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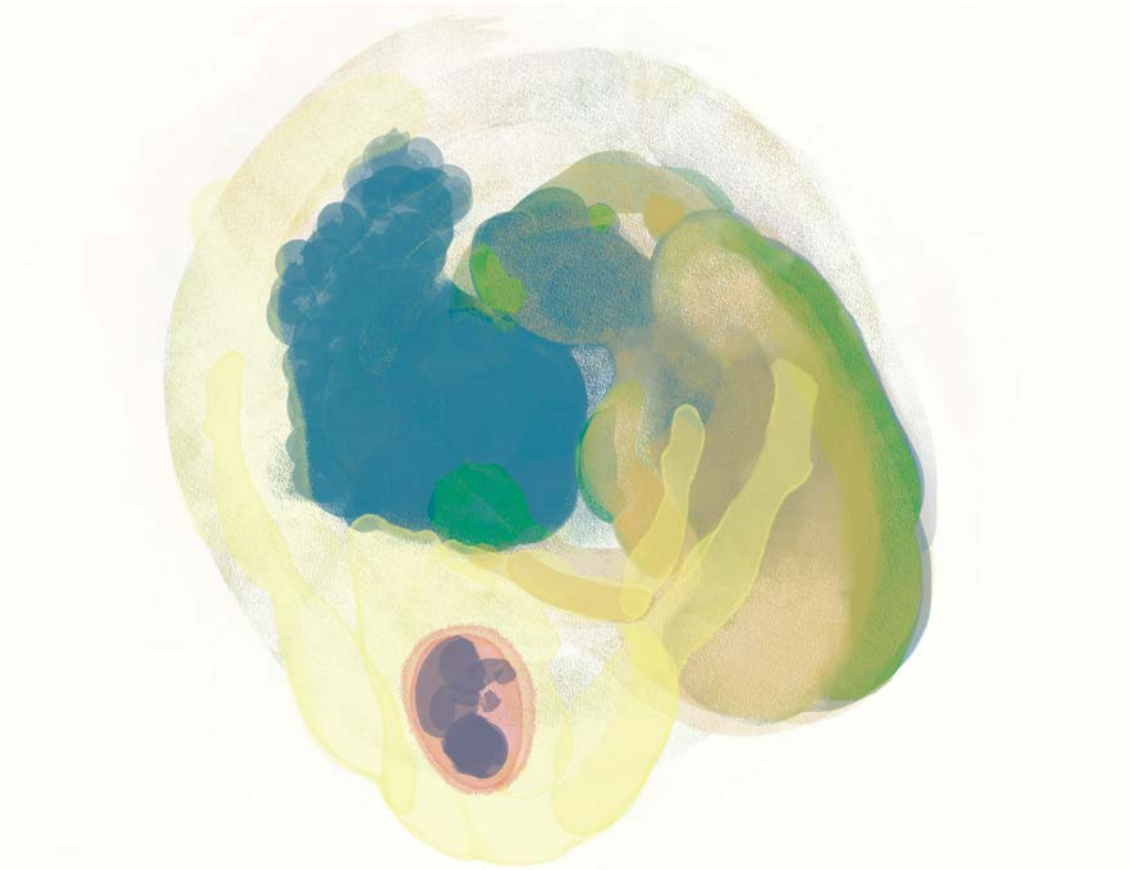
Ovarian response to vascular and toxic insults in early life: focus on ovarian reserve and fertility preservation



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OVARIAN RESPONSE TO VASCULAR AND TOXIC INSULTS IN EARLY LIFE: FOCUS ON OVARIAN RESERVE AND FERTILITY PRESERVATION

Valentina Pampanini

Karolinska Institutet

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Ovarian response to vascular and toxic insults in early life: focus on ovarian reserve and fertility preservation

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To Leon and Fiamma, with love

You travel to search and you come back home to find yourself there

Chimamanda Ngozi Adichie

Keep Ithaka always in your mind.
Arriving there is what you're destined for.
But don't hurry the journey at all.
Better if it lasts for years,
so you're old by the time you reach the island,
wealthy with all you've gained on the way,
not expecting Ithaka to make you rich.
Ithaka gave you the marvelous journey.
Without her you wouldn't have set out.
She has nothing left to give you now.
And if you find her poor, Ithaka won't have fooled you.
Wise as you will have become, so full of experience,
you'll have understood by then what these Ithakas mean.

Constantine Cavafy

ABSTRACT

The ovarian reserve is defined as the number of eggs embodied in primordial follicles. Every woman is born with a fixed number of primordial follicles, which are formed during fetal development. Pathological events can affect the ovarian reserve both pre- and postnatally and cause a premature ovarian insufficiency (POI), i.e. a precocious exhaustion of the endocrine and reproductive potential of the ovaries. In prenatal life, intrauterine growth restriction (IUGR) caused by poor maternal nutrition or insufficient placental flow to the fetus, has been associated with alterations in pubertal development and follicle number in animals and humans, with inconclusive evidence. In postnatal life, major iatrogenic causes of POI are chemo- and radiotherapeutic treatments administered to cancer patients. Fertility preservation strategies, such as ovarian tissue cryopreservation (OTC), are of fundamental importance to preserve fertility in these patients. These procedures are still experimental and in continuous evolution as more data are acquired.

In this thesis, we aimed at investigate the impact of IUGR on ovarian follicles and gene expression in neonatal, juvenile and peripubertal rats, using a model of surgically-induced placental insufficiency. Secondly, we focused on the effects of chemotherapy exposure on ovarian follicles and stroma in ovarian tissues from cancer patients who underwent OTC for fertility preservation purposes. Additionally, we analyzed the efficacy of fertility preservation criteria in selecting the patients at high risk of infertility, by analysis of ovarian function during follow-up, the fruition rate and the timing of OTC in a large pediatric oncology unit in Finland. Timing of OTC was analyzed in relation to exposure to chemotherapy.

A reduced primordial and total follicle count was found in the ovaries of neonate and juvenile rats. Follicle count normalized at peripubertal ages, alongside with the recovery of body weight. The expression of 24 genes was modified in the rat ovaries in response to the placental insufficiency. We interpreted the gene reprogramming as compensatory, possibly explaining the recovery of follicle number (by a downregulation of the physiological rates of follicle activation and/or depletion). The gene modifications could also have unknown long-term consequences on ovarian function, whose definition was beyond the aims of this work. An increased number of atretic follicles and a reduced size and number of residual intact follicles were evidenced in the ovaries of patients exposed to chemotherapy before OTC. Exposure to chemotherapy also reduced the secretion of steroid hormones in culture and damaged the ovarian stroma, by increasing collagen deposition and apoptosis. Finally, analysis of OTC data from Helsinki Children's Hospital revealed that patients at high risk of infertility were correctly identified by the selection criteria, despite fruition rate was not optimal. We observed an increased risk of ovarian failure in older patients, which is in line with the age-related decline of the ovarian reserve. We also reported longer time frames between indication to OTC and execution of the procedure in patients with malignant diseases, which was associated to increased exposure to alkylating agents. Delays in the execution of OTC should be limited in order to avoid further damage to the ovarian tissue as a consequence of chemotherapy exposure, especially in older patients.

LIST OF SCIENTIFIC PAPERS

I. **Valentina Pampanini**, Kirsi Jahnukainen, Lena Sahlin, Daniela Germani, Antonella Puglianiello, Stefano Cianfarani and Olle Söder. Impact of uteroplacental insufficiency on ovarian follicular pool in the rat. Reproductive Biology and Endocrinology 2019 Jan 10;17(1):10.

II. **Valentina Pampanini**, Magdalena Wagner, Babak Asadi-Azarbaijani, Irma Caroline Oskam, Mona Sheikhi, Marcus Sjödin, Johan Lindberg, Outi Hovatta, Lena Sahlin, Richelle Duque Björvang, Marjut Otala, Pauliina Damdimopoulou and Kirsi Jahnukainen. Impact of first-line cancer treatment on the follicle quality in cryopreserved ovarian samples from girls and young women. Human Reproduction 2019 Sep 29;34(9):1674-1685.

Appendix: Reply: Impact of first-line cancer treatment on follicle quality in cryopreserved ovarian samples. Human Reproduction 2020 Apr 13; 0(0): 1-2.

III. **Valentina Pampanini**, Lena Sahlin, Elina Holopainen, Mervi Taskinen, Kim Vettenranta, Jaana Vettenranta, Tiina Laine and Kirsi Jahnukainen. Effect of primary diagnose and planned gonadotoxic therapy on patient selection and timing of fertility preservation in young girls. Manuscript

Additional publication (not included in the thesis)

I. **Valentina Pampanini**, Daniela Germani, Antonella Puglianiello, Jan-Bernd Stukenborg, Ahmed Reda, Iuliia Savchuk, Kristin Ròs Kjartansdóttir, Stefano Cianfarani and Olle Söder. Impact of uteroplacental insufficiency on postnatal rat male gonad. Journal of Endocrinology 2017 Feb;232(2):247-257.

CONTENTS

LIST OF ABBREVIATIONS

AFC	Antral follicle count
AKT	Protein kinase B
AMH	Anti-Müllerian hormone
BAX	B-cell lymphoma 2 protein associated X protein
BCL2	B-cell lymphoma 2 protein
BW	Birth weight
CCSS	Childhood cancer survivor study
CED	Cyclophosphamide equivalent dose
CL	Corpus luteum
CNS	Central nervous system
DIE	Isotoxic dose equivalent
DOHAD	Developmental origins of health and disease
DPC	Days post-conception
DPP	Days post-partum
E2	Estradiol
FOXL2	Forkhead box L2
FOXO3A	Forkhead box O3A
FSH	Follicle-stimulating hormone
FST	Follistatin
GCs	Granulosa cells
GnRH	Gonadotropin-releasing factor
HCG	Human chorionic gonadotropin
HPG	Hypothalamic-pituitary-gonadal
HRT	Hormone replacement therapy
HSCT	Hematopoietic stem cell transplantation
IHC	Immunohistochemistry
IUGR	Intrauterine growth restriction
LH	Luteinizing hormone

MDR	Minimal residual disease
MTOR	Mammalian target of rapamycin
NGF	Non-growing follicles
NOPHO	Nordic society for pediatric hematology and oncology
OS	Overall survival
OTC	Ovarian tissue cryopreservation
PDK	Phosphoinositide-dependent protein kinase
PFA	Paraformaldehyde
PFs	Primordial follicles
PGCs	Primordial germ cells
PI3K	Phosphatidylinositol-3-kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PND	Postnatal day
PTEN	Phosphatase and tensin homolog
RPS6	Ribosomal protein S6
RT	Radiotherapy
RT-qPCR	Real-time quantitative polymerase chain reaction
S6K	Ribosomal protein S6 kinase
SDs	Standard deviations
SGA	Small for gestational age
SRY	Sex-determining region on the Y chromosome
TAp63	Isoform of p53 homolog p63
TBI	Total body irradiation
TBS	Tris-buffered saline
TCs	Theca cells
TSC1	Tuberin/tuberous sclerosis complex 1
TSC2	Tuberin/tuberous sclerosis complex 2
WNT4	Wingless-type MMTV integration site family, member 4
γH2AX	H2A histone family member X phosphorylated on serine 139

1 INTRODUCTION

The mammalian ovary is a highly organized organ that harbors the female gametes and provide fertilizable oocytes throughout the reproductive lifespan. The ovary also produces sex steroid hormones that support the oocyte maturation, the development of female secondary sexual characteristics and the establishment of pregnancy after oocyte fertilization. Genetic and environmental factors can affect the ovarian reserve, defined as the non-renewable pool of immature oocytes surrounded by flattened granulosa cells (GCs) forming the primordial follicles (PFs), the basic functional unit of the ovary. The precocious exhaustion of the ovarian reserve is known as premature ovarian insufficiency (POI) and can derive from the exposure to insults during the life of an individual, starting from fetal life. Any insult that alters the fetal environment leads to adaptive responses of the fetus that often translate into diseases in later life. Exposure to environmental chemicals, drugs, infections, poor or excessive maternal nutrition and placental dysfunction have been shown to be detrimental to the fetal ovary [1]. In postnatal life, gonadotoxic therapies are the most relevant iatrogenic causes of POI, which is becoming increasingly important given the growing number of long-term childhood cancer survivors. Fertility preservation methods have been developed during the last decades to spare fertility in patients treated with gonadotoxic cancer therapies.

This thesis describes the consequences of two different environmental insults on the ovarian reserve: placental insufficiency during fetal life in rat ovaries and chemotherapy treatment during childhood and adolescence in human ovarian tissues harvested for fertility preservation. In addition, we investigated the appropriateness of the selection criteria for fertility preservation in children and the efficacy of their implementation.

1.1 Ovarian physiology in humans and rodents

1.1.1 Oogenesis

At birth the mammalian ovary contains a finite number of oocytes enclosed into PFs. In humans and rodents, the oocytes arise during fetal life from primordial germ cells (PGCs). These cells reside in the extra-embryonic mesoderm and migrate to the genital ridge at around 5-6 weeks of fetal development in humans [2, 3] and between 9 and 10.5 days post-conception (dpc) in mice [4]. The gonad is 'bipotential' until week 7 in humans and 11.5 dpc in mice. Subsequently, PGCs differentiate into oogonia and the gonad becomes an ovary in the process called sex differentiation. These events can take place in the absence of the sex-determining region on the Y chromosome (SRY) gene and under the drive of female-specific genes, such as forkhead box L2 (FOXL2), wingless-type MMTV integration site family, member 4 (WNT4) and follistatin (FST) [1]. From week 7 in humans and

from 10.5 dpc in mice, the oogonia undergo multiple divisions by mitosis and start to arrange in cyst-like clusters known as germ cell cysts, structures containing pre-granulosa cells and germ cells [1, 5]. The oogonia enter the first steps of meiosis by 13.5 dpc in mice and week 10 of gestation in humans and become oocytes. Oocytes proceed through prophase I of meiosis and arrest at the diplotene stage, remaining quiescent until ovulation [1, 5]. A large number of oocytes undergo cell death during this phase. In mice two main waves of apoptosis have been documented, between 13.5 and 15.5 dpc and from 17.5 dpc until the day of birth, when cyst breakdown takes place allowing the assembly of PFs. In humans, waves of germ cell apoptosis occur during midgestation, with highest rates between 14 and 28 weeks. Cyst breakdown starts at 20 weeks of gestation, determining the formation of PFs already during fetal life in humans [1] (Figure 1).

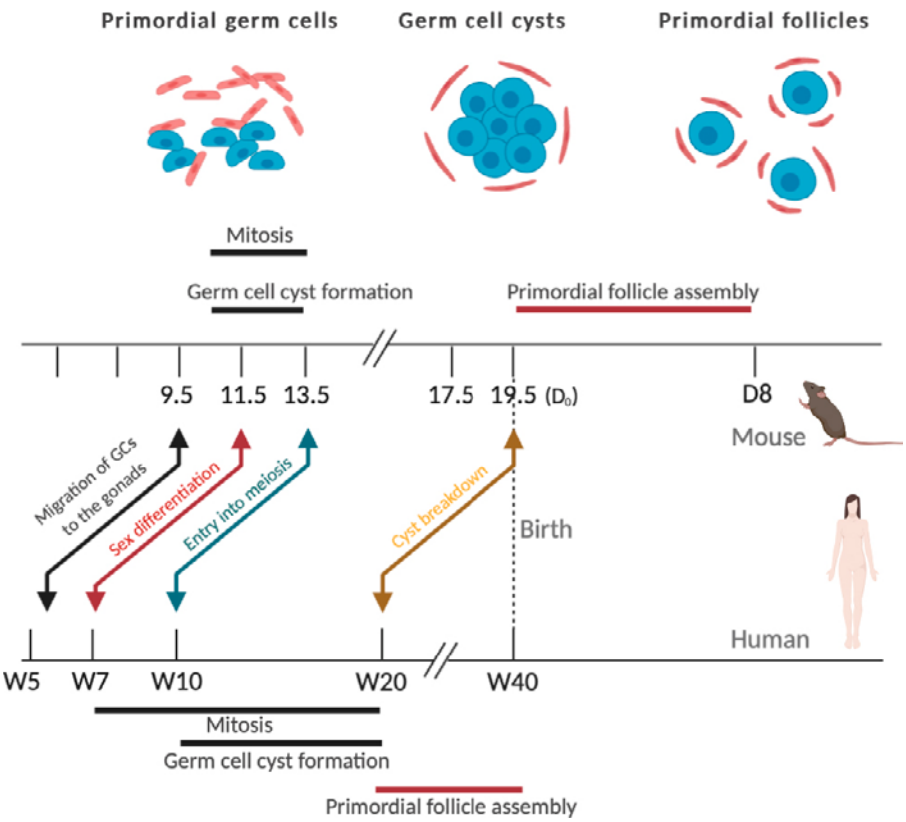

Figure 1. Timelines of major events during ovarian fetal development in mouse and human. Redrawn based on [1].

As a result of these complex processes of cell proliferation and apoptotic depletion, the number of oocytes in humans decline from several million at around five months of gestational age to less than 1 million at birth. This is followed by a continuous decline in the number of PFs that will be depleted by apoptosis until the age of menopause, that occurs at around 50 years of age, when only 1000 follicles are left in the ovaries [6]. In the rat ovary, around two-thirds of the oocytes are loss from the onset of meiosis until two days after birth [7].

1.1.2 Folliculogenesis

The age-related decline of PFs in postnatal life occurs as a result of different processes: the follicles can be depleted already in their quiescent state or they can start to mature and then undergo apoptosis at different stages during their development. Around 1000 follicles are lost every month until 37 years of age and then the decay accelerates until the pool is almost completely depleted and menopause occurs [8, 9].

During childhood the ovary is quiescent and oocytes within PFs are arrested in the diplotene stage of meiosis I. The PFs at this stage are defined as non-growing (NGF) or dormant follicles. Although no ovulation will occur until puberty, PFs are already activated and grow up to the antral stage in childhood. Antral follicles have been described throughout infancy and childhood in human ovaries [10] and during the early infantile period (postnatal day (PND) 8-14) in rats and mice [7, 11]. The developmental dynamics of PFs have been the subject of recent studies in mice. These studies led to the identification of two different classes of PFs: a ‘first wave’ and an ‘adult wave’. The ‘first wave’ is activated immediately after birth, persists in the mouse ovary up to ~ 3 months of age and appears to be responsible for the onset of puberty and for the early fertility. The ‘adult wave’ of PFs is activated gradually during reproductive life and provides fertility until the end of the reproductive life [12-14] (Figure 2). Structural and dynamic differences in the PFs have been observed also in humans. A study from Anderson *et al.* reported the presence of morphologically abnormal PFs in prepubertal human ovaries that were absent in adult ovaries, and could represent the human equivalent of the first wave identified in mice studies [15].

With the activation of the hypothalamic-pituitary-gonadal (HPG) axis at puberty, under the drive of gonadotropins, one oocyte is selected at every cycle to complete meiosis I and be ovulated. After ovulation the oocyte remains arrested in metaphase of meiosis II until fertilization.

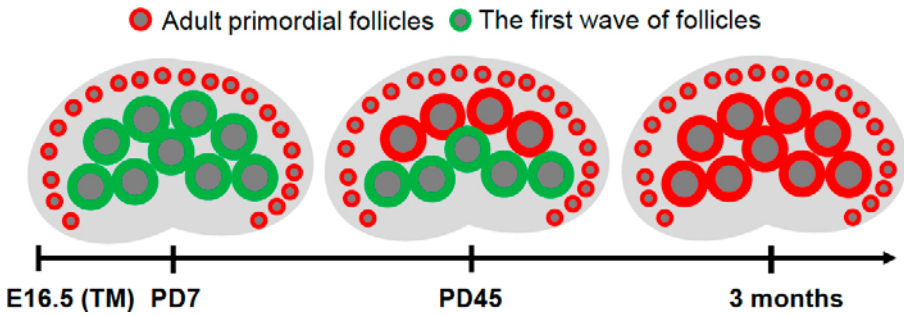

Figure 2. The two waves of follicles traced by tamoxifen-inducible reporters specific of primordial follicles, Foxl2 for the first wave and Sohlh1 for the adult wave. Adapted with permission from [14].

From the activation of a PF to the ovulation, the oocyte and the follicle undergo dramatic morphological changes in the process known as folliculogenesis. The recruitment of NGF into the growing pool is marked by the proliferation and differentiation of the surrounding pre-granulosa cells followed by a remarkable growth of the oocyte [8]. Pre-granulosa cells shift from flattened to cuboidal shape and studies have shown that the oocyte growth starts when a critical number of cuboidal GCs is reached, that is 10 cuboidal cells in mice [16] and 15 cuboidal cells in humans [17]. When a complete layer of cuboidal GCs surrounds the oocyte, it becomes a primary follicle. The follicle diameter increases from 40 μm to 50-60 μm during this transition [18]. At this stage, a zona pellucida is formed around the oocyte and GCs proliferate building multiple layers, marking the transition into secondary follicles (80-100 μm in early stage) [9]. The secondary follicles receive blood supply by one or two arterioles, becoming exposed to the blood circulation [19]. When the follicles contain three to six layers of GCs (103-163 μm), stromal cells near the basal lamina align parallel to each other and differentiate into theca cells (TCs) marking the transition into a preantral follicle. Subsequently, antral (or tertiary) follicles develop following the formation of small antral cavities filled with fluid that progressively aggregate to form a single antrum (180-250 μm). The antral follicles enlarge by further proliferation of GCs and increase of the antrum's size until they reach a diameter of 2 mm. From this stage onwards, follicles become selectable for ovulation. Most of these follicles undergo atresia, whereas the follicle selected for ovulation continues to grow and increases its size up to 20 mm in humans [9] (Figure 3).

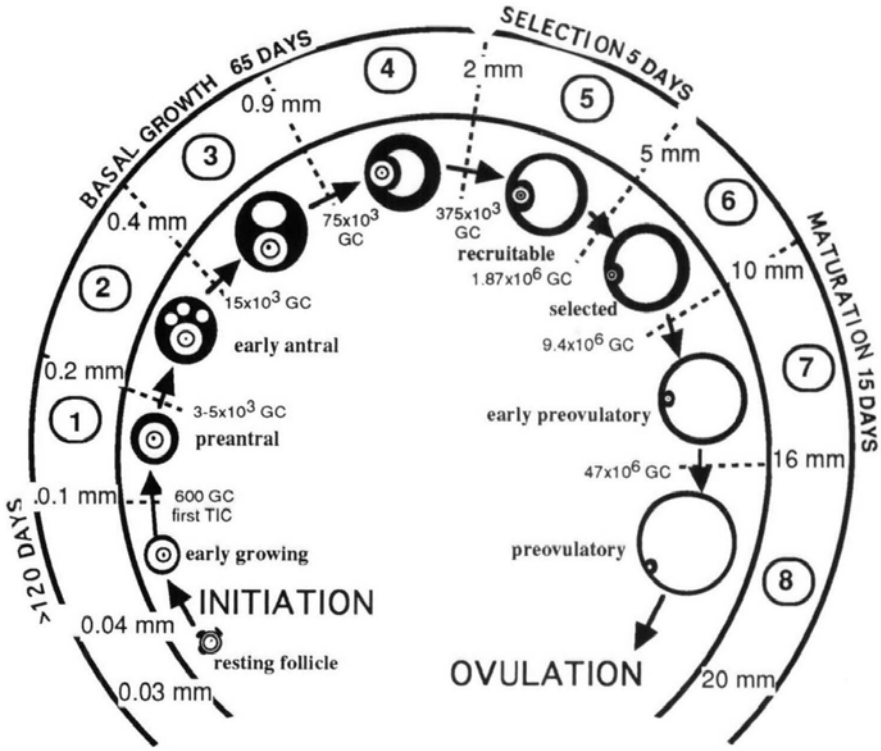

Figure 3. Schematic representation of follicle development in the human ovary. Reproduced with permission from [9].

Follicles in the antral stage have about the same size in rats and humans, whereas pre-ovulatory follicles in rats are only 0.9-1.0 mm in diameter [20]. Under the drive of the midcycle gonadotropin surge, the follicles increase their progesterone production and start to luteinize. This process ultimately leads to ovulation through the rupture of the follicle wall and the release of the oocyte [9, 21]. Luteinization of the GCs and TCs defines the formation of a corpus luteum that will be responsible for progesterone secretion during the second half of the menstrual cycle. If pregnancy occur, the human chorionic gonadotropin (HCG) will maintain the corpus luteum and production of progesterone by GCs will support the early stages of pregnancy. In the absence of fertilization the corpus luteum will involute into a corpus albicans and eventually be absorbed.

The above described process of folliculogenesis is regulated by the two gonadotropins secreted from the pituitary, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), under the control of the hypothalamic gonadotropin-releasing factor (GnRH) and ovarian factors such as activin and inhibin. Although preantral follicle growth is gonadotropin-independent, FSH promotes follicle growth also at preantral stages and is necessary for the formation of antral follicles. From the

antral stage, FSH sustains follicle growth, stimulates estradiol production by GCs and induces expression of LH receptors in GCs [22]. With the progression of the follicle growth, the sensitivity of GCs to FSH decline simultaneously with increasing sensitivity to LH, which maintains follicle growth and allows luteinization upon LH surge [21]. During follicular growth LH stimulates the production of androgens from TCs and the expression of aromatase in GCs, thus doubly contributing to estrogens production [21, 22].

1.1.3 Regulation of primordial follicle activation

The activation of PFs, that marks their entry into the growing phase from the dormant stage, is not yet completely understood. In particular, a major challenge is to understand the factors that regulate PFs' fate towards activation rather than quiescence or apoptosis. Two main signaling pathways involved in PF activation and tightly interconnected with each other have been intensively investigated in recent years, the phosphatidylinositol 3 kinase (PI3K) pathway and the mammalian target of rapamycin (MTOR) pathway (Figure 4).

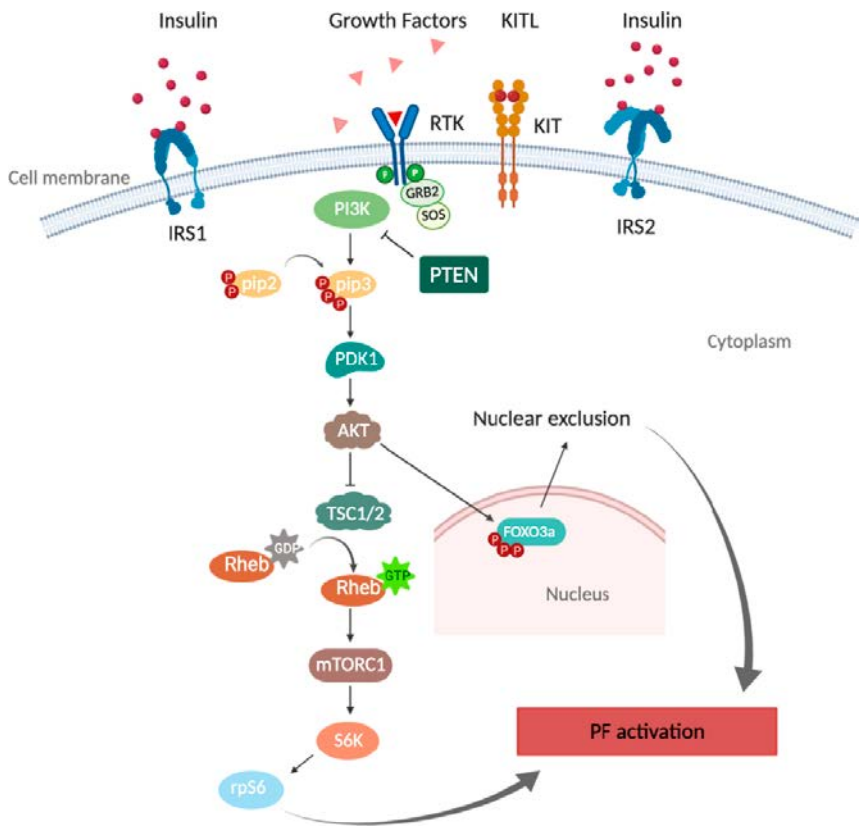

Figure 4. Schematic illustration of PI3K/PTEN/AKT and MTOR signaling pathways in the mammalian ovary.

The PI3K pathway is constituted by different members including phosphatases, kinases and transcription factors and it is involved in processes of cell proliferation and survival in all organs and tissues [8]. Upon binding of different ligands (such as insulin and growth factors) with the respective receptors located on the cell membrane, PI3K activates and converts phosphatidylinositol-4,5-bisphosphate (PIP_2) into phosphatidylinositol-3,4,5-trisphosphate (PIP_3). The latter molecule acts as a messenger and recruits the phosphoinositide-dependent protein kinase (PDK) to phosphorylate protein kinase B (AKT). The AKT is a serine/threonine kinase that can in turn phosphorylate many substrates. Among those, a crucial one is the forkhead box O3A (FOXO3A) [8]. FOXO3A is a transcription factor residing in the cell nucleus in its unphosphorylated state and is translocated into the cytoplasm upon phosphorylation thereby losing its transcriptional function. Studies in mice models have demonstrated that FOXO3A is expressed in the nucleus of PFs and becomes downregulated as soon as the PFs start to grow and transform into primary follicles [23]. Moreover FOXO3A knock-out in mice resulted in massive activation of PFs, followed by depletion and precocious exhaustion of the PF reserve [24]. Thus, FOXO3A acts as a suppressor of PF activation by transcriptional control of genes involved in apoptosis and cell cycle arrest in the oocyte nucleus. Another key molecule of the PI3K pathway is the phosphatase and tensin homolog (PTEN) that regulates the PI3K pathway by inhibition of PI3K, thereby contributing to the suppression of PF activation. Indeed, deletion of PTEN in mice oocytes also resulted in premature activation and depletion of the entire PF pool [25]. Other two well-defined substrates of AKT are the tuberin/tuberous sclerosis complexes 1 and 2 (TSC1 and TSC2) that are inactivated upon phosphorylation by AKT. Inactivated TSCs cannot longer suppress mTOR, which subsequently activates downstream molecules such as ribosomal protein S6 kinase (S6K) and ribosomal protein S6 (RPS6), responsible for translation of proteins involved in cell survival and growth. Deletion of both TSCs from the oocytes of mice PFs produced the premature activation and depletion of all PFs [26, 27]. The role of several other molecules in the maintenance of PFs dormancy has been elucidated [28]. Among them, anti-Müllerian hormone (AMH) secreted by growing follicles [29] and FOXL2 expressed in pre-granulosa cells of PFs play an important role in maintaining the quiescence of PFs [30]

1.1.4 Postnatal developmental stages and hormonal patterns in rats and humans

The neonatal period corresponds to PND 0-7 in rats and to the first month of life in humans. During this period a transient activation of the hypothalamic-pituitary-gonadal (HPG) axis causes a rise of gonadotropins that peaks at PND 12 in rats (infantile period) and between 1 week and 3 months of age in humans [31]. This neonatal surge marks the so-called mini-puberty, which corresponds also to an increase in circulating sex steroid hormones, with preponderance of estradiol in

females and testosterone, inhibin B and AMH in males. In humans, the HPG axis then enters a quiescent phase during which gonadotropins' levels approach the zero. On the contrary, in rats, although FSH and LH significantly decline during the juvenile period, they never reach zero and a pulsatile activity of LH begins already at around PND 24 and rises progressively until puberty onset. FSH levels increase only after PND 35 when the peripubertal stage starts in rats. Detailed frames of postnatal development in rats and humans are presented in Table 1.

Table 1. Time frames of postnatal development in rats and humans. Adapted with permission from [32].

Stage	Rats	Humans
Neonatal	0-6 PND	0-1 mo
Infantile	7-17 PND	2 mo - 2 y
Juvenile	18-34 PND	3-9 y
Peripubertal	35-42 PND	10-15 y
Late adolescent	43-70 PND	16-21 y

1.2 Effects of intrauterine growth restriction (IUGR) on the female reproductive organs

1.2.1 IUGR and the Developmental Origins of Health and Disease

The term IUGR describes an impairment of growth occurring during fetal life and can be caused by maternal and environmental factors, such as chronic undernutrition, placental insufficiency, infections, and other toxic exposures. When IUGR occurs, the newborn will often be small for gestational age (SGA) at birth, defined as a weight and/or length at birth of at least 2 SD below the mean for gestational age. SGA is often improperly used as a synonym for IUGR, although the term also includes newborns who are constitutionally small and whose growth has not been compromised during fetal life. During the late ´80s the retrospective cohort studies of Barker and Hales showed a significant association between patterns of fetal growth and risk of diseases later in life, such as type 2 diabetes and cardiovascular diseases. Many studies in the following decades confirmed these findings, giving origin to the so called Developmental Origins of Health and Disease (DOHaD) hypothesis. According to the hypothesis, when the fetus is exposed to an inadequate supply of oxygen and/or nutrients, survival of vital organs, such as brain, is preserved at the expense of whole body growth and development of other organs. In response to this, the organism actuates adaptive responses, which are driven by changes in gene expression, in a process known as fetal programming. However, in postnatal life, the adaptive genetic asset clashes with an adequate

or even opposite environment, resulting in an increased susceptibility to various diseases. Experimental studies have provided evidence of the fetal programming of gene expression as a consequence of IUGR in many organs and tissues.

Fetal life is a critical window of exposure for the reproductive system and although differences exist between humans and animals used in experimental studies, such animal models are extremely useful in assessing the risk of exposure to pathological events on key human reproductive and developmental processes. An insufficient blood and/or nutrient supply to the fetus can induce a reprogramming of the endocrine axes and a change in endocrine set points that will persist throughout life. Moreover, as gametogenesis takes place during fetal life, an altered fetal environment may affect gonadal development and function, having a potential impact on future fertility and/or on the quality of gametes.

1.2.2 Human studies

Some epidemiological studies have focused on long-term health outcomes in children born SGA, as surrogate measure of IUGR. In terms of pubertal development, the results are controversial and difficult to compare for the variability of selection criteria and outcome measures. However, most of the studies concluded that puberty in SGA children begins relatively early but within the normal range and at a shorter height compared to children born with a normal weight/length [33]. This seems to be particularly true for the small percentage of SGA children who fail in attaining catch-up growth during postnatal life [34]. A lower number of primordial follicles has been observed in the ovaries of severely growth-restricted fetuses who died between 20 and 40 weeks of gestation because of placental insufficiency [35] and smaller ovaries and uterus and a reduced ovulation rate have been reported in post-menarcheal girls born SGA [36, 37]. The influence of IUGR on future fertility is still controversial. An association between IUGR and risk of infertility was reported in a study including women selected from infertility clinics [38]. SGA women did not have a longer time to pregnancy as compared to AGA women in a questionnaire-based study [39] and in another recent publication including more than 10,000 men and women, a higher risk of infertility was found in SGA men but not in SGA women [40].

1.2.3 Animal studies

Animal models are a fundamental tool to infer data relevant to human diseases and have been extensively used to explore the consequences of IUGR in various organs and tissues. Several experimental models have been designed to restrict fetal growth [41, 42]. Prenatal nutrient manipulation is the most common approach, whereas uterine arteries ligature is the most frequently used among surgical models. Uterine arteries ligature produces a reduction of the placental blood flow

to the fetuses that mimics the pathogenetic mechanism of placental insufficiency in human pregnancies. This model is particularly relevant to study human fetal growth restriction in developed countries, where a reduced placental blood flow is the most frequent cause of IUGR [43]. Most of the data generated from animal models have focused on the metabolic effects of IUGR whereas only a few studies have investigated the impact on the reproductive system. DNA damage in fetal oogonia and altered folliculogenesis have been described after IUGR induced by maternal undernutrition in murine and ovine models [44-46]. Only two studies using uterine arteries ligature reported a reduced number of follicles and delayed onset of puberty in adult IUGR female rats compared to controls [47, 48].

1.3 Effects of cancer treatments on the female reproductive organs

Over the past five decades the percentage of children surviving cancer has dramatically increased. According to the last reports, the 5-year survival rate after a cancer diagnosis has exceeded the 80% among children and young adolescents in Europe and in the USA [49]. As a result of the increased life expectancy, many more children are becoming adult survivors and facing the chronic consequences of cancer treatments. In females, one of the major late effects of cancer treatments is premature ovarian insufficiency (POI). POI is characterized by the precocious exhaustion of the ovarian follicles that leads to premature menopause and infertility. Clinically POI is characterized by different features according to the patient's pubertal development at the onset of POI: i) lack of pubertal onset in prepubertal girls (absent Tanner stage 2 breast development by age 13), ii) lack of pubertal progression in pubertal girls, iii) absent menarche by age 16 (primary amenorrhea) in pre-menarcheal patients, iv) disappearance of menstrual cycles (secondary amenorrhea) for ≥ 4 months in post-menarcheal girls. These clinical features are accompanied by elevated gonadotropins and low levels of estrogens, AMH and inhibin. Twice elevated serum levels of FSH in the menopausal range (usually > 25 IU/l) support the diagnosis of POI [50]. A report from the childhood cancer survivor study (CCSS) in 2006 estimated that the risk of POI in childhood cancer survivors is increased compared to their siblings, with a cumulative incidence of 8% by age 40 years [51]. More recently, Barton and co-workers analyzed data from 3,531 female cancer survivors and 1,366 unaffected siblings enrolled in the CCSS and found that 16% of the survivors experienced infertility, corresponding to a relative risk of 1.54, compared to siblings. Among them, 64% eventually achieved a pregnancy, even though with an increased time to pregnancy [52].

The entity of ovarian damage varies according to two main variables: the patient age and the type of treatment.

1.3.1 Age

Studies have shown that the likelihood of severe ovarian failure following cancer treatments increases with the age of the patient [53]. This is in agreement with the physiological decline in the number of PFs with age, well shown by prediction models of the ovarian reserve [6]. The higher the amount of PFs at start of gonadotoxic treatments, the less severe will be the damage. However, variations in the amount of PFs present at birth as well as genetic polymorphisms likely play an important role in the individual susceptibility to damage [54].

1.3.2 Chemotherapy

The gonadotoxic potential of chemotherapeutic agents is determined by the type, the cumulative dose and the treatment duration [55]. Despite all cytotoxic drugs have a negative impact on the ovary, alkylating agents such as cyclophosphamide, busulfan and dacarbazine, are associated with the highest risk of infertility, with an effect that increases proportionally with increasing doses [56]. The individual gonadotoxic potential of chemotherapeutic drugs has been established for the most commonly used ones [57, 58] (Table 2).

Table 2. Risk of ovarian insufficiency for common chemotherapeutic drugs. Adapted with permission from [57, 58].

High (> 80%)	Intermediate	Low (< 20%)	Very low/no risk
Cyclophosphamide	Cisplatin	Bleomycin	Vincristine
Ifosphamide	Carboplatin	Dactinomycin	Vinblastine
Procarbazine Dacarbazine	Doxorubicin	Mercaptopurine	Methotrexate
Chlorambucil			5-fluorouracil
Busulfan			
Melphalan			
Chlormethine			

However, as chemotherapeutic regimens usually combine multiple drugs, the gonadotoxicity of every individual agent is difficult to ascertain. Therefore, a better risk assessment for infertility in clinical settings has been predicted taking into account the type of malignant disease and the associated treatments [58] (Table 3). For example, conditioning regimens used before hematopoietic stem cell transplantation (HSCT) and total body irradiation (TBI) carry the highest risk of ovarian insufficiency, together with treatment of metastatic sarcomas [58].

Table 3. Risk of ovarian insufficiency based on treatment protocols for common cancers in childhood and adolescence. Adapted with permission from [58].

High (> 80%)	Intermediate	Low (< 20%)
Total-body irradiation (TBI) or pelvic radiotherapy	Acute myeloblastic leukemia	Acute lymphoblastic leukemia
Hematopoietic stem-cell transplantation (HSCT)	Hepatoblastoma	Wilms' tumor
Hodgkin's disease: treatment with alkylating-drugs	Osteosarcoma	Stage I soft-tissue sarcoma
Metastatic soft-tissue sarcoma	Non-metastatic Ewing's sarcoma	Retinoblastoma
Metastatic Ewing's sarcoma	Stage II-III soft-tissue sarcoma	Brain tumor: surgery only, cranial irradiation < 24 Gy
	Neuroblastoma	Germ-cell tumor (no radiotherapy)
	Non-Hodgkin lymphoma	
	Hodgkin's disease: alternating treatment	
	Brain tumor: craniospinal radio-therapy, cranial irradiation > 24 Gy	

A variety of malignant and non-malignant conditions require treatment protocols that expose the patients to a high risk of infertility. The most frequent malignant diseases in children include hematologic cancers, such as Hodgkin's lymphoma, non-Hodgkin's lymphoma, and leukemia, together with central nervous system (CNS) tumors [59]. Depending on the disease stage and evolution, these patients can become eligible for HSCT (allogenic or autologous) and thus be exposed to a high risk of infertility. Non-malignant systemic conditions, such as primary immunodeficiency, sickle cells anemia, thalassemia, aplastic anemia and other inherited bone marrow failure syndromes are also candidates to allogenic HSCT and constitute an increasingly prevalent indication for OTC in pediatric patients, as a consequence of the improvement of diagnostic tools and earlier referral.

1.3.2.1 Mechanisms involved in chemotherapy-induced PF loss

Exposure to chemotherapeutic drugs induces depletion of the ovarian follicles (both PFs and growing follicles) and ovarian atrophy [60-63]. The mechanisms by which cytotoxic drugs deplete PFs are not yet fully understood but during the last decade some of the pathways involved have been clarified. The two main

researched mechanisms are follicle apoptosis and PF activation followed by depletion, the latter known as the 'burn-out' theory:

i) Follicle apoptosis is triggered by DNA damage following exposure to cytotoxic drugs. Dividing cells are particularly sensitive, as shown by apoptotic markers' positivity in granulosa cells of exposed growing follicles [63]. In the mature oocyte, that is a non-dividing cell, apoptosis is mediated by several molecules including B-cell lymphoma 2 protein (BCL2) associated X protein (BAX) and caspases [64]. Alkylating agents have been shown to cause DNA damage even in non-dividing cells, thereby affecting also dormant PFs. In recent years, some of the pathways responsible for PF apoptosis have been unraveled. A homolog of the anti-oncogene P53, and specifically the p63 isoform TAp63, has been shown to mediate DNA damage and apoptosis of PF oocytes following DNA injury [65].

ii) The 'burn-out' theory suggests that chemotherapy agents trigger the activation of dormant PFs, which is followed by their massive depletion [66]. Alteration of pathways controlling PF dormancy/activation has been shown after exposure to chemotherapeutic agents: cyclophosphamide can upregulate PI3K/PTEN/AKT signaling pathway thereby causing activation and growth of PFs followed by their depletion [66] (Figure 5). Furthermore, in physiological conditions, growing follicles secrete AMH that contributes to the maintenance of PFs' dormancy [29]. The destruction of growing follicles caused by chemotherapy provokes a reduction of AMH secretion and a loss of its suppressive action on the activation of PFs [67].

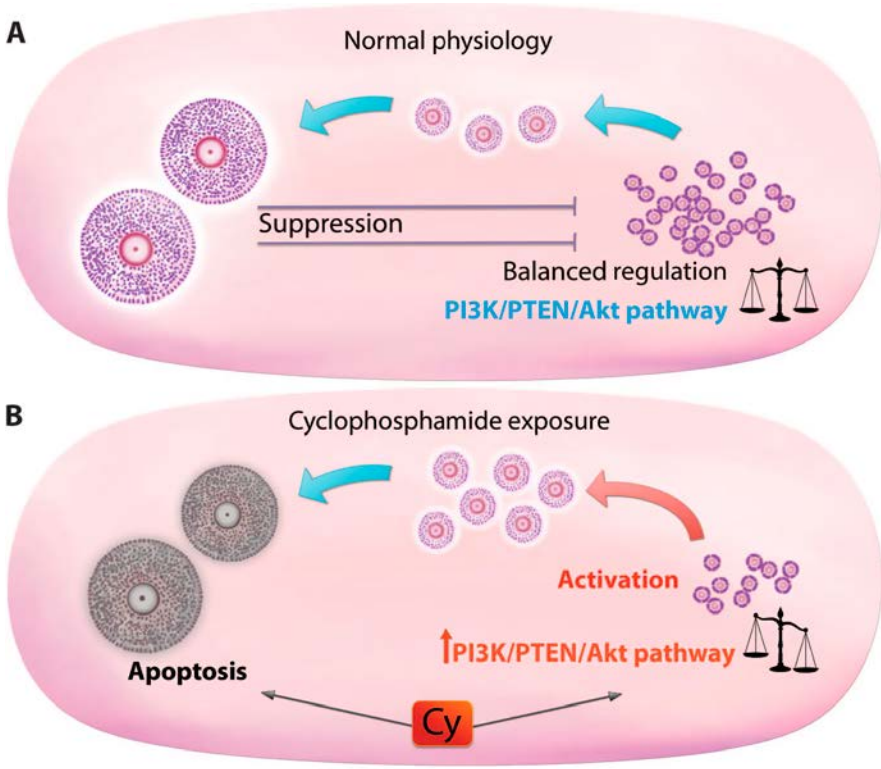

Figure 5. Regulation of primordial follicle activation in physiological conditions (A) and its dysregulation following exposure to cyclophosphamide (Cy) (B). Adapted with permission from [66].

1.3.3 Radiotherapy

Whole-body, abdominal, or pelvic irradiation cause damage to the ovary and to the uterus [68]. Oocytes are very sensitive to radiation and both PFs and growing follicles can be affected by radiotherapy (RT). As for chemotherapy, the extent of damage depends on the dose, the fractionation schedule and the age at treatment. The median lethal dose (the dose needed to destroy 50% of the PFs present in the ovary) is estimated to be less than 2 Gy [69] and a dose greater than 5 Gy is sufficient to cause ovarian insufficiency, regardless of the age [70]. This is meaningful considering that doses of TBI administered before HSCT range from 2 to 16 Gy. Fractionated doses are more tolerable than high single doses, even though the repetitive nature of fractionated RT might interfere with cell repair [58]. Regarding the age at treatment, older patients have a lower quantity of PFs and are therefore less tolerant to the ovarian insult than younger patients. This has been clearly demonstrated by Wallace *et al.* who have shown that, as the age

at treatment increases, a progressively lower dose of RT is required to induce POI in the 97.5% of the patients. The sterilizing dose of RT is estimated to be 20.3 Gy for a newborn child and 14.3 Gy for a 30-year-old woman [58]. Cranial or craniospinal irradiation, frequently used in the treatment of CNS tumors and in acute lymphoblastic leukemias, can disrupt the hypothalamic-pituitary-gonadal axis and cause a central hypogonadism, especially for doses > 30 Gy [58]. As a result of the above described mechanisms, it is understandable that the combination of TBI and alkylating agents, used in conditioning regimens preceding HSCT, bears the highest risk, leaving almost 100% of patients infertile. Previous research conducted by Vatanen *et al.* showed that only 25% of girls conditioned with TBI for HSCT had a residual ovarian function at long-term follow-up [71].

1.3.4 Options for fertility preservation

1.3.4.1 Embryo cryopreservation and oocyte cryopreservation

Embryo cryopreservation and oocyte cryopreservation are established techniques for preservation of fertility in adult women. Embryo cryopreservation requires a stable relationship and both procedures are performed by controlled ovarian stimulation and transvaginal oocyte retrieval. Therefore, the patient should be post-pubertal and it should be possible to postpone the start of chemotherapy of at least 10-12 days [72].

1.3.4.2 Ovarian tissue cryopreservation (OTC)

When the patient is prepubertal or when chemotherapy start cannot be delayed, cryopreservation of the ovarian tissue is the only option to preserve fertility [58, 72, 73]. Over the last 15 years, OTC has become an established technique in many countries but it is still considered experimental for prepubertal girls and is often performed in the context of a research protocol (Figure 6).

Wallace et al. developed [58] and validated [74] selection criteria for OTC that are nowadays applied in many centers across Europe:

- Age younger than 35 years
- No previous chemotherapy or radiotherapy if aged 15 years or older at diagnosis, but mild, non-gonadotoxic chemotherapy acceptable if younger than 15 years
- A realistic chance of surviving for 5 years
- A high risk of premature ovarian insufficiency (>50%)
- Informed consent (from parents and, where possible, patient)
- Negative serology results for HIV, syphilis, and hepatitis B
- Not pregnant and no existing children

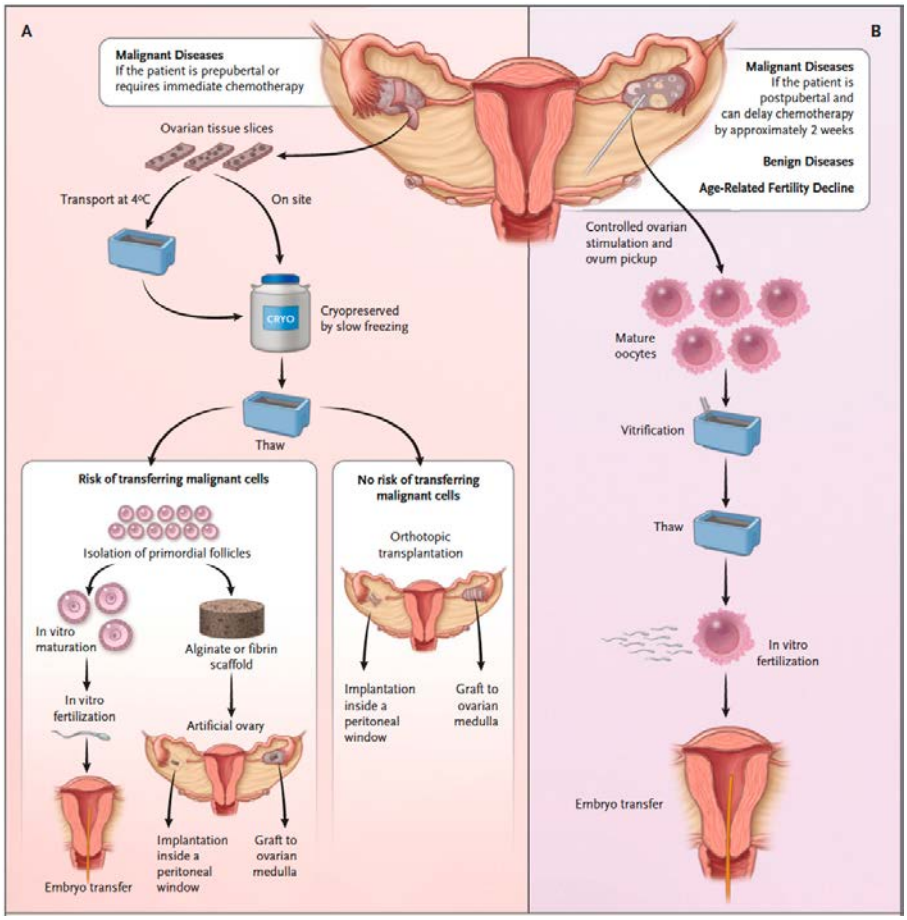

Figure 6. Options for fertility preservation in prepubertal patients and patients that require immediate chemotherapy (A) and in postpubertal patients or patients that can delay the start of chemotherapy (B). Reproduced with permission from [72], Copyright Massachusetts Medical Society.

However, selection criteria are not completely homogeneous among different centers, especially regarding the definition of high risk of infertility, the upper age limit and the previous exposure to chemotherapy. In Nordic countries, recommendations on fertility preservation for girls and young women with childhood cancer have been established by the Nordic Society for Pediatric Hematology and Oncology (NOPHO) in 2013:

i) All girls should be examined regarding pubertal development (Tanner stage and menstrual history) at diagnosis and should be informed of the risk for impaired fertility following the planned treatment

ii) If there is a very high risk of infertility, the patient and her parents should be informed of possibilities for fertility preservation by a professional, specially assigned for this purpose, e.g. a pediatric endocrinologist, a gynecologist or fertility specialist, according to local availability and routines. It is important that the autonomy of the girl is respected and that she, if mature enough, is offered the opportunity of individual consultation.

iii) After treatment, all girls who have received alkylating agents or abdominal irradiation should after sexual maturation be offered referral to a gynecologist or fertility specialist for evaluation, counseling and for considering the possibility for ovarian hyperstimulation and cryopreservation of oocytes.

iv) If the girl is menstruating, mature enough to give informed consent, is facing cancer therapy with very high risk of infertility AND therapy can be delayed 1-2 weeks, ovarian hyperstimulation and cryopreservation of oocytes may be considered. The responsible oncologist must be consulted to make sure that no contraindications, such as bleeding disorders or too long delay of cancer therapy, to such procedures are present. At present, cryopreservation of non-fertilized oocytes is routine among older women. The girl should get information adjusted to her age.

v) All girls regardless of maturational stage who are facing or receiving oncological treatments associated with a very high risk of infertility, could be offered the experimental procedure of ovarian cortical tissue cryopreservation.

vi) In menstruating girls, cryopreservation of ovarian tissue can precede controlled ovarian hyperstimulation (see above). The responsible oncologist must be consulted to make sure that no contraindications to such procedures are present.

vii) Adaptation to adult guidelines should be considered when the girl has reached the maturity of a young adult.

viii) Patients eligible for cryopreservation of ovarian cortical tissue are those undergoing allogeneic/autologous HSCT, radiotherapy with ovary in the field, ovariectomy due to any cause.

At diagnosis, patients should be informed about their risk of infertility and those with a high risk should be offered fertility preservation [6]. Ovarian cortex stripes are isolated from cortical biopsies or from the whole ovary after oophorectomy and cryopreserved by slow-freezing method. The procedure is, when possible, combined with other surgical procedures under general anesthesia, such as positioning of a central venous catheter (CVC) or surgery for removal of tumor mass. Once the patient has been cured and a wish for childbearing arises, reimplantation of the ovarian tissue can be performed. The most common sites of reimplantation are peritoneal pockets inside the pelvic cavity or the ovarian medulla of the remaining ovary [75]. Starting from 2004, more than 130 live births have been reported worldwide following reimplantation of ovarian tissue stored from adult

women, showing that cryopreserved ovarian tissue is able to restore ovarian function in more than 95% of cases [72, 76].

However, there are still many uncertainties, especially for application in pediatric patients. First, knowledge of how patients respond to reimplanted tissue collected prior to puberty is limited. Some recent reports provide the proof of principle that this technique may work also in such cases. Ovarian tissue cryopreserved from two pre-pubertal patients restored puberty when reimplanted back [77, 78] and two successful pregnancies have been reported after reimplantation of ovarian tissue harvested at 9 and 13 years of age [79, 80]. With increasing time from OTC establishment in many centers, more reports are expected in the coming years.

A debated issue regards whether OTC should be performed before or after the patient has been exposed to chemotherapy and which extent of exposure is acceptable. Ideally, OTC should be performed before initiation of chemotherapy, to avoid damage to the follicles and guarantee the best quality of the harvested ovarian tissue [74, 81-83]. However, this is only possible when the patient falls in a definite risk category for infertility already at the time of diagnosis. In many cases, the prognosis is uncertain or the course of the disease increases the risk category time after the initial diagnosis. In this case, fertility preservation becomes an option only after the first cycles of treatment have been performed [74]. First-line cancer treatments are considered to have a small effect on follicles, but this assumption has been recently questioned. A deterioration of the follicular intracellular quality, such as increase in abnormal granulosa cell nuclei and oocyte vacuolization, was evidenced in the ovaries of patients exposed to chemotherapy (both with and without alkylating agents) before OTC compared to non-exposed ones [81]. Another study explored cryopreservation of *in-vitro* matured oocytes as an additional technique to ovarian tissue cryopreservation in pediatric patients exposed and not exposed to chemotherapy before OTC and concluded that exposure to chemotherapy significantly reduced the number of collected oocytes and the amount of *in-vitro* matured oocytes [84]. El Issaoui *et al.* reported a significant reduction of the ovarian volume in a cohort of girls below 18 years who were exposed to first-line chemotherapy before OTC, compared to those that were not exposed to any chemotherapy, to an extent that was directly proportional to age [82]. Finally, previous exposure to chemotherapy significantly reduced the survival and development of follicles in culture, and increasing doses of alkylating agents were identified as major predictor of follicle atresia [83]. Despite evidence of an effect of first-line chemotherapy on the quality of ovarian follicles, reimplantation of harvested ovarian tissues after exposure to chemotherapy is equally effective in terms of restoration of the ovarian function, at least in adult patients [85-87].

Another major concern is that reimplantation of ovarian tissue involves the risk of seeding malignant cells back to the patient, in case the ovary had been contaminated

at the time of active disease [88-93]. This is particularly true for certain types of malignancies, such as leukemia and neuroblastoma, which are highly prevalent categories of tumors among children [90, 94, 95]. For this reason, in most clinical settings, only patients with cancers associated with a low risk of ovarian metastasis are currently considered for reimplantation [94, 95]. For leukemias, if the patient is in complete remission at the time of OTC, the risk of ovarian contamination is very low [91]. However, the bone marrow minimal residual disease (MRD) does not always corresponds to the ovarian MRD and a significant contamination has been found in ovarian tissues of patients suffering from leukemia even at the time of bone marrow remission [96]. Despite these uncertainties, reimplantation of ovarian tissue from patients affected by leukemia has been performed in a few cases, where the tissue was harvested during full remission and after extensive tissue evaluation to exclude ovarian contamination [85, 87, 97, 98]. It is important to stress that tissue evaluation cannot be performed in the same fragment of tissue that will be used for reimplantation, because the tissue will not survive the procedure. Moreover, detection of ovarian MDR by real-time quantitative polymerase chain reaction (RT-qPCR) is the most sensitive way to detect malignant contamination but can only be applied when a disease-specific molecular marker is identified [94, 99].

Alternative strategies to overcome the risk of reseeding the disease in these patients are under investigations and include transplantable artificial ovary and in vitro development of primordial follicles. A protocol for the isolation of primordial follicles to obtain a disease-free follicle suspension has been developed recently [100] and development of PFs up to the antral stage has been reported after transplantation and xenografting in mice [101-103]. Restoration of fertility in ovariectomized mice has been shown using a 3D-printed ovary seeded with follicles [104]. A multistep culture system has been developed by McLaughlin *et al.* and was shown to support follicle maturation from primordial to metaphase II stage in vitro [105]. However, the efficiency is low and several issues remain to be solved. Suboptimal culture conditions, cryogenic cell damage and chemotherapeutic agents to which patients have been exposed before harvesting the tissue may have an impact on the oocyte viability and maturing potential in vitro and the effects of the artificially-induced maturation on the genomic stability of the oocytes need to be assessed [76].

1.3.5 Follow-up programs for cancer survivors and assessment of ovarian reserve

Ovarian and/or hypothalamic-pituitary damage following cancer treatments can result in hypergonadotropic or hypogonadotropic hypogonadism respectively. In prepubertal patients hypogonadism translates clinically into failure of pubertal onset, whereas in patients that already started puberty hypogonadism will manifest as

lack of pubertal progression, primary or secondary amenorrhea. Recommendations for premature ovarian insufficiency surveillance for childhood, adolescent and young cancer survivors have been proposed by the International Late Effects of Childhood Cancer Guideline Harmonization Group in collaboration with the European Union-founded PanCareSurFup consortium and published in 2016 [50].

Monitoring of growth and puberty by Tanner stage is recommended annually or as clinically indicated for prepubertal girls treated with potentially gonadotoxic treatments. Girls with no signs of puberty by age 13, failure in pubertal progression, primary amenorrhea by age 16 or menstrual cycle dysfunction should be referred to pediatric endocrinology or gynecology. FSH and estradiol should be tested in prepubertal girls treated with potentially gonadotoxic treatments who fail to initiate or progress through puberty, starting from 11 years of age. AMH measurement for assessment of the ovarian reserve is only recommended after 25 years of age in conjunction with FSH and estradiol [50]. In pediatric patients, AMH levels decline already at cancer diagnosis [106] and soon after treatment, showing a recovery timing that is inversely correlated with the intensity of the gonadotoxic treatment [107]. However, its utility as marker of ovarian reserve in prepubertal girls is still uncertain and requires further research [108]. Antral follicle count (AFC) by vaginal ultrasound is the most reliable way for prediction of the ovarian reserve, but is not indicated in young patients [109]. No consensus guidelines exist for the pubertal induction and maintenance with hormone replacement therapy (HRT) in this specific population, therefore guidelines for Turner syndrome are usually applied [59, 110, 111].

1.4 Future perspectives

Future research efforts should focus on the elucidation of these areas of uncertainty. In particular, the potential of prepubertal ovarian tissue to restore fertility after reimplantation, the consequences of chemotherapy exposure before OTC on the fertility-restoring potential and quality of the ovarian tissue and the effectiveness of alternative strategies such as the artificial ovary or PF maturation in vitro for patients with diseases at high risk of ovarian contamination are major areas of interest. Moreover, the establishment of harmonized selection criteria for fertility preservation is an important issue that would allow to target the patients that will benefit the most from the procedure and optimize the risk-benefit ratio. Further understanding of genetic variations responsible for different individual susceptibility to ovarian gonadotoxic insults as well as investigation of the gonadotoxic potential of novel cancer treatments must be guaranteed to improve patient-tailored counselling.

2 AIMS

2.1 Overall aims

The overall objectives of this thesis are:

- to define the impact of IUGR on the ovary using a rat model of placental insufficiency
- to define the impact of first-line chemotherapy on the ovary using ovarian tissues harvested from cancer patients for fertility preservation
- to assess the efficacy of fertility preservation criteria in selecting patients at high risk of infertility
- to analyze the implementation of the OTC service in a large pediatric oncology unit

2.2 Specific aims

The specific objectives of this thesis are:

- to study the effects of IUGR on ovarian follicles and gene expression in ovaries of rats from neonatal to peripubertal ages
- to study the effects of exposure to first-line chemotherapy on pre-antral follicles and ovarian stroma in a cohort of girls and young women with onco-hematological diseases
- to study the clinical outcomes of a cohort of patients who underwent OTC, in terms of ovarian function (pubertal development, hormonal profiles and need for estrogen replacement therapy) and put them in relation to age, diagnosis and treatment in order to verify if the selection criteria for OTC target the right patients
- to study the fruition rate of the OTC service and analyze the timing of OTC, i.e. the time from indication to execution of the procedure, in relation to the exposure to chemotherapy

3 MATERIAL AND METHODS

3.1 Ethics

3.1.1 Paper I

The experimental animal model was approved by the Committee for Animal Research of Tor Vergata University in Rome, Italy and was performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publication No. 85–23, revised 1996). All the procedures complied with the Italian regulations for laboratory animal care.

3.1.2 Paper II

Ovarian tissues were donated by patients who underwent OTC at the Helsinki University Central Hospital (Finland), the Tampere University Hospital (Finland) and the Oslo University Hospital (Norway). For pediatric patients (referred to Helsinki University Central Hospital), OTC was performed in the context of a research project approved by the Ethics Committee of Helsinki University Central Hospital (Dnr 340/13/03/03/2015). Written informed consents for pediatric patients were given by their guardians and by the patients when appropriate. Adult patients (referred to Tampere and Oslo University Hospitals) were offered OTC as part of a fertility preservation program. Written informed consent for the quality control of ovarian tissue, including morphological analysis and in vitro culture, was signed by all patients. The need of further ethical licenses were discussed with the Oslo University Hospital, the Finnish National Supervisory Authority for Welfare and Health and the Finnish Medicines Agency. In accordance with the Medial Research Act (488/1999) and Act on the Medical Use of Human Organs, Tissues and Cells (101/2001), written informed consent for the use of ovarian tissues for research purposes was sufficient and no further ethical permits were required. All the patients' data handled in Sweden were anonymized.

3.1.3 Paper III

The collection of register data of patients with indication for OTC was approved by the Helsinki University Central Hospital (Hospital license nr HUS 248/2019). Data handled in Sweden were anonymized. OTC was offered as part of a research protocol approved by the Ethics Committee of Helsinki University Central Hospital (Dnr 340/13/03/03/2015). For patients who underwent OTC, the parents and the patients, when appropriate, gave their written informed consent to the OTC procedure and the related research.

3.2 Animals

IUGR was induced by bilateral ligature of the uterine arteries on day 19 of gestation in time-dated Sprague-Dawley pregnant rats (Charles River Laboratories Inc., Italy) [112]. Control animals were obtained from pregnant rats undergoing a 'sham' procedure, in order to equalize the effects of anesthesia and surgical stress. After spontaneous delivery (day 22-23 of gestation) IUGR animals were selected at birth according to a body weight (BW) of less than 2 standard deviations (SDs) compared to the mean BW of the control litter. Newborn pups were assigned to feeding dams with a maximum number of six *per* litter to equalize postnatal nutrition. At 21 days post-partum (dpp), pups were weaned onto standard rat chow diet (Mucedola S.R.L., Milano, Italy) and placed in groups of three to five, with the males and females separated. The animals were sacrificed by cervical dislocation at postnatal days 5, 20 and 40 dpp, representing the neonatal, juvenile and peripubertal periods, respectively. Body weight and ovary weight were measured weekly and at sacrifice, respectively. Each group of IUGR and shams at 20 and 40 dpp included six animals from at least three different litters to ensure biological variability. For the 5 dpp old animals, 8 IUGR and 4 sham animals from three different litters were included. Gene expression analysis was performed in 6 IUGR and 3 sham animals and histological evaluation was performed in 4 IUGR and 4 sham animals.

3.3 Tissue processing (Paper I)

Ovaries excised from euthanized animals were processed as follows. For histological analysis, one gonad was fixed in 4% paraformaldehyde (PFA) (P/N15812–7, Sigma-Aldrich, MO, USA) overnight at 4 °C, dehydrated in ethanol at increasing concentrations followed by butyl acetate (P/N 45860, Sigma-Aldrich, MO, USA), and embedded in paraffin (Paraplast X-TRA®; P/N P3808, Sigma-Aldrich) at 61 °C overnight. Ovaries were cut serially to a thickness of 5 µm, mounted on microscope slides (P/N10143352, Superfrost Plus, Thermo Scientific, MA, USA) and placed at 37 °C overnight.

3.4 Immunohistochemistry (Paper I)

For immunohistochemical (IHC) analysis, three sections per ovary were stained and evaluated. Chosen sections corresponded to approximatively the 25, 50 and 75% of the entire tissue block. Tissue sections were dewaxed in xylene (P/N 02080, HistoLab, Gothenburg, Sweden) for 10 minutes and then rehydrated in ethanol at decreasing concentrations. Antigen retrieval was performed using citrate buffer at pH 6.0 at 95 °C for 10 minutes. Slides were incubated first in methanol + 3%

H_2O_2 for 10 minutes at room temperature for endogenous peroxidase blocking, and then in tris-buffered saline (TBS) + 2% horse serum for 30 minutes at room temperature to prevent nonspecific antibody binding. Samples were then incubated overnight at 4 °C with primary antibody against AMH (P/N MCA2246, Bio-Rad, CA, USA) or unspecific IgGs (for negative control) dissolved in TBS + 2% horse serum. After washing with TBS + 0.01% Tween20 (P/N P1379, Sigma Aldrich,), slides were incubated with biotinylated secondary antibody (P/N BA1400, Vector laboratories, CA, USA), and then with avidin-biotin-peroxidase complex prepared using Vectastain ABC kit (P/N PK-6100, Vector Laboratories, CA, USA) for 30 minutes each at 37 °C. After washing, slides were stained with DAB (P/N SK-4105, Vector Laboratories) and then with hematoxylin solution modified according to Gill III, followed by tap water rinsing for 5 minutes. Finally, slides were dehydrated with ethanol at increasing concentrations, cleared with xylene and mounted with Pertex (Cell Path, Hemel Hempstead, UK) and cover slips.

3.5 Histological analysis (Paper I)

For histological evaluations, rat ovarian sections were stained with hematoxylin and eosin. In brief, after dewaxing in xylene and rehydration in ethanol, samples were incubated for 3 minutes with hematoxylin solution modified according to Gill III, rinsed with tap water for 20 minutes, and soaked in 70 and 95% ethanol for 5 minutes each. Slides were then incubated with eosin for 30 seconds, dehydrated with 100% ethanol and xylene and finally mounted with Pertex (Cell Path, Hemel Hempstead, UK) and cover slips. Three sections per ovary, corresponding to approximatively the 25, 50 and 75% of the entire tissue block, were selected for follicle quantification. For each section, only follicles with a visible nucleus were counted and classified as follows: (1) PFs, oocyte surrounded by a single layer of flattened pre-GCs; (2) primary follicles, oocyte surrounded by two or more cuboidal GCs up to one complete layer of cuboidal GCs; (3) secondary follicles, oocyte surrounded by at least two layers of GCs but no antrum; and (4) tertiary follicles, follicles with a visible antrum. The follicle numbers were reported as follicular density (calculated as number of follicles /mm^3 of ovarian tissue). Two blinded observers evaluated the same sections independently and the results were compared showing an inter-observer concordance above the 90%. As indirect measure of the ovulation rate, corpora lutea (CL) were quantified in the same three sections per animal. Total ovarian volume (mm^3) was calculated using the ellipsoid formula $4/3 * \pi * a * b2$, where 'a' corresponds to the length of the ovary (a = total number of sections (n) multiplied by 0.005 (representing the thickness of each section)), and 'π b2' is the area of the middle section (Am) of the ovary. Therefore, total ovarian volume (V) = $4/3 * Am * n * 0.005$.

3.6 RNA isolation and cDNA synthesis

Total RNA was extracted from entire frozen ovaries using an RNeasy Mini Kit (P/N 74104, Qiagen, Veno, Netherlands). Samples were dissolved in Qiagen lysis buffer and then homogenized twice for 30 seconds in an ULTRA-TURRAX T25 homogenizer (JANKE and KUNKEL, Staufen, Germany). The supernatant was mixed with half of its volume amount of 70% ethanol, the mixture was transferred to RNeasy spin columns and processed according to the manufacturer's protocol. The extracted RNA was stored at − 80 °C after quantification of RNA concentrations with a single-beam UV/vis spectrophotometer (P/N 6132000032, Eppendorf, Hamburg, Germany). To generate cDNA, the IScript™ cDNA synthesis kit (P/N 170–8891, Bio-Rad, CA, USA) was used according to the manufacturer's protocol. One µg of RNA was reverse-transcribed on a thermocycler (P/N 4359659, Applied Biosystems, MA, USA) using random hexamers in a total reaction volume of 20 µl.

3.7 Taqman low-density arrays (TLDAs)

Comparative analysis of gene expression of IUGR and control animals was performed by TLDA cards (P/N 4342259, ABI, Hilden, Germany) and TaqMan Gene expression Master Mix (P/N 4369510, Applied Biosystems, MA, USA), according to the manufacturer's protocol. The TLDA cards were pre-loaded with 96 TaqMan gene expression assays of importance for germ cell and gonadal somatic cell function and differentiation, cell proliferation, apoptosis and metabolism, and six endogenous controls assigned for normalization. Gene expression was normalized to the mean of five out of six endogenous controls (Actin beta, Beta-2-microglobulin, Catenin beta 1, Eukaryotic translation elongation factor 1 alpha 1 and Glyceraldehyde-3-phosphate dehydrogenase) of the same sample (dCt), selected according to their stability. Data from one randomly chosen animal were used as a calibrator (ddCT) and data from all other animals were normalized to it. Gene expression was finally presented as relative expression, using the fold change (2 − ddCT) method for calculation.

3.8 Serum amh measurement

Serum levels of AMH were measured by Gen II ELISA Reagent kit REF A79765 and Calibrator kit A79766 (Beckman Coulter) according to the manufacturer's protocol. The detection limit was 0.08 ng/mL and the intra and inter-assay coefficients of variation were 4.3 and 9.8%, and 1.4 and 4.3%, for the low and high levels of the standard curve, respectively.

3.9 Tissue processing (Paper II)

Ovarian cortical tissue was collected from 43 patients. For immunohistochemical analysis, ovarian tissues were freshly fixed in PFA (27 patients). For histological analysis, cryopreserved ovarian tissues were thawed and fixed in Bouin's solution (27 patients). Eleven patients had ovarian tissue fixed both in PFA and Bouin, whereas in the other cases one patient had the ovarian tissue fixed either in PFA or in Bouin.

3.10 Immunohistochemistry (Paper II)

Immunohistochemical analyses were performed using PFA-fixed samples. FOXO3A analysis was performed in one section/patient in 8 patients (3 treated and 5 untreated) and H2A histone family member X phosphorylated on serine 139 (γH2AX) analysis in one section/patient in 18 patients (8 untreated and 10 treated). After dewaxing and rehydration of the slides, antigen retrieval was performed with citrate buffer + 0.05% Tween20, pH 6.0 at 95 °C for 45 minutes. Slides were then blocked in 5% BSA and normal donkey serum (S30, EMD Millipore, Darmstadt, Germany) and then incubated with polyclonal rabbit FOXO3A antibody (PA5-27145, Thermo Fisher Scientific, MA, USA), polyclonal rabbit γH2AX antibody (ab2893, Abcam, Cambridge, UK) or polyclonal rabbit IgG antibody (ABIN376827, antibodies-online, Aachen, Germany) for negative control overnight at 4 °C. Donkey anti-rabbit Alexa Fluor 555 conjugated antibody (A31572, Life Tech, MA, USA) diluted in 5% BSA and normal donkey serum (S30, EMD Millipore, Darmstadt, Germany) was used for detection of the primary antibodies (1 hour at room temperature). Sections were mounted using VECTASHIELD mounting medium with DAPI (H-1200, Vector Labs, CA, USA).

3.11 Histological analysis (Paper II)

A subset of 10 sections was evaluated by two blinded observers independently, showing an inter-observer concordance above the 90%. Subsequently, each observer evaluated half of the study material. Bouin's fixed sections stained with hematoxylin–eosin were digitalized using a spectral scanner (Vectra 3 system, Perkin Elmer, MA, USA) and every fifth section was evaluated using Pannoramic Viewer software (3DHistech, Budapest, Hungary). Follicles with a visible oocyte nucleus were counted and classified according to established morphological criteria [9], as follows: (1) primordial follicles, oocyte surrounded by a single layer of flattened granulosa cells; (2) intermediary follicles, oocyte surrounded by a single layer of flattened and at least two cuboidal granulosa cells; (3) primary follicles, oocyte surrounded by one complete layer of cuboidal granulosa cells; and (4) secondary follicles, oocyte surrounded by more than one layer of cuboidal granulosa cells.

A follicle was considered intact and healthy if the round shape was preserved, the oocyte nucleus was central, round and not pyknotic, there was no contraction or eosinophilia of the cytoplasm, and there was full contact between the oocyte and the surrounding granulosa cells. On the opposite, follicles were classified as atretic follicles if they had a distorted follicle shape, non-circular or pyknotic oocyte nucleus, eosinophilic cytoplasm or dislocated/detached GCs. Follicle diameter and oocyte nucleus diameter were calculated for each follicle as the arithmetic mean of two perpendicular measurements.

Detection of fibrotic areas in the ovarian stroma was performed in one section/patient in 12 patients (6 untreated and 6 treated patients) using Bouin's fixed tissues. The trichrome stain kit (ab150686, Abcam, Cambridge, UK) was used according to the manufacturer's instructions, as follows: tissue sections were incubated in Weigert's iron hematoxylin for 5 minutes, followed by Biebrich scarlet-acid fuchsin solution (15 minutes), phosphomolybdic/phosphotungstic acid solution (10 minutes), aniline blue solution (5 minutes) and acetic acid solution (3 minutes). Slides were then dehydrated in increasing ethanol concentration, cleared with xylene and mounted with Pertex® (Histolab, Askim, Sweden).

For stromal cell death detection, TUNEL staining assay (11684795910, Roche, Penzberg, Germany) was performed in one section/patient in 13 patients (6 untreated and 7 treated) using PFA-fixed samples. De-paraffinized and rehydrated tissue sections were incubated with proteinase K followed by TUNEL reaction mixture for 1 hour at 37 °C. Positive controls were treated with DNase I for 20 minutes at room temperature before TUNEL reaction. For negative controls incubation without detection enzyme was performed. Samples were mounted using VECTASHIELD mounting medium with DAPI (H-1200, Vector Labs, CA, USA).

3.12 Steroid production in culture

Steroid hormones were measured in used culture media where thawed ovarian tissue pieces were cultured for 7 days in a previous study from our group [83]. Cortisol, corticosterone, estradiol, androstenedione, 17α-OH progesterone, progesterone, dehydroepiandrosterone, dihydrotestosterone, and testosterone were quantified in media samples from 27 patients on days 4 and 7 of culture using ultra-performance liquid chromatography tandem mass spectrometry [113]. Nine blank media samples were used as controls.

3.13 Study population (Paper III)

Data were collected from a total of 206 patients who were eligible for OTC between 2002 and 2019 and were referred to the Helsinki University Central Hospital (Finland) from different centers, including municipality hospitals belonging to the

Helsinki University Hospital. For patients with allogenic HSCT, referral centers were all five University hospitals in Finland. The OTC procedure was offered to patients at very high risk of POI (i.e. HSCT or radiotherapy with ovary in the field). In 90 of the 206 patients OTC was successfully performed, when possible at the same time of another operation under general anesthesia (central venous catheter positioning or removal of tumor mass). Different proportions of the ovary were harvested, from a minimum of the 25% of the ovary to the entire organ. Fourteen patients underwent unilateral oophorectomy based on the predicted risk of irreversible damage due to ovarian irradiation. The slow-freezing method was used to cryopreserve the ovarian tissue pieces [114]. Two-thirds of the tissue were stored in biobank facilities at the Helsinki University Central Hospital for the patients' future use. The remaining third was anonymized and cryopreserved for quality control of ovarian tissue, including morphological analysis and in vitro culture. For the 90 patients who underwent OTC, age and pubertal status at OTC, diagnosis, indication for OTC, time from OTC indication to OTC execution, treatment characteristics and survival time were obtained from the hospital records. For the other 116 patients, who had indication to OTC but did not undergo the procedure, age and pubertal status at treatment, diagnosis, treatment characteristics and survival time were retrieved from the hospital records.

Of the 90 girls who underwent OTC, 65 became long-term survivors and were followed-up at the outpatient clinic. Follow-up included pubertal evaluation, menstrual history and laboratory examinations and was performed initially every six months and later annually. Occurrence of spontaneous puberty and menarche, need of hormone replacement therapy (HRT), measurements of FSH, LH, AMH and estradiol (E2) were collected retrospectively. Prepubertal hormonal levels and those measured during HRT were excluded from the analysis. POI requiring estrogen substitution was defined as follows: absence of spontaneous puberty and FSH levels > 25 IU/l after gonadotoxic cancer treatment in girls that were prepubertal at the time of treatment; lack of pubertal progression, primary or secondary amenorrhea and FSH levels > 25 IU/l in girls that had already entered puberty or started menarche at the time of treatment.

Indication for OTC was subclassified as follows: HSCT or abdominal irradiation for (1) correction of severe hematological or immunological disease (i.e. bone marrow failure syndromes, immunodeficiencies, myelodysplastic syndromes), (2) metastatic cancer, (3) high risk treatment protocol according to stratifying factors at diagnosis (high leucocyte count, genetic subtypes or tumor histology), (4) poor response to induction therapy, (5) relapse, (6) second tumor. A last category of patients, referred in the rest of the thesis as 'patient's decision', included 3 patients that expressed their will to have OTC performed because of planned treatment with high-dose alkylating agents that put them at high risk of POI, even though they did not meet the strict criteria for OTC (HSCT or radiotherapy with ovary in the field).

Cyclophosphamide equivalent doses (CED) were calculated as described by Green *et al.* [115]. Isotoxic dose equivalents (DIE) for anthracycline equivalent doses were calculated using a conversion factor of 1 for doxorubicin, 0.833 for daunorubicin, 5.0 for idarubicin and 4.0 for mitoxantrone [116]. Exact gonadal irradiation dose was available for all cases exposed to whole abdomen irradiation and TBI. Gonadal irradiation was estimated as equal to the 50% of the local irradiation dose for cases who had RT to the retroperitoneum, kidney, spine or tumor area. In one case the exact dose to the ovaries was available from dosimetry.

3.14 Statistical analysis

3.14.1 Paper I

Analyses were performed using the SigmaStat (v 11.00) package (SPSS, Inc., IL, USA). The continuous variables among groups were compared using Student's T-test, based on Shapiro-Wilk normality test. Results are shown as mean ± SD. Differences were considered statistically significant for P values < 0.05.

3.14.2 Paper II

Statistical analyses were performed using the SigmaStat (v 11.00) package (SPSS, Inc., IL, USA) and R statistical software [117]. Mann–Whitney *U* test was used to compare follicle densities, follicle and oocyte nucleus diameters, steroid concentrations, fibrotic areas, TUNEL signal and γH2AX foci numbers between the groups. χ^2 test was used to compare the proportions of different follicle classes between the groups. Correlation between the morphological measures, age, CED, DIE and steroid concentrations was performed using Spearman's rank correlation. Forward stepwise regression analysis was used for the evaluation of the major predictor when more than one variable correlated with morphological measures. Results are shown as medians and interquartile range. Differences were considered statistically significant for P values < 0.05.

3.14.3 Paper III

Statistical analyses were performed using the SigmaStat (v 11.00) package (SPSS, Inc., IL, USA), SPSS statistical software (v 25.0) and R statistical software [118]. Mann-Whitney *U* test or Student's t-test were used to compare continuous variables between groups, after performing Shapiro-Wilk test for normal distribution. Fisher's exact test and χ^2 test were used for comparison of categorical variables. ANOVA on ranks was used to compare the time from OTC indication to OTC execution for different diagnoses, indication for OTC and Tanner stage at OTC. Spearman's rank correlation was used to analyze the association between time

from OTC indication to OTC execution, age at diagnosis, chemotherapy exposures before OTC in malignant cases and between hormonal measurements, age at gonadotoxic treatment, time from gonadotoxic treatment, chemotherapy and radiotherapy exposures. Overall survival (OS) was defined as time from gonadotoxic treatment until date of death or until December 1st, 2019 for survivors, and illustrated using the Kaplan-Meier method [119]. Differences in the OS between patients with OTC performed and not performed were tested with the log-rank test. Time to HRT was defined as the number of years between gonadotoxic treatment and HRT start, for patients treated with HRT, and between gonadotoxic treatment and December 1st, 2019 for those who did not receive HRT. The cumulative incidence of HRT was illustrated using Kaplan-Meier curves. The association between Tanner stage at OTC, age at OTC, chemotherapy and radiotherapy exposures, and time to HRT was evaluated using Cox proportional hazards models. The differences in cumulative incidence of HRT between prepubertal and pubertal patients at OTC were tested using the log-rank test.

4 RESULTS AND DISCUSSION

4.1 Uteroplacental insufficiency affects follicles and gene expression in the rat ovary (Paper I)

Placental insufficiency is the common denominator of different diseases that can complicate human pregnancies. Placental insufficiency is characterized by a reduced placental flow that results in a low blood supply to the fetus and intrauterine growth restriction (IUGR). The fetal period is a very sensitive window of development when the formation and maturation of organs takes place and gametogenesis is established. Some studies suggest that IUGR can affect the ovarian follicles and alter pubertal milestones but the evidence is inconclusive. Therefore, we investigated the impact of IUGR in a rat model of placental insufficiency induced by uterine arteries ligature on the ovarian follicle density and gene expression profile.

4.1.1 Decreased follicle density in the ovaries of IUGR rats

IUGR rats had a significantly lower body weight at 5 dpp compared to sham rats. At 20 dpp body weight became comparable to that of sham rats, as a result of catch-up growth. At 5 dpp, PFs and total follicle density tended to be lower in IUGR rats as compared to controls, although the comparison did not reach statistical significance. At 20 dpp, PFs and total follicles densities were significantly reduced in the ovaries of IUGR rats compared to sham, but this difference was no longer present at 40 dpp. The ratio between growing follicles (primary, secondary and tertiary) and total follicles was evaluated as indirect measure of an increased activation of PFs. No difference was detected in the ratio between the groups at any age. Ovarian weight (both simple and normalized to body weight) and ovarian volumes were not different between the groups at any age. Corpora lutea number, as indirect measure of ovulation, was also not different between IUGR and sham animals.

Our study is the first exploring the impact of IUGR on the ovary in a rat model of placental insufficiency throughout postnatal development. We found that the IUGR phenotype was associated with a reduction of PFs at birth that persisted until 20 dpp and was no longer evident at 40 dpp, when normalization of the body weight had occurred. These findings are in partial agreement with a previous study from Engelbregt *et al.* that reported a decrease in total, primordial and growing follicles in IUGR rats at the time of their first cycle (~ 40 dpp) [48]. Despite these results were obtained using the same animal model as in our study, the uterine arteries ligature was performed 2 days earlier (at 17 dpc) and the animals had a longer catch-up growth interval before normalization of their weight at ~ 38 dpp.

The normalization in follicle number could be explained in the light of the theory of two waves of PFs [13] explained before in this thesis. Based on this theory, we speculate that the placental insufficiency in our model damaged only the first wave of PFs, while PFs from the adult wave were unaffected, possibly protected by their dormant status. The first wave is the prevalent population of PFs in the ovary until ~3 months of age, and their depletion would have caused the lower number of PFs observed at 20 dpp. At 40 dpp, PFs from the adult wave start to be activated and gradually become the most prevalent population of follicles in the ovary. This dynamic process could account for the comparable amount of follicles between IUGR and control rats observed at that age. This could imply that the ovary has a certain capacity to compensate ovarian damages during childhood which is lost as soon as the first wave of follicles is exhausted (i.e. after puberty). As there are no histological markers to distinguish the two populations of follicles, this hypothesis remains unproven.

4.1.2 Altered gene expression in the ovaries of IUGR rats

Among genes involved in the regulation of cell proliferation and apoptosis, Proliferation-related Ki67 antigen (*MKi67*), Proliferating cell nuclear antigen (*Pcna*), Cyclin-dependent kinase inhibitor 1B (*Cdkn1b*), DNA topoisomerase II (*Top2a*) and Thymidine kinase 1 (*Tk1*) were significantly downregulated in IUGR rats at 40 dpp. Furthermore, IUGR animals showed a downregulation of genes belonging to the insulin-like growth factor (IGF) system, whose components are fundamental growth factors in the ovary. In particular, a lower expression of IGF-I receptor (*Igf1r*) both at 20 and 40 dpp, and an increased expression of IGF binding protein (*Igfbp3*) at 40 dpp were detected in IUGR animals. Altogether, these findings point towards a lower proliferation that could interest both germ and somatic cell in the ovary and could reflect hypothetically a lower activation rate of PFs, to compensate the PFs loss induced by placental insufficiency during fetal life. In parallel to this, the downregulation of Transforming growth factor beta 2 (*Tgfb2*) expression at 20 dpp and of Caspase 9 (*Casp9*) at 40 dpp could negatively regulate follicle depletion, lowering the rate at which follicles are physiologically depleted during postnatal life and providing a further compensatory mechanism.

4.2 First-line chemotherapy affects follicles and ovarian stroma in the human ovary (Paper II)

OTC is offered to patients at high risk of POI based on the planned treatment. However, many patients will receive low/medium risk therapies as initial treatment of their cancer disease (first-line treatment) before shifting to high risk or second line treatments and will therefore be exposed to several cycles of conventional

chemotherapy before OTC is performed. Reports on the impact of chemotherapy on the quality of ovarian tissue in pediatric and young adult populations are limited [61, 62, 81-83, 120, 121]. Therefore, we investigated the impact of chemotherapy exposure on quantitative and qualitative measures of preantral follicle health and on the ovarian stroma in a cohort of young cancer patients who received OTC in comparison to patients who were not exposed to any treatment before OTC.

Some issues in our paper II were discussed by Shapira et al. [122] and responded to by us in the appendix to paper II.

4.2.1 Altered follicle density and size of ovarian follicles in the ovaries of cancer patients exposed to chemotherapy

A higher density of atretic primordial and intermediary follicles and a lower density of intact primary follicles were found in exposed patients compared to non-exposed ones. In exposed patients, intact primordial follicle and oocyte nucleus diameters were significantly smaller compared to those of non-exposed ones. Intact intermediary follicles had also a significantly lower follicle diameter in exposed patients.

These findings could indicate that the follicles that survive after exposure to chemotherapy are nevertheless affected by the treatment (reduced dimensions of intact follicles in the earliest stages) and have a lower maturation potential (lower density of primary follicles). A lower maturation potential was already reported after culture of ovarian tissues exposed to chemotherapy [83]. Both CED and DIE showed a direct association with the reduced diameters of primordial and intermediary follicles and with the lower density of primary follicles.

4.2.2 Reduced steroid hormone production during culture from ovarian tissues of patients exposed to chemotherapy

To evaluate the effects of chemotherapy on the functionality of ovarian tissue, we quantified the production of steroid hormones in the media of previously cultured ovarian samples, cryopreserved from exposed and non-exposed patients. Estradiol, androstenedione, 17α-OH-progesterone, dehydroepiandrosterone, testosterone and progesterone were significantly lower in exposed patients compared to non-exposed ones. Similar findings were also reported in another study, in which estradiol production was reduced in cultured tissues from adult patients who had received chemotherapy [123]. Steroid hormones in our media samples could be produced by pre-antral follicles or by stromal cells. Therefore, our findings of reduced steroid production could reflect both a functional damage to residual pre-antral follicles and/or a damage to the stromal compartment after exposure to chemotherapy, as detailed in the following paragraph.

4.2.3 Increased collagen deposition and DNA fragmentation in ovarian stroma of patients exposed to chemotherapy

Trichrome assay was used to detect newly formed collagen-rich areas of connective tissue, representing fibrotic areas in the stromal compartment of the ovary. Patients exposed to chemotherapy showed significantly larger areas of collagen than non-exposed ones and with a higher degree of intensity. DNA fragmentation, evaluated by TUNEL staining, was increased in the stroma of exposed patients compared to non-exposed ones. However, 2 out of 7 exposed patients had low levels of TUNEL signal, comparable to that of non-exposed ones, which smoothed the difference when statistical analysis was performed. After these two patients were removed from the analysis, the comparison between exposed and non-exposed became statistically significant. Stromal apoptosis has been detected after exposing stromal cells derived from cryopreserved ovarian tissues to doxorubicin and cisplatin [124]. Stromal fibrosis and damage to the microvasculature have been reported as additional mechanisms responsible for the PFs loss induced by chemotherapy [64, 125, 126]. Ovarian stromal cells express steroidogenic enzymes and a dysregulated synthesis of steroid hormones from these cells might contribute to the pathogenesis of different conditions, such as ovarian tumors and PCOs [127, 128]. However, a physiological function of stromal-derived steroid hormones has not yet been identified. Stromal cells are also the source for recruitment of theca cells around early follicle, which is an essential step for follicle development [129]. Implications of a compromised stromal compartment might therefore be wider.

4.2.4 No increase in primordial follicle activation nor in oocyte DNA fragmentation in ovaries of patients exposed to chemotherapy

Massive PF activation followed by apoptosis, defined as 'burn-out' hypothesis, has been proposed as a mechanism for the depletion of the ovarian reserve induced by chemotherapy [66]. We evaluated PF activation by two measures: the ratio between intact growing follicles and total follicles (PFs + growing) and the localization of FOXO3A in PFs. In contrast to what expected in case of increased activation, the ratio growing/total follicles was decreased in exposed patients compared to non-exposed patients, suggesting a decreased activation of PFs, which is in line with the reduced density of intact primary follicles observed in the ovaries of these patients. FOXO3A, which is translocated from the nucleus to the cytoplasm when a PF is activated, was expressed in the nucleus of all the intact PFs evaluated, regardless of the exposure to chemotherapy. While growing follicles/total follicles ratio is a solid morphological measure of PF activation [66], FOXO3A results should be interpreted cautiously. Ovarian tissues were harvested 7-55 days after exposure to chemotherapy, when detectable signs of follicle activation could have already

vanished. DNA fragmentation was explored in the oocyte nuclei by staining for phosphorylated H2AX (γH2AX). No difference in the amount of γH2AX was observed between exposed and non-exposed cases. Again, the timing of sample collection after treatment exposure could justify the lack of difference, as phosphorylation of H2AX is an early phenomenon after DNA damage [130-132].

In summary, exposure to first-line chemotherapy before OTC caused significant alterations in the quality of ovarian tissue, both on morphological and functional parameters. Ovarian tissues harvested after exposure to chemotherapy are widely used for restoration of fertility by reimplantation in various centers worldwide. However, as discussed in the appendix to paper II, there are no reports on CED exposure in relation to parameters of efficacy after reimplantation (i.e. pregnancy rates, duration of the functionality of the transplanted tissue). Furthermore, only two published cases reported restoration of fertility after reimplantation of ovarian tissue stored during childhood/adolescence and in none of them the patients were exposed to chemotherapy before OTC. Moreover, exposure to chemotherapy before OTC could lower the in vitro maturation potential of follicles [81, 83, 84], a strategy of great importance for fertility restoration when reimplantation is contraindicated for the risk of malignant contamination. Setting-up OTC is not straightforward and limiting the procedure before start of chemotherapy would not be a realistic end point. However, it is of vital importance to concentrate efforts in reducing unnecessary delays in the execution of OTC.

4.3 Patients at high risk of infertility are correctly identified by selection criteria but fruition rate and timing of otc are not optimal (Paper III)

In pediatric settings, OTC is indicated when there is a high (> 50%) or very high (> 80%) estimated risk of infertility based on the treatments planned for a certain disease. Continuous monitoring of follow-up data is essential in order to verify the efficacy of OTC selection criteria, especially in pediatric patients, whose outcomes in terms of ovarian function become evident years after the treatment. Furthermore, analysis of the modalities by which OTC service is performed allows to identify critical issues and adopt corrective strategies.

We analyzed pubertal development and need for hormonal replacement therapy in a cohort of patients eligible for OTC between 2002 and 2019 at the Helsinki Children's Hospital. We also evaluated the fruition rate of OTC service and the timing required to perform OTC after the indication was established. Our aims were i) to analyze if the selection criteria in use in Nordic countries target the patients at high risk of infertility, ii) to identify the factors that influence OTC execution, timing and extent of chemotherapy exposure.

4.3.1 Fruition rate and timing of OTC

Between 2002 and 2019, 44% of all eligible patients received OTC at Helsinki Children's Hospital (90/206). The proportion of patients who received OTC increased from 35% to 47% during time, possibly indicating an improvement of the service. Different referral centers did not affect the proportion of performed OTC. Patients who received OTC were comparable to those who did not receive it in terms of age and pubertal stage at the time of treatment, diagnosis and indication to OTC and survival rate. A sub-analysis of patients who underwent HSCT, revealed that OTC was performed more frequently if the conditioning regimen contained TBI than if it did not contain TBI. Clinician's perception of an increased risk of infertility for patients exposed to TBI could have influenced this result. Moreover, a significantly higher proportion of OTC was performed in patients who had a matched unrelated donor (MUD) for allogenic HSCT than in those with a matched related donor (MRD). HSCT from MUD usually require longer time to be organized, which could facilitate setting up OTC procedure.

Timing of OTC, i.e. time from when the indication was established until OTC was performed, was significantly longer in malignant diseases than in non-malignant ones. Among malignant conditions, timing of OTC was significantly longer in metastatic diseases as compared to relapsed cases. Patients with metastatic diseases (mostly neuroblastomas and Ewing sarcomas) undergo several courses of induction chemotherapy followed by surgery or radiation on the tumor mass. After this, patients receive high-dose consolidation chemotherapy followed by autologous HSCT. In these patients OTC is probably often combined with the operation to remove the tumor mass, or with the CVC positioning performed later on. This justify a delay in OTC of several weeks from the diagnosis. On the other hand, relapsed cases in our series were mostly represented by hematological malignancies and for these patients preparation for HSCT starts immediately after diagnosis of the relapse, providing a shorter window to set up OTC.

In agreement with this hypothesis, patients with metastatic diseases had a significantly higher CED exposure before OTC and a significant direct association between CED exposure before OTC and time to OTC emerged in correlation analysis. We have previously shown that exposure to first-line chemotherapy damage the ovarian tissue and might reduce the quality of the tissue harvested for fertility preservation. Based on the results of our analysis, future directions in OTC practice should point at performing OTC at the earliest possible time, in order to limit chemotherapy exposure. For patients with metastatic diseases, for example, it could be more beneficial that OTC is performed earlier as a separate procedure rather than it is postponed to combine it with the primary operation.

4.3.2 Pubertal development and need for hormonal replacement therapy (HRT)

Forty patients were selectable for analysis of pubertal development and need of HRT. The 50% of them (20/40) needed HRT during follow-up after gonadotoxic treatment. Reasons for HRT prescription were induction or maintenance of pubertal development, primary or secondary amenorrhea. We divided the 40 patients based on their pubertal status at OTC and analyzed the frequency of HRT administration: HRT was necessary for 46% of patients who were prepubertal at OTC (11/24) and for 56% of those that were pubertal at OTC (9/16).

The cumulative incidence of HRT reached 61% 10 years after the gonadotoxic treatment (HSCT or abdominal irradiation) and it was significantly higher in patients that were pubertal at OTC compared to prepubertal ones. After correction for age as a confounding factor, pubertal stage was no longer significant and age at OTC appeared as the main factor accounting for the increase of the cumulative incidence of HRT. These results are in agreement with the well-documented physiological decline of PFs, that makes older patients more susceptible of premature ovarian insufficiency after gonadotoxic damage [6, 133, 134].

In agreement with the literature [115, 135-137], we observed a higher proportion of HRT administration in patients treated with irradiation involving the ovarian field and with high doses of alkylating agents. Sixty percent of patients exposed to irradiation with ovaries in the field (17/29) needed HRT during follow-up. Among patients treated with chemotherapy-based regimens and no irradiation to the ovarian field (11) only 3 out of 11 (~30%) required HRT during follow-up. The low number of patients in this group did not allow a statistical determination of the impact of different doses of alkylating agents on the risk of needing HRT. However, the 3/11 patients that needed HRT in this group had received CED > 20 g/m^2, whereas none of the patients exposed to CED < 20 g/m^2 (5/11) needed HRT during follow-up. Furthermore, patients treated with CED above 20 g/m^2 (6/11) had significantly higher levels of FSH and significantly lower levels of AMH than patients exposed to CED below 20 g/m$_2$.

5 CONCLUSIONS

In this thesis, we investigated the consequences of two pathological events on different measures of ovarian health. In the first study, we analyzed the impact of a reduced blood flow to the fetus and intra-uterine growth restriction (IUGR) on ovarian development. For this purpose, we evaluated ovarian follicles and gene expression in the ovaries of IUGR rats born after surgically-induced placental insufficiency. In the second study, we aimed at defining the effects of chemotherapy treatment received before fertility preservation on the quality of ovarian tissues. Ovarian follicles and stromal compartment were investigated in the ovaries of young patients exposed to gonadotoxic treatment before undergoing fertility preservation. Finally, we analyzed the fertility preservation service in a large pediatric oncology unit in terms of appropriateness of selection criteria and performance of the service. Ovarian function of young patients who received ovarian tissue cryopreservation (OTC) during follow-up was evaluated as a measure of the appropriateness of indications for fertility preservation. Fruition rate and timing of OTC in relation to chemotherapy exposure before fertility preservation were analyzed.

The main conclusions are listed below:

- IUGR following placental insufficiency reduced primordial and total follicle count in the ovaries of neonate and juvenile rats. Follicle count was no longer reduced in peripubertal rats, alongside with the recovery of body weight. The expression of 24 genes was modified in IUGR rat ovaries, with a potential compensatory significance. However, the long-term course and consequences of these genetic alterations remain to be elucidated.
- Exposure to chemotherapy before OTC increased the amount of atretic follicles, reduced the size of residual intact follicles in the earliest stages of development (primordial and intermediary) and reduced the amount of intact follicles at more advanced stages of development (primary follicles). The secretion of steroid hormones from cultured ovarian tissue pieces was reduced and ovarian stroma was significantly damaged by exposure to chemotherapy before OTC.
- The implementation of selection criteria for OTC was not optimal and the fruition rate for eligible patients was around the 50%. A fraction of patients are excluded from OTC because of their clinical conditions, which is a non-modifiable factor. Interventions should focus on controllable factors (such as appropriate counselling to the parents and the patients) aiming at increasing the fruition rate of this fundamental service. The timing of OTC was longer for patients with malignant diseases, in particular those affected by cancers in metastatic stage. A longer time before OTC increases the exposure to chemotherapy, thus lowering the quality of the ovarian tissue harvested for fertility preservation. Optimal slots for OTC should be defined for the different indications in order to implement the guidelines.

- OTC execution should be prioritized, compatibly with clinical conditions, and all efforts should be done to limit unnecessary exposure to chemotherapy before OTC and guarantee the best quality of the ovarian tissue harvested for fertility preservation.
- Fifty percent of the patients who received OTC needed hormonal replacement therapy during a follow-up of more than 10 years, a figure set to increase with time. Hence, our conclusion is that the selection criteria for fertility preservation adopted by Nordic Countries correctly identify the patients at high risk of infertility. We suggest that these guidelines are implemented with specific considerations about the age of the patient. Older patients are at increased risk of infertility than younger ones for a given gonadotoxic treatment. It is therefore essential that these patients get access to the service and in a short time frame from when the indication is established. This precaution would prevent unnecessary exposure to chemotherapy that we proved to be detrimental for the quality of the harvested ovarian tissues.

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