separating the granulosa and theca cell layers (Hennet and Combelles, 2012).

2.1.1.4. Cyclic recruitment and ovulation

The cyclic recruitment phase is coordinated with the menstrual cycle. During the follicular phase, antral follicles continue to grow and mature under gonadotropin influence until ovulation. This phase begins with the completion of the previous menstrual cycle, which is marked by a decline in oestrogen, progesterone, inhibin A and the shedding of the uterine lining.

The decrease in these hormones reduces negative feedback on the hypothalamus and anterior pituitary, leading to increased secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus, and subsequently, follicle-stimulation hormone (FSH) from the pituitary gland (Groome *et al.*, 1996; Welt *et al.*, 2003).

The development of the dominant follicle (DF) can be described in three stages: recruitment, selection and dominance, which are coordinated by endocrine and intra-ovarian mechanisms. During the recruitment phase (days 1–4 of the cycle), rising circulating FSH levels stimulate the growth of a cohort of antral follicles in each ovary (Monis and Tetrokalashvili, 2022).

As follicular growth proceeds, negative feedback from increasing oestrogen (E2) levels and inhibin B secretion by the growing follicles leads to a decline in circulating FSH concentration (Groome *et al.*, 1996; Sawetawan *et al.*, 1996; Welt *et al.*, 1997). This reduction in FSH marks the onset of the selection phase (days 5–7), during which only one follicle is selected to continue development while the remaining follicles undergo atresia (Reed and Carr, 2000). Follicle fate during this stage is influenced by intra/ovarian factors, including AMH, secreted by GCs, which contributes to reduced FSH responsiveness and promotes atresia of supportive follicles (Durlinger *et al.*, 1999; Hampl, Šnajderová and Mardešić, 2011).

The follicle that becomes dominant is distinguished by enhanced sensitivity to FSH. As GCs proliferate within the DF, FSH receptor expression increases, resulting in higher FSH receptor density and sustained responsiveness despite declining circulating FSH levels (Baerwald, Adams and Pierson, 2012). This enhanced FSH signalling promotes increased E2 production by GCs, further suppressing FSH secretion and reinforcing dominance by inhibiting the growth of competing follicles (Edson, Nagaraja and Matzuk, 2009).

By approximately day 8, the dominance phase is established. Continued E2 production from the dominant follicle ultimately triggers the preovulatory luteinizing hormone (LH) surge via positive feedback on the hypothalamic-pituitary axis (Edson, Nagaraja and Matzuk, 2009). The LH surge occurs ~34–36 hours before

ovulation (Hoff, Quigley and Yen, 1983), reaching peak levels ~10–12 hours prior to ovulation (Pauerstein *et al.*, 1978), therefore allowing precise timing of ovulation. The number of oocyte that reach dominance is species-specific; in humans this process typically results in the ovulation of a single oocyte per menstrual cycle (Gougeon, 1996).

Ovulation results in the release of the cumulus-oocyte complex (COC) and resumption of meiosis in the oocyte up to meiosis II (where it is again halted), leading to the formation of the first polar body. Following ovulation, the remaining mural granulosa cells (MGCs) and theca cells differentiate to form the corpus luteum (CL), a transient endocrine structure responsible for progesterone production, which prepares the endometrium for implantation (Edson, Nagaraja and Matzuk, 2009).

2.2. Female infertility and factors affecting DNA integrity

According to the World Health Organization (WHO), infertility is defined as the inability to achieve pregnancy after 12 months or more of regular unprotected sexual intercourse, and it is estimated to affect approximately 10% of couples of reproductive age (World Health Organization, 2023). Recent evidence also suggests that the COVID-19 pandemic may have negatively influenced fertility in both males and females, warranting further investigation (Săndulescu *et al.*, 2022).

Female fertility strongly depends on the ovarian reserve, which is established during foetal life and determines reproductive lifespan. A diminished ovarian reserve or poor oocyte quality – often associated with compromised DNA integrity – can adversely affect female fertility. Several factors influence female fertility, including:

- 1) Ageing (Li *et al.*, 2021);
- 2) Pathological conditions such as endometriosis, polycystic ovarian syndrome (PCOS), premature ovarian insufficiency (POI) and thyroid disorders (Weiss and Clapauch, 2014);
- 3) Lifestyle factors, including smoking, recreational drug use, alcohol consumption and obesity (Shelling and Ahmed Nasef, 2023);
- 4) Exposure to certain industrial chemicals (Arya *et al.*, 2020);
- 5) Life-saving medical treatments, such as chemotherapy and radiotherapy (Hanson *et al.*, 2017; Pampanini *et al.*, 2019; Poorvu *et al.*, 2019).

These factors can significantly impact fertility, and in some cases, fertility preservation (FP) may be the only option to safeguard future reproductive potential (HFEA, 2025).

2.2.1. Ageing

The female reproductive system ages more rapidly than most other systems in the human body, with the ovary being one of the earliest and most rapidly ageing organs. This ageing occurs continuously due to the ongoing recruitment of PMFs from the follicular pool throughout reproductive life (Baird *et al.*, 2005). Menopause, which typically occurs between 45 and 55 years of age, marks the end of ovarian function (McKinlay, Brambilla and Posner, 2008; Gold, 2011; El Khou-dary *et al.*, 2019). The timing of menopause is influenced by genetic factors, lifestyle and diet (Gold, 2011). Recent genetic studies have also highlighted the importance of DNA damage response (DDR) pathways, apoptosis and metabolic signalling in determining menopausal age (Day *et al.*, 2015; Ruth *et al.*, 2021).

Ovarian ageing affects both the quantity and quality of follicles. Around the fourth month of foetal development, the ovary contains approximately 6–7 million oocytes surrounded by flattened GCs, forming PMFs (Baker, 1963; Baker and Zuckerman, 1997). Apoptosis rapidly reduces this number, leaving approximately 1–2 million PMFs at birth (Markström *et al.*, 2002). After birth, follicular loss slows, with an estimated 300,000–400,000 PMFs present at menarche (te Velde and Pearson, 2002). This decline continues throughout reproductive life and falls below the threshold of approximately 1000 PMFs at menopause (Richardson, Senikas and Nelson, 1987; Faddy and Gosden, 1996; Faddy, 2000) (Figure 3).

In addition to the decline in follicular numbers, oocyte quality also decreases with age, particularly after the age of 30, contributing to a shortened reproductive lifespan (Hamatani *et al.*, 2004). Oocyte development is vulnerable to errors at multiple stages, including synapsis formation and meiotic recombination, follicle formation, follicle growth, meiotic resumption, and fertilization (Hunt and Hassold, 2008; Mikwar, MacFarlane and Marchetti, 2020). These errors help explain the higher incidence of chromosomal abnormalities observed in women of advanced maternal age (AMA).

Oocytes are especially susceptible to DNA damage due to their prolonged arrest in meiotic prophase I, and this vulnerability increases with age (Sasaki *et al.*, 2019; Rodríguez-Nuevo *et al.*, 2022; Ferreira *et al.*, 2023). With advanced age, mitochondrial dysfunction, increased oxidative stress, and declining DNA repair efficiency lead to the accumulation of DNA damage (Gorbunova *et al.*, 2007; Wong *et al.*, 2016; Sasaki *et al.*, 2019). Recent findings show that the suppression of mitochondrial activity in early-stage oocytes limits oxidative stress generation (Rodríguez-Nuevo *et al.*, 2022). Moreover, aged oocytes exhibit reduced expression of key DNA repair genes, such as *BRCA1*, *BRCA2*, *RAD51* and *ATM* (Xu *et al.*, 2024), resulting in a compromised DDR in older females (Titus *et al.*, 2013; Horta *et al.*, 2020; Rémillard-Labrosse *et al.*, 2020). Taken together, the progressive accumulation of DNA damage and diminished repair capacity contribute significantly to the age-related decline in oocyte quality (Figure 3).

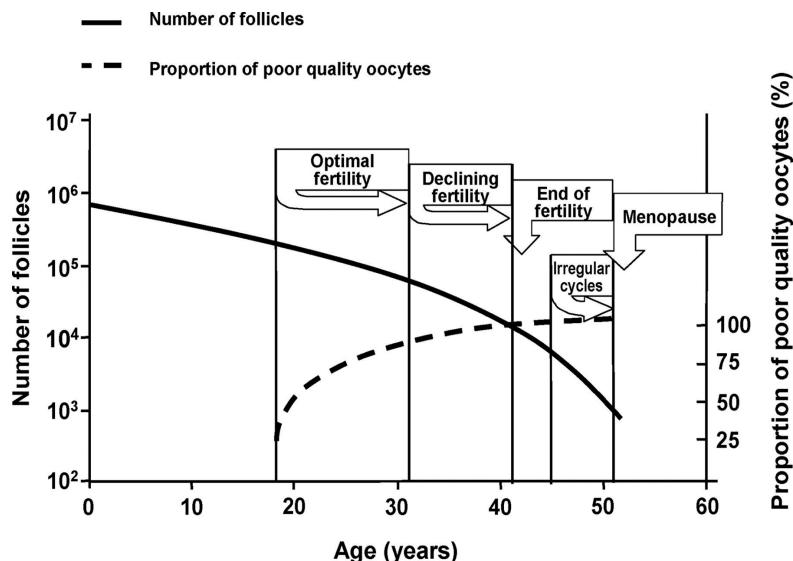


Figure 3: Overview of follicle loss and oocyte quality during female reproductive ageing. The diagram illustrates the age-related decline in the number of primordial follicles and the increase in the proportion of poor-quality oocytes, together with key reproductive milestones such as optimal fertility, declining fertility, irregular cycles and menopause. Graph reproduced by Broekmans *et al.*, 2009 (DOI: <https://doi.org/10.1210/er.2009-0006>) under the terms of the Creative Commons CC-BY license. Copyright 2009 by The Endocrine Society. License Number: 6200600025515.

2.2.2. Gonadotoxic treatment

In recent years, cancer treatments for women at reproductive age have significantly improved survival rates (Miller *et al.*, 2022). However, this increased survival often comes at the cost of impaired reproductive function due to gonadal toxicity. Chemotherapy – particularly regimens containing high doses of alkylating agents – and ionising radiation are especially damaging to ovarian function and can lead to permanent infertility in a significant proportion of patients (Roberts *et al.*, 2024).

2.2.2.1. Chemotherapy treatment

The impact of chemotherapy on female reproductive health varies depending on cancer type, the agents used, cumulative dose, treatment protocol and the patient's fertility status at the time of treatment (Goodwin *et al.*, 1999; Arnon *et al.*, 2001). Alkylating agents are particularly toxic to the ovaries, causing both direct cytotoxic damage to GCs and oocytes (Meirow *et al.*, 1999; Walshe, Denduluri and Swain, 2006) and indirect damage through accelerated PMF recruitment due to reduced AMH production (Meirow *et al.*, 2010; Rosendahl *et al.*, 2010). The severity of ovarian damage ranges from mild impairment to complete ovarian failure and POI (Goodwin *et al.*, 1999; Lee *et al.*, 2006; Hickey *et al.*, 2009).

Apoptosis is the primary mechanism behind PMF loss following chemotherapy. This was demonstrated in a human ovarian xenograft model, where a single dose of cyclophosphamide caused a peak in apoptosis 12 h after treatment and significant PMF depletion within 48 h (Oktem and Oktay, 2007). Chemotherapy-induced DNA double-strand breaks (DSBs) activate the oocyte's DDR, which can initiate DNA repair or lead to apoptotic cell death (Soleimani *et al.*, 2011; Bedoschi, Navarro and Oktay, 2016). The ataxia-telangiectasia mutated (ATM)-mediated DNA repair pathway plays a central role in the oocyte's attempt to repair such damage (Figure 4) (Oktay *et al.*, 2015).

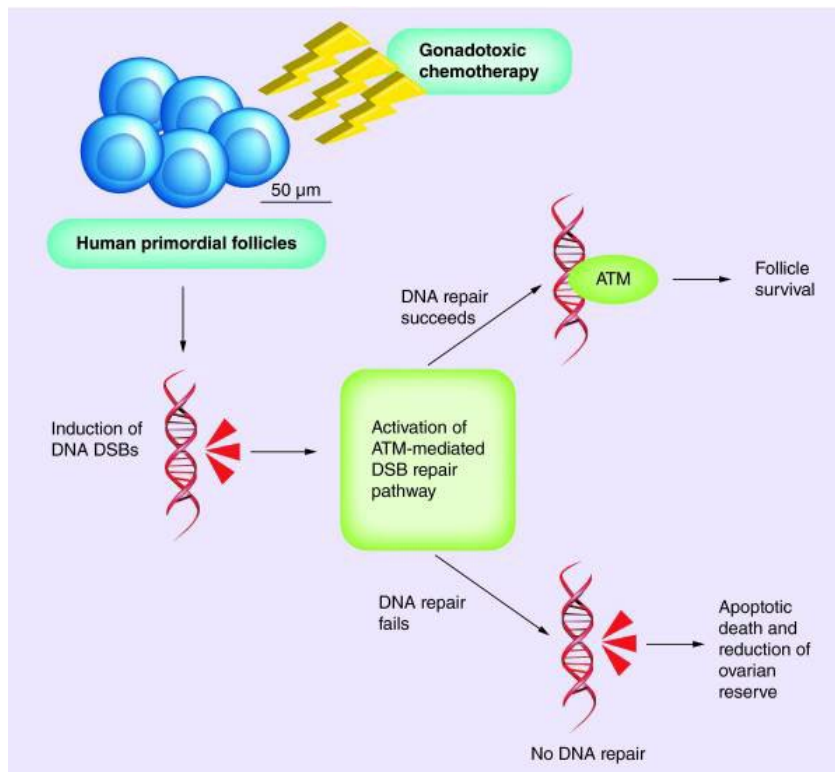


Figure 4: Mechanism of chemotherapy-induced damage in ovarian tissue. Chemotherapy agents such as cyclophosphamide and doxorubicin induce DNA double-strand breaks (DSBs) in the oocytes of primordial follicles. In response, the ATM-mediated DNA repair pathway is activated to promote DNA repair. Oocytes that successfully repair the damage may survive, while those unable to do so undergo apoptosis or enter cellular senescence. Figure reproduced by Bedoschi *et al.*, 2016 (DOI: <https://doi.org/10.2217/fo-2016-0176>) under the terms of the Creative Commons CC-BY license. Copyright 2016 by Future Medicine Ltd

A mouse study has suggested that chemotherapy-induced injury to growing follicles may reduce their inhibitory effect on PMF recruitment, leading to over-activation of PMFs in an attempt to replace lost antral follicles (Durlinger *et al.*, 1999). However, this hypothesis is primarily supported by a single rodent study, while multiple other studies have reported direct PMF apoptosis following chemotherapy exposure instead (Gonfloni *et al.*, 2009; Kerr *et al.*, 2012). Chemotherapy-induced ovarian damage also affects the ovarian stroma (Marcello *et al.*, 1990) and vascular components (Doll, Ringenberg and Yarbrow, 1986), resulting in blood vessel injury, cortical fibrosis and disruption of sex steroid production (Oktay *et al.*, 2006). Collectively, these changes impair ovarian blood supply and contribute to endocrine dysfunction.

2.2.2.2. Radiation therapy and mechanisms of DNA damage

Ionising irradiation, or radiotherapy (RT), is widely used in cancer treatment. It is often applied in combination with chemotherapy and surgery, and it is estimated that 30–50% of patients receive RT as part of their first-line treatment (Barton *et al.*, 2014). Its widespread use reflects its relatively low cost – accounting for only 5% of total cancer care expenditures – as well as its significant contribution to cancer survival, being responsible for approximately 40% of all cancer cures (Hanna *et al.*, 2018; Mee *et al.*, 2023).

However, the cytotoxic effects of RT are not limited to malignant cells; neighbouring non-malignant tissues may also be affected, leading to long-term collateral damage and compromised tissue integrity. RT-induced tissue injury involves multiple mechanisms, including inflammation, oxidative stress, cell death, extracellular matrix (ECM) remodelling and fibrosis (Zhou *et al.*, 2024).

RT damages cellular DNA either directly, through the generation of single- or double-strand breaks (SSBs or DSBs), or indirectly, via irradiation-induced free radicals and reactive oxygen species (ROS) (Figure 5) (Baskar *et al.*, 2014; Wang, Wang and Qian, 2018; Kim *et al.*, 2021).

The DDR begins with damage sensing by the protein kinases ATM, which primarily detects DNA DSBs (Stracker *et al.*, 2013), and ataxia-telangiectasia and RAD3-related protein (ATR), which mainly detects SSBs or DNA crosslinks (Stokes *et al.*, 2007). ATM and ATR initiate downstream signalling pathways by phosphorylating specific target proteins (Abraham, 2001; Shiloh, 2003). Following DDR activation, cells may undergo cell cycle arrest, allowing time for DNA repair and maintenance of genome stability (Lukas, Lukas and Bartek, 2004), or, if the damage is irreparable, initiate apoptosis (Vousden, 2000; Takasawa *et al.*, 2005).

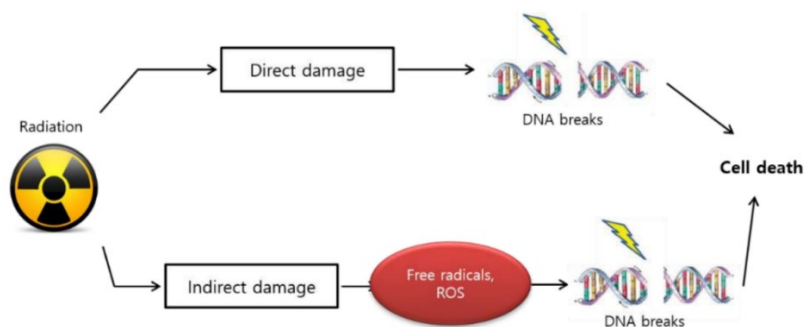


Figure 5: Biological effects of radiation on tissues. Radiation causes direct DNA damage by inducing single strand breaks (SSBs) or double strand breaks (DSBs), or indirect damage through the generation of free radicals and reactive oxygen species (ROS). Figure reproduced by Kim et al., 2021(DOI: <https://doi.org/10.3390/ijms22147484>) under the terms of the Creative Commons CC-BY license. Copyright 2021 by the authors. Published by MDPI on behalf of International Journal of Molecular Sciences.

Key regulators of cell cycle arrest include checkpoint kinases 1 and 2 (CHK1 and CHK2) (Zhou and Bartek, 2004), which modulate the activity of the tumour suppressor protein p53 (Mirzayans et al., 2012; Reinhardt and Schumacher, 2012). The extent and pattern of p53 phosphorylation determine cell fate: phosphorylation at Ser15 promotes cell cycle arrest and survival, while phosphorylation at Ser 46 triggers apoptosis (Zhang, Liu and Wang, 2011).

When p53 is phosphorylated at Ser15, it induces the expression of cyclin-dependent kinase inhibitor 1A (CDKN1A/p21), which blocks the cell cycle progression at the G1 (Weinberg and Denning, 2002) and G2 phases (Smits *et al.*, 2000) by inhibiting CDK complexes. In addition, CHK1 and CHK2 rapidly halt the cell cycle by promoting phosphorylation, ubiquitination and degradation of the CDC25 family of phosphatases, leading to cell cycle arrest (Boutros, Lobjois and Ducommun, 2007) (Figure 6).

If DNA damage is beyond repair and p53 is phosphorylated at Ser46, cells undergo apoptosis. This process is tightly regulated by members of the B-cell lymphoma 2 (BCL2) family (Cory and Adams, 2002). Nuclear accumulation of p53 induces the transcription of pro-apoptotic BCL2 family members, including p53-upregulated modulator of apoptosis (*PUMA*), BCL2-associated X protein (BAX) and NOXA (Oda *et al.*, 2000; Dogu and Díaz, 2009; Kuribayashi *et al.*, 2011). This activation leads to mitochondrial outer membrane permeabilisation and the subsequent release of cytochrome c (Dejean *et al.*, 2006; Dogu and Díaz, 2009). Cytochrome c then triggers apoptosome formation (Cain *et al.*, 2000) and activation of downstream caspases (Cain *et al.*, 1999).

This pathway may be further amplified by indirect effects of RT, causing the production of $O_2^{\cdot-}$ and other reactive oxygen species (ROS), such as H_2O_2 or $OH^{\cdot}$ radicals. These ROS further promote cytochrome c release and mitochondrial-mediated apoptosis (Richter and Frei, 1988; Ogura *et al.*, 2009) (Figure 6).

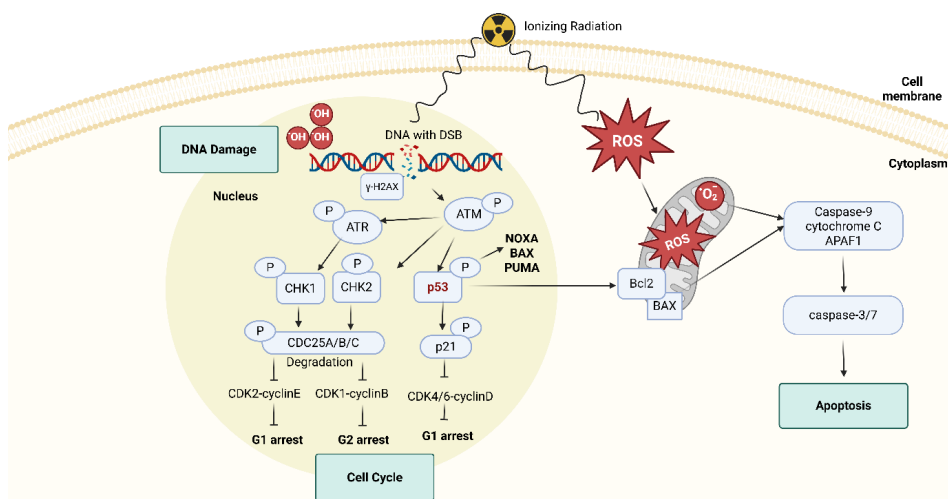


Figure 6: Schematic overview of DNA damage response (DDR) and cell fate decisions following ionising radiation. DNA lesions are sensed by the ATM and ATR kinases, which activate checkpoint proteins CHK1 and CHK2 and stabilise p53, leading to either cell cycle arrest or apoptosis. p53 induces p21 to block cell cycle progression at the G1/S or G2/M phases. If the damage is irreparable, p53 promotes apoptosis through the intrinsic mitochondrial pathway by inducing pro-apoptotic BCL2 family members such as PUMA, BAX and NOXA, resulting in cytochrome c release, apoptosome formation and caspase activation. Figure adapted by Maier et al., 2016 and modified in BioRender (DOI: <https://doi.org/10.3390/ijms17010102>) under the terms of the Creative Commons CC-BY license. Copyright 2016 by the authors. Published by MDPI on behalf of International Journal of Molecular Sciences.

2.2.2.2.1. Effect of irradiation on the ovary

Ovarian tissue is among the most radiosensitive organs of the female reproductive system, and RT-induced damage can cause long-term side effects in women of reproductive age (Shin *et al.*, 2020). The severity of RT-induced damage depends on multiple factors, including patient age, infertility history, cumulative radiation dose and the anatomical location of irradiation (Meirow and Nugent, 2001).

Dividing GCs within growing follicles appear to be the primary targets of RT, with marked cell death observed within hours after irradiation (Stroud *et al.*, 2009). Oocytes are also highly sensitive to radiation and other stressors. Pelvic irradiation at doses below 2 Gray (Gy) is estimated to deplete approximately half of the ovarian follicle reserve (Wallace, Thomson and Kelsey, 2003), while doses of around 10 Gy may result in complete ovarian failure in adults (Irtan *et al.*, 2013). Radiosensitivity varies by follicle stages, with quiescent PMF being more radioresistant than larger, actively growing follicles (Wallace, Thomson and Kelsey, 2003).

RT also affects the ovarian stroma, causing tissue shrinkage (Stroud *et al.*, 2009) and contributing to further follicular loss (Kimler *et al.*, 2018). Damage to

the ECM microenvironment has also been reported (Bohn, Zhao and Jones, 2011; Kimler *et al.*, 2018). Moreover, RT induces injuries to small blood vessels, leading to thrombosis and further impairment of ovarian function (Stroud *et al.*, 2009). Despite these observations, the specific effects of RT on distinct ovarian somatic cell populations remain poorly understood.

2.3. Fertility preservation strategies

The integrity of oocyte DNA is essential for female fertility, yet it can be compromised by various internal and external factors across a woman's life. As these risks accumulate over time, fertility preservation (FP) has become increasingly important, particularly for women undergoing gonadotoxic treatments or facing conditions associated with accelerated ovarian ageing.

Current FP strategies include oocyte cryopreservation, embryo cryopreservation and ovarian tissue cryopreservation (OTC). In addition, GnRH agonists are sometimes administered during chemotherapy treatment to suppress ovarian activity and potentially reduced treatment-related ovarian damage. However, compared with established cryopreservation methods, the effectiveness of GnRH agonists for fertility preservation remains controversial. Consequently, they are generally considered an adjunct to, rather than a replacement for, established FP strategies. While GnRH agonists may provide benefit in certain clinical settings, they do not preserve fertility as reliable as oocyte, embryo, or ovarian tissue cryopreservation (Donnez and Dolmans, 2013). Understanding the advantages and limitations of each approach is essential for selecting the most appropriate option for individual patients seeking to preserve fertility.

2.3.1. Oocyte cryopreservation

Oocyte cryopreservation is one of the most widely used FP methods. Initially, slow-freezing protocols were employed, but the introduction of the vitrification technique in 1985 (Rall and Fahy, 1985) marked a major breakthrough. Unlike slow freezing, which relies on a controlled and gradual decrease in temperature using relatively low concentrations of cryoprotective agents, vitrification employs ultra-rapid cooling and higher cryoprotectant concentrations to prevent ice crystal formation. Because slow freezing allows more time for intracellular ice crystals to develop, it is associated with a greater risk of cryoinjury compared with vitrification (Shi *et al.*, 2017).

The first successful pregnancy resulting from vitrified oocytes was reported in 1999 (Kuleshova *et al.*, 1999). Since then, vitrification has achieved post-thaw survival rates of approximately 85%, with fertilisation and live birth rates comparable to those obtained with fresh oocytes – 79% and 33%, respectively (Cobo and Diaz, 2011; Cobo *et al.*, 2013). However, these outcomes are highly age-dependent and decline with AMA. In women over 35, fertilisation rates fall to approximately 59%, and live birth rates to 17% (Borini *et al.*, 2004; Pai *et al.*,

2021; Kasaven *et al.*, 2022; Kakkar *et al.*, 2023). In 2013, based on evidence from several clinical trials, the American Society of Reproductive Medicine recognised oocyte cryopreservation as a standard clinical method for FP (Cobo *et al.*, 2008, 2010; Rienzi *et al.*, 2010; Parmegiani *et al.*, 2011).

Despite its effectiveness and suitability for both social and medical FP, this technique has important limitations. It requires ovarian stimulation and the retrieval of a sufficient number of mature oocytes for freezing, which may delay the initiation of cancer treatment in oncology patients (Mahajan, 2015; Specchia *et al.*, 2019). Moreover, the elevated hormone levels associated with stimulation may pose risks in those with hormone-sensitive tumours (Kim *et al.*, 2023).

Additionally, the removal of cumulus cells before oocyte cryopreservation may alter the properties of the *zona pellucida*, reducing fertilisation efficiency when using conventional insemination. Consequently, intracytoplasmic sperm injection (ICSI) is often the preferred method for fertilising frozen/thawed oocytes (Sánchez *et al.*, 2008; Donnez *et al.*, 2013). Crucially, oocyte cryopreservation is not suitable for prepubertal girls, for whom hormonal stimulation for is not effective for the retrieval of oocytes in a suitable maturation stage for fertilization (Resetkova *et al.*, 2013; Chen, Dong and Chen, 2023).

2.3.2. Embryo Cryopreservation

Embryo cryopreservation is considered the most established FP method (Gook and Edgar, 2007). It is a safe and effective option for many patients (Devroey, Polyzos and Blockeel, 2011; Cakmak and Rosen, 2013), with the first live birth from a cryopreserved embryo reported in 1990 (Gordts *et al.*, 1990). As with oocyte cryopreservation, vitrification yields better outcomes than slow-freezing (Debrock *et al.*, 2015), achieving post-thaw survival rates of approximately 90% and live birth rates of around 36% (ter Welle-Butalid *et al.*, 2024).

While embryo cryopreservation shares many of the same limitations as oocyte cryopreservation – including the need for ovarian stimulation and its unsuitability for prepubertal girls – it generally demonstrates slightly higher success rates. Therefore, it is particularly suitable for patients who have sufficient time to undergo ovarian stimulation and who have a male partner or access to donor sperm for fertilisation (Lee *et al.*, 2012). However, embryo cryopreservation raises ethical concerns regarding the ownership and future use of stored embryos, particularly in cases involving the death of one partner or the dissolution of the relationship (Stegmann, 2010).

2.3.3. Ovarian Tissue Cryopreservation

OTC is an advanced FP strategy and is the only available option for prepubertal girls with cancer or for patients who require immediate treatment and cannot delay therapy for ovarian stimulation (Haering *et al.*, 2024). Unlike oocyte or embryo cryopreservation, OTC preserves a large pool of PMFs within the ovarian

cortex and offers the additional benefit of potentially restoring both fertility and endocrine function following transplantation (Khattak *et al.*, 2022).

Ovarian tissue can be retrieved by biopsy, partial or total oophorectomy. The excised cortical tissue is then processed and cryopreserved in liquid nitrogen using either slow-freezing or vitrification protocols before initiation of cancer therapy (Corkum, Laronda and Rowell, 2017). Since the first reported live birth after OTC in 2004 (Donnez *et al.*, 2004), the slow-freezing method has remained the preferred approach, with more than 200 live births reported to date (Dolmans and Donnez, 2021), compared with only five reported live births reported from vitrified ovarian tissue (Suzuki *et al.*, 2015; Arapaki *et al.*, 2022; Karimizadeh *et al.*, 2025). OTC achieves live birth rates of approximately 25–33% (Jensen *et al.*, 2015; Van der Ven *et al.*, 2016; Jadoul *et al.*, 2017) and restores ovarian activity in around 92.9% of transplantations (Donnez *et al.*, 2013). Hormonal recovery is typically observed within three months, and graft function generally persists for around five years, with some reports describing activity lasting up to ten years (Andersen *et al.*, 2012).

Despite its promise, OTC has several limitations, most of which relate to surgical and biological risks. The procedure requires two surgeries, one for tissue retrieval and another for reimplantation, each carrying operative risks. Successful graft survival depends heavily on adequate vascularisation and tissue viability. The most significant concern occurs in patients with haematological malignancies or ovarian cancers, where autotransplantation may carry the risk of reintroducing malignant cells (Dolmans *et al.*, 2010). Thus, while OTC offers distinct advantages, careful patient selection and continued development of alternative strategies remain essential.

2.3.3.1. Follicle isolation, *in vitro* maturation and artificial ovaries

OTC is currently the only FP option available for prepubertal girls, among whom acute lymphoblastic leukaemia and acute myeloid leukaemia are the most frequently diagnosed cancers (Ward *et al.*, 2014). However, as discussed earlier, autotransplantation of ovarian tissue in these patients carries the risk of reintroducing malignant cells. Several studies suggest that the basement membrane surrounding ovarian follicles may act as a physical barrier that protects them from malignant infiltration (Irving-Rodgers and Rodgers, 2006). Therefore, isolating malignant cell-free follicles followed by *in vitro* maturation (IVM) represents a promising future FP strategy, particularly for this high-risk patient group.

Numerous studies have explored methods for isolating preantral follicles across different species, including rodents (Pangas *et al.*, 2003; Hornick *et al.*, 2013; Mochida *et al.*, 2013), domestic animals (Xu *et al.*, 2006; McLaughlin and Telfer, 2010; Silva *et al.*, 2015), non-human primates (Xu *et al.*, 2010) and humans (Telfer *et al.*, 2008; Kristensen *et al.*, 2011; Wang *et al.*, 2016; Yin *et al.*, 2016).

One of the major challenges is selecting an optimal follicle isolation technique. Follicles can be isolated mechanically, enzymatically, or through a combina-

tion of both. Mechanical isolation is considered safer but is technically demanding and time-consuming (Tajbakhsh *et al.*, 2024). Previous studies indicate that combining mechanical and enzymatic approaches results in higher survival rates of PMFs compared to mechanical isolation alone (Dong *et al.*, 2014).

The second challenge involves selecting an appropriate scaffold that can mimic the native ovarian microenvironment. Initially, isolated follicles were cultured in two-dimensional (2D) systems (Eppig and O'Brien, 1996), which later evolved into three-dimensional (3D) alginate-based cultures (Pangas *et al.*, 2003). However, these approaches have shown limited success; to date, only a single mouse study has reported live offspring using such systems (Eppig and O'Brien, 1996; Mochida *et al.*, 2013). These limitations have driven the development of artificial ovaries, and multiple efforts have been made to engineer constructs that more closely replicate the structure and function of the natural ovarian microenvironment (Dolmans and Amorim, 2019).

The final major challenge relates to the growth dynamics of isolated follicles. Studies have demonstrated that follicle-follicle interactions play an essential role in supporting normal follicular development (Hornick *et al.*, 2013). Furthermore, recent work has indicated the presence of two distinct follicle populations within the ovarian cortex (Rooda *et al.*, 2024). Taken together, these observations – along with the coordinated growth of multiple follicles before DF selection – suggest that identifying reliable biomarkers for DF determination may be critical for overcoming current limitations in follicle isolation and *in vitro* maturation strategies.

2.4. The microbiome and its contribution to female reproduction

In recent years, the microbial composition of the female reproductive tract has received increasing attention due to its emerging role in reproductive health and disease. Several studies have shown that the female reproductive tract harbours a diverse bacterial community (Evans *et al.*, 2016; Benner *et al.*, 2018; Koedooder *et al.*, 2019), and microbial dysbiosis has been associated with several infertility-related conditions (Moreno *et al.*, 2016; Molina *et al.*, 2020).

The reproductive tract microbiome has also been linked to embryo implantation success. Patients with a *Lactobacillus*-dominant endometrium show significantly higher implantation and pregnancy rates compared to those who have non-*Lactobacillus*-dominated profiles (Moreno *et al.*, 2016; Moreno and Frana-siak, 2017). Emerging evidence suggests that the microbiome may also influence ovarian reserve. Certain microbial metabolites – particularly fatty acids – may regulate the expression of key genes such as *Nobox* and *Amh*, which are critical for early folliculogenesis and primordial follicle activation (Munyoki *et al.*, 2024). However, the specific effects of reproductive tract microbiomes on oocyte quality remain largely unexplored.

Studies have further suggested the existence of an ovary-specific microbiome, although most research so far has focused on ovarian cancer tissues (Qin *et al.*, 2022). The microbial composition of healthy ovarian tissue remains poorly characterised, largely because of the technical challenges in obtaining contamination-free samples (Canha-Gouveia *et al.*, 2023).

Finally, emerging evidence suggests that maintaining a balanced ovarian microbiome – or its interaction with the gut microbiome – may help delay ovarian ageing (Huang *et al.*, 2024; Munyoki *et al.*, 2024). A deeper understanding of the healthy ovarian microbiome could therefore support the development of future strategies to preserve ovarian function and improve outcomes in fertility preservation procedures, including ovarian tissue cryopreservation, transplantation and ovarian stimulation.

2.5. Summary of the literature review

The ovary is a highly dynamic organ. Within this organ, one of the primary functions are the establishment, maintenance and depletion of the ovarian reserve. These functions are regulated by complex interactions between germ cells, somatic cell populations and the surrounding microenvironment. Folliculogenesis is a highly optimised process occurring from foetal stage until menopause, and its successful progression is essential for female fertility. However, this process is highly vulnerable to multiple stressors occurring throughout life. Ageing, pathological conditions, lifestyle factors, environmental exposures and life-saving treatments contributed to compromised ovarian function, ultimately affecting reproductive potential.

Gonadotoxic cancer treatments, such as chemotherapy and radiotherapy, represent a major threat to ovarian health. The DNA damage induced triggers the DNA damage response pathways, resulting in either repair or apoptosis. While these mechanisms are essential for maintaining genomic integrity, they also contribute to the depletion of ovarian reserve and disruption of the ovarian microenvironment. Despite advance in understanding these processes, the specific responses of distinct ovarian somatic cell population, as well as their capacity to recover or adapt, remain insufficiently characterised. Furthermore, current knowledge is derived from bulk or single-layer analyses, which fail to capture the complexity of the cell-cell interaction within the ovary.

Fertility preservation strategies have been developed to minimise these side effects, yet each approach presents important limitations. In particular, ovarian tissue cryopreservation offers unique advantages but remains associated with challenges related to tissue viability, follicle survival and safe transplantation. At the same time, emerging areas such as *in vitro* follicle culture and artificial ovarian constructs highlight advances in fertility preservation. Moreover, the growing recognition of the microbiome in reproduction raises the possibility that the ovarian microbiome may further contribute to improved fertility preservation strategies. Altogether, these aspects highlight the need for a more integrated

understanding of ovarian biology. Multi-omics approaches offer a powerful framework to comprehensively investigate cellular heterogeneity, molecular responses to damage and previously unexplored aspects such as ovary-specific microbiomes. Bridging these knowledge gaps is essential for improving current fertility preservation strategies and for developing novel approaches aimed at maintaining ovarian function and reproductive health.

3. AIMS OF THE STUDY

The main aim of the study was to investigate how ovarian tissue responds to cancer treatments and fertility preservation approaches.

The specific aims of the study were:

1. To explore the response of human primary ovarian somatic cells (POCs) derived from cortex (cPOCs) and from medulla (mPOCs) to an acute dose of X-ray irradiation.
2. To assess the molecular and functional impact of irradiation on ovarian somatic cells, including DNA damage response activation, cell cycle regulation and the ability to form a novel 3D ovarian model (Silk-Ovarioid).
3. To investigate the effect of vitrification on ovarian cortex structure and the survival of preantral follicles following isolation using bovine model.
4. To examine vitrification-induced molecular alterations, focusing on changes that could be targeted in further developments of vitrification protocol.
5. To examine the presence or absence of microbiome-derived DNA in healthy human ovarian tissue.

4. MATERIALS AND METHODS

4.1. Ethical statement (Study I–III)

Study I and Study III involved human ovarian tissue and were approved by the Stockholm Regional Ethical Review Board (Study I: license number 2024-08606-01; Study III: license number 2015/798-31). Written informed consent was obtained from all participants, and all procedures were conducted in accordance with ethical guidelines for research involving human subjects.

Study II used ovarian tissue obtained from a slaughterhouse; therefore, no ethical approval was required.

All samples were pseudonymised, and all personal data were handled in compliance with the European General Data Protection Regulation (GDPR).

4.2. Study participants, sample collection and processing (Study I–III)

In studies I and III, human ovarian tissue was obtained from patients undergoing gender-affirming surgery at Karolinska University Hospital Huddinge, Sweden:

- Study I: n=8 patients; ages 23–36.
- Study III: n=11 patients; ages 23–37, plus 6 follicular fluid samples.

Additionally, for study III, ovarian cortical tissue was collected from women undergoing caesarean section at term (n=5; ages 26–31). All patients provided written informed consent before surgery, in accordance with the Declaration of Helsinki. Bilateral oophorectomy was performed during gender-affirming surgery to obtain ovarian tissue.

For study II, bovine ovaries were collected from a local slaughterhouse in collaboration with the Institute of Veterinary Medicine and Animal Sciences at the Estonian University of Life Sciences in Tartu, Estonia (n=19; ages 2–8).

Across all studies, tissue was transported to the laboratory within 30 minutes in the following media:

- Studies I and III: Dulbecco's Phosphate Buffered Saline (DPBS) supplemented with calcium, magnesium, glucose and sodium pyruvate (Life Technology).
- Study II: L-15 Leibowitz's L-15 medium (Sigma-Aldrich).

Upon arrival, ovarian tissue was processed by removing the medulla and isolating the cortex at a depth of 1–1.5 mm. Cryopreservation strategies differed between studies:

- Study I: Cortex and medulla were cryopreserved separately using a slow-freezing protocol (Schmidt *et al.*, 2003).
- Study II: Ovarian cortex was cryopreserved using a vitrification protocol (Sheikhi *et al.*, 2013).

- Study III: Samples were snap-frozen and stored at -80 °C for downstream analyses.

4.3. Study design (Studies I-III)

In Study I, the objective was to assess the effects of X-ray irradiation on primary ovarian cells (POCs). Following an initial dose-finding experiment in KGN cells, cortical (cPOCs) and medullary (mPOCs) were exposed to an acute 10 Gy irradiation dose. Post-irradiation analyses included:

- Metabolic activity assays: intracellular ATP levels and mitochondrial dehydrogenase activity were measured over a 7-day culture period.
- Immunofluorescence staining: performed at 1 h, 4 h, and 24 h post-irradiation, with non-irradiated controls collected at 1 h, targeting established irradiation-responsive markers in cPOCs and mPOCs.
- Transcriptomic and proteomic profiling: conducted at 1h, 4 h and 24 h post-irradiation, with non-irradiated controls sampled at 1 h and 24 h.
- Functional assessment: the ability of irradiated cPOCs and mPOCs to generate Silk-Ovaroids was evaluated as a measure of their regenerative and organisational capacity (Figure 7).

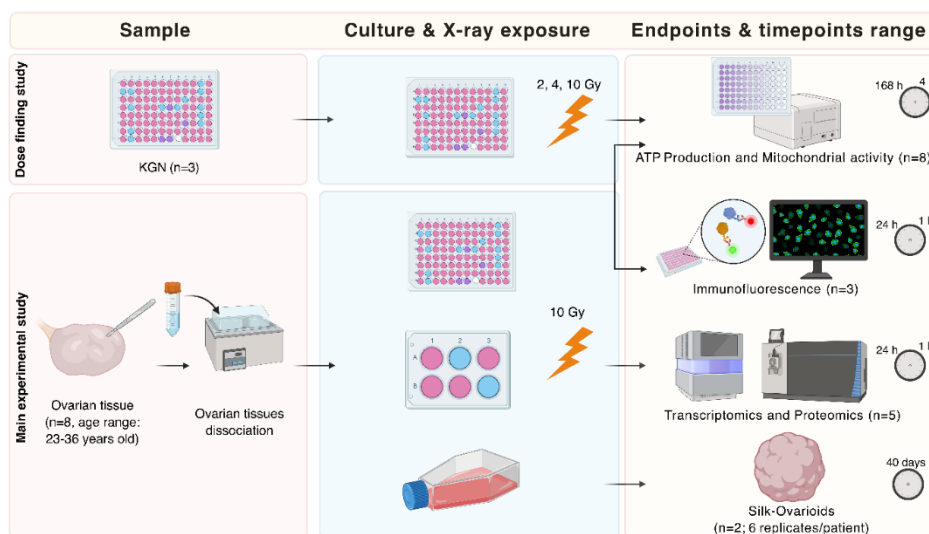


Figure 7. Schematic overview of the experimental design for Study I. The figure illustrates the workflow from primary ovarian cell dissociation and *in vitro* culture to X-ray irradiation and subsequent downstream analyses. cPOCs and mPOCs were exposed to 10 Gy (following a dose-finding study in KGN cells), after which mitochondrial function (ATP levels and mitochondrial dehydrogenase activity), immunofluorescence, transcriptomic and proteomic profiling, and Silk-Ovarioid formation were assessed. **Abbreviations:** ATP – adenosine triphosphate; cPOCs – cortex-derived primary ovarian cells; Gy – Gray; mPOCs – medulla-derived primary ovarian cells. Figure created with BioRender.com.

In Study II, the primary objective was to evaluate the effects of vitrification on the ovarian cortex and isolated follicles, using bovine ovaries as a model system. The ovarian cortex (depth ~1–1.5 mm) was dissected from the medulla and subsequently cut into small fragments (~2 × 5 × 1–1.5 mm). These fragments were assigned to four experimental groups:

- Fresh tissue (FT)
- Vitrified–thawed tissue (VT)
- Fresh tissue cultured for six days (FTC)
- Vitrified tissue cultured for six days (VTC).

All samples were analysed using the following approaches:

- Hematoxylin and eosin (H&E) staining and follicle density assessment to evaluate the morphological characteristics and compare follicular integrity across groups.
- Immunofluorescence staining, including Ki67 for assessing cell proliferation and TUNEL for detecting apoptosis within the four groups.
- Functional assessment, in which isolated follicles from FT and VT samples were cultured and monitored for viability over six days.
- Transcriptomics profiling, performed through bulk RNA sequencing of all four groups to identify vitrification-associated changes in gene expression (Figure 8).

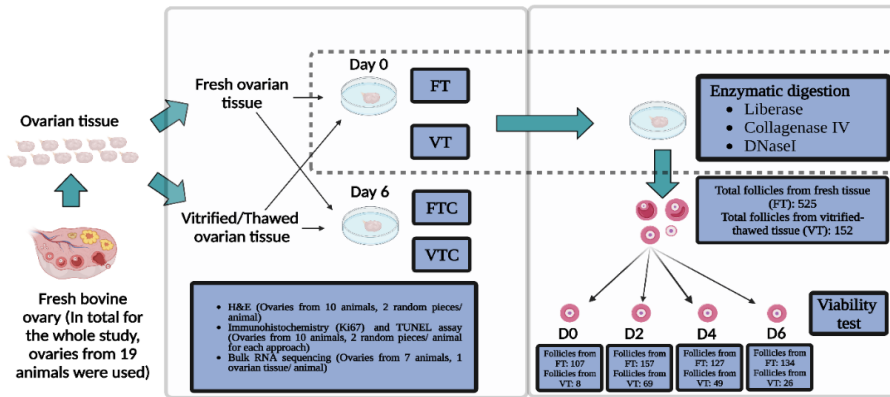


Figure 8. Schematic representation of the experimental design for Study II. Bovine ovarian tissue from 19 animals was collected, and after separation of the cortex and medulla, only the cortical tissue was used. Cortex fragments were assigned to four groups: FT (fresh tissue), VT (vitrified tissue), FTC (fresh tissue cultured for 6 days), and VTC (vitrified-thawed tissue cultured for 6 days). Tissue samples were analysed using H&E staining (n = 10 animals), Ki67 immunohistochemistry (n = 10 animals), TUNEL assay (n = 10 animals), and RNA sequencing (n = 7 animals). For FT and VT, samples from all 19 animals underwent enzymatic follicle isolation followed by follicle culture at multiple time points (day 0: n = 3 animals; day 2: n = 3 animals; day 4: n = 3 animals; day 6: n = 10 animals) to assess follicle survival using the LIVE/DEAD assay. **Abbreviations:** FT – fresh tissue; FTC – fresh tissue cultured for 6 days; VT – vitrified–thawed tissue; VTC – vitrified–thawed tissue cultured for 6 days; H&E – hematoxylin and eosin. Figure created with BioRender.com.

In Study III, the primary aim was to determine whether an ovary-specific microbiome exists. Human ovarian tissue was obtained from patients undergoing gender-affirming surgery and from patients delivering by caesarean section, together with follicular fluid samples from the gender-affirming cohort.

In the gender-affirming cohort, ovarian tissue was dissected into cortex and medulla, and follicular fluid was aspirated from visible antral follicles. From caesarean section patients, small cortical biopsies (approximately 5×2 mm) were collected. All samples were snap-frozen and stored at -80°C .

To control the potential environment or procedural contamination, multiple negative controls were included: saline storage buffer, laboratory air, operating room air, clinical staff hand swabs during sample handling, and researcher hand swabs. DNA was extracted from all samples and all negative controls, followed by 16S rRNA sequencing to characterise microbial DNA profiles.

4.4. Ovarian tissue cryopreservation and thawing

Ovarian tissue was separated into cortex and medulla before processing. In study I, both cortical and medullary tissue were cryopreserved using a slow-freezing protocol (Schmidt *et al.*, 2003). In study II, only cortical tissue was cryopreserved using a vitrification protocol (Sheikhi *et al.*, 2013). In study III, cortical biopsies were snap-frozen and stored at -80°C for downstream analyses.

Slow-freezing protocol (Study I):

Cortical and medullary tissue pieces ($5 \times 5 \times 1$ mm) were equilibrated on ice for up to 30 min in freezing solution containing 7.5% ethylene glycol (Sigma-Aldrich, USA), 33.9 mg/ml sucrose (Sigma-Aldrich), 10 mg/ml human serum albumin (HSA, Vitrolife), prepared in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline (PBS^{-/-}; Thermo Scientific). After equilibration, samples were transferred into Nunc™ CryoTube™ vials (ThermoFisher Scientific) containing 1 ml of the same freezing medium, and cryopreserved using a controlled-rate freezer (Kryo 230-1.7, Planer PLC).

Thawing procedure for slow-frozen tissue:

Vials were warmed at a 37°C water bath and tissue pieces were sequentially transferred to three thawing solutions (room temperature), each for 10 min with gentle agitation:

1. Thawing Medium I: 85 mg/ml sucrose, 1.26% ethylene glycol, and 1% HSA in PBS^{-/-}
2. Thawing Medium II: 85 mg/ml sucrose and 1% HSA in PBS^{-/-}
3. Thawing Medium III: 1% HSA in PBS^{-/-}

Vitrification protocol (Study II):

Cortical fragments were incubated twice in vitrification solution at room temperature – first for 2 min, then for 3 min. The solution consisted of 40% (v/v)

ethylene glycol (ThermoFisher Scientific), 30% (v/v) Ficoll 70 (Sigma-Aldrich), 0.5 M sucrose, and 10 mg/ml bovine serum albumin (BSA, Sigma-Aldrich), prepared in PBS^{-/-}. After equilibration, tissue fragments were transferred into Nunc™ CryoTube™ vials and stored in liquid nitrogen.

Thawing procedure for vitrified tissue:

Samples were warmed in a 37°C water bath, followed by sequential incubation in

1. 1 M sucrose solution for 2 min
2. 0.5 M sucrose solution for 3 min

Both diluted in PBS^{-/-} supplemented with glucose and pyruvate.

Finally, tissues were washed for 5 min in PBS^{-/-} containing glucose, pyruvate, and 10 mg/ml BSA.

4.5. Tissue culture and fixation (Study II)

For study II, two randomly selected replicates from both fresh and vitrified tissues were fixed overnight in Bouin's solution (VWR Chemicals), 4% paraformaldehyde (PFA; ThermoFisher Scientific), or RNAlater (ThermoFisher Scientific). Additionally, two replicates of fresh and vitrified cortical fragments were cultured in 96-well plates containing 150 µl of α MEM medium supplemented with 0.1 mg/ml penicillin–streptomycin (ThermoFisher Scientific), 1× insulin–transferrin–selenium (ITS; ThermoFisher Scientific), 50 mg/ml L-ascorbic acid, 10 mg/ml BSA (Sigma-Aldrich), and 3 mM glutamine (ThermoFisher Scientific). Cultures were maintained for up to six days at 37°C in a humidified incubator with 5% CO₂. Half of the medium was replaced every two days with pre-equilibrated cultured medium. After the six-day culture period, the tissue fragments were fixed as described above.

4.6. KGN cell culture (Study I)

KGN cells, a human granulosa cell line derived from an ovarian tumour (Nishi *et al.*, 2001), were used for irradiation dose screening due to their high proliferation rate and transcriptomic similarity to POCs (Tarvainen *et al.*, 2023). The KGN cell line was obtained from the RIKEN Cell Bank (RBRC-RCB1154, Japan).

Cells were cultured in DMEM/F12 medium (Life Technologies) supplemented with 10% heat-inactivated foetal bovine serum (HI-FBS; Life Technologies), 1% penicillin–streptomycin (Life Technologies), and 1% GlutaMAX™ (100X; Life Technologies). Cultures were maintained at 37°C in a humidified 5% CO₂ incubator, with medium refreshed every two days. Seeding densities varied depending on the downstream experimental setup and are detailed in Table 1.

Table 1. Seeding densities of KGN cells and primary ovarian cells (cPOCs and mPOCs) used in Study I. The table summarises the plate or flask formats, experimental setups and the corresponding seeding densities used for each cell type.

Type of cells	Plate/flask	Experiment set up	Seeding density
KGN cPOCs m POCs	96-well plate	CellTiter and MTT	1.5×10^4 cells/well
	T75 flask	Thawing cells	2×10^6 cells/flask
cPOCs mPOCs	96-well plate	Immunofluorescence	1.5×10^4 cells/well
	6-well plate	Transcriptomic/proteomics	1.5×10^5 cells/well

4.7. Ovarian tissue dissociation and primary ovarian cell culture (Study I)

Primary ovarian cells (POCs) were isolated separately from the ovarian cortex and medulla using an enzymatic dissociation protocol adapted from Wagner et al. (2020). Briefly, tissue pieces were finely minced with scalpels and digested in an enzymatic solution containing 1 mg/ml collagenase IA (Sigma Aldrich), 50 µg/ml LiberaseTM (Roche Diagnostics), and 10 IU/ml DNase I (Roche Diagnostics), diluted in Dulbecco's Modified Eagle Medium F12 (DMEM/F12; Life Technology) supplemented with 2.5% HI-FBS (Life Technology). Digestion was carried out at 37 °C in a shaking water bath for up to 50 min. Enzymatic activity was stopped by adding an equal volume of DMEM/F12 (Life Technology) supplemented with 10% HI-FBS (Life Technology). The suspension was centrifuged at 300 x g (centrifuge 5702R; Eppendorf) for 5 min, and the pellet was resuspended in low glucose Dulbecco's Modified Eagle Medium containing pyruvate (DMEM low glucose, pyruvate; Gibco, Life Technology), supplemented with 10% HI-FBS and 1% penicillin-streptomycin.

The resulting cell suspension was filtered through a 40 µm cell strainer (VWR), and cell numbers were quantified using the MOXI mini automated counter (ORFLO Technologies), counting cells between 7.5 and 25 µm in diameter. Cortex-derived primary ovarian cells (cPOCs) and medulla-derived primary ovarian cells (mPOCs) were maintained at 37 °C in a humidified incubator with 5% CO₂, with medium changed every two days. Seeding densities used for each assay are listed in Table 1.

4.8. KGN and POCs X-ray irradiation (Study I)

KGN cells, cPOCs, and mPOCs were irradiated using a CIX2 X-ray irradiator cabinet (Xstrahl, Surrey, England) at the X-ray Irradiation Core Facility, Karolinska Institutet (Huddinge, Sweden). Single-dose exposures were delivered at a dose rate of 1.33 Gy/min (dosimetry uncertainty approximately 1.7%) using 195

kV and 10 mA settings. The focus-to-skin distance (FSD) was set to 40 cm, and a 3.0 mm aluminium filter was applied, resulting in a half-value layer (HVL) of 9.0436 mm Al. A rotating platform ensured uniform dose distribution across all samples. Control samples were transported and handled identically but were not exposed to X-rays.

To determine the optimal irradiation dose for POCs, KGN cells were exposed to 2, 4, or 10 Gy. The upper limit of 10 Gy was selected based on clinical survivorship guidelines recommended by the PanCareLIFE Consortium (Mulder *et al.*, 2021).

4.9. Histological analysis (Study I-II)

4.9.1. Histological analysis of ovarian tissue post-vitrification/thawing and culture (Study II)

For histological analysis, ovarian cortical tissue fixed in Bouin's solution was collected from 10 animals, with two randomly selected technical replicates per animal. After fixation, tissues were dehydrated through graded ethanol (EtOH) series, followed by two sequential xylene incubation (each 1.5 h), and subsequently embedded in paraffin. Paraffin blocks were sectioned at 4 µm thickness using a rotary microtome (MICROM HM 355S; ThermoFisher Scientific). Sections were stained with H&E following a standard protocol (Cardiff, Miller and Munn, 2014), scanned at 20× magnification using a slide scanner (Leica SCN400), and analysed using Aperio ImageScope software (Leica Biosystems).

Follicle stages in the bovine ovarian cortex were classified based on GC morphology as follows:

- Monolayered follicles – one layer of flattened or cuboidal GCs surrounding the oocyte
- Secondary follicles – multiple GC layers surrounding the oocyte
- Antral follicles – presence of an antral cavity with multiple GC layers.

Atretic follicles was identified based on the presence of pyknotic oocytes or GCs (Rodgers and Irving-Rodgers, 2010). Counting was performed independently and blindly by two researchers, and only follicles with a clearly visible oocyte nucleus were included to avoid double-counting. Follicle density was calculated as the number of follicles per analysed tissue area.

4.9.2. Immunofluorescence staining (Study I-II)

Immunofluorescence staining was performed on two randomly selected technical replicates of ovarian cortical tissue from 10 animals to assess cell proliferation (Study II), and on irradiated cPOCs (n = 3) and mPOCs (n = 3) to evaluate activation of irradiation-responsive pathways, including DNA damage, apoptosis, and cell cycle (Study I). Primary antibodies used are listed in Table 2.

For Study I, irradiated and non-irradiated cPOCs and mPOCs were fixed with 4% methanol-free formaldehyde (FA; Sigma-Aldrich) at 1 h, 4 h, and 24 h post-

irradiation. For Study II, ovarian tissue sections were prepared following fixation in 4% PFA, paraffin embedding, and microtome sectioning as described previously.

Paraffin-embedded sections were deparaffinised and rehydrated through a graded ethanol series, followed by heat-mediated antigen retrieval at ~98 °C in 10 mM sodium citrate buffer (pH 6.0) for up to 20 min. Sections were then permeabilised for 20 min in 0.1% Triton X-100 and incubated for 1 h in 5% goat serum. POCs were permeabilised for 1 h in blocking buffer containing 0.3% Triton X-100 (Sigma-Aldrich), 3% normal donkey serum (NDS; Nordic Biosite), and 5% BSA (Sigma-Aldrich). All samples were incubated overnight at 4 °C with primary antibodies (Table 2). Negative controls were incubated in the relevant antibody diluent: 1% BSA/PBS for bovine ovarian tissue and blocking buffer for POCs.

Table 2: Overview of primary antibodies used for immunofluorescence staining in Study I (POCs) and Study II (bovine ovarian tissue).

Type of marker	Antibody	Host	Company	Catalog Number	Dilution
DNA Damage	p-Histone H2AX (Ser139)	Mouse	Cell Signaling Technology	80312	1: 200
	p-Chk1 (Ser345)	Rabbit		2348	1: 1000
Apoptosis	p-p53 (Ser15)	Mouse	Invitrogen	MA5-15229	1: 200
	Cleaved caspase 3	Rabbit	Cell Signaling Technology	9661	1: 400
	Bcl-2	Mouse	Santa Cruz Biotechnology	sc-7382	1: 50
Cell cycle	Cyclin D1	Rabbit	Cell Signaling Technology	555065	1: 1000
	Cyclin E	Mouse	Invitrogen	32-1500	1: 250
	p-p21 (Ser146)	Mouse	Santa Cruz Biotechnology	sc-377515	1: 250
Proliferation	Ki67 monoclonal antibody (SolA15)	Mouse/Rat	Invitrogen	14-5698-82	1:200

After overnight incubation with primary antibodies, both tissue sections and POCs were washed three times in PBS and subsequently incubated for 1 h at room temperature with the appropriate secondary antibodies (Table 3). Following secondary antibody incubation, samples were treated with 4',6-diamidino-2-

phenylindole dihydrochloride (DAPI; Sigma-Aldrich) for 20 min for nuclear counterstaining, and were then washed to remove excess dye. Tissue sections were mounted using Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich), whereas POC slides were mounted with DAKO Fluorescence Mounting Medium (Agilent Technologies). All mounted slides were stored at 4 °C in the dark until imaging.

Table 3: Overview of secondary antibodies used for immunofluorescence staining in Studies I and II.

Primary antibody used	Secondary Antibody	Host	Company	Dilution
p-Histone H2AX (Ser139)	Anti-mouse Alexa Fluor 647	Donkey	Invitrogen	1:200
p-Chk1 (Ser345)	Anti-rabbit Alexa Fluor 594		Santa Cruz Biotechnology	
p-p53 (Ser15)	Anti-mouse Alexa Fluor 647		Invitrogen	
Cleaved caspase 3	Anti-rabbit Alexa Fluor 594		Santa Cruz Biotechnology	
Bcl-2	Anti-mouse Alexa Fluor 488		Invitrogen	
Cyclin D1	Anti-mouse Alexa Fluor 647		Invitrogen	
Cyclin E	Anti-rabbit Alexa Fluor 594		Santa Cruz Biotechnology	
p-p21 (Ser146)	Anti-mouse Alexa Fluor 488	Goat	Invitrogen	1:1000
Ki67	Anti-mouse Alexa Fluor 488		ThermoFisher Scientific	

Tissues stained for Ki67 (Study II) were imaged at 10× magnification using a fluorescence microscope. Ki67-positive (Ki67⁺) cells were counted in a blinded manner, and Ki67⁺ cell density was calculated by dividing the number of positive cells by the total ovarian tissue area. Data were reported in arbitrarily normalised units.

For markers related to DNA damage, apoptosis, and cell cycle regulations (Study I), imaging was performed using a Nikon Eclipse Ti2 confocal microscope equipped with a 20× air objective and an additional 1.5× magnification lens. Emission filters were configured to detect blue (438/24), green (511/20), red (560/25) and far-red (685/40) fluorescence, and images were acquired using a Kinetix sCMOS camera (Crest Optics). All images were processed uniformly using Omero Figure software, applying consistent adjustments to contrast and brightness across experimental groups.

Quantification of immunofluorescence was conducted using pipelines developed in CellProfiler (version 4.2.5), following a previously described approach (Stirling *et al.*, 2021). In brief, uneven illumination in the DAPI channel was corrected using a Gaussian filter. Nuclei were segmented based on DAPI fluorescence, and marker-specific fluorescence intensity was measured per cell. Data extraction and downstream analyses were performed in RStudio, and protein expression levels were expressed as fluorescence intensity ratios.

4.9.3. TUNEL assay (Study II)

To detect fragmented DNA in ovarian tissue following vitrification/thawing and culture, the DeadEnd™ Fluorometric TUNEL System (Promega) was used according to the manufacturer's instructions. The assay was performed on two randomly selected 4% PFA-fixed replicates collected from 10 animals.

Briefly, tissue sections were deparaffinized and rehydrated, followed by a 15-minute incubation at room temperature in equilibration buffer. Sections were then incubated with a reaction mixture containing equilibration buffer, biotinylated nucleotide mix, and recombinant terminal deoxynucleotidyl transferase (rTdT) at 37 °C for 60 minutes. Negative controls were treated identically, except that rTdT was omitted and replaced with Milli-Q water. The reaction was terminated using 2× saline sodium citrate (SSC) for 15 minutes at room temperature, and nuclei were counterstained with DAPI (1:1000). Slides were mounted with Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich) and stored at 4 °C.

TUNEL-positive cells were visualised at 10× magnification and semi-automatically quantified using the TUNEL Cell Counter tool in Fiji (ImageJ), following previously established guidelines (Maidana *et al.*, 2015). The number of TUNEL-positive cells was normalised to the tissue area, and results were reported in arbitrary normalised units.

4.10. Retrieval and *in vitro* maintenance of follicles from ovarian cortex (Study II)

To ensure successful follicle isolation and minimise contamination, all solutions were freshly prepared every two weeks and filtered using 0.22 µm GVS syringe filters (Novatech International).

Pre-antral follicles were isolated from fresh and vitrified ovarian cortex using 20 technical replicates from 19 biological replicates (animals), following the previously described protocol (Kristensen *et al.*, 2011). Briefly, ovarian cortex tissue was manually minced into fragments of approximately 0.1 mm³ in handling medium composed of McCoy's 5A with 25 mM HEPES (ThermoFisher Scientific), 0.5 mg/ml BSA (Sigma-Aldrich), 2 mM GlutaMAX (ThermoFisher Scientific), 100 U/ml penicillin/streptomycin (ThermoFisher Scientific), and 1× ITS (ThermoFisher Scientific).

Manual disaggregation was followed by enzymatic digestion using the same handling medium supplemented with 50 mg/ml Neutral Red (NR; Sigma-Aldrich), 0.04 mg/ml Liberase™ (Sigma-Aldrich), 0.2 mg/ml collagenase IV (ThermoFisher Scientific), and 0.2 mg/ml DNase I (Roche). Digestion was performed at 37 °C with gentle agitation for up to 60 minutes. The reaction was stopped by adding an equal volume of termination medium consisting of handling medium supplemented with 10% FBS (ThermoFisher Scientific) and 100 U/ml penicillin/streptomycin.

NR-positive viable follicles were collected using an EZ-range type stripper pipette (Cooper Surgical), washed in fresh handling medium, and subsequently encapsulated. For encapsulation, 4–7 NR-positive follicles were suspended in 0.5% alginate solution (Sigma-Aldrich), prepared as described by Hornick *et al.*, (2013), and dispensed into a cross-linking solution (140 mM NaCl and 50 mM CaCl₂ in Milli-Q water) to form alginate beads. After 2 minutes, beads were rinsed in pre-warmed α MEM culture medium (Sigma-Aldrich) supplemented with 0.1 mg/ml penicillin–streptomycin, 1 $\times$ ITS, 50 mg/ml L-ascorbic acid (Sigma-Aldrich), 100 ng/ml recombinant Activin A (Bio-Techne), 50 ng/ml recombinant human FSH (ProSpec), and 3 mg/ml BSA.

Encapsulated follicles were transferred to a 96-well plate containing 150 μ l of culture medium and maintained at 37 °C in a humidified incubator with 5% CO₂ for up to six days. Every two days, half of the medium was replaced with freshly prepared, pre-warmed culture medium.

4.10.1. LIVE/DEAD staining for isolated follicle viability assessment

Follicle viability was assessed immediately after isolation (Day 0), and on Days 2, 4, and 6 of *in vitro* culture using a double fluorescent staining approach with Calcein AM and ethidium homodimer-1 (EthD-1), following the manufacturer's instructions (LIVE/DEAD™ Viability/Cytotoxicity Kit for Mammalian Cells; ThermoFisher Scientific) and previously published protocols (Dolmans *et al.*, 2006). Alginate-encapsulated follicles were incubated with 1.6 mM Calcein AM and 0.5 mM EthD-1 for 20–45 minutes at 37 °C in the dark.

Fluorescence signals were examined using a Nikon Eclipse TS100 microscope. Green fluorescence indicated viable cells, while red fluorescence indicated membrane-compromised (dead) cells. Follicles with green-stained oocytes and $\geq 60\%$ viable GCs were classified as viable; those with red-stained oocytes and $\geq 60\%$ non-viable GCs were categorised as atretic.

4.11. RNA sequencing (Study I-II)

4.11.1. RNA extraction

Bulk RNA sequencing was performed on cPOCs and mPOCs isolated from $n = 5$ patients (study I) and bovine ovarian cortex from $n = 7$ animals (study II). RNA was extracted using the RNeasy Micro Kit (Qiagen) for study I and the RNeasy mini kit (Qiagen) for study II.

For RNA extraction from ovarian cortex (Study II), tissue samples were mechanically minced using sterile scalpels and transferred into 600 μ l of RLT buffer supplemented with β -mercaptoethanol. Samples were homogenised using a TissueRuptor (Qiagen). For Study I, irradiated and control cPOCs and mPOCs were lysed directly in 75 μ l of Buffer RLT (Qiagen). Genomic DNA was removed using on-column DNase I treatment (Qiagen). Subsequent steps, including ethanol addition, centrifugation, and washes with buffer RW1 and RPE, followed the manufacturer's protocol. RNA was eluted in 14 μ l RNase-free water (Study I) and 30 μ l RNase-free water (Study II).

RNA concentration and purity were assessed using a NanoDrop™ 2000/2000c spectrophotometer (ThermoFisher Scientific), and RNA integrity was evaluated using the Agilent Bioanalyzer 2000 (Agilent Technologies). Only samples meeting the following quality criteria were used for library preparation:

- Study I: RNA Integrity Number (RIN) > 9 and $A_{260}/A_{280} > 1.8$
- Study II: RIN > 7

4.11.2. RNA sequencing and data analysis

Library preparation and sequencing:

For Study I, RNA libraries were prepared using the Illumina Stranded mRNA Prep Ligation protocol (Illumina). Sequencing was performed on the Illumina NovaSeq 6000 platform, generating 150 bp paired-end reads (~42 million reads/ sample).

For Study II, libraries were prepared using the NEXTFLEX Rapid Directional RNA-seq Kit 2.0 (Perkin Elmer) and sequenced on the Illumina HiSeq 4000, generating 75 bp single-end reads (~13 million reads/sample).

Quality control and trimming:

Study I: Low-quality bases and adapter sequences were removed using Trim Galore (version 0.6.1).

Study II: Raw reads were assessed using FASTQC (v0.11.8)(Brown, Pirrung and McCue, 2017), followed by trimming with FASTP (Chen *et al.*, 2018).

Alignment and quantification:

Trimmed reads were aligned to:

- Human genome (hg38) for Study I
 - Bos Taurus genome (ARS-UCD1.2) for Study II
- using the STAR aligner (version 2.7.11a) (Yates *et al.*, 2020).

For Study I, BAM files were quantified using the *featureCount* function from (subread v2.0.3), with gene annotation from the UCSC gtf file. All preprocessing steps (trimming, alignment, quantification) were executed within a Snakemake workflow (v5.10.0).

For Study II, the STAR program was run using default parameters (Dobin *et al.*, 2013), and gene counts were generated using *featureCount* (Liao, Smyth and Shi, 2014).

Differential expression analysis:

Differential expression was performed using the DESeq2 R package (Love, Huber and Anders, 2014).

1. Study I comparisons (analysed separately for cPOCs and mPOCs):

- 24 h control vs 1 h control
- 1 h irradiated vs 1 h control
- 4 h irradiated vs 1 h control
- 24 h irradiated vs 24 h control

Two outlier samples (one 24 h control and one 24 h irradiated cPOCs) were excluded following quality control.

2. Study II comparisons:

- VT vs FT
- FTC vs FT
- VTC vs FTC
- VTC vs VT

P-values were adjusted using Benjamini-Hochberg's (BH) method (Benjamini and Hochberg, 1995). Differentially expressed genes (DEGs) with a false discovery rate (FDR) < 0.05 were considered statistically significant.

Principal component analysis (PCA)

PCA was performed with the *plotPCA* function in DESeq2 (Love, Huber and Anders, 2014). To minimise clustering bias, a variance stabilising transformation (VST) was applied to raw counts before PCA.

Pathway and gene set analysis

Study I:

Gene pattern analysis for DEGs in cPOCs and mPOCs was performed using the DEGREport package (Pantano, 2024). Identified gene clusters were subjected to Gene Ontology (GO) over-representation analysis using the clusterProfiler (Yu *et al.*, 2012; Wu *et al.*, 2021).

To assess broader biological processes, gene set enrichment analysis (GSEA) was performed on all expressed genes ranked by log₂ fold change, using the MSigDB hallmark gene sets. Analyses were carried out using clusterProfiler together with pathview (Luo and Brouwer, 2013), DOSE (Yu *et al.*, 2015), and apegglm (Zhu, Ibrahim and Love, 2019). Heatmaps were generated with the ComplexHeatmap package (Gu, Eils and Schlesner, 2016; Gu, 2022).

Study II:

Pathway analysis was performed using the g:Profiler (Raudvere *et al.*, 2019), configured for *Bos taurus*. GSEA was carried out using the g:GOST function with g:SCS correction. Pathways with adjusted p-value < 0.05 were considered significant. Enriched KEGG pathways were visualised using pathview (Bioconductor, v1.43.0).

4.12. DNA isolation and 16s rRNA sequencing (Study III)

4.12.1. Microbial DNA isolation from ovarian tissue

Microbial DNA was isolated from ovarian cortex and medulla tissues from 11 gender-affirming patients, follicular fluid samples from 6 patients, and ovarian cortex biopsies from 5 caesarean-section patients using the QIAamp DNA Microbiome Kit (Qiagen).

Briefly, tissue samples were mixed with Buffer AHL and incubated at room temperature for 30 min. Samples were centrifuged at $10,000 \times g$ for 10 min, the supernatant was discarded, and the pellet was incubated with 190 μ l Buffer RDD and 2.5 μ l Benzonase at 37 °C for 30 min at 600 rpm. Subsequently, 20 μ l Proteinase K was added, followed by incubation at 56 °C for 30 min.

After a brief centrifugation to remove condensation, 200 μ l Buffer ATL (containing Reagent DX) was added. The mixture was transferred to Pathogen Lysis Tubes L and processed according to the manufacturer's lysis protocol, then centrifuged at $10,000 \times g$ for 1 min. The resulting supernatant was transferred to a clean tube, avoiding bead carry-over. Next, 40 μ l Proteinase K was added, and samples were incubated at 56 °C for 30 min with shaking. An additional 200 μ l Buffer APL2 was added, vortexed thoroughly, and incubated at 70 °C for 10 min.

Lysates were combined with 200 μ l ethanol and applied to QIAamp UCP Mini Columns in 700 μ l aliquots, followed by centrifugation at $6,000 \times g$ for 1 min. Columns were washed with 500 μ l Buffer AW1 ($6,000 \times g$, 1 min) and 500 μ l Buffer AW2 ($20,000 \times g$, 3 min). A final dry spin at $20,000 \times g$ for 1 min was performed before DNA was eluted in 50 μ l Buffer AVE and stored at -20 °C until further processing.

4.12.2. 16s rRNA sequencing and data analysis

The universal primer pair 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3') was used to amplify the 16S rRNA gene from isolated genomic DNA. Sequencing on the amplified DNA was performed on the Illumina MiSeq platform.

Raw sequence data were processed using QIIME2 (v2022.11). Low-quality bases (quality score < 25) were trimmed using the *q2-dada2* plugin with the following parameters: *trunc_len_f* = 287; *trunc_len_r* = 247; *trim_left_f* = 9; and *trim_left_r* = 9.

Taxonomic assignment was carried out using the SILVA 16S rRNA database (version 138.1), and sequences were collapsed to the genus level for downstream analyses. To identify and remove potential contaminants, the microDecon R package (McKnight *et al.*, 2019) was applied using default settings, based on the proportion abundance of reads detected in negative control samples.

4.13. Proteomics sample preparation and data analysis (Study I)

4.13.1. Proteomic sample preparation

Irradiated cPOCs and mPOCs collected from $n = 4$ patients, together with their matched controls, were snap-frozen, thawed on ice, and lysed in 10 μL of 8 M urea. The lysates were sonicated for 5 min in a water bath. Subsequently, 70 μL of 0.5 M NaCl prepared in 50 mM Tris-HCl buffer (pH 8.5), and 0.8 μL of 100 x protease inhibitor cocktail (Roche Diagnostics) were added, followed by 5 min sonication. Samples were then centrifuged at $13,000 \times g$ for 10 min at 4°C , and protein concentrations were quantified using a BCA assay (Pierce).

For each sample, 7.7 μg of total protein was transferred to a fresh tube and adjusted to a 75 μL final volume using 1 M urea and 438 mM NaCl in Tris-HCl buffer. Reduction was performed by adding 2.8 μL of 250 mM dithiothreitol (DTT) (Sigma-Aldrich) and incubating at 37°C for 45 min with agitation at 400 rpm on a block heater. Proteins were subsequently alkylated by adding 3.1 μL of 500 mM iodoacetamide (Sigma-Aldrich) and incubating for 30 min at room temperature in the dark with continuous shaking.

Proteolytic digestion was carried out with 0.4 μg of sequencing-grade modified trypsin (Promega) and incubating at 37°C for 16 h. The reaction was quenched by adding 4.5 μL of concentrated formic acid (FA) and incubating for 5 min at room temperature. Peptides were purified using a C18 HyperSep plate (40 μL bed volume; Thermo Fisher Scientific) and dried in a vacuum concentrator (Eppendorf).

For quantitative proteomics, peptides were labelled with TMT-10plex reagents (Thermo Fisher Scientific). Dried peptides were dissolved in 70 μL of 50 mM triethylammonium bicarbonate and incubated for 2 h at room temperature with 100 μg of TMT-10plex reagent dissolved in anhydrous acetonitrile (ACN). Remaining reagents were quenched by adding 6 μL hydroxylamine and incubating for 15 min. Labelled peptides were pooled, dried under vacuum, and purified again using a second C18 cleanup.

4.13.2. Liquid chromatography-tandem mass spectrometry (LC-MS)

TMT-10plex-labelled peptide mixtures were dissolved in solvent A, and approximately 2 μg of peptides per sample was loaded onto a 50 cm EASY-Spray C18

analytical column (Thermo Fisher Scientific) connected to an Ultimate 3000 nanoUPLC system (Thermo Fisher Scientific). Peptides were separated using a 90-min gradient at a flow rate of 300 nL/min, starting from 4% to 26% of solvent B (98% acetonitrile, 0.1% formic acid), followed by an increase to 95% solvent B over 5 min, and held at 95% for an additional 5 min.

Mass spectrometric analysis was performed using a Q Exactive HF hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific). Full MS scans were acquired from m/z 375–1700 at a resolution of 120,000 (at m/z 200), with an automatic gain control (AGC) target of 1×10^6 ions and a maximum injection time of 80 ms.

Data-dependent higher-energy collisional dissociation (HCD) was applied to the top 18 most intense precursor ions, selected within a charge state range of 2+ to 7+, with a dynamic exclusion of 45 s. MS/MS (tandem mass spectra) were recorded at a resolution of 60,000, using an AGC target of 2×10^5 ions, a maximum injection time of 54 ms, a quadrupole isolation window of 1.4 Th, and a normalised collision energy (NCE) of 34%.

4.13.3. Proteomics data analyses

Raw mass spectrometry data were processed using Proteome Discoverer v3.0 (Thermo Fisher Scientific) with the MS Amanda v2.0 search engine. Spectra were searched against the human Swiss-Prot database (20,022 entries; downloaded February 9th, 2023). Up to two missed cleavages were allowed. Mass tolerances were set to 10 ppm for precursor ions and 0.02 Da for fragment ions. Carbamidomethylation of cysteine was specified as a fixed modification, while oxidation of methionine, deamidation of asparagine and glutamine, and TMT6plex labelling (+229.163 Da) on lysine and peptide N-termini were included as variable modifications.

Peptide-spectrum matches were filtered to a 5% FDR using Percolator. Protein quantification was performed based on reporter ion intensities from the TMT labelling. Further analyses were conducted in R (version 4.3.2) using RStudio. One sample each from the cPOCs 1 h irradiated and mPOCs 4 h irradiated groups was excluded due to insufficient protein yield. Proteins with missing values were removed, after which the data were median-normalised, batch-corrected using the *removeBatchEffect* function from the limma package (Ritchie *et al.*, 2015), and log₂-transformed before differential expression analysis (DEA).

Fold changes were calculated by comparing mean protein expression in irradiated samples with their respective controls. Comparisons included: 1 h and 4 h irradiated vs. 1 h control, and 24 h irradiated vs. 24 h control, analysed separately for cPOCs and mPOCs. Statistical significance was evaluated using t-tests with BH correction. Proteins with FDR < 0.1 were considered differentially expressed (DEPs). Functional analysis of DEPs was performed using g:Profiler, focusing on Gene Ontology (GO) biological processes (Kolberg *et al.*, 2023).

4.14. Formation and culture of 3D Silk-Ovarioids

Monolayer cultures of cPOCs and mPOCs derived from $n = 2$ gender-affirming patients were expanded to confluency and exposed to a single 10 Gy irradiation dose. At 24 h post-irradiation, POCs were detached using TrypLE (Thermo Fisher Scientific) and used for the generation of Silk-Ovarioids, following the protocol described by Di Nisio et al., (2025).

Briefly, 20 μ l droplets of Biosilk™ were dispensed into the wells of a 24-well plate, and air bubbles were introduced to create compact silk foams. Irradiated and non-irradiated (control) cells were seeded onto the foams at a density of 1.2×10^5 cells per foam. Constructs were incubated at 37°C for 20 min to allow structural stabilisation, after which 700 μ l of culture medium was added. Cultures were maintained at 37°C in a humidified 5% CO₂ incubator for 14 days, with medium replacement daily.

On day 14, the foams were carefully lifted using a spatula, bisected, and transferred into an ultra-low attachment 24-well flat-bottom plate (Corning) for suspension culture. Silk-Ovarioids were maintained under these conditions for an additional 40 days, with daily medium changes. At the end of the culture period, constructs were fixed in 4% formaldehyde for downstream morphological analyses.

5. RESULTS

5.1. Immunofluorescence analysis of canonical pathways induced by irradiation

To investigate how POCs respond to irradiation, we performed immunofluorescence staining for key signalling pathways involved in the cellular response to DNA damage. These pathways are well-characterised in the context of irradiation and include markers associated with the DNA damage response, apoptosis, and cell cycle checkpoint regulation.

Effects of irradiation on DNA damage in cPOCs and mPOCs

We first examined the activation of DNA damage markers in cPOCs and mPOCs by assessing the expression of phosphorylated Chk1 (Ser345) (p-Chk1) and γ H2AX (Ser139), as shown in Figure 9. In cPOCs, both markers exhibited a rapid and robust increase at 1 h post-irradiation ($p < 0.001$). Their expression began to decline by 4 h, although levels remained significantly elevated ($p < 0.001$), and returned to baseline levels by 24 h (Figure 9A).

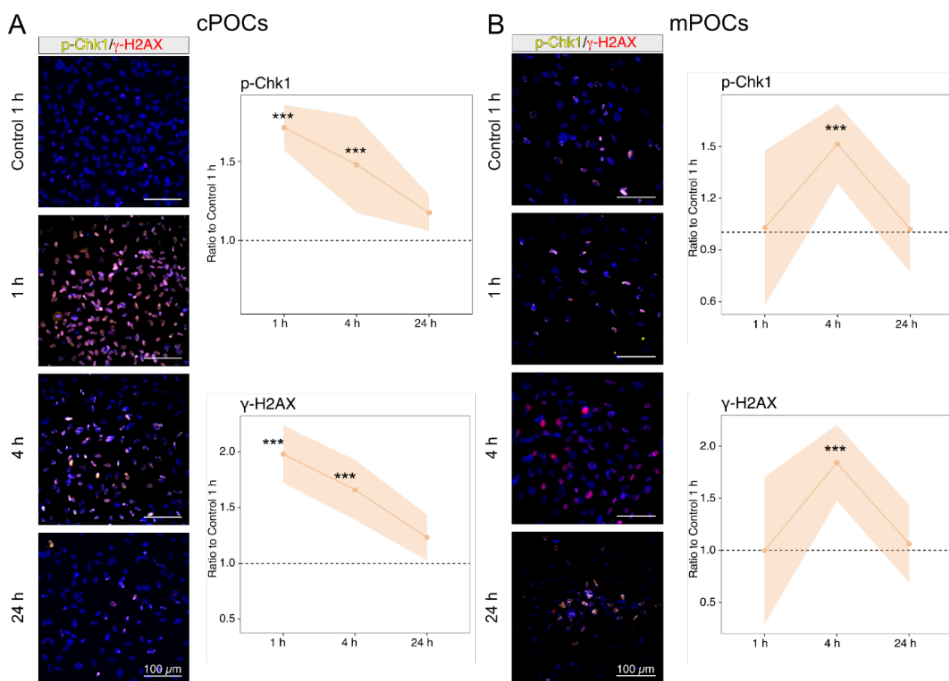


Figure 9: Expression of DNA damage markers in cPOCs and mPOCs. Representative images and fluorescence intensity ratios of irradiated samples relative to the 1 h control in (A) cPOCs and (B) mPOCs. Cells were stained for the DNA damage markers γ H2AX (red) and p-Chk1 (yellow). The graphs display the mean fluorescence intensity ratios, with shaded regions indicating the standard deviation. Ratios were calculated by dividing the mean intensity of each irradiated sample by that of the corresponding control.

Statistical significance was assessed using the Kruskal-Wallis test followed by Dunn's post hoc correction; significance levels are indicated by asterisks (***) $p < 0.001$). Scale bars = 100 μm . **Abbreviations:** cPOCs – cortex-derived primary ovarian cells; mPOCs – medulla-derived primary ovarian cells.

In contrast, mPOCs displayed a delayed response. p-Chk1 and γH2AX levels remained comparable to baseline at 1 h, but both markers showed a significant peak at 4 h post-irradiation ($p < 0.001$), followed by a return to baseline at 24 h (Figure 9B).

Despite the temporal differences in activation, these findings demonstrate that both cPOCs and mPOCs mount a rapid DNA damage response following irradiation, characterised by the transient activation of key DDR proteins.

Effects of irradiation on cell cycle regulation in cPOCs and mPOCs

We next assessed key regulators of cell cycle progression, including cyclin D1, cyclin E, and phosphorylated p21 at Ser146 (p-p21), in both cPOCs and mPOCs (Figure 10). Cell cycle arrest is a central component of the response to irradiation, allowing time for DNA repair before proceeding with the cell cycle. In somatic POCs, we observed distinct temporal patterns of cell cycle regulation between cPOCs and mPOCs.

In cPOCs, cyclin D1 levels remained stable across all time points. In contrast, cyclin E expression increased significantly at 1 h ($p < 0.01$), remained elevated at 4 h ($p < 0.05$), and persisted at 24 h ($p < 0.05$). p-p21 levels increased significantly at 1 h ($p < 0.05$) but returned to baseline by 4 h and remained unchanged at 24 h (Figure 10A).

In mPOCs, no significant changes were detected in cyclin D1 and cyclin E expression at the early time points (1 and 4 h), but both markers were significantly reduced at 24 h ($p < 0.01$). Importantly, p-p21 levels remained significantly elevated throughout the entire 24 h period (1 h, $p < 0.001$; 4 h, $p < 0.01$; 24 h, $p < 0.001$) (Figure 10B).

Together, these findings indicate that cPOCs recover more rapidly from irradiation-induced stress, exhibiting only transient perturbations in cell cycle regulation. In contrast, mPOCs show sustained cell cycle arrest, likely reflecting more extensive DNA damage or reduced repair efficiency. The extended arrest observed in mPOCs may indicate a greater irradiation-induced damage or reduced efficiency in repair mechanisms.

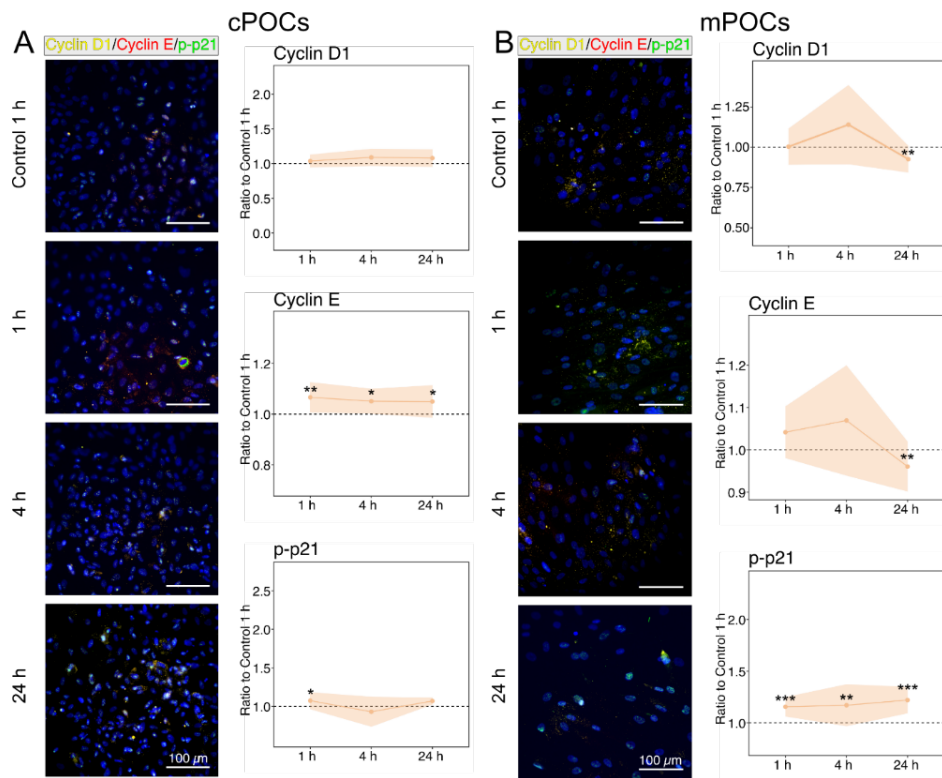


Figure 10. Expression of cell-cycle related markers in cPOCs and mPOCs. Representative immunofluorescence images and fluorescence intensity ratios of irradiated cPOCs and mPOCs relative to the 1 h control in (A) cPOCs and (B) mPOCs. Cells were stained for the cell cycle-associated markers cyclin D1 (yellow), cyclin E (red), and phosphorylated p21 (p-p21, green). Graphs represent mean fluorescence intensity ratios, with shaded areas indicating the standard deviation. Ratios were calculated by dividing the mean fluorescence intensity of each irradiated sample by that of the corresponding control. Statistical significance was evaluated using the Kruskal-Wallis test followed by Dunn's post hoc correction. Significance levels indicated by asterisks: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Scale bars = 100 μm . **Abbreviations:** cPOCs – cortex-derived primary ovarian cells; mPOCs – medulla-derived primary ovarian cells.

Effects of irradiation on the activation of apoptosis signalling in cPOCs and mPOCs

To investigate the activation of apoptotic pathways, we assessed the expression of cleaved caspase-3 (cl-Cas3), phosphorylated p53 at Ser15 (p-p53), and Bcl-2 (Figure 11). In cPOCs, p-p53 showed a slight but non-significant increase at 1 h, followed by significant reductions at 4 h ($p < 0.05$) and 24 h ($p < 0.001$) post-irradiation. cl-Cas3 levels remained unchanged at 1 and 4 h but were significantly decreased at 24 h ($p < 0.01$). In contrast, Bcl-2 expression increased significantly at 1 h ($p < 0.05$), peaked at 4 h ($p < 0.001$) and returned to baseline by 24 h (Figure 11A).

In mPOCs, p-p53 expression remained stable across all time points. cl-Cas3 levels were significantly elevated at 1 h ($p < 0.01$), followed by a gradual decline to baseline by 24 h. Bcl-2 levels also increased significantly at 1 h ($p < 0.001$) and 4 h ($p < 0.001$), returning to baseline at 24 h (Figure 11B).

Together, these results indicate a dynamic balance between apoptotic activation and survival signalling. In cPOCs, the early and sustained increase of Bcl-2 suggests a predominantly protective response to irradiation-induced DNA damage. In contrast, mPOCs exhibit an early transient apoptotic response, reflected by cl-Cas3 upregulation, accompanied by a short-lived Bcl-2 increase that may help to limit apoptosis and support recovery.

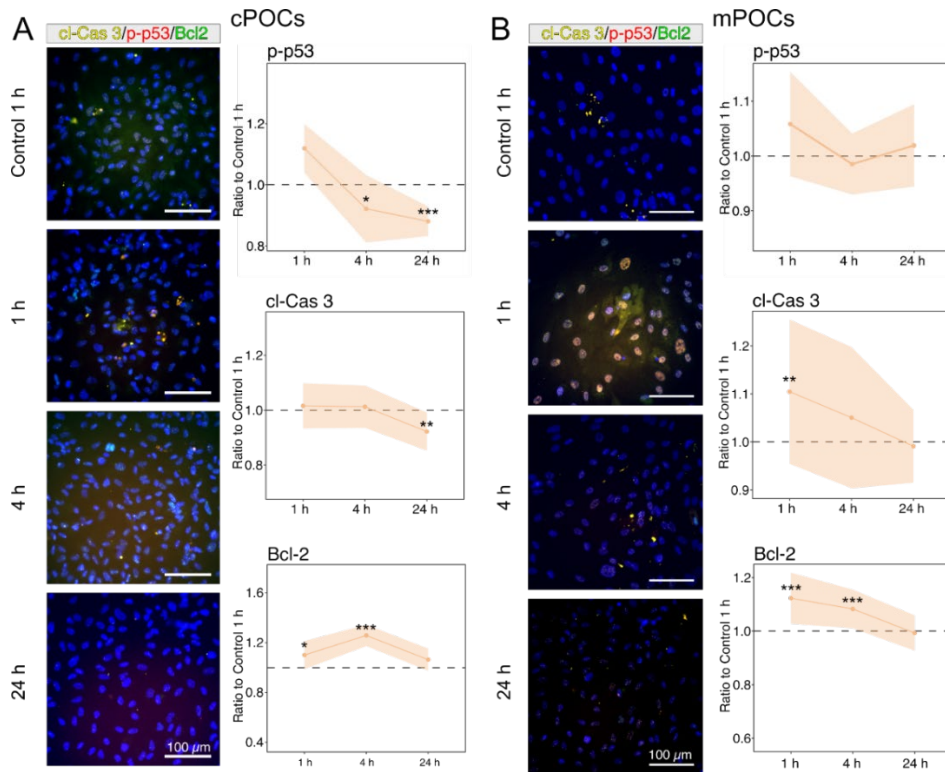


Figure 11. Expression of apoptosis-related markers in cPOCs and mPOCs. Representative images and fluorescence intensity ratios of apoptosis-related markers in irradiated samples relative to the 1 h control for (A) cPOCs and (B) mPOCs. Cells were immunostained for the phosphorylated p53 (p-p53, red), Bcl-2 (green), and cleaved caspase-3 (cl-Cas3, yellow). The graphs display mean fluorescence intensity ratios, with shaded regions indicating standard deviation. Ratios were calculated by dividing the mean fluorescence intensity of each irradiated sample by that of the control. Statistical significance was assessed using the Kruskal-Wallis test with Dunn's post-hoc correction. Significance levels are indicated by asterisks: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Scale bars = 100 μ m. **Abbreviations:** cPOCs – cortex-derived primary ovarian cells; mPOCs – medulla-derived primary ovarian cells.

5.2. Transcriptome Profiling Reveals Distinct Irradiation-Responsive Genes in cPOCs and mPOCs

To examine the effects of X-ray irradiation on gene expression in POCs, we performed bulk RNA sequencing on cPOCs and mPOCs (n = 5 each) collected at 1, 4, and 24 h post-irradiation, together with non-irradiated controls at 1 and 24 h. We first assessed baseline transcriptional differences between control samples. PCA revealed clear separation between the 1 h and 24 h control groups in both cell types (Figure 12A–B), indicating that time in culture contributes substantially to transcriptomic variation.

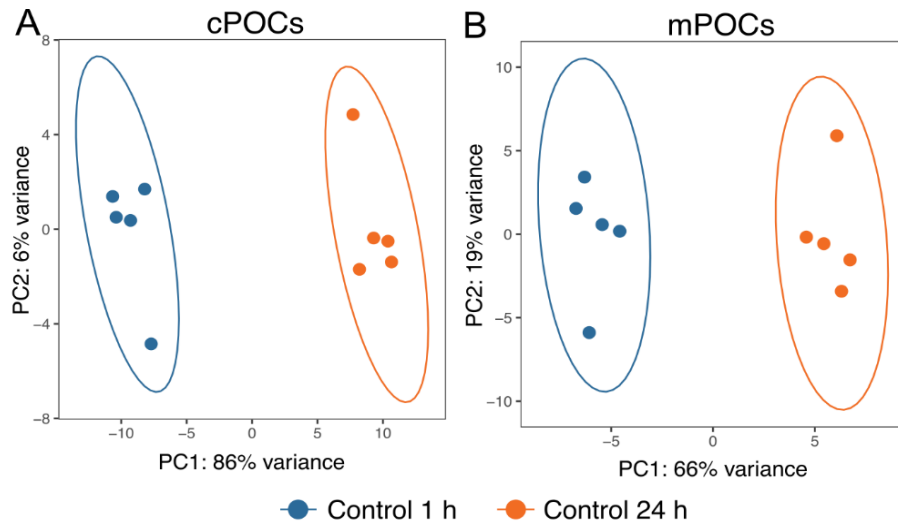


Figure 12. Baseline transcriptomic variation in control cPOCs and mPOCs. PCA was performed using the top 500 most variable genes after batch correction to assess baseline transcriptomic differences between 1 h and 24 h control samples in (A) cPOCs and (B) mPOCs. In both cell types, 1 h and 24 h controls formed clearly separated clusters, indicating time in culture contributes substantially to transcriptomic variation. **Abbreviations:** cPOCs – cortex-derived primary ovarian cells; mPOCs – medulla-derived primary ovarian cells; DEGs – differentially expressed genes; PCA – principal component analysis.

Based on these observations, irradiated samples at 1 h and 4 h were compared to the 1 h control, whereas the 24 h irradiated samples were compared to the corresponding 24 h controls. Following quality control, two cPOCs samples (one irradiated and one 24 h control) were removed due to outlier behaviour. PCA performed on all remaining samples demonstrated clear separation based on both irradiation status and timepoint (Figure 14A), although early transcriptomic shifts appeared less pronounced when visualised together. To better resolve early

dynamics, PCA plots were stratified into early (1 h, 4 h) and late (24 h) responses (Figure 13A-B). This stratified analysis revealed detectable transcriptomic alterations emerge as early as 4 h post-irradiation in both cPOCs and mPOCs.

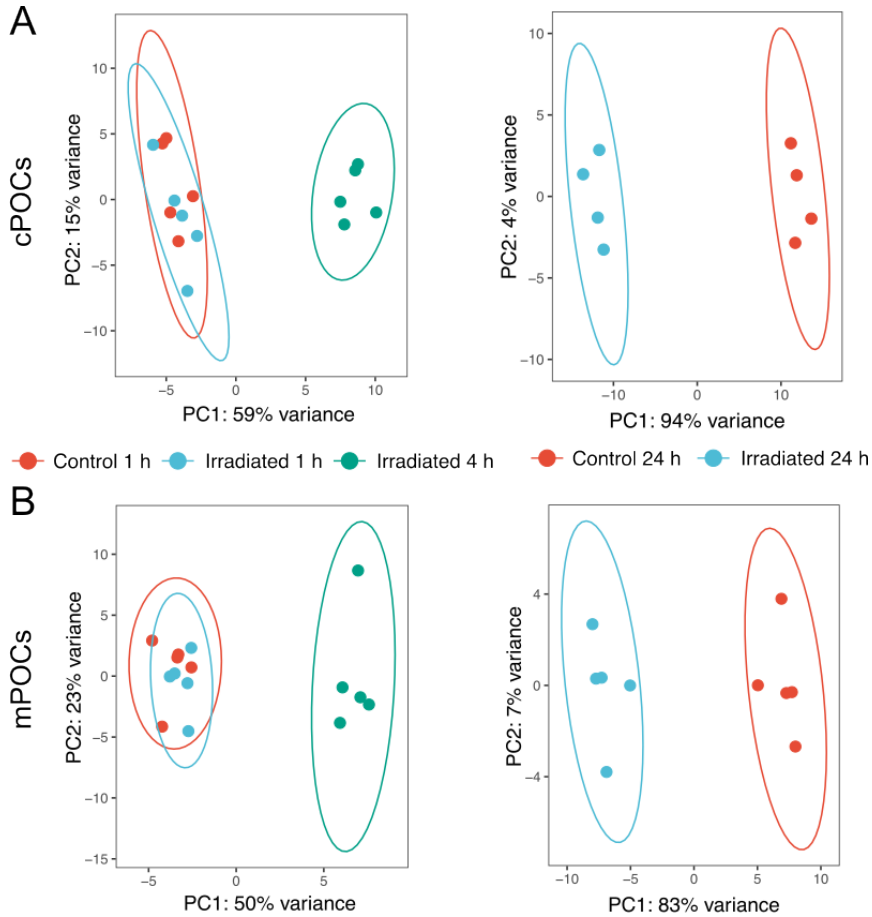


Figure 13. Transcriptomic alterations following irradiation. PCA was performed using the top 500 most variable genes after batch effect correction. For the early timepoints (1 h and 4 h), irradiated samples were compared with the 1 h control; for the late timepoint (24 h), irradiated samples were compared with corresponding 24-h control. PCA plots are shown for **(A)** cPOCs and **(B)** mPOCs. Two cPOC samples (one irradiated and one 24-h control) were excluded due to outlier behaviour. **Abbreviations:** cPOCs – cortex-derived primary ovarian cells; mPOCs – medulla-derived primary ovarian cells; PCA – principal component analysis.

Differential expression analysis (DEA) revealed only minimal transcriptional changes at 1 h post-irradiation, with 3 DEGs detected in cPOCs and 1 DEG in mPOCs relative to the 1 h control. By 4 h, however, both cell types exhibited widespread transcriptomic remodelling, with 2,810 DEGs identified in cPOCs and 2,540 DEGs in mPOCs. A similarly extensive response was observed at 24 h, with 2,462 DEGs in cPOCs and 2,802 DEGs in mPOCs. Several key regulators of the cell cycle and p53 signalling pathway, including *CENPF*, *PLK1*, *CDKN1A*, *PCNA*, and *TP53INP1*, were significantly upregulated at 24 h (Figure 14B). To uncover more subtle transcriptomic changes, particularly at the 1 h timepoint, we performed GSEA to ranked gene lists and performed gene set variation analysis (GSVA) to examine pathway-level dynamics. GSEA revealed upregulation of MYC targets, E2F targets, and G2M checkpoint genes in both cell types; however, statistical significance at 1 h was observed only in cPOCs (Figure 14D). As expected, p53 signalling was strongly induced at 4 h and 24 h in both cPOCs and mPOCs (Figure 14C-D), consistent with activation of the canonical DNA damage response. At 4 h, both cell types increased activity in PI3K-AKT-mTOR signalling, DNA repair pathways, and oestrogen response pathways (Figure 14D). By 24 h, a broader transcriptional reprogramming had emerged, characterised by downregulation of gene sets associated with cell cycle progression and glycolysis (Figure 14C-D), suggesting a delayed transition towards cell cycle arrest and metabolic adaptation following irradiation-induced stress.

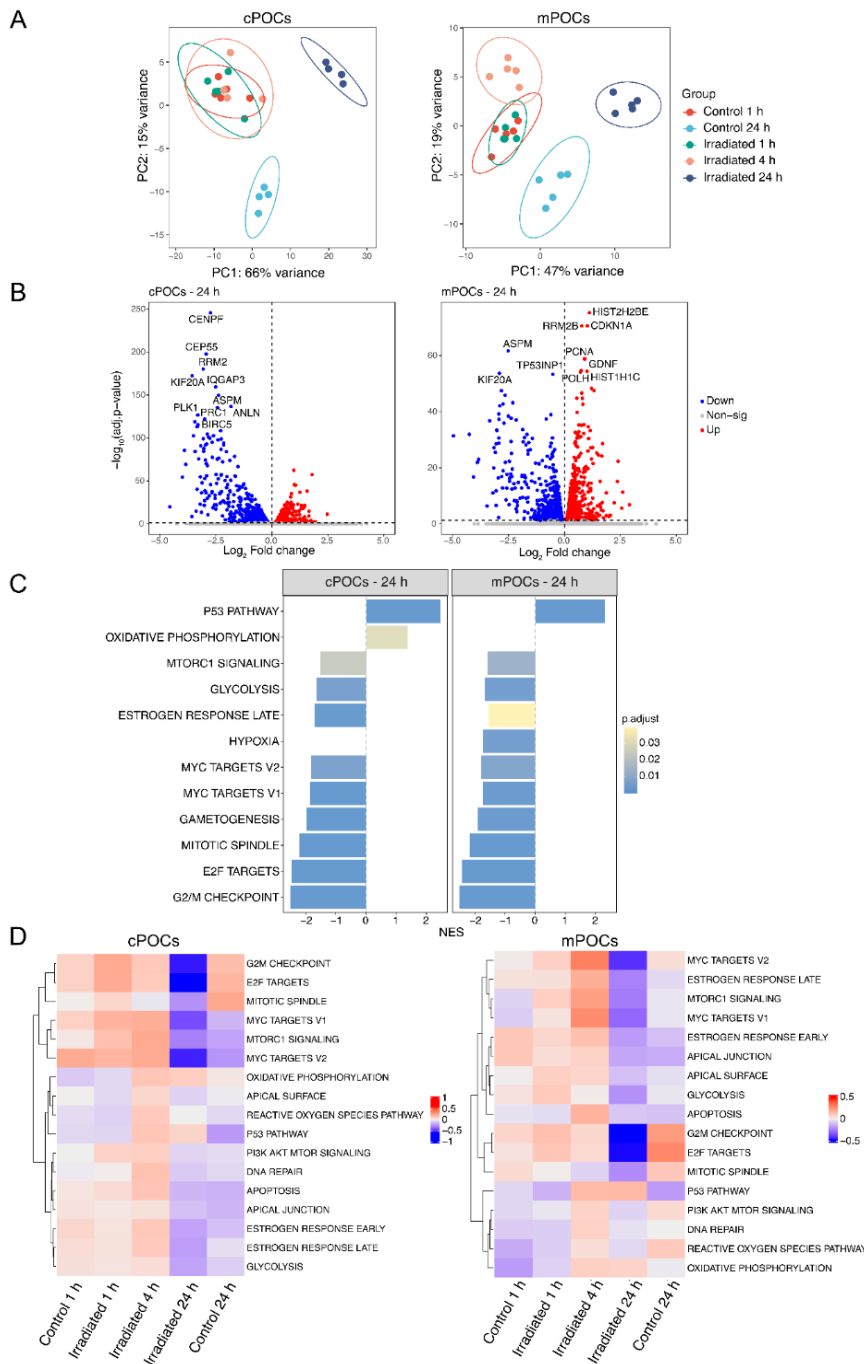


Figure 14. Irradiation-induced transcriptomic alterations in cPOCs and mPOCs. (A) PCA based on the 500 most variable genes after batch effect correction in cPOCs and mPOCs. Two cPOCs samples (one 24 h control and one 24 h irradiated) were removed due to outlier behaviour. (B) Volcano plots illustrating the DEGs at 24 h post- irradiation

in cPOCs and mPOCs. The ten most statistically significant DEGs are highlighted. Colours represent gene regulation status: blue = downregulated, red = upregulated, and grey = non-significant. **(C)** Significantly enriched Hallmark pathways identified by GSEA using genes ranked by $\log_2$ fold change at 24 h. The x-axis shows the normalised enrichment score (NES). **(D)** GSVA-based heatmap displaying enrichment dynamics of selected Hallmark pathways at 1 h, 4 h, and 24 h in cPOCs and mPOCs. **Abbreviations:** cPOCs – cortex-derived primary ovarian cells; mPOCs – medulla-derived primary ovarian cells; PCA – principal component analysis; DEGs – differentially expressed genes; GSEA – gene set enrichment analysis; GSVA – gene set variation analysis; NES – normalised enrichment score.

5.2.1. Gene pattern analysis and GO enrichment reveal irradiation-induced activation of DNA damage and chromosome segregation pathways in cPOCs

To assess whether X-ray induced transcriptomic changes followed coordinated temporal patterns during the 24 h recovery period, we analysed DEGs from all pairwise comparisons and examined their expression trajectories. For both cPOCs and mPOCs, genes were grouped into a maximum of six clusters based on shared expression profiles (Figure 15–16). In cPOCs, clusters 3, 4, and 5 were selected for detailed interpretation, as they exhibited comparable expression levels between the two baseline controls (1 h and 24 h) but showed clear deviations following irradiation (Figure 15A).

Cluster 3 contained genes associated with apoptosis signalling and autophagy, which were consistently upregulated at both 4 h and 24 h post-irradiation (Figure 15A–B). Cluster 4 demonstrated a biphasic pattern, with an initial downregulation at 4 h followed by a rebound upregulation at 24 h. GO enrichment indicated that this cluster was enriched for ubiquitin-related biological processes (Figure 15C). In contrast, cluster 5 showed a progressive reduction in expression across all irradiated timepoints, with functional enrichment highlighting pathways involved in nuclear chromosome organisation and sister chromatid segregation (Figure 15D).

Together, these findings demonstrate that cPOCs undergo structured and coordinated transcriptional remodelling following irradiation, characterised by early activation of apoptosis and autophagy programs, a delayed engagement of ubiquitin-mediated processes, and sustained suppression of chromosome segregation pathways.

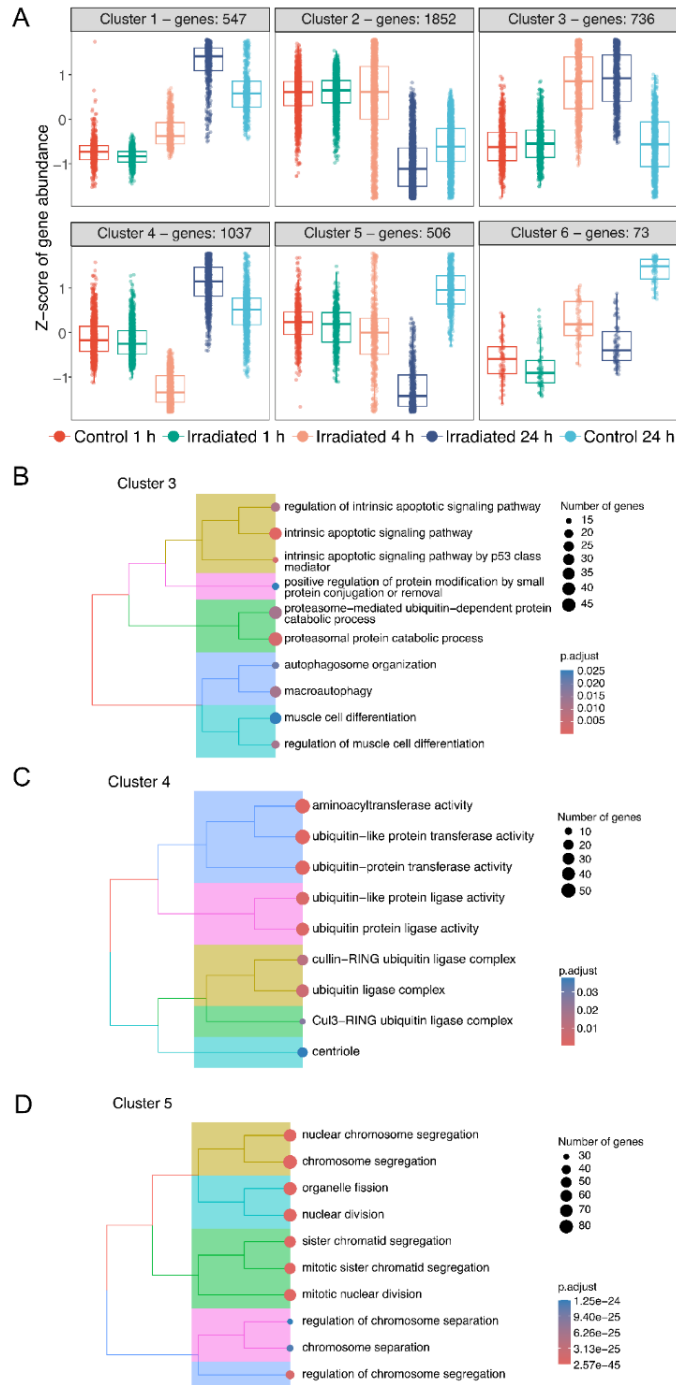


Figure 15. Gene pattern analysis and GO enrichment in cPOCs following irradiation. (A) Gene pattern analysis based on pooled DEGs from all pairwise group comparisons. The y-axis represents Z-scores of gene expression for each cluster. (B–D) GO

enrichment analysis for DEGs in **(B)** cluster 3, **(C)** cluster 4, and **(D)** cluster 5. GO terms were grouped using the ward.D clustering method according to gene overlap and network proximity. Dot size corresponds to the number of genes per GO term, and dot colour indicates the adjusted p-value. **Abbreviations:** cPOCs – cortex-derived primary ovarian cells; DEGs – differentially expressed genes; GO – gene ontology.

5.2.2. Gene pattern and GO enrichment analysis identify DNA damage, G1/S transition and chromosome segregation effects in mPOCs

For mPOCs, the same clustering strategy was applied, and four clusters (1, 2, 3, and 4) of the six identified expression patterns were selected for interpretation (Figure 16A). Cluster 1 demonstrated strong upregulation across all irradiated timepoints, mirroring the behaviour of cluster 3 in cPOCs. GO enrichment indicated associations with cell death pathways, apoptotic signalling and DNA damage-related processes (Figure 16B). Clusters 2 and 3 displayed opposing temporal dynamics. Cluster 2 exhibited a progressive decrease in expression from 1 h to 24 h and was enriched for GO terms linked to the G1/S transition, DNA replication and mRNA transport (Figure 16C). In contrast, cluster 3 contained genes related to histone modification and ubiquitin-mediated pathways, exhibited an initial in downregulation at 4 h followed by a marked upregulation at 24 h post-irradiation (Figure 16A). Cluster 4 was consistently downregulated at all timepoints. GO enrichment showed associations with chromosome segregation, mitotic nuclear division and cell cycle phase transitions (Figure 16D).

Overall, these results indicate that mPOCs engage a coordinated transcriptional response to irradiation, characterised by early activation of DNA damage and apoptotic pathways, suppression of G1/S transition and replication processes, dynamic regulation of histone and ubiquitin-associated genes, and sustained downregulation of chromosome segregation machinery.

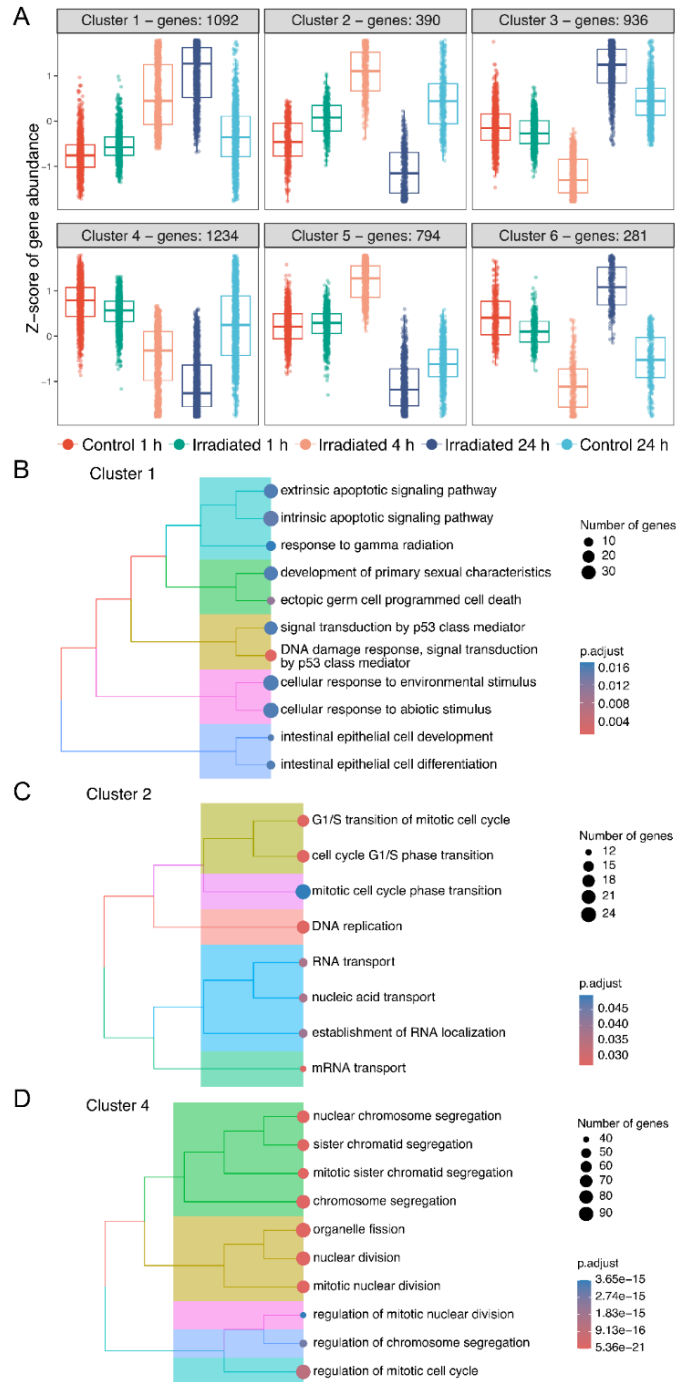


Figure 16. Gene pattern analysis and GO enrichment in mPOCs following irradiation. (A) Gene pattern analysis was performed using pooled DEGs from all pairwise group comparisons. Boxplots show Z-scored gene expression levels across experimental

conditions. Tree plots display the top ten significantly enriched GO terms for DEGs within **(B)** cluster 1, **(C)** cluster 2, and **(D)** cluster 4. GO terms were grouped using ward.D hierarchical clustering based on shared gene membership and functional similarity within the GO network. Dot size represents the number of genes associated with each GO term, while dot colour indicates the adjusted p-value. **Abbreviations:** DEGs – differentially expressed genes; GO – gene ontology; mPOCs – medulla-derived primary ovarian cells.

5.3. Proteomic profiling revealed alterations in cell adhesion mechanisms

To complement the transcriptomic analysis, we next investigated irradiation-induced proteomic changes. Quantitative proteomic profiling was performed using LC-MS/MS on cPOCs (n = 4) and mPOCs (n = 4). Two samples, one cPOC sample at 1 h and one mPOC sample at 4 h post-irradiation, were excluded due to insufficient protein yield. After adjusting for patient-specific effects, DEPs (FDR < 0.1) were identified by comparing mean peptide abundances in irradiated groups with their respective controls. As in the transcriptomic analysis, irradiated samples collected at 1 h and 4 h were compared with the 1 h control, while samples irradiated for 24 h were compared with the corresponding 24 h control. No significant DEPs were detected at 1 h or 4 h post-irradiation in either cell type. In contrast, substantial proteomic alterations were observed at 24 h, with 443 DEPs identified in cPOCs and 110 DEPs in mPOCs. In cPOCs, DEPs were predominantly enriched for proteins associated with cell adhesion (e.g., TPM1, PKP2), whereas mPOCs enrichment was observed for metabolic proteins (e.g., HK2, ACSL4) (Figure 17A).

To determine whether the DEPs identified at 24 h exhibited earlier directional trends, their expression patterns were visualised across all timepoints by plotting mean log₂ fold changes (log₂FC) at 1 h and 4 h. Although these early changes did not reach statistical significance, consistent directional shifts were already apparent at these earlier timepoints (Figure 17B).

To further characterise the affected biological processes, DEPs identified at 24 h were separated into up- and downregulated groups based on log₂FC and subjected to GO over-representation analysis. In line with RNA-seq findings, downregulated DEPs were enriched for GO terms related to cell adhesion and focal adhesion, whereas upregulated DEPs were associated with ubiquitin-protein binding and extracellular vesicle-related processes in both cPOCs and mPOCs (Figure 17B).

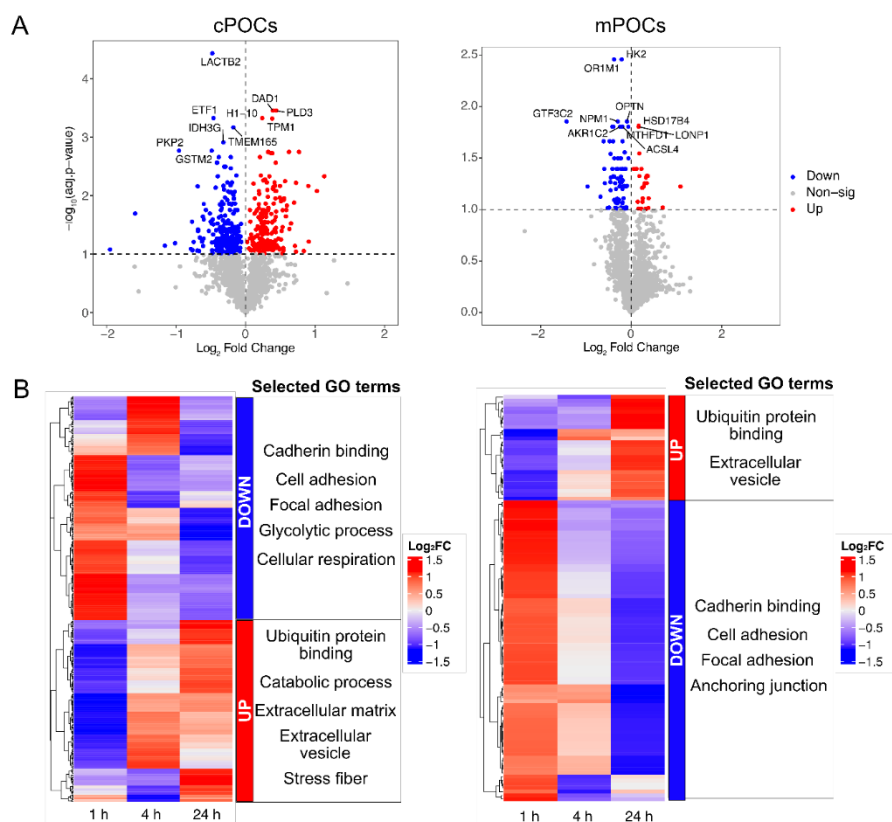


Figure 17. Irradiation-induced alterations in the proteomic profile of cPOCs and mPOCs. (A) Volcano plots showing up- and downregulated proteins in cPOCs and mPOCs at 24 h post-irradiation, with the ten most statistically significant DEPs annotated. Protein regulation is colour-coded: red indicates upregulation, blue indicates down-regulation, and grey represents proteins without significant expression changes. (B) Heatmaps display the mean log₂ fold change of DEPs identified at the 24 h timepoint across all recovery timepoints (1h, 4 h and 24 h), together with selected GO terms enriched among up- and downregulated DEPs in cPOCs and mPOCs. **Abbreviations:** cPOCs – cortex-derived primary ovarian cells; mPOCs – medulla-derived primary ovarian cells; DEPs – differentially expressed proteins; GO – gene ontology; Log₂FC – log₂ fold change.

5.4. Irradiation impairs the capacity of cPOCs and mPOCs to generate Silk-Ovarioids *in vitro*

Transcriptomic analyses indicated that X-ray irradiation disrupts pathways associated with chromatin segregation, extracellular matrix (ECM) organisation, and cytoskeletal structure in both cPOCs and mPOCs, while proteomic profiling revealed significant alterations in cell adhesion-related proteins. Together, these

findings suggested that irradiation may compromise the ability of POCs to support cell-cell and cell-matrix interaction, which are critical processes for 3D tissue formation. To evaluate the functional consequences of these molecular changes, we assessed the capacity of irradiated cPOCs ($n = 2$) and mPOCs ($n = 2$) to generate 3D Silk-Ovarioids *in vitro*. Cells were seeded onto Biosilk scaffolds 24 h post-irradiation, with non-irradiated cells used as controls. Cultures were maintained on the scaffolds for 14 days and then transferred to a floating culture system, where they were monitored for an additional 40 days (Figure 18A). In cPOCs, all 12 control-seeded scaffolds formed Silk-Ovarioids (100%), whereas 0 of 12 scaffolds (0%) produced Silk-Ovarioids in the irradiated group. The effect in mPOCs was similar: 10 of 12 control scaffolds (83%) successfully formed Silk-Ovarioids, while none (0%) of the irradiated mPOCs generated Silk-Ovarioids during the entire culture period (Figure 18C). Inverted phase-contrast microscopy revealed that non-irradiated cPOCs and mPOCs produced well-defined, stable Silk-Ovarioids within three days of free-floating culture, retaining their structure for up to 40 days (Figure 18B). In contrast, irradiated cells displayed impaired aggregation capacity; cells were poorly aggregated and distributed across the silk fibres, and where aggregates did form, they lacked structural integrity. Histological analysis confirmed these observations: H&E-stained sections from control constructs exhibited cohesive, organised tissue-like structures, while constructs derived from irradiated cells showed fragmented, poorly organised architecture with few intact cells (Figure 18B).

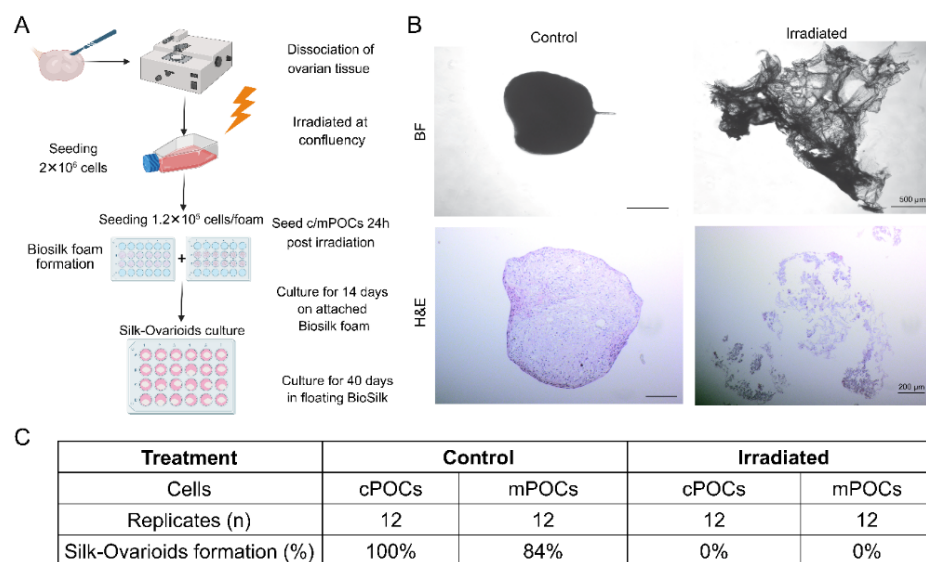


Figure 18: *In vitro* functional assessment of Silk-Ovarioid formation in control and irradiated cPOCs and mPOCs. (A) Schematic overview of the experimental workflow for Silk-Ovarioid generation ($n = 2$ patients; 6 Biosilk scaffolds per patient). Graphic created with BioRender. (B) Representative brightfield and H&E-stained images of Silk-Ovarioids derived from control and irradiated POCs after 40 days in free-floating culture.

(C) Summary table showing the number of successfully formed Silk-Ovarioids from non-irradiated and irradiated cPOCs and mPOCs. Scale bars: brightfield = 500 μm , H&E = 200 μm . **Abbreviations:** BF – brightfield; cPOCs – cortex-derived primary ovarian cells; mPOCs – medulla-derived primary ovarian cells; H&E – Hematoxylin and Eosin.

5.5. Histological analysis of ovarian tissue after vitrification

To determine whether the vitrification protocol could serve as an effective alternative to ovarian tissue cryopreservation (OTC), ovarian tissue from ten bovine specimens was analysed histologically. Follicle density and structural integrity were evaluated across four experimental groups: fresh tissue (FT), fresh tissue with culture (FTC), vitrified tissue (VT), and vitrified tissue with culture (VTC) (Figure 19A). Follicles were classified as monolayered, secondary, antral, or atretic according to established criteria (Rodgers and Irving-Rodgers, 2010). The impact of vitrification and subsequent culture was assessed by examining both follicular and stromal morphology. In FT samples, ovarian architecture appeared intact, with well-organised granulosa cell layers, preserved stromal structure, and absence of atretic follicles. FTC samples retained overall structural organisation but displayed a significant increase in atretic follicles, characterised by pyknotic nuclei or disorganised GCs ($p=0.0017$; Figure 19A, yellow arrowheads; quantified in Figure 19C), compared to FT. VT samples showed normal stromal and follicular morphology, with no detectable increase in atresia (Figure 19A, VT; quantified in Figure 19C), indicating that vitrification alone did not compromise tissue integrity. In contrast, VTC samples exhibited a pronounced decline in healthy follicle density and a significant increase in atretic follicles compared to VT ($p=0.0051$, Figure 19B; $p=0.0016$, Figure 19C; yellow arrowheads in Figure 19A). For quantitative analysis, follicles were further stratified by developmental stage into monolayered (primordial and primary), secondary, and antral phases. Monolayered follicle density did not differ significantly among FT, FTC, and VT groups but was significantly reduced in the VTC group ($p<0.0001$; Figure 19D). Secondary follicle density remained comparable across all conditions (Figure 19D). In contrast, antral follicle density was significantly reduced in FTC ($p=0.0122$) and VTC ($p=0.0190$) compared with FT and VT, respectively (Figure 19D), suggesting that short-term culture, rather than the vitrification itself, primarily impacts follicle survival.

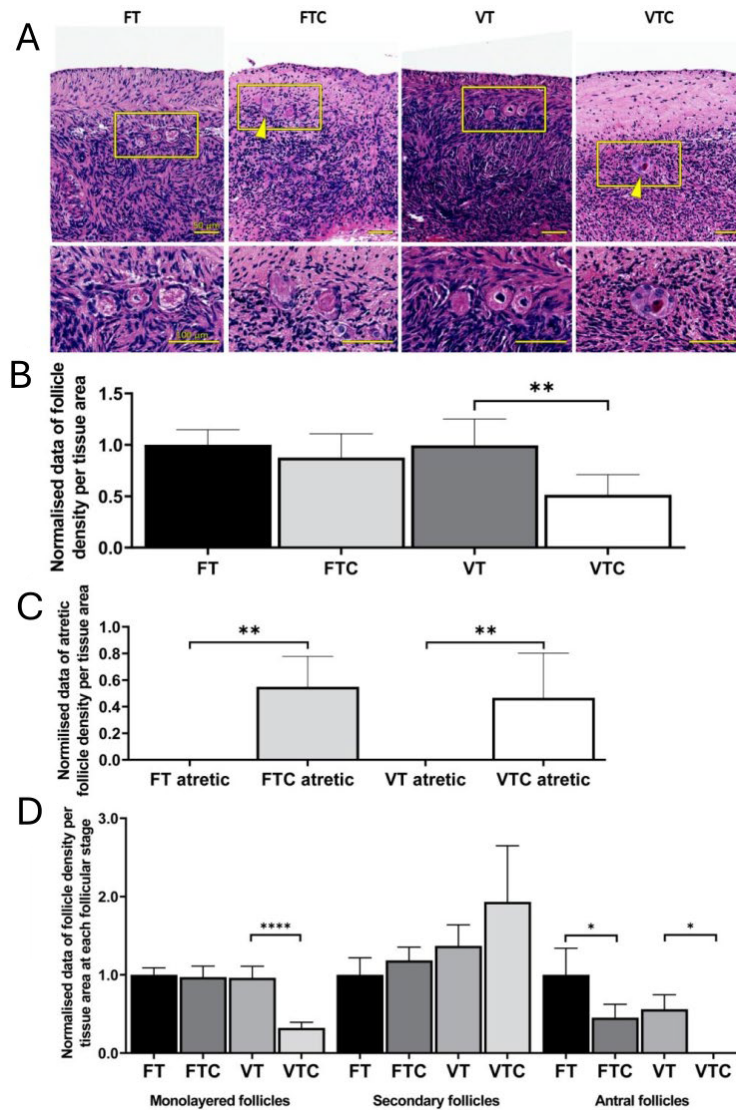


Figure 19: Histological assessment of ovarian tissue. (A) Representative H&E-stained sections from the four experimental groups – FT, FTC, VT, and VTC. Yellow arrowheads indicate atretic follicles, and yellow boxes highlight regions shown at higher magnification in the lower image panels. (B) Bar chart showing the normalised density of viable follicles (combined counts of monolayered, secondary, and antral follicles) across experimental groups. Follicle density was determined from four randomly selected sections per animal, including only follicles with a visible nucleus. (C) Normalised density of atretic follicles across groups. (D) Normalised density of follicles categorised by developmental stage. Data are presented as mean $\pm$ SEM ($n = 10$ animals per group). Statistical analysis was performed using two-way ANOVA. * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$. Scale bars: 50 μm / 100 μm . **Abbreviations:** H&E – Hematoxylin & Eosin; FT – fresh tissue; FTC – fresh tissue after culture; VT – vitrified – thawed tissue; VTC – vitrified – thawed tissue after culture.

5.6. Ovarian tissue culture enhances ovarian stromal proliferation and increases DNA double-strand breaks

To assess the impact of vitrification and subsequent culture on cellular proliferation, immunohistochemical staining for the monoclonal mouse/rat anti-human Ki67 antibody was performed on ovarian tissue from ten bovine samples. Proliferating cell density did not differ significantly between the FT and VT groups ($p = 0.8605$; Figure 20A, B), indicating that vitrification alone did not alter basal proliferative activity. In contrast, short-term culture substantially increased the number of Ki67⁺ cells in both FTC ($p = 0.0030$) and VTC ($p = 0.0027$) relative to their respective non-cultured controls (FT and VT) (Figure 20A, B).

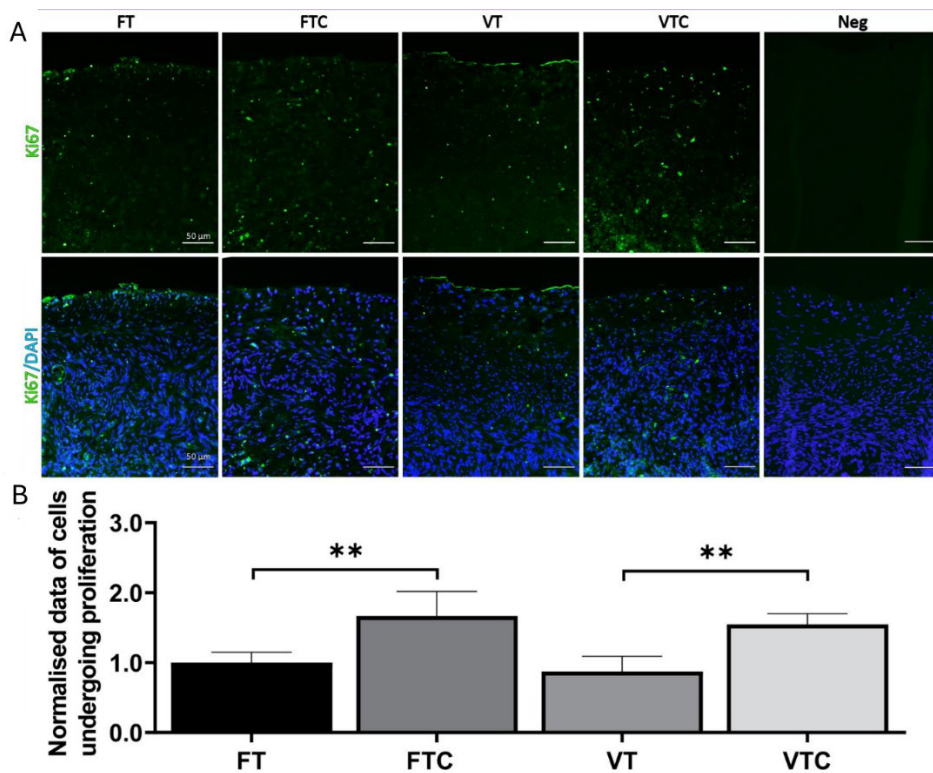


Figure 20. Cell proliferation before and after ovarian tissue culture. (A) Representative immunofluorescence images showing Ki67-positive proliferating cells (green) and nuclei counterstained with DAPI (blue) across the experimental groups. A negative control (Neg) is shown for reference. (B) Quantification of Ki67-positive cells. Four random sections per animal were analysed, and Ki67-positive cell counts were normalised to the corresponding tissue area. Data are presented as mean $\pm$ SEM ($n = 10$ animals per group). Statistical analysis by two-way ANOVA. $**p \leq 0.01$. Scale bars: 50 μ m. **Abbreviations:** FT – fresh tissue; FTC – fresh tissue after culture; VT – vitrified – thawed tissue; VTC – vitrified – thawed tissue after culture.

Moreover, the same ovarian pieces were used to evaluate the impact of vitrification and subsequent culture on DNA damage by the TUNEL assay. Although vitrification resulted in a higher number of TUNEL-positive cells compared with fresh tissue, this difference was not statistically significant ($p = 0.6837$ for VT vs FT; Figure 21A, B). In contrast, in vitro culture induced a pronounced increase in TUNEL-positive cells in both FTC and VTC groups, with both comparisons reaching high statistical significance ($p < 0.0001$; Figure 21A, B). These findings indicate that DNA fragmentation is primarily driven by in vitro culture rather than by vitrification itself.

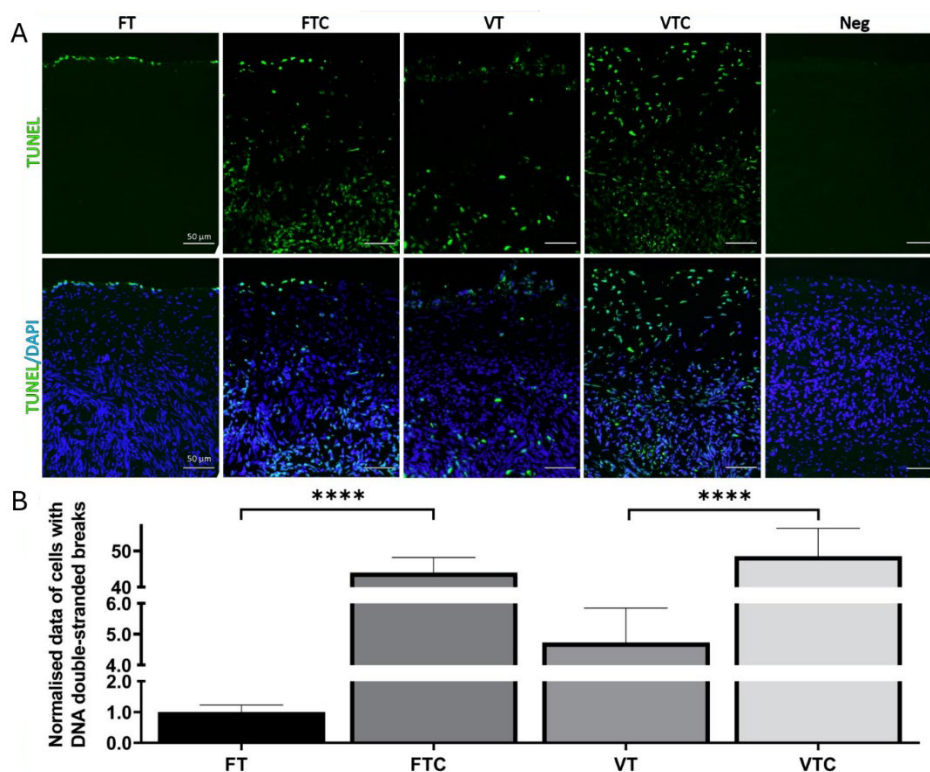


Figure 21. DNA fragmentation in ovarian tissue before and after culture. (A) Representative images showing TUNEL-positive (TUNEL⁺) cells (green) and nuclei counterstained with DAPI (blue) across the experimental groups. **(B)** Quantification of TUNEL⁺ cells. Counts were obtained from four randomly selected sections per animal and normalised to the analysed tissue area. Data are presented as mean ± SEM (n = 10 animals per group). Statistical analysis by two-way ANOVA. **** $p < 0.0001$. Scale bars: 50 μm. **Abbreviations:** FT – fresh tissue; FTC – fresh tissue after culture; VT – vitrified – thawed tissue; VTC – vitrified – thawed tissue after culture.

5.7. Survival rate of isolated follicles during a 6-day culture period

Morphological assessment indicated that vitrification did not cause immediate damage to ovarian tissue upon thawing; however, a marked reduction in follicle density emerged during subsequent culture in the vitrified group. This suggests that cryopreservation-induced effects may become evident only in functional assays such as ovarian tissue culture. To investigate this further, follicles were isolated from FT and VT groups and monitored over a 6-day period to evaluate their growth and viability *in vitro* (Figure 22A). Viability was assessed using Calcein AM/EthD-1 double fluorescent staining, whereby viable follicles exhibited green fluorescence in the oocyte and $\geq 60\%$ of GCs, while non-viable follicles showed red fluorescence in the oocyte and $\geq 60\%$ of GCs (Figure 22B, C). A total of 525 follicles from fresh tissue and 152 follicles from vitrified-thawed tissue were successfully isolated from 19 bovine ovaries. Follicle viability was assessed every second day during culture. On day 0, viability did not differ significantly between groups: 93.5% (100/107) of follicles from FT and 87.5% (7/8) of follicles from VT were viable (Figure 22D, E). In the FT group, follicle viability remained stable throughout the 6-day culture period (day 2, $p = 0.85$; day 4, $p = 0.8808$; day 6, $p = 0.3913$) (Figure 22D). In contrast, follicles from the VT group maintained high viability up to day 4 (day 2, $p = 0.8487$; day 4, $p = 0.7133$), but viability declined sharply by day 6, with only 23.1% (6/26) remaining alive ($p = 0.0199$) (Figure 22E). Overall, these results indicate that follicles isolated from vitrified-thawed ovarian tissue can be maintained in culture with high viability up to 4 days; however, their survival decreases significantly thereafter compared with follicles derived from fresh tissue.

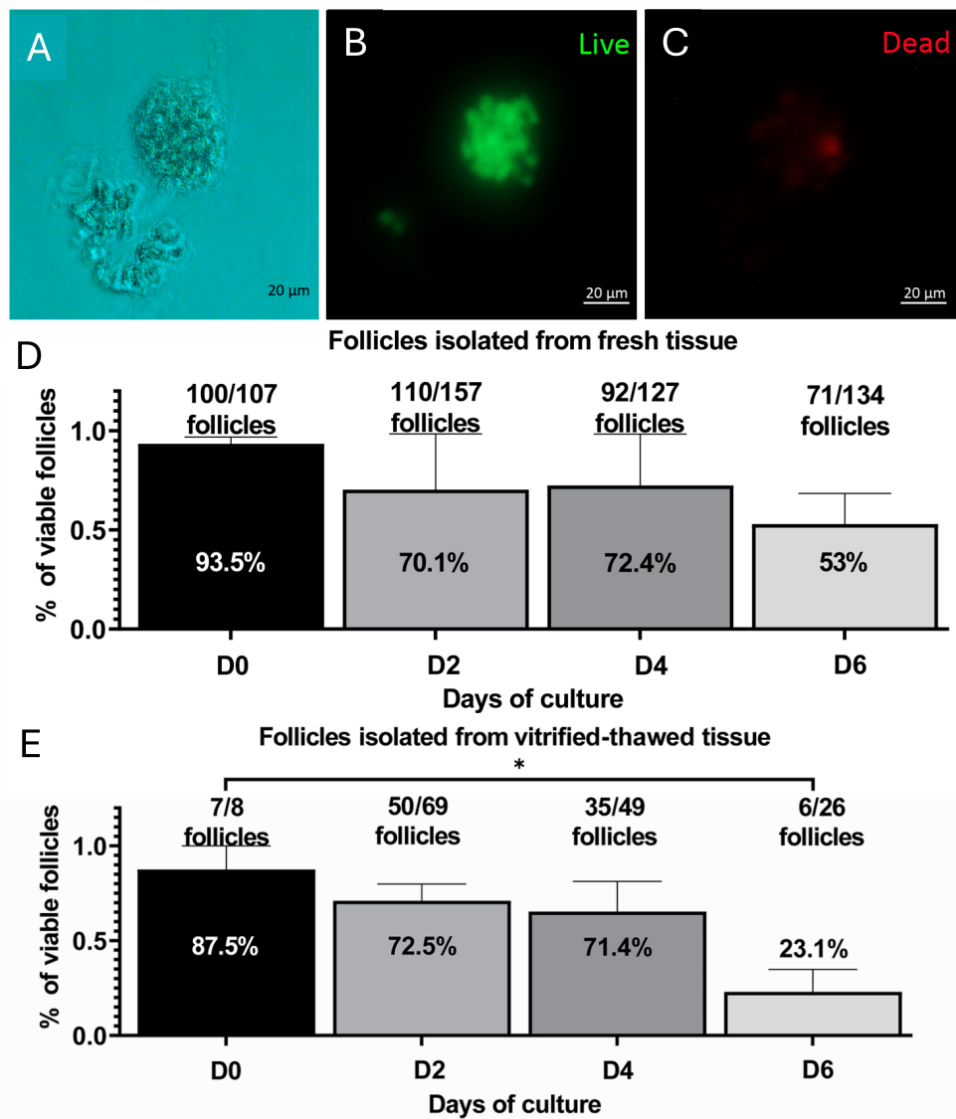


Figure 22: Viability of isolated follicles during culture. (A) Representative bright-field image of a freshly isolated follicle. (B) Example of a viable follicle showing green fluorescence in the oocyte and GCs following Calcein AM staining. (C) Example of the same follicle shown in (B), displaying red fluorescence in non-viable cells following EthD-1 staining. (D) Viability of follicles isolated from FT over a 6-day culture period. In total, 525 follicles were isolated from FT, with subgroups assessed for viability at baseline (day 0) and on days 2, 4, and 6 of culture. (E) Viability of follicles isolated from VT during a 6 days culture period. A total of 152 follicles were isolated from VT and assessed at the same time points. Data are presented as mean percentage viability $\pm$ SEM, with the number of analysed follicles indicated above each bar. Statistical analysis was performed using one-way ANOVA; * $p \leq 0.05$. **Abbreviations:** GCs – granulosa cells; FT – fresh tissue; VT – vitrified – thawed tissue.

5.8. Vitrification/thawing exerts little influence on ovarian tissue gene expression

To evaluate the impact of vitrification on ovarian tissue after thawing, transcriptomic profiling was performed on cortical fragments from FT, VT, FTC, and VTC groups ($n = 7$ animals). PCA showed that PC1, explaining 70% of total variance, clearly segregated uncultured (FT and VT) from cultured (FTC and VTC) samples (Figure 23A), indicating that culture status was the dominant source of transcriptional variation. PC2 further separated fresh from vitrified samples, although this effect was comparatively modest. Overall, vitrification exerted only a limited influence on basal gene expression relative to the extensive shifts induced by culture. Differentially expression analysis, applying $|FC| > 2$ and $FDR < 0.05$, identified 175 DEGs between uncultured fresh and vitrified tissue (VT vs FT), with 128 genes upregulated in vitrified samples. Similarly, comparison of vitrified and fresh cultured tissue (VTC vs FTC) yielded 158 DEGs, of which 13 were upregulated. Intersection of these datasets revealed 29 shared DEGs, including *CCNA2*, *GSTA2*, *INSL3*, *IRS4*, *MMP3*, *OVOL1*, and *RELN*, representing a small set of vitrification-associated transcriptional alterations that persisted after culture (Figure 23B).

To characterise the effects of culture itself, we compared FTS vs FT and VTC vs VT samples. Culture induced extensive transcriptomic reprogramming, with 1,042 DEGs identified in fresh tissue and 1,181 DEGs in vitrified tissue. Both comparisons showed a balanced distribution of up- and downregulated genes. While a substantial core set of DEGs was shared between the two groups ($n = 725$), several hundred genes were uniquely affected in either fresh ($n = 317$) or vitrified ($n = 456$) tissue during culture (Figure 23C), indicating that vitrification modulates certain culture-induced responses.

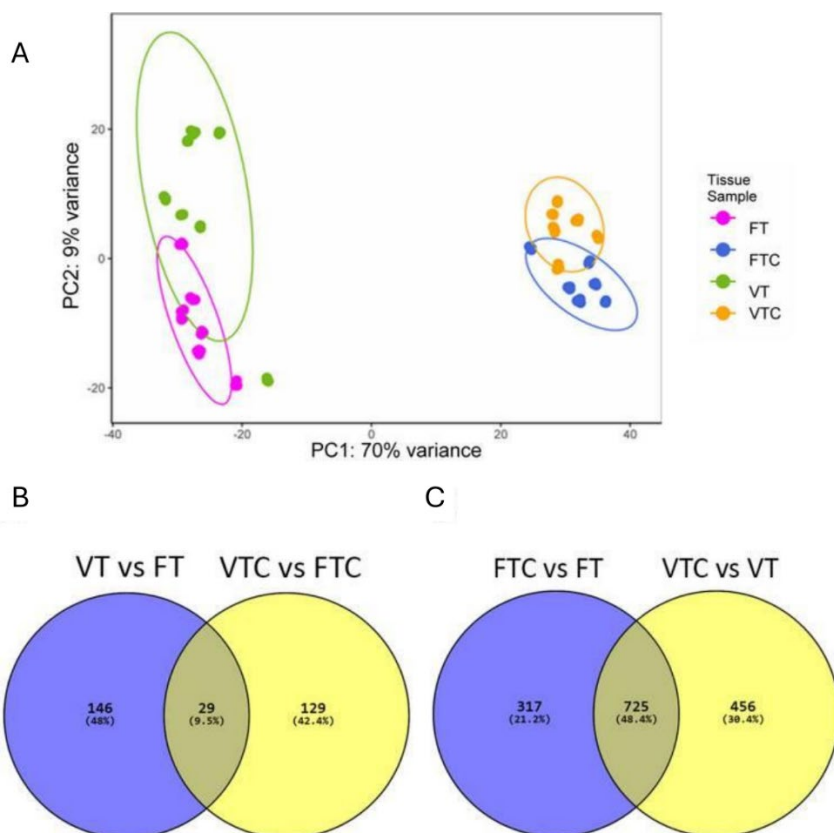


Figure 23: Overview of transcriptomic alterations associated with vitrification and culture: (A) PCA of bulk RNA-seq data from ovarian cortical tissue in four experimental groups: fresh tissue (FT; purple), fresh tissue after culture (FTC; blue), vitrified-thawed tissue (VT; green), and vitrified-thawed tissue after culture (VTC; orange). Shaded ellipses represent the confidence intervals for each group, illustrating variance and covariance; larger ellipses reflect greater variability within the group, while smaller ellipses indicate tighter clustering. (B) Venn diagram showing the overlap of differentially expressed genes (DEGs) between fresh and vitrified tissue in uncultured (VT vs FT) and cultured (VTC vs FTC) comparisons. (C) Venn diagram depicting the overlap of DEGs between cultured and uncultured tissue for both fresh (FTC vs FT) and vitrified (VTC vs VT) samples. **Abbreviations:** FT – fresh tissue; FTC – fresh tissue after culture; VT – vitrified – thawed tissue; VTC – vitrified – thawed tissue after culture; PCA – principal component analysis.

5.8.1. Functional pathway alterations associated with culture and vitrification

To investigate the biological significance of these transcriptional changes, gene set enrichment analysis (GSEA) was performed using KEGG pathway annotations for *Bos taurus* via the g:Profiler platform, applying a significance threshold of $FDR < 0.05$. No significantly enriched pathways were identified in

vitrification-related comparison (VT vs FT and VTC vs FTC), consistent with the relatively small number of DEGs ($n < 200$). In contrast, culture-induced transcriptional changes were associated with broad and highly significant pathway enrichment. In fresh tissue (FTC vs FT), 24 pathways were enriched among upregulated DEGs and 34 among downregulated DEGs. Prominently affected pathways included HIF-1 signalling, glycolysis/gluconeogenesis, and amino acid biosynthesis (Figure 24). Similarly, in vitrified tissue (VTC vs VT), 18 pathways were enriched for upregulated DEGs and 38 among downregulated DEGs, with HIF-1 signalling and glycolysis/gluconeogenesis again among the most significantly altered pathways (Figure 24).

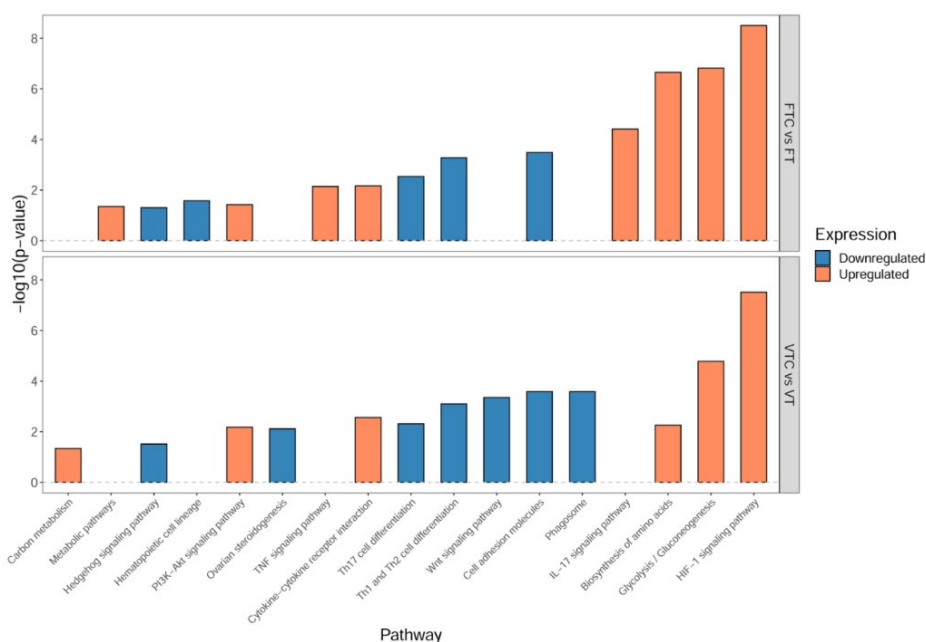


Figure 24: Signalling pathways enriched during culture. KEGG pathway analysis was performed to identify signalling pathways enriched during in vitro culture of fresh and vitrified ovarian tissue, based on differentially expressed genes. The most significantly enriched pathways are shown for each comparison, with orange bars representing pathways enriched among upregulated genes and blue bars representing pathways enriched among downregulated genes. The y-axis shows the $-\log_{10}$ adjusted p-values calculated using the Benjamin-Hochberg (BH) method, while the x-axis lists the corresponding KEGG pathways. The upper panel presents results for FTC vs FT, and the lower panel shows VTC vs VT. **Abbreviations:** FT – fresh tissue; FTC – fresh tissue after culture; VT – vitrified – thawed tissue; VTC – vitrified – thawed tissue after culture; BH – Benjamin-Hochberg.

Comparative pathway analysis revealed extensive overlap between cultured fresh and vitrified tissue, but also identified vitrification-associated alterations. These included the Wnt signalling pathway, ovarian steroidogenesis, carbon metabolism, and phagosome-related functions (Figure 24). Given the essential roles of Wnt signalling and steroidogenesis in follicular development and tissue integrity, these pathways were examined in greater detail. KEGG pathway mapping revealed disruptions of both canonical and Ca^{2+} -dependent Wnt signalling cascades in VTC, suggesting potential downstream consequences for cell cycle regulation, cytoskeletal organisation, and cell-cell adhesion (Figure 25).

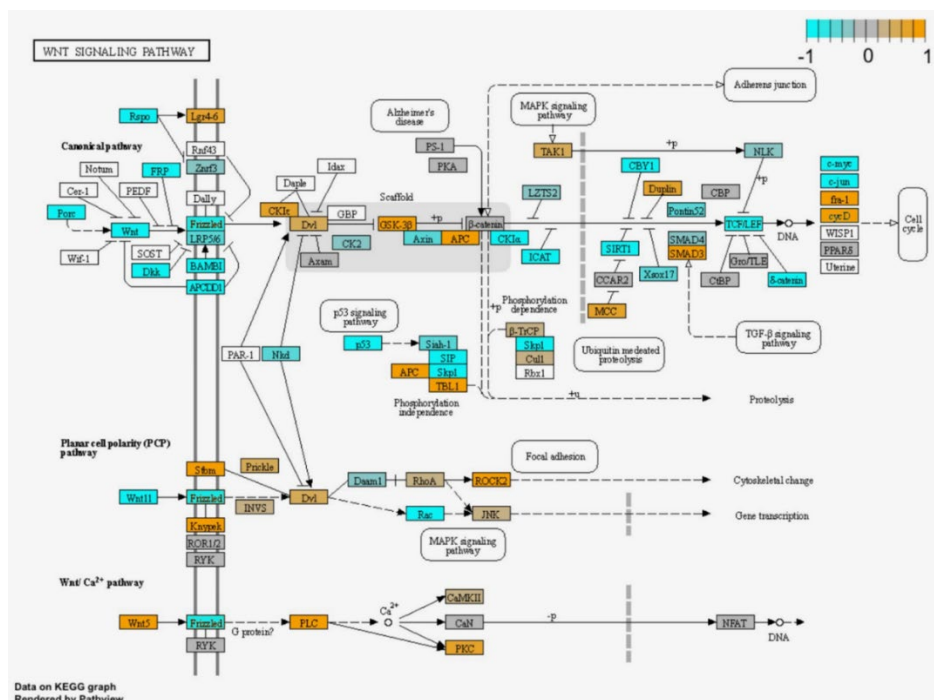


Figure 25: Alterations in WNT signalling during culture of vitrified ovarian tissue. KEGG pathway mapping illustrates relative gene expression changes within the WNT signalling pathway for the VTC vs VT comparison. Genes upregulated during culture are shown in orange, while downregulated genes are shown in blue. Genes with no detectable expression are depicted in white, and genes without significant expression changes are shown in grey. **Abbreviations:** VT – vitrified – thawed tissue; VTC – vitrified – thawed tissue after culture.

In the ovarian steroidogenesis pathway, key theca cell genes, including *LHCGR*, *STAR*, *CYP11A1*, and *HSD3B*, were markedly downregulated in VTC (Figure 26), indicating impaired hormone biosynthesis. Given the functional interplay between Wnt signalling and steroidogenesis, these coordinated disruptions may contribute to the reduced follicle viability observed in vitrified ovarian tissue.

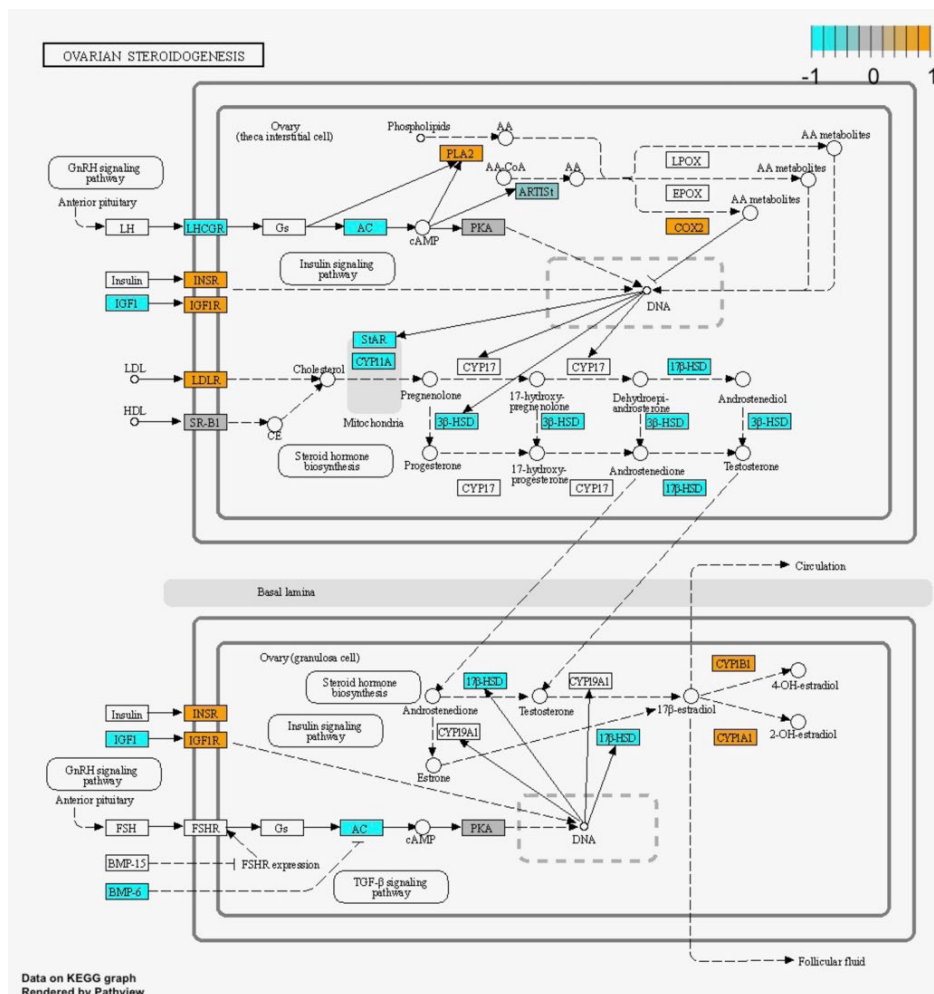


Figure 26: Changes in the ovarian steroidogenesis pathway during culture of vitrified tissue. KEGG pathway mapping illustrates relative gene expression changes in the ovarian steroidogenesis pathway for the VTC vs VT comparison. Genes upregulated during culture are shown in orange, while downregulated genes are shown in blue. The upper panel depicts alterations in theca interstitial cells, while the lower panel illustrates changes in GCs. Genes with no detectable expression are shown in white, and genes with unchanged expression are shown in grey. **Abbreviations:** VT – vitrified – thawed tissue; VTC – vitrified – thawed tissue after culture; GCs – granulosa cells.

5.9. Microbial DNA detection in ovarian tissue and follicular fluid

To investigate whether microbial composition might contribute to the resilience of ovarian tissue during cryopreservation, we assessed the presence and diversity of bacterial DNA in healthy fresh and vitrified ovarian tissue. In total, 27 ovarian tissue samples and 6 follicular fluid samples were analysed. PCR amplification targeting bacterial 16S rRNA detected no bacterial DNA in 16 ovarian tissue samples (59.3%) and in 5 follicular fluid samples (83.3%). Eleven ovarian tissue samples, one follicular fluid sample, and all six negative controls were subsequently processed for 16S rRNA gene sequencing. Following sequencing and quality filtering, two ovarian tissue samples and the single follicular fluid sample were excluded from diversity analyses due to insufficient numbers of high confidence reads. Mock community sequencing confirmed successful DNA extraction, library preparation, and sequencing performance. Despite rigorous contamination-control procedures, a substantial proportion of reads (30.3% – 100%, median 62.1%) were identified as contaminants (Figure 27A). Although alpha-diversity values decreased following decontamination, no statistically significant differences were observed between ovarian samples and negative controls (Shannon diversity: $P = 0.784$; richness: $P = 0.453$) (Figure 27B).

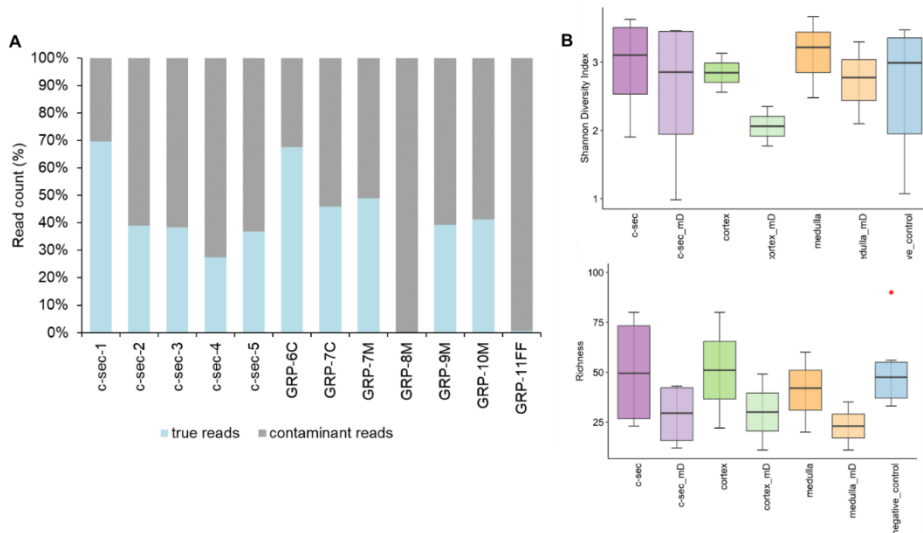


Figure 27: Microbial profiling of ovarian samples from gender-affirming patients and c-sec cases. Ovarian cortex (C; $n = 2$), medulla (M; $n = 4$), and FF ($n = 1$) samples from gender-affirming patients, together with ovarian cortex samples from c-sec patients ($n = 5$), were analysed. **(A)** Proportion of contaminant versus true microbial reads identified following in-silico decontamination. **(B)** Alpha-diversity measured by Shannon index (top) and richness (bottom). Box-and-whisker plots show the median, interquartile range, and full range; a red dot in the richness plot indicates an outlier. **Abbreviations:** c-sec – Caesarean section; FF – Follicular Fluid.

Across ovarian tissue and follicular fluid samples, 227 bacterial genes were detected, of which 83 were classified as contaminants and removed before downstream analysis. After decontamination, microbial profiles of ovarian tissue samples exhibited high inter-individual variability (Figure 28).

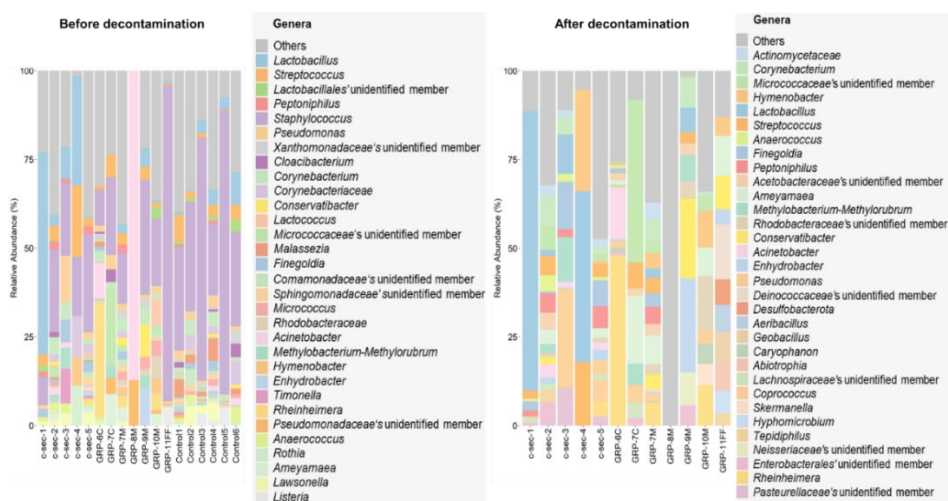


Figure 28: Microbial relative abundance of ovarian samples from gender-affirming patients and c-sec cases. Ovarian cortex (C; n = 2), medulla (M; n = 4), and FF (n = 1) samples from gender-affirming patients, together with ovarian cortex samples from c-sec patients (n = 5), were analysed. The graph displays the relative abundance (%) of bacterial taxa before (left panel) and after (right panel) decontamination, shown as stacked bar plots. **Abbreviations:** c-sec – Caesarean section; FF – Follicular Fluid.

Beta-diversity analysis was performed to evaluate differences in microbial community structure across groups (Figure 29). After correction for multiple comparisons, the microbial composition of decontaminated ovarian tissue samples differed significantly from negative controls ($P = 0.003$) (Figure 29 right). However, no significant differences were detected between decontaminated and non-decontaminated ovarian tissue samples ($P = 0.194$) (Figure 29, left). Collectively, these data do not support the presence of a distinct ovarian-specific microbiome. Instead, the bacterial DNA detected in ovarian tissue and follicular fluid is most consistent with environmental or reagent contamination, insufficient sequencing depth, or the presence of minimal and biologically insignificant bacterial material.

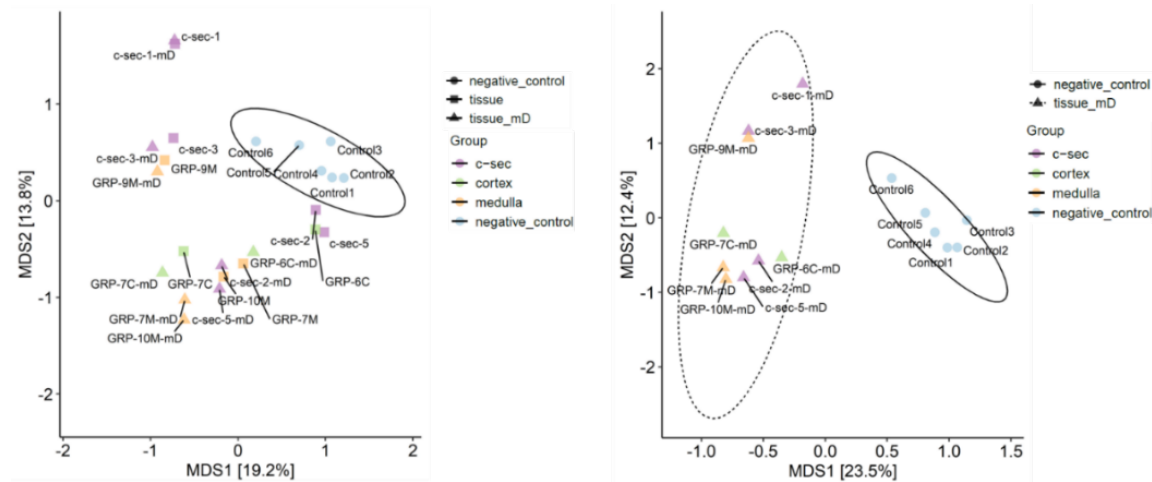


Figure 29: Microbial profiling of ovarian samples from gender-affirming patients and c-sec cases. Ovarian cortex (C; n = 2), medulla (M; n = 4), and FF (n = 1) samples from gender-affirming patients, together with ovarian cortex samples from c-sec patients (n = 5), were analysed. Beta-diversity visualised by PCoA using Bray–Curtis distances, comparing samples before and after decontamination (left) and after decontamination only (right), including negative controls. Alpha-diversity comparisons were performed using Kruskal–Wallis and Wilcoxon tests; and beta-diversity differences were assessed via permutational multivariate analysis of variance with FDR-adjusted p-values. In the PCoA plots, point shape indicates processing stage (circles = negative controls, squares = pre-decontamination, triangles = post-decontamination), and colours indicate sample type (violet = c-sec, green = cortex, orange = medulla, blue = negative control). Ellipses outline negative controls (solid line) and post-decontamination ovarian samples (dotted line). **Abbreviations:** c-sec – Caesarean section; FF – Follicular Fluid; PCoA – Principal Coordinate Analysis; FDR – False Discovery Rate.

6. DISCUSSION

This thesis addresses key aspects of fertility preservation by investigating the response of ovarian tissue somatic cells to irradiation (Study I), evaluating alternative protocols for ovarian tissue cryopreservation (OTC) (Study II), and assessing the presence of potential ovarian tissue microbiome (Study III) as a factor that might influence fertility preservation outcomes.

6.1. cPOCs and mPOCs exhibit distinct DNA damage responses following irradiation

Although ovarian tissue contains a diverse mixture of somatic germ cells, most previous studies have predominantly focused on the radiosensitivity of oocytes, GCs, and follicles (Larsen *et al.*, 2003; Chemaitilly *et al.*, 2006; Irtan *et al.*, 2013; Mishra *et al.*, 2017; Kimler *et al.*, 2018; Immediata *et al.*, 2022; Wang *et al.*, 2023), or ovarian cancer cell lines (Langland *et al.*, 2010; Li *et al.*, 2020; Wang *et al.*, 2020). In contrast, Study I specifically examined the response of healthy primary ovarian cells (POCs) from cortex (cPOCs) and medulla (mPOCs) to an acute 10 Gy dose of X-ray irradiation. We demonstrated a rapid activation of DNA damage markers in cPOCs (Figure 9A), whereas mPOCs displayed a delayed response (Figure 9B). While cPOCs recovered more quickly from irradiation-induced cell cycle arrest (Figure 10A), mPOCs exhibited a more persistent arrest (Figure 10B). In both cell types, irradiation triggered sustained activation of the p53 pathway and downregulation of proliferative signalling (Figure 14), together with pronounced alterations in proteins regulating cell adhesion and ECM organisation (Figure 17). These molecular changes ultimately compromised the ability of irradiated cells to assemble functional Silk-Ovarioids (Figure 18).

Ionising radiation induces DNA damage through both direct strand breaks and oxidative stress, with approximately 70% of the damage resulting from free radical formation (Baskar *et al.*, 2014; Wang, Wang and Qian, 2018). This damage activates the canonical DNA damage response (DDR), beginning with phosphorylation of histone H2A2 (γ H2AX) at sites of double-strand breaks (Siddiqui *et al.*, 2015), followed by Chk1 activation to promote DNA repair and cell cycle checkpoint control (Patil, Pabla and Dong, 2013). In Study I, cPOCs exhibited early DDR activation at 1 h post-irradiation, characterised by a robust increase in γ H2AX and p-Chk1 (Figure 9A), whereas mPOCs showed delayed activation, peaking at 4 h (Figure 9B).

Importantly, around 5% of DNA double-strand breaks remain unrepaired following irradiation (Minchom, Aversa and Lopez, 2018), which can lead to prolonged cell cycle arrest to prevent replication of damaged DNA. This arrest is critical determinant of post-irradiation cell fate, providing time for DNA repair; when damage is irreparable, cells may undergo apoptosis or senescence to

prevent the propagation of genomic instability (Hein, Ouellette and Yan, 2014; Shim *et al.*, 2014). Such protective mechanisms operate in both somatic and germline cells (Shim *et al.*, 2014). Consistent with these principles, our findings revealed distinct cell cycle responses in cPOCs and mPOCs. cPOCs exhibited a transient cell cycle arrest and resumed proliferation relatively quickly, as indicated by early cyclin E activation at 1 h post-irradiation and subsequent normalisation of p-p21 levels by 4 h (Figure 10A). In contrast, mPOCs showed a stronger and more sustained cell cycle block, characterised by reduced cyclin E and cyclin D1 expression at 24 h (Figure 10B). These differences may reflect higher levels of accumulated DNA damage, reduced repair efficiency, or cell-cycle-phase-dependent radiosensitivity, as cells in S-phase has proven to be relatively more radioresistant (Hill *et al.*, 2023).

6.2. cPOCs and mPOCs exhibit persistent cell cycle arrest and p-53 mediated stress response following irradiation

Apoptosis represents another key outcome of unrepaired DNA damage. The p53 signalling pathway plays a central role in coordinating DNA damage responses, including cell cycle arrest and apoptosis, depending on the site and context of p53 phosphorylation (Reinhardt and Schumacher, 2012). Both cPOCs and mPOCs exhibited an early increase (1 h) in pro-apoptotic markers (cl-Cas3 and p53), followed by a decline at later timepoints (4 and 24 h), accompanied by transient upregulation of Bcl-2 (Figure 11A, B). This pattern suggests a shift away from apoptosis toward sustained cell cycle arrest, likely driven by the selective survival of more resistant cells following irradiation. Such dynamics mirror the p53-dependent delayed mitotic recovery previously described in irradiated retinal pigment epithelial cells (Reyes *et al.*, 2018).

Transcriptomic analysis further supported these observations, revealing a persistent activation of the p53 pathway alongside a broad suppression of the cell cycle associated signalling, including E2F targets, mTORC1 signalling, the G2/M checkpoint, and MYC target genes at 24 h post-irradiation in both cell types (Figure 14C). A prolonged p53 activity after irradiation has been reported previously (Stewart-Ornstein *et al.*, 2021) and has been identified as a marker of radiation sensitivity in cancer patients (Stewart-Ornstein *et al.*, 2021; Thariat *et al.*, 2021). Consistent with the mechanistic model proposed by Reyes *et al.* (Reyes *et al.*, 2018), we observed increased p-p21 levels and reduced cyclin E expression in both cell types (Figure 10A, B), despite the relatively low detection level of p-p53 protein at later timepoints (Figure 11A, B). In addition, downregulated DEGs highlighted disruptions in chromatin segregation and mitotic machinery (Figures 15 and 16), suggesting unresolved DNA damage and impaired genomic stability (Lonati *et al.*, 2021).

Furthermore, cyclin D1 expression remained stable in cPOCs but progressively decreased in mPOCs, further supporting the idea that mPOCs are more radiosensitive. The higher sensitivity may reflect their higher proliferative capa-

city, as rapidly dividing cells are typically more vulnerable to radiation-induced damage (Panyutin *et al.*, 2017). Alternatively, it may be exacerbated by normoxic culture conditions, which are known to enhance radiation-induced damage, whereas lower oxygen tension can mitigate such effects (Koch, Kim and Wiersma, 2023).

The role of MYC further supports these interpretations. MYC is a key driver of cell cycle progression (García-Gutiérrez, Delgado and León, 2019), acting primarily by suppressing cell cycle inhibitors such as p15, p21, and p27 (Serrano, Hannon and Beach, 1993; Gartel *et al.*, 2001) and by regulating the G1/S checkpoint (Sheen and Dickson, 2002). In line with this, we observed a pronounced downregulation of gene patterns associated with the G1/S phase transition particularly in mPOCs at 24 h post-irradiation (Figure 16), indicative of cell cycle disruption and potential genomic instability (Sheen and Dickson, 2002). Thus, the combined downregulation of MYC target genes (Figure 14) together with the upregulation of p-p21 at 1 h in cPOCs (Figure 10A) and throughout the entire recovery period in mPOCs (Figure 10B) is consistent with the induction of cell cycle arrest. These findings suggest that MYC-dependent signalling may play a key role in the adaptive response to irradiation in cPOCs – and especially in mPOCs – by reinforcing cell cycle arrest mechanisms and potentially contributing to long-term genomic instability.

6.3. Irradiation-induced damage disrupts cell adhesion, extracellular matrix organisation and Silk-Ovarioids formation

Proteomic analyses revealed that irradiation markedly affected cell adhesion pathways. Proteins involved in focal adhesion and cadherin-mediated binding were significantly downregulated in both cell types (Figure 17B). These processes are essential components of ECM organisation, which provides both structural support and biochemical cues that regulate tissue integrity and cellular behaviour (Frantz, Stewart and Weaver, 2010). Integrins and cadherins act as the central regulators of cell-ECM and cell-cell interactions (Harburger and Calderwood, 2009; Xian, Gopal and Couchman, 2010; Weber, Bjerke and DeSimone, 2011), and their disruption has been shown to enhance radiosensitivity (Babel *et al.*, 2017) or, in certain contexts, facilitate adaptive radioresistance (Osuka *et al.*, 2021).

Beyond their structural role, cell adhesion mechanisms critically influence cytoskeletal organisation and cell migration (Schmidt and Friedl, 2010). Accordingly, the impaired Silk-Ovarioid formation observed in irradiated POCs (Figure 18) is consistent with defective ECM deposition and compromised cell-cell communication.

Previous studies have demonstrated that a successful Silk-Ovarioids assembly depends on the tightly coordinated interactions between ovarian somatic cells and

de novo ECM formation within the biomaterial scaffold (Di Nisio *et al.*, 2025). Overall, our findings demonstrate that exposure to a 10 Gy dose of X-ray irradiation induces profound and persistent alterations in both cortical and medullary ovarian somatic cells. Beyond activating the canonical DDR, irradiation disrupts fundamental processes governing cell cycle progression, survival signalling, and especially cell adhesion. Together, these alterations compromise the structural organisation required for ovarian tissue regeneration and limit the capacity of ovarian cells to support biomaterial-based reconstructive approaches.

6.4. Vitrification preserves follicle density, whereas *in vitro* culture promotes follicular atresia and DNA damage

The lack of a standardised ovarian tissue cryopreservation (OTC) protocol remains a major challenge in fertility preservation, as a wide range of slow-freezing and vitrification methods are used across laboratories and clinical centres (Kagawa, Silber and Kuwayama, 2009; Andersen, Bollerup and Kristensen, 2018; Silber *et al.*, 2018). The heterogeneous composition of ovarian tissue further complicates cryopreservation and thawing procedures, and several studies have suggested that vitrification may better preserve stromal morphology compared with slow freezing (Keros *et al.*, 2009; Schallmoser *et al.*, 2023).

In Study II, we examined the effects of vitrification on bovine ovarian cortex, evaluated the viability of follicles isolated from fresh and vitrified tissue, and assessed potential refinements to current vitrification approaches. Bovine ovaries were selected as a model system due to their availability and strong physiological similarities to human ovaries, including comparable folliculogenesis dynamics and age-related functional changes (Malhi, Adams and Singh, 2005; Rodgers and Irving-Rodgers, 2010; Sirard, 2017; Roberts and Jeff Huang, 2022).

Because follicles represent the functional unit of the ovary, their preservation is the primary objective of OTC. In Study II, vitrification alone did not significantly affect follicle density (Figure 19A). However, subsequent *in vitro* culture led to a pronounced decline in total follicle numbers (Figure 19A) and a corresponding increase in the number of atretic follicles (Figure 19C) in both fresh and vitrified cultured tissue, consistent with previous findings using comparable protocols (Salehnia *et al.*, 2012; Abdollahi *et al.*, 2013; Sheikhi *et al.*, 2013; Ramezani, Salehnia and Jafarabadi, 2017).

Notably, increased follicular atresia was observed exclusively in cultured tissue, indicating that this effect is likely driven by culture-induced stress, including oxygen deprivation resulting from the absence of vascular support. Further analysis revealed that VTC exhibited reduced densities of monolayered and antral follicles (Figure 19D), along with a modest increase in secondary follicle density (Figure 19D). This pattern may reflect the premature activation of primordial follicles triggered by the mechanical stress during cortical fragmentation (De Roo *et al.*, 2020), potentially mediated through the disruption of the

Hippo signalling pathway and reciprocal activation of Akt signalling (Liu-Chittenden *et al.*, 2012; Kawamura *et al.*, 2013).

Previous studies have also linked reduced follicle density to increased ovarian stromal proliferation (McLaughlin *et al.*, 2014). In line with these reports, proliferation indices did not differ between FT and VT samples, whereas both cultured groups exhibited significantly higher proliferation compared with their respective non-cultured controls (Figure 20B). While increased proliferation could suggest enhanced regenerative activity, it more plausibly reflects a compensatory response to cellular stress or cell loss under suboptimal culture conditions (Yang, Ray and Krafts, 2014). This interpretation is supported by the significant increase in DNA DSBs observed in cultured groups (Figure 21B), indicating that in vitro culture conditions, rather than vitrification, are the primary drivers of the damage responses in ovarian cortex.

6.5. Vitrification reduces follicle recovery and long-term survival following isolation

Although vitrification did not significantly affect follicle density (Figure 19A) or DNA damage levels (Figure 21B), vitrified samples yielded fewer isolated follicles compared with fresh tissue (Figure 22D, E), even when using an optimised enzymatic isolation protocol validated in previous studies (Wary *et al.*, 1996; Hovatta *et al.*, 1999; Dolmans *et al.*, 2006). This reduction may result from increased mechanical fragility or cryo-induced microdamage that compromises follicle integrity during the isolation process.

Initial post-isolation viability was high in both fresh (93.5%) and vitrified (87.5%) tissue (Figure 22D, E), consistent with earlier reports (Martinez-Madrid *et al.*, 2004; Amorim *et al.*, 2009; Vanacker *et al.*, 2011; Yin *et al.*, 2016). Nevertheless, follicle survival declined markedly by day 6, particularly in the VT group (53% in FT vs 23.1% in VT).

Recent evidence suggests the presence of distinct follicle subpopulations within the ovarian cortex (Rooda *et al.*, 2024), which may respond differently to encapsulation and in vitro culture. Thus, selection bias during isolation may contribute to a reduced long-term survival. While alginate scaffolds maintained structural stability throughout the culture, more physiologically relevant matrices, such as Silk-Ovarioids, may provide improved support for follicular growth and development. These findings highlight the need for further optimisation of both follicle isolation procedures and long-term culture systems.

6.6. In vitro culture, rather than vitrification, drives major transcriptomic changes in ovarian cortex

Transcriptomic analysis supported the histological and functional findings. *In vitro* culture induced substantial transcriptional reprogramming compared with

non-cultured controls. However, direct comparisons between vitrified and fresh tissue (VT vs FT and VTC vs FTC) did not yield significantly enriched KEGG pathways, indicating that vitrification alone has minimal impact on the ovarian tissue transcriptome.

In contrast, GSEA comparing cultured versus non-cultured tissues (FTC vs FT and VTC vs VT) identified both shared and distinct transcriptional responses, with 725 overlapping DEGs detected between comparisons (Figure 23C). Pathways enriched exclusively in vitrified-culture comparisons therefore reflect vitrification-specific vulnerabilities that become apparent only after subsequent *in vitro* culture.

6.7. Vitrification alters WNT signalling pathways associated with follicle development and hormone responsiveness

Among the pathways identified, WNT signalling emerged as particularly affected (Figures 24 and 25). In vitrified samples, *WNT11* was downregulated and *WNT5A* upregulated, demonstrating an expression pattern opposite to that observed in fresh tissue. WNT signalling plays a critical role in follicle development, cellular proliferation, and migratory behaviour (Anastas and Moon, 2013).

Importantly, *WNT5A* expression has been further associated with the differentiation status of GC: it is upregulated in luteinised human GCs (Kenigsberg *et al.*, 2009; Sanchez *et al.*, 2014) and inversely correlated with FSH levels in bovine GCs (Abedini *et al.*, 2015). The downregulation of *WNT5A* in VT compared to FT (Figure 25) may therefore contribute to the altered follicular dynamics observed following culture (Figure 19B). Conversely, the marked upregulation of *WNT5A* in VTC may impair FSH responsiveness, potentially contributing to the lower live birth rates reported after transplantation of vitrified ovarian tissue (Arapaki *et al.*, 2022).

6.8. Altered LHCGR signalling may influence post-thaw follicle activation and ovarian function

We also identified opposing patterns of *LHCGR* expression between VT and VTC relative to their respective controls (FT and VT). *LHCGR*, primarily expressed in theca cells, is a key regulator of steroidogenesis and is essential for ovulation and luteinisation (Stocco, Telleria and Gibori, 2007).

The increased recruitment of secondary follicles in VTC (Figure 19D) aligned with the initial upregulation of *LHCGR* in VT and its subsequent downregulation following culture, suggesting that vitrification may transiently promote follicle activation, whereas culture conditions subsequently suppress *LHCGR* expression. In addition, *GSK3 β* , a known regulator to promote β -catenin degradation (Wang, Li and Kidder, 2010), was elevated in VTC (Figure 25). Because β -catenin promotes *LHCGR* expression (Law *et al.*, 2013), increased *GSK3 β* activity may

further attenuate *LHCGR* signalling, despite the absence of significant changes in β -catenin itself.

Taken together, these transcriptomic and functional findings indicate that while vitrification preserves follicular structure and overall transcriptomic integrity, it may subtly modulate pathways related to WNT signalling and ovarian hormone responsiveness. These pathways represent key molecular determinants of post-thaw follicle function and may ultimately influence clinical outcomes following ovarian tissue transplantation.

6.9. Assessment of the Ovarian Microbiome Reveals No Ovary-Specific Microbes

Growing interest in the reproductive tract microbiome and its potential influence on reproductive health and pregnancy outcomes (Moreno *et al.*, 2016; Moreno and Franasiak, 2017) has driven investigations into whether the ovary harbours a resident microbial community. Such knowledge could potentially propose improvements to fertility preservation protocols.

In Study III, we analysed ovarian tissue obtained from patients undergoing gender-affirming surgery and caesarean section to characterise the microbial environment of healthy ovaries. Microbial DNA sequences were detected in 36.4% of samples; however, these samples contained low microbial read counts and a high proportion of microbial contaminant sequences. These findings differ from earlier reports proposing the presence of an ovary-specific microbiome (Walsh *et al.*, 2019; Qin *et al.*, 2022), but are consistent with a recent investigation of neoplastic ovarian tissue using both 16S DNA sequencing and bacterial culture, which found no evidence of a distinct ovarian microbiome (Ingerslev *et al.*, 2025).

Disagreements between studies may arise from methodological variations. Although 16S rRNA gene sequencing remains a cornerstone of microbiome research due to its efficiency and phylogenetic resolution, it provides limited functional insight and may underestimate microbial diversity (Johnson *et al.*, 2019). These limitations are particularly important when analysing low-biomass tissues, where the risk of contamination is substantial and significantly influence study outcomes.

Indeed, the existence and biological significance of microbial communities in the upper female reproductive tract remain subjects of ongoing debate. While the vaginal microbiome is well established, evidence supporting resident microbial communities in low-biomass sites, *i.e.*, uterus, fallopian tubes and ovaries is less consistent. Although microbial signals have been detected in these tissues, there is always the possibility that such findings are influenced by contamination introduced during sample collection, DNA extraction, library preparation or sequencing. As a result, it remains unclear whether the reported microbiomes in the upper reproductive tract represent true microbiome existence or instead reflects to contamination and technical artefacts.

Overall, our findings do not support the existence of a stable, ovary-specific microbiome in healthy tissue. The bacterial DNA detected in our samples most likely reflects background contamination or brief microbial presence rather than a genuine resident microbial community.

6.10. Study limitations and future directions

Although this thesis presents several strengths, it is important to acknowledge its limitations. A key limitation across all three studies relates to the composition of the study population. Ovarian tissue for Study I and Study III was primarily obtained from patients undergoing gender-affirming surgery. While previous work suggests that ovarian tissue from gender-affirming patients is comparable in cellular composition to tissue collected during caesarean section (Wagner *et al.*, 2020), the potential influence of prior androgen therapy cannot be entirely excluded. Hormonal treatment may have affected cellular functionality in Study I and could have contributed to the low microbial detection observed in Study III. In addition, Study II was conducted using bovine ovarian tissue. Although bovine ovaries share several anatomical physiological, and folliculogenesis similarities with human ovaries and are therefore widely used as translational model in reproductive research (Baerwald, 2018; Roberts and Jeff Huang, 2022), species specific differences in ovarian biology (Roberts and Jeff Huang, 2022) may influence tissue responses to cryopreservation and *in vitro* culture. Therefore, these findings should be compared further to human ovarian tissue before translated to human clinical setting. Additional limitations related to the inherent heterogeneity of ovarian tissue in Studies I and II, as different cortical fragments may exhibit variable biological responses.

Study I employed a monolayer cell culture model to assess the effects of X-ray irradiation. While this approach allowed controlled investigation of cellular responses, it does not fully recapitulate the three-dimensional ovarian micro-environment and the complex cell-cell interactions that may influence tissue repair mechanisms. Moreover, this study focused exclusively on somatic cells, leaving the impact of irradiation on follicles and their functional competence unaddressed.

In Study II, the size of ovarian tissue fragments used for cryopreservation may also represent a confounding factor, as differences in strip dimensions could influence cryosurvival and subsequent culture outcomes. Future research should therefore investigate the effects of irradiation using fully developed Silk-Ovaroids in parallel with ovarian tissue to provide a more physiologically relevant comparison. In addition, a systemic evaluation of ovarian tissue strip size may help identify optimal dimensions for cryopreservation, thereby improving both the reproducibility and translational relevance of fertility preservation protocols.

7. CONCLUSION

Based on the principal findings presented in this thesis, the following conclusions can be drawn:

1. **There are regional differences in ovarian somatic cells radiosensitivity.**

Somatic cells derived from ovarian cortex and medulla exhibit distinct responses to X-ray irradiation. cPOCs show an early and robust activation of DNA damage response markers, followed by an efficient recovery from irradiation-induced cell-cycle arrest.

In contrast, mPOCs demonstrate a delayed DNA damage response leading to more extensive DNA damage or reduced repair efficiency as indicated by the prolonged cell-cycle arrest. These findings indicate intrinsic, region-specific differences in ovarian radiosensitivity, with potential implications for tissue resilience following radiotherapy.

2. **Irradiation induces molecular stress responses and disrupts tissue architecture.**

X-ray irradiation induced similar transcriptomic responses in both cPOCs and mPOCs. These responses are mainly characterised by the sustained activation of the p53 signalling pathway and concomitant suppression of proliferation-associated signalling. In addition to these transcriptomic changes, proteomic analyses reveal disruption of proteins involved in cell adhesion and ECM organisation. Collectively, these alterations impair cell – cell communication and tissue organisation, resulting in compromised structural integrity and an inability to form compact ovarian-like structures, as evidenced by the lack of Silk-Ovarioids formation following irradiation.

3. **Vitrification preserves ovarian structure and follicular density but reveals limitations in long-term follicle culture**

Vitrification preserves ovarian tissue architecture and follicle density, as neither overall tissue morphology nor follicle density differs significantly between vitrified and fresh ovarian tissue. These findings support vitrification as a viable alternative approach for OTC. However, despite the effective preservation of tissue structure and follicle abundance, long-term culture of isolated follicles remains challenging, with inconsistent follicle survival throughout culture. These limitations may arise from suboptimal vitrification conditions and/or insufficiently optimised follicle culture systems.

4. **Vitrification alters key signalling pathways**

Although vitrification effectively preserves ovarian tissue structure and follicular density, molecular alterations remain evident following cryopreservation. Changes in Wnt signalling and ovarian steroidogenesis were identified as vitrification-associated effects, indicating disruptions of pathways essential for post-thaw tissue functionality. These findings highlight the molecular impact of vitrification on ovarian tissue and identify potential targets for further optimisation of vitrification protocols.

5. Lack of evidence for an ovary-specific microbiome limits microbiome-based interventions

Although the microbiome plays an established role in reproductive health, no evidence supporting the presence of an ovary-specific microbiome in healthy ovaries was identified. Consequently, microbiome-based strategies are unlikely to represent a viable approach for improving ovarian tissue cryopreservation or vitrification outcomes.

8. SUMMARY IN ESTONIAN

Mitmeoomilised vaated munasarjade tervise säilitamisele

Sissejuhatus

Vähiravi tulemuste paranemine tuleneb peamiselt keemiaravi, kiiritusravi ja kirurgiliste sekkumiste arengust. Kuigi kiiritusravi on vähiravi üks keskseid põhi-komponente, on selle mõju munasarjade somaatilistele rakkudele seni vähe uuritud, kuna valdav osa teadusuuringutest on keskendunud peamiselt folliikulite kahjustustele. Arvestades ulatuslikku tõendusmaterjali kiiritusest põhjustatud folliikulikao kohta, on gonadotoksilist ravi saavate patsientide viljakuse säilitamine kliiniliselt ülioluline.

Munasarjakoe külmutamine (*ovarian tissue cryopreservation*, OTC) on üks olulisemaid viljakust säilitavaid meetodeid, kuna see võimaldab koguda suure folliikulite varu, toetada endokriinset funktsiooni ning pakkuda viljakuse säilitamise võimalust ka prepuberteedie eas tüdrukutele. Hoolimata OTC kasvavast kliinilisest kasutusest puuduvad teadus- ja meditsiinikeskustes endiselt universaalselt aktsepteeritud OTC protokollid. Munasarjakoe keerukas ja heterogeenne struktuur muudab optimeeritud, tõhusa ja kuluefektiivse külmutamise meetodi väljatöötamise keerukaks. Täiendav teadmiste lünk puudutab küsimust, kas munasari võib sisaldada ainuomast mikrobioomi. Arvestades, et reproduktiivtrakti mikroobikooslused võivad mõjutada viljakust, on võimalik, et munasarja mikrobioom — juhul kui see eksisteerib — võiks toetada follikulogeneesi või mõjutada krüopreservatsiooni edukust. Käesolev doktoritöö käsitleb seega kolme omavahel seotud uurimisküsimust: kiirituse mõju munasarja somaatilistele rakkudele, vitrifitseerimise sobivust alternatiivse OTC meetodina ning terve munasarjakoe võimaliku mikrobioomi olemasolu ja selle potentsiaalset rolli viljakuse säilitamise strateegiate täiustamisel.

Uurimisfookus

Käesoleva doktoritöö üldeesmärgiks oli uurida, kuidas laialdaselt kasutatavad viljakuse säilitamise meetodid mõjutavad munasarja kude rakulisel ja molekulaarsel tasandil ning kinnitada täheldatud muutuste bioloogilist olulisust funktsionaalsete analüüside abil.

Doktoritöö põhieesmärk oli iseloomustada vähiravi ja viljakuse säilitamise strateegiate mõju munasarja koele.

Spetsiifilisemad eesmärgid olid:

1. Kirjeldada inimese munasarja koore ja säsiosa primaarsete somaatiliste rakkude vastust röntgenkiirgusele

2. Hinnata kiirituse molekulaarset ja funktsionaalset mõju munasarja somaatilistele rakkudele, sealjuures nende DNA kahjustuse vastusele, rakutsükli kontrollile ja võimekusele moodustada 3D organoide.
3. Kirjeldada vitrifitseerimise mõju veise munasarja koore struktuurile ning preantraalsete folliikulite elulemusele
4. Kirjeldada vitrifitseerimise poolt põhjustatud molekulaarseid muutusi munasarjas.
5. Kinnitada või ümber lükata hüpotees munasarja spetsiifilisest mikro-bioomist tervetel inimestel.

Materjal ja meetodid

Kõik patsientide kudedega teostatud uuringud kiitis heaks Stockholmi piirkondlik eetikakomitee ning kõik uuringutes osalenud isikud andsid kirjaliku informeeritud nõusoleku. Veise munasarjakude hangiti kohaliku tapamaja kaudu ning selle kasutamine ei vajanud eetilist heakskiitu.

Uuring I: Kaheksalt soovahetusoperatsioonil osalenud patsiendilt kogutud munasarjakudedest eraldati kortikaalsed ja medullaarsed munasarja somaatilised rakud, mida kiiritati 10 Gy röntgendoosiga. DNA kahjustust, apoptoosi ja rakutsükli dünaamikat hinnati immunofluorestsentsanalüüsi abil. Eraldatud täiskoe RNA sekveneeriti (Illumina Stranded mRNA Prep Ligation; NovaSeq 6000) ja valke mõõdeti kvantitatiivse proteoomika analüüsil (LC-MS/MS). Rakkude funktsionaalset võimekust hinnati nende võimes moodustada siidimaatriksil ovarioide.

Uuring II: Üheksateistkümne veise munasarja korteks vitrifitseeriti, sulatati ja kasvatati *in vitro* kultuuris. Kudede morfoloogiat hinnati hematoksüliin-eosiini (H&E) värvimise, Ki67 immunofluorestsentsvärvimise ja TUNEL-testi abil ning täiskoest eraldatud RNA sekveneeriti (NEXTFLEX Rapid Directional RNA-seq Kit 2.0; Illumina HiSeq 4000). Folliikulid isoleeriti ensümaatilisel viisil värsketest kui ka vitrifitseeritud koest ning nende eluvõimet hinnati 6-päevase kasvu-perioodi jooksul.

Uuring III: Kahekümne seitsme munasarjakoe proovi (korteks ja medulla), soovahetusoperatsioonide käigus kogutud folliikulaarvedeliku ning keisrilõike patsientidelt saadud munasarjakoe proovide mikroobset DNA-d analüüsiti 16S rRNA sekveneerimise abil (Illumina MiSeq). Negatiivsed kontrollid hõlmasid füsioloogilise lahuse, keskkonna ja personali proove.

Tulemused

Uuring I: Kiiritus põhjustas nii cPOC-ides kui ka mPOC-ides ulatuslikke muutusi vastuses DNA kahjustustele, apoptoosi ja rakutsükli regulatsioonis. Geeniekspressiooni analüüs näitas märkimisväärsed muutusi juba 4 tunni järel (2810 erineva ekspressiooniga geeni cPOC-ides; 2540 mPOC-ides), mis püsisid ka 24 tunni möödudes (vastavalt 2462 ja 2802 erineva ekspressiooniga geeni). Täheldati tugevat p53 signaaliraja aktivatsiooni ning rakkude jagunemisega seotud signalisatsiooniradade (MYC, E2F, G2/M, mTORC1) alla surumist. Proteoomika analüüs tuvastas 24 tunni järel häireid rakkude adhesiooni ja ekstratsellulaarse maatriksi regulatsiooniga seotud valkudes. Funktsionaalselt avaldus see kiiritatud rakkude oluliselt vähenenud võimes moodustada ovarioide. **Uuring II:** Nii värske kui ka vitrifitseeritud munasarjakoe morfoloogia oli vahetult pärast sulatamist säilinud ning erinevusi folliikulite tiheduses, proliferatsioonis ega DNA terviklikkuses ei täheldatud. Seevastu *in vitro* kultiveerimise järel ilmnis vitrifitseeritud ja sulatatud koes primordiaalse/primaarse ja antraalse folliikulite tiheduse vähenemine, koos strooma proliferatsiooni ja DNA kahjustuste suurenemisega. Isoleeritud folliikulite esialgne elulemus oli värskes ja vitrifitseeritud koes sarnane, kuid vitrifitseeritud koest pärit folliikulite eluvõime langes oluliselt 6. kasvupäevaks. Transkriptoomi analüüs näitas, et vitrifitseerimine üksi põhjustas vaid minimaalseid muutusi, samas kui *in vitro* kultiveerimine kutsus esile ulatuslikke transkriptoomi erinevusi. Muutused WNT signaalirajas ja hormonaalse regulatsiooni radades tõid esile potentsiaalseid sihtmärke OTC protokollide edasiseks täiustamiseks. **Uuring III:** Bakteriaalset DNA-d tuvastati vaid 36,4% proovides ning pärast kvaliteedi alusel filtreerimist olid mikroobsed lugejad äärmiselt madalad. Hoolimata rangetest saaste vältimisest ja tõrjemeetmetest oli proovides kõrge kontaminatsioonimäär (30,3–100%; mediaan 62,1%). Munasarja proovid ei erinenud statistiliselt negatiivsetest kontrollidest, mis viitab sellele, et tervel munasarjakoel puudub spetsiifiline mikrobiom.

Kokkuvõte

Käesoleva doktoritöö tulemused näitavad, et munasarja somaatilised rakud reageerivad kiiritusele ulatusliku ja püsiva molekulaarse ümberprogrammeerimisega, mis mõjutab rakutsükli regulatsiooni ja koe organiseeritust ning võib seeläbi kaasa aidata munasarja düsfunktsiooni tekkele sõltumatult folliikulite kaost.

Vitrifitseerimine säilitab munasarjakoe morfoloogia ja transkriptoomi profiili vahetult pärast sulatamist, kuid funktsionaalne *in vitro* kultuuris kasvatamine toob esile olulised piirangud, sealhulgas folliikulite vähenenud eluvõime ja muutused olulistest signaaliradades nagu WNT signaalirada ja hormonaalne regulatsioon. Need tulemused osutavad konkreetsetele molekulaarsetele mehhanismidele, mis võivad suunata OTC protokollide täiustamisele.

Munasarja-spetsiifilise mikrobioomi puudumine viitab sellele, et mikroobide sihipärane manipuleerimine ei ole tõenäoliselt efektiivne viis krüopreservatsiooni tulemuste parandamiseks. Siiski pakuvad need uuringud olulisi teadmisi mehha-

nismide, metodoloogiate ja funktsionaalsete arengute osas, mis toetavad viljakuse säilitamise strateegiate edasist arendamist gonadotoksilist ravi saavate patsientide jaoks.

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During my time in Estonia, I had the privilege to meet, work with, laugh with, and spend time alongside many special people. My sincere gratitude goes to **Keiu Kask**, **Vijayachitra Modhukar**, **Hindre Teder**, **Merli Saare**, **Maire Peters**, **Marina Loid**, **Apostol Apostolov**, **Amruta Pathare**, **Hanna Maria Kariis**, **Niels Fritsma**, and **Eric Roldan Roa**. We shared many great moments and unforgettable experiences, and I am truly grateful to have met each of you on this journey.

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Valentina Di Nisio, Laura Baque Vidal, Elisabeth Moussaoud-Lamodière, Nikoletta Drazinaki, Fredrik Lanner, Cheng Zhao, Sanjiv Risal, Sarah Saietz, Shivam Chandel, Eda Erbil, and Celia Mesias Casares for the shared work, conversations, and moments that made this period so enriching.

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To my parents, and my brother: thank you for your endless and unconditional love and support. Thank you for always standing by my side, believing in me, and cheering me on throughout this journey. No words can fully express my gratitude, but I know you understand how much this means to me.

Above all, I wish to express my deepest gratitude to my wife, **Marietta**, for her unwavering love, patience, and support throughout this journey. Her strength, encouragement, and steadfast belief in me sustained me through both the challenges and the triumphs of this work. I am also profoundly grateful to my newborn son, whose arrival transformed my world, giving this work a deeper meaning and my life a new sense of purpose. Together, they have been my greatest inspiration, reminding me daily of what truly matters. I love you both more than words can express.

PUBLICATIONS

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EDUCATION

01/09/2020–Present **Ph.D in Female Reproductive Care in European Training Network “MATER”** – The University of Tartu (Tartu, Estonia) & The University of Helsinki (Helsinki, Finland)
03/09/2018–23/08/2019 **Master of Science by Research in Reproductive Science** – The University of Edinburgh, Scotland, United Kingdom
05/09/2016–14/08/2017 **Master of Science in Human Clinical Embryology and Assisted Conception** – The University of Dundee, Scotland, United Kingdom
13/09/2010–16/03/2016 **Bachelor of Science in Molecular Biology and Genetics** – Democritus University of Thrace, Alexandroupolis, Greece

SCIENTIFIC FELLOWSHIPS, NOMINATIONS, AWARDS AND HONORS

29/06/2025 Cancer Foundationi reisistipendium
01/01/2024 Orion Research Foundationi individuaalne teadusgrant
25/06/2023 Helsingi Ülikooli doktoriõppe kooli reisistipendium
01/11/2022 Erasmus+ koostöösleping teaduslähetusks Karolinska Instituudis

WORK EXPERIENCE

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- 01/11/2022–30/09/2023 Research internship, Chemical and Female Fertility, Karolinska Institutet, Stockholm, Sweden
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- 01/11/2019–15/03/2020 Research Assistant, Centre for Reproductive Health, University of Edinburgh, Scotland, United Kingdom
- 01/06/2013–31/08/2016 Trainee Embryologist, General University Hospital of Alexandroupolis, Alexandroupolis, Greece

SUPERVISING AND TEACHING ACTIVITIES

I have supervised two bachelor students (one at university of Tartu and one at Karolinska Institutet). I have given three lectures at a master program (two at University of Tartu and one at National and Kapodistrian University of Athens)

- 13/01/2026 **Lecture Title:** Gametogenesis and the Female Reproductive System
Master Program: Reproductive and Regenerative Medicine, Department of Medicine, National and Kapodistrian University of Athens
- 10/05/2023 **Lecture Title:** Male Reproductive Biology: Fertility and Infertility
Course: Reproductive Biology and Medicine (*Reproduktiivbioloogia ja meditsiin*)
- 01/03/2023–31/05/2023 **Bachelor Student:** Fanny Hölcke
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- 18/05/2023 **Lecture Title:** Male Reproductive Biology: Fertility and Infertility
Course: Reproductive Biology and Medicine (*Reproduktiivbioloogia ja meditsiin*)
- 01/01/2022–31/06/2022 **Bachelor Student:** Dana-Karolin Rand
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PUBLISHED AND ACCEPTED ARTICLES

Deligiannis SP, Li T, Moussaud-Lamodière E, Végvári A, Damdimopoulos A, Lavogina D, Papaikonomou K, Zubarev R, Acharya G, Velthut-Meikas A, Damdimopoulou P, Salumets A*, Di Nisio V*. Acute high-dose irradiation disrupts cell adhesion and Silk-Ovarioid formation in human primary ovarian cells. *J Ovarian Res.* 2026 Jan 2;19(1):79. doi: 10.1186/s13048-025-01932-8.
* Co-senior authors

Sola-Leyva A, Pérez-Prieto I, Di Nisio V, **Panagiotis-Deligiannis S**, Blanco-Rodríguez L, Acharya G, Saare M, Papaikonomou K, Damdimopoulou P,

- Salumets A, Altmäe S. Assessing the ovarian microbiome: lack of a distinguishable microbial signature beyond contamination. *Reprod Biomed Online*. 2025 Sep;51(3):104988. doi: 10.1016/j.rbmo.2025.104988.
- Deligiannis SP**, Kask K, Modhukur V, Boskovic N, Ivask M, Jaakma Ü, Damdimopoulou P, Tuuri T, Velthut-Meikas A, Salumets A. Investigating the impact of vitrification on bovine ovarian tissue morphology, follicle survival, and transcriptomic signature. *J Assist Reprod Genet*. 2024 Apr;41(4):1035-1055. doi: 10.1007/s10815-024-03038-4.
- Essers R, Lebedev IN, Kurg A, Fonova EA, Stevens SJC, Koeck RM, von Rango U, Brandts L, **Deligiannis SP**, Nikitina TV, Sazhenova EA, Tolmacheva EN, Kashevarova AA, Fedotov DA, Demeneva VV, Zhigalina DI, Drozdov GV, Al-Nasiry S, Macville MVE, van den Wijngaard A, Dreesen J, Paulussen A, Hoischen A, Brunner HG, Salumets A, Zamani Esteki M. Prevalence of chromosomal alterations in first-trimester spontaneous pregnancy loss. *Nat Med*. 2023 Dec;29(12):3233-3242. doi: 10.1038/s41591-023-02645-5.
- Boskovic N, Yazgeldi G, Ezer S, Tervaniemi MH, Inzunza J, **Deligiannis SP**, Yaşar B, Skoog T, Krjutškov K, Katayama S, Kere J. Optimized single-cell RNA sequencing protocol to study early genome activation in mammalian preimplantation development. *STAR Protoc*. 2023 Sep 15;4(3):102357. doi: 10.1016/j.xpro.2023.102357.

SUBMITTED MANUSCRIPTS

- Li T, Wehrli L, Antonouli S, Panagiotou EM, **Deligiannis SP**, Liu X, Hassan J, Moussaud E, Arnelo C, Pettersson K, Papaikonomou K, Salumets A, P Damdimopoulou P*, Di Nisio V*. Hypoxia enhances follicle survival and partially restores metabolic function in human ovarian cortical tissue culture. *Reprod Biomed Online* [under revision]

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CONFERENCE PROCEEDINGS

Selected Oral and Poster Presentations:

- Spyridon Panagiotis Deligiannis et al.**, (2025). Molecular and functional impact of X-ray on primary ovarian cells. 7th von Baer Seminar, 7th von Baer Seminar. Tartu, Estonia, September 11-12, 2025. **Oral presentation.**
- S. P. Deligiannis**, et al., (2025). Molecular and functional impact of X-ray on primary ovarian cells. ESHRE annual meeting 2025. ESHRE 41st annual meeting 2025. Paris, France, June 29 – July 2, 2025. **Poster presentation.**
- Deligiannis SP** (2025). Deep dive into emerging fertility preservation strategies. Thessaloniki, Greece, 3rd Department of Obstetrics and Gynecology at Aristotle University of Thessaloniki, Ippokrateio Hospital, May 22, 2025. **Invited speaker.**
- Deligiannis SP** (2025). Deep dive into emerging fertility preservation strategies. Athens, Greece, 1st Department of Obstetrics and Gynecology at National and Kapodistrian University of Athens, School of Medicine, May 16, 2025. **Invited speaker.**

- Spyridon Panagiotis Deligiannis**, et al., (2025). Molecular and functional impact of X-ray irradiation on primary ovarian cells. 1st European Ovary Workshop 2025. Lagos, Portugal, February 17 – 20, 2025. **Poster presentation.**
- Deligiannis SP** (2025). Molecular and morphological characteristics of cryo-preserved ovarian tissue. 6th Karl Ernst Von Baer’s seminar, Tartu, Estonia, October 19 -21, 2023. **Invited speaker.**
- S P Deligiannis**, et al., (2023). Ovarian tissue vitrification as a cost-effective protocol for female fertility preservation: a bovine study. ESHRE 39th annual meeting 2023. Copenhagen, Denmark, June 25–28, 2023. **Poster presentation.**
- Deligiannis Spyridon Panagiotis**, et al., (2020). Novel causes of 46XY DSD: The role of DHX37 in human fetal testis development. British Fertility conference. Edinburgh, Scotland, United Kingdom, January 9 – 11, 2020. **Oral presentation**

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HARIDUS

01/09/2020–Praegu **Doktoriõpe (PhD) naiste reproduktiivtervise valdkonnas Euroopa koolitusvõrgustikus MATER** – Tartu Ülikool, Tartu, Eesti ja Helsingi Ülikool, Helsingi, Soome
03/09/2018–23/08/2019 **Teadusmagister (MSc by Research) reproduktiivteadustes** – Edinburghi Ülikool, Šotimaa, Ühendkuningriik
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13/09/2010–16/03/2016 **Loodusteaduste bakalaureus (BSc) molekulaarbioloogias ja geneetikas** – Traakia Demokritose Ülikool, Alexandroupoli, Kreeka

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29/06/2025 Cancer Foundation travel grant for the ESHRE 41st Annual Meeting 2025
01/01/2024 Orion Research Foundation personal grant
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- 01/11/2022–30/09/2023 Teaduspraktikant, Chemical and Female Fertility, Karolinska Instituut, Stockholm, Rootsi
- 01/08/2022–31/10/2022 Doktorandi lähetus, Tervisetehnoloogia Arenduskeskus, Tartu, Eesti
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- 01/06/2013–31/08/2016 Embrüoloogi praktikant, Alexandroupolise Ülikooli Üldhaigla, Alexandroupoli, Kreeka

JUHENDAMIS- JA ÕPPETEGEVUS

Olen juhendanud kahte bakalaureusetudengit (üks Tartu Ülikoolis ja üks Karolinska Instituudis). Samuti olen pidanud kolm loengut magistriõppe programmi- des (kaks Tartu Ülikoolis ja ühe Ateena Rahvuslikus ja Kapodistriase Ülikoolis).

13/01/2026 **Loeng:** *Gametogenees ja naiste reproduktiivsüsteem*

Magistriõppekava: Reproduktiivne ja regeneratiivne meditsiin
Ateena Rahvuslik ja Kapodistriase Ülikool

10/05/2023 **Loeng:** *Meeste reproduktiivbioloogia: viljakus ja viljatus*

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Kursus: Reproduktiivbioloogia ja meditsiin

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PUBLIKATSIOONID

Avaldatud ja vastu võetud artiklid

Deligiannis SP, Li T, Moussaud-Lamodière E, Végvári A, Damdimopoulos A, Lavogina D, Papaikonomou K, Zubarev R, Acharya G, Velthut-Meikas A, Damdimopoulou P, Salumets A*, Di Nisio V*. Acute high-dose irradiation disrupts cell adhesion and Silk-Ovarioid formation in human primary ovarian cells. *J Ovarian Res.* 2026 Jan 2;19(1):79. doi: 10.1186/s13048-025-01932-8.

* Co-senior authors

Sola-Leyva A, Pérez-Prieto I, Di Nisio V, **Panagiotis-Deligiannis S**, Blanco-Rodríguez L, Acharya G, Saare M, Papaikonomou K, Damdimopoulou P, Salumets A, Altmäe S. Assessing the ovarian microbiome: lack of a distinguishable microbial signature beyond contamination. *Reprod Biomed Online.* 2025 Sep;51(3):104988. doi: 10.1016/j.rbmo.2025.104988.

Deligiannis SP, Kask K, Modhukur V, Boskovic N, Ivask M, Jaakma Ü, Damdimopoulou P, Tuuri T, Velthut-Meikas A, Salumets A. Investigating the

- impact of vitrification on bovine ovarian tissue morphology, follicle survival, and transcriptomic signature. *J Assist Reprod Genet.* 2024 Apr;41(4):1035-1055. doi: 10.1007/s10815-024-03038-4.
- Essers R, Lebedev IN, Kurg A, Fonova EA, Stevens SJC, Koeck RM, von Rango U, Brandts L, **Deligiannis SP**, Nikitina TV, Sazhenova EA, Tolmacheva EN, Kashevarova AA, Fedotov DA, Demeneva VV, Zhigalina DI, Drozdov GV, Al-Nasiry S, Macville MVE, van den Wijngaard A, Dreesen J, Paulussen A, Hoischen A, Brunner HG, Salumets A, Zamani Esteki M. Prevalence of chromosomal alterations in first-trimester spontaneous pregnancy loss. *Nat Med.* 2023 Dec;29(12):3233-3242. doi: 10.1038/s41591-023-02645-5.
- Boskovic N, Yazgeldi G, Ezer S, Tervaniemi MH, Inzunza J, **Deligiannis SP**, Yaşar B, Skoog T, Krjutškov K, Katayama S, Kere J. Optimized single-cell RNA sequencing protocol to study early genome activation in mammalian preimplantation development. *STAR Protoc.* 2023 Sep 15;4(3):102357. doi: 10.1016/j.xpro.2023.102357.

ESITATUD KÄSIKIRJAD

- Li T, Wehrli L, Antonouli S, Panagiotou EM, **Deligiannis SP**, Liu X, Hassan J, Moussaud E, Arnelo C, Pettersson K, Papaikonomou K, Salumets A, P Damdimopoulou P*, Di Nisio V*. Hypoxia enhances follicle survival and partially restores metabolic function in human ovarian cortical tissue culture. *Reprod Biomed Online* [under revision]
- * Co-senior authors

KONVERENTSID JA ETTEKANDED

Valitud suulised ja posterettekanded

- Spyridon Panagiotis Deligiannis** et al., (2025). Molecular and functional impact of X-ray on primary ovarian cells. 7th von Baer Seminar, 7th von Baer Seminar. Tartu, Estonia, September 11-12, 2025. **Suuline ettekanne.**
- S. P. Deligiannis**, et al., (2025). Molecular and functional impact of X-ray on primary ovarian cells. ESHRE annual meeting 2025. ESHRE 41st annual meeting 2025. Paris, France, June 29 – July 2, 2025. **Poste ettekanne.**
- Deligiannis SP** (2025). Deep dive into emerging fertility preservation strategies. Thessaloniki, Greece, 3rd Department of Obstetrics and Gynecology at Aristotle University of Thessaloniki, Ippokrateio Hospital, May 22, 2025. **Kutsutud esineja.**
- Deligiannis SP** (2025). Deep dive into emerging fertility preservation strategies. Athens, Greece, 1st Department of Obstetrics and Gynecology at National and Kapodistrian University of Athens, School of Medicine, May 16, 2025. **Kutsutud esineja.**
- Spyridon Panagiotis Deligiannis**, et al., (2025). Molecular and functional impact of X-ray irradiation on primary ovarian cells. 1st European Ovary Workshop 2025. Lagos, Portugal, February 17 – 20, 2025. **Poste ettekanne.**

- Deligiannis SP** (2025). Molecular and morphological characteristics of cryo-preserved ovarian tissue. 6th Karl Ernst Von Baer's seminar, Tartu, Estonia, October 19–21, 2023. **Kutsutud esineja.**
- S P Deligiannis**, et al., (2023). Ovarian tissue vitrification as a cost-effective protocol for female fertility preservation: a bovine study. ESHRE 39th annual meeting 2023. Copenhagen, Denmark, June 25–28, 2023. **Poste ettekanne.**
- Deligiannis Spyridon Panagiotis**, et al., (2020). Novel causes of 46XY DSD: The role of DHX37 in human fetal testis development. British Fertility conference. Edinburgh, Scotland, United Kingdom, January 9–11, 2020. **Suuline ettekanne.**

DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

1. **Heidi-Ingrid Maaroos.** The natural course of gastric ulcer in connection with chronic gastritis and *Helicobacter pylori*. Tartu, 1991.
2. **Mihkel Zilmer.** Na-pump in normal and tumorous brain tissues: Structural, functional and tumorigenesis aspects. Tartu, 1991.
3. **Eero Vasar.** Role of cholecystokinin receptors in the regulation of behaviour and in the action of haloperidol and diazepam. Tartu, 1992.
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5. **Ants Peetsalu.** Vagotomy in duodenal ulcer disease: A study of gastric acidity, serum pepsinogen I, gastric mucosal histology and *Helicobacter pylori*. Tartu, 1992.
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7. **Hele Everaus.** Immuno-hormonal interactions in chronic lymphocytic leukaemia and multiple myeloma. Tartu, 1993.
8. **Ruth Mikelsaar.** Etiological factors of diseases in genetically consulted children and newborn screening: dissertation for the commencement of the degree of doctor of medical sciences. Tartu, 1993.
9. **Agu Tamm.** On metabolic action of intestinal microflora: clinical aspects. Tartu, 1993.
10. **Katrin Gross.** Multiple sclerosis in South-Estonia (epidemiological and computed tomographical investigations). Tartu, 1993.
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18. **Aavo Lang.** The role of dopamine, 5-hydroxytryptamine, sigma and NMDA receptors in the action of antipsychotic drugs. Tartu, 1995.
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20. **Tõnis Karki.** Quantitative composition of the human lactoflora and method for its examination. Tartu, 1996.
21. **Reet Mändar.** Vaginal microflora during pregnancy and its transmission to newborn. Tartu, 1996.
22. **Triin Remmel.** Primary biliary cirrhosis in Estonia: epidemiology, clinical characterization and prognostication of the course of the disease. Tartu, 1996.
23. **Toomas Kivastik.** Mechanisms of drug addiction: focus on positive reinforcing properties of morphine. Tartu, 1996.
24. **Paavo Pokk.** Stress due to sleep deprivation: focus on GABA_A receptor-chloride ionophore complex. Tartu, 1996.
25. **Kristina Allikmets.** Renin system activity in essential hypertension. Associations with atherothrombogenic cardiovascular risk factors and with the efficacy of calcium antagonist treatment. Tartu, 1996.
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27. **Svetlana Päi.** Factors promoting heterogeneity of the course of rheumatoid arthritis. Tartu, 1997.
28. **Maarike Sallo.** Studies on habitual physical activity and aerobic fitness in 4 to 10 years old children. Tartu, 1997.
29. **Paul Naaber.** *Clostridium difficile* infection and intestinal microbial ecology. Tartu, 1997.
30. **Rein Pähkla.** Studies in pinoline pharmacology. Tartu, 1997.
31. **Andrus Juhan Voitk.** Outpatient laparoscopic cholecystectomy. Tartu, 1997.
32. **Joel Starkopf.** Oxidative stress and ischaemia-reperfusion of the heart. Tartu, 1997.
33. **Janika Kõrv.** Incidence, case-fatality and outcome of stroke. Tartu, 1998.
34. **Ülla Linnamägi.** Changes in local cerebral blood flow and lipid peroxidation following lead exposure in experiment. Tartu, 1998.
35. **Ave Minajeva.** Sarcoplasmic reticulum function: comparison of atrial and ventricular myocardium. Tartu, 1998.
36. **Oleg Milenin.** Reconstruction of cervical part of esophagus by revascularised ileal autografts in dogs. A new complex multistage method. Tartu, 1998.
37. **Sergei Pakriev.** Prevalence of depression, harmful use of alcohol and alcohol dependence among rural population in Udmurtia. Tartu, 1998.
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39. **Vallo Matto.** Pharmacological studies on anxiogenic and antiaggressive properties of antidepressants. Tartu, 1998.
40. **Maire Vasar.** Allergic diseases and bronchial hyperreactivity in Estonian children in relation to environmental influences. Tartu, 1998.
41. **Kaja Julge.** Humoral immune responses to allergens in early childhood. Tartu, 1998.

42. **Heli Grünberg.** The cardiovascular risk of Estonian schoolchildren. A cross-sectional study of 9-, 12- and 15-year-old children. Tartu, 1998.
43. **Epp Sepp.** Formation of intestinal microbial ecosystem in children. Tartu, 1998.
44. **Mai Ots.** Characteristics of the progression of human and experimental glomerulopathies. Tartu, 1998.
45. **Tiina Ristimäe.** Heart rate variability in patients with coronary artery disease. Tartu, 1998.
46. **Leho Kõiv.** Reaction of the sympatho-adrenal and hypothalamo-pituitary-adrenocortical system in the acute stage of head injury. Tartu, 1998.
47. **Bela Adojaan.** Immune and genetic factors of childhood onset IDDM in Estonia. An epidemiological study. Tartu, 1999.
48. **Jakov Shlik.** Psychophysiological effects of cholecystokinin in humans. Tartu, 1999.
49. **Kai Kisand.** Autoantibodies against dehydrogenases of α -ketoacids. Tartu, 1999.
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51. **Ants Kask.** Behavioural studies on neuropeptide Y. Tartu, 1999.
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