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Guest Editors: I. Demeestere, O. Basso, F. Moffa, F. Peccatori, C. Poirot, and E. Shalom-Paz

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Editorial Board

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Editorial

Fertility Preservation in Female Cancer Patients

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Thanks to the progress in oncology research, the cure rate of patients with cancer has greatly improved, reaching 70% or higher in children and adolescents. Consequently, the long-term side effects of the treatment, including chemotherapy-induced premature ovarian failure, have received broader attention. The prospect of definite infertility can induce major psychological distress in cancer patients, as the inability to reproduce can represent a profound loss for women, affecting their self-esteem as well as their relationships. During the last decade, information and management of fertility issue before oncological treatment have become part of the guidelines that should be considered by all oncological units. Fertility preservation is, however, an area that needs specific attention and knowledge by dedicated physicians working in this field. Therefore, collaboration between oncological units and fertility clinics is necessary to adequately counsel each patient with a short delay.

The papers in this special issue have examined the impact of chemotherapy and radiotherapy on ovarian function and reviewed the different fertility preservation strategies in light of the current clinical and experimental advances. For the occasion, 8 papers were accepted for publication, dealing with the different approaches to preserve fertility, with a special attention on children and adolescents.

The first step of fertility preservation management is the evaluation of the individual risk of premature ovarian failure according to the patient's age and type of treatment. Many factors affect the risk of injury to the ovaries, including the specific chemotherapeutic agents, the cumulative dose, and whether or not radiotherapy is administered. These issues were discussed by different authors, and specifically addressed for children by S. Gnaneswaran et al. According to the potential level of gonadal damage, different options to preserve fertility can be proposed. A review article by some of the editors of this special issue discusses the different approaches to preserve fertility, focusing on their advantages and limitations. Vitrification of embryos is a well-established procedure and is currently performed in many IVF laboratories with a high success rate for adults. However, not all cancer patients can benefit from stimulation protocols for embryo and, more recently, for oocyte freezing. The effectiveness of slow freezing versus vitrification techniques for oocytes storage is well reviewed by A. Revelli et al. Cryopreservation of ovarian tissue offers an attractive alternative for many patients. The experience in pediatrics patients of the Orsola-Malpighi Hospital in Bologna was reported by R. Fabbri et al. J. Michaeli et al. report new revised guidelines for providing ovarian cryopreservation to premenarcheal girls with cancer and address the medical and ethical aspects of the procedure.

The safety of autotransplantation of cryopreserved ovarian cortex remains, however, a major concern for both adults and children undergoing the procedure. The paper of L. Bockstaele et al. discusses this important issue, focusing

on the main tools for detecting disseminated cancer cells currently available, their limitations, and clinical relevance.

Future options related to the possibility of postnatal neogenesis in the ovary are subjected to many debates in the literature and discussed in the paper by D. Bhartiya et al.

Finally, the risks associated with conceiving after cancer were addressed by M. L. Matthews et al. in a review discussing also the different aspects of fertility preservation management.

Through these 8 papers, the issue aims to provide an overview of the different available approaches to preserve fertility before oncological treatment in female adults and children.

Authors' Contribution

The coauthors are listed alphabetically and equally contributed to the editorial.

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Review Article

Reproductive Late Effects in Female Survivors of Childhood Cancer

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Childhood cancer treatments can cause female reproductive late effects. Radiation to the hypothalamic-pituitary-ovarian axis is associated with altered menarche, miscarriage, and implantation failure. Patients who receive chemotherapy and/or ovarian radiation are at risk of premature ovarian failure; the risk increases with increasing radiation dose, alkylating agent score, combination therapy, and older age at treatment. Ovarian reserve may be assessed using antimullerian hormone assay and ultrasound measurements of ovarian volume and antral follicle count; however, their efficacy is poorly established in this cohort. Fertility preservation options including cryopreservation, oophoropexy, and gonadotropin-releasing hormone analogues may be initiated prior to treatment, although most are still considered experimental. Uterine radiation has been linked to pregnancy complications including miscarriage, premature delivery, stillbirth, low-birth-weight and small-for-gestational-age infants. This paper summarises the literature on female reproductive late effects. The information should facilitate counseling and management of female survivors throughout their reproductive lives.

1. Introduction

Cancer is the second commonest cause of death in children in developed countries [1]. Common childhood cancers include leukaemia, lymphoma, rhabdomyosarcoma, neuroblastoma, Wilms' tumour, central nervous system tumours, and germ cell tumours [2, 3]. Most of these cancers are curable using chemotherapy, radiotherapy, or surgery, either alone or in combination [2, 4]. More aggressive or treatment-refractory cancers require intensive multimodal therapies involving multiagent chemotherapy [4].

As a result of advances in paediatric cancer treatment protocols, survival rates from childhood cancers have improved dramatically over the past 3 decades [2]. The expected 5-year survival rate for newly diagnosed patients is at least 70% [2, 5].

Despite being highly successful in treating cancers, therapies such as chemotherapy and radiotherapy have also produced complications referred to as late effects [6]. Late effects can either arise during treatment or shortly thereafter to persist as chronic conditions. They may also manifest years after the completion of therapy [4]. Late effects encompass a range of clinical conditions including neurocognitive deficits, skeletal deformities, cardiopulmonary, and renal and hepatic damage, as well as endocrine and reproductive dysfunction. It is estimated that 60–75% of survivors of childhood cancer will develop at least one late effect as a direct result of their treatment [7].

The female reproductive system is especially vulnerable to late effects of cancer therapy. Normal hypothalamic, pituitary, ovarian, and uterine functions as well as adequate ovarian reserve are required for pubertal progression, fertility and pregnancy [2]. Potential late effects on the female reproductive system can therefore occur as a result of chemotherapy and/or radiation to the hypothalamic-pituitary-ovarian (HPO) axis, ovaries or uterus [2, 4]. Although the late effects show individual variation, there is a strong relationship with the treatment received [2].

The purpose of this paper is to summarise the literature regarding the influence of childhood cancer therapies on female reproductive late effects, measures to assess ovarian reserve, and options for fertility preservation. Literature was obtained from electronic resources including Medline, Embase, PubMed Central, Journals@Ovid, and The Cochrane Library (including The Cochrane Database Of Systematic Reviews). The Medical Subject Headings (MeSH) and keywords "childhood cancer," "pediatrics/child/paediatric," "neoplasms/malignancy," "menarche," "menopause/premature menopause/amenorrhea/female infertility/ovarian failure," "pregnancy/pregnancy complications/pregnancy outcome," "fertility preservation," "GnRH analogues/GnRH agonist,' "ovarian tissue cryopreservation," "embryo freezing/em-bryo cryopreservation," and "oocyte cryopreservation" were used. Relevant references cited by the obtained literature were also acquired independently.

2. Late Effects of Radiotherapy to the Hypothalamic-Pituitary-Ovarian Axis

Children who receive radiation to the brain are at risk of damage to the hypothalamus and pituitary with subsequent changes in the release of pituitary gonadotropins to stimulate the ovaries [8].

Earlier timing of menarche has an association with cranial radiation. Low dose radiation (18–24 Gy), as part of treatment for acute lymphoblastic leukaemia (ALL), and early menarche has been reported in previous studies [9–14]. Currently, although many ALL treatment protocols favour more intensive alkylating agent chemotherapy, 10–15% of patients continue to receive cranial radiation upfront [15]. Within this cohort there remains a link with early menarche; however, there is no increased risk amongst patients treated for ALL with chemotherapy only [15]. Patients treated with higher doses of radiation for CNS tumours are also at risk of an earlier menarche, with one study of 235 survivors exposed to cranial radiation prior to menarche determining doses >50 Gy to be a significant independent risk factor [16]. Furthermore, a younger age at cranial radiation has been independently associated with early menarche in patients treated for ALL and CNS tumours (<5 years and <4 years, resp.) [15, 16].

Patients who receive radiation to the lumbar-sacral spine have an increased risk of delayed menarche [11–13, 15]. This is presumably due to indirect radiation effects on the ovaries. However, as some of these patients also receive radiation to the brain, it is possible that gonadotropin deficiency may contribute to their delayed menarche. Radiation doses >50 Gy to the hypothalamus/pituitary in combination with spinal radiation have been reported to increase the risk of delayed menarche 12-fold compared to patients who do not receive radiation [16]. Although alkylating agent exposure is known to increase the risk of gonadal damage, no such associations have been noted to date.

Lower pregnancy rates have been described in patients who received cranial radiation, although the radiation doses reported to decrease the risk of a pregnancy are variable. In one study of 5149 female survivors [17] radiation doses >30 Gy to the hypothalamus/pituitary were a significant risk factor for not having a pregnancy, whilst in another study [18] a decreased risk of pregnancy was noted in patients receiving >22 Gy to the hypothalamus/pituitary. Lower dose exposures (18–24 Gy) used in the treatment of ALL have also been shown to decrease fertility rates in female survivors compared to sibling controls [19, 20], particularly in patients who receive 18–24 Gy to the brain within two years of menarche and whose age at first pregnancy is 18–21 years [19]. Further studies of proven fertility are limited to clarify these findings; however, Bath et al. [21] have observed decreased luteinizing hormone (LH) excretion, decreased LH surge, and high frequency of short ($\leq$11 days) luteal phase (despite regular (26–30 day) ovulatory menstrual cycles) in 12 ALL survivors treated with 18–24 Gy cranial irradiation. These findings could suggest subnormal mid-cycle LH surge and decreased progesterone production by the corpus luteum as one of the causal factors for delayed endometrial maturation and subsequent implantation failure and/or infertility [18].

It has been suggested that cranial radiation increases the risk of miscarriage (<24 weeks) possibly through impairment of HPO axis function [22]. Two studies have shown some support for this hypothesis reporting 1.8- and 1.4-fold significantly increased risks, respectively, amongst survivors treated with cranial radiation only [23, 24]. Another study reported an increased risk amongst patients treated with cranial and craniospinal radiation; however, there was no distinction in the risk of first-trimester miscarriage (<12 weeks) between these treatment groups [25].

3. Late Effects of Treatment to the Ovaries

There is a relationship between age and the number of primordial follicles in human ovaries [2]. At 5-6 months gestation, the number of follicles reaches a maximum of approximately 7×10^6. Thereafter, there is an exponential decline with approximately 400 follicles released as mature oocytes during the reproductive lifespan. Accelerated decline in follicle number occurs after 35 years until menopause occurs. This occurs at an average age of 50.4 years in the Western world [2, 26–30].

Any radiation or chemotherapy will deplete the number of follicles and induce damage to the ovaries [2]. Patients who are older at the time of either treatment have an increased risk of ovarian damage as there is a greater reserve of primordial follicles in younger patients [2, 25, 28]. It has also been suggested that the oocytes present in older patients are more vulnerable to gonadal toxins [31, 32]. Accordingly the mean sterilising dose of radiation to the ovary at 12 years of age has been estimated at 18 Gy compared to 9.5 Gy at 45 years of age [33].

4. Direct Radiotherapy to the Ovaries

Additional to scatter from lumbar-sacral radiation, the ovaries can also be irradiated directly as part of abdominal, pelvic, or total body irradiation. Premature ovarian failure may take the form of either acute ovarian failure (AOF), where there is a loss of ovarian function during or

shortly after the completion of cancer therapy, or premature menopause, defined as menopause younger than 40 years in survivors who retain ovarian function following treatment [28, 33]. The specific risk of premature ovarian failure after direct radiation to the ovaries is site and dose-dependent [28, 34]. Stillman et al. [35] reported ovarian failure in none of 34 survivors who had both ovaries outside of the field of abdominal radiation, in 14% of 35 survivors whose ovaries were at the edge of the radiation field, and in 68% of 25 survivors who had both ovaries entirely within the field of irradiation. Previous studies have demonstrated ovarian doses >10 Gy to be linked to a high risk of AOF, especially doses >20 Gy which are associated with the highest rate, with over 70% of a study cohort of 3390 survivors developing AOF [34]. Recently, doses as low as 5 Gy to the ovaries have been identified as a significant risk factor for not having a pregnancy, presumably due to ovarian failure [17]. The LD_{50} (the radiation dose required to kill 50% of oocytes) of the human oocyte has been estimated at <2 Gy [25, 28].

5. Chemotherapy

Factors affecting the risk of ovarian injury in children treated with chemotherapy include the specific agent, the number of agents, and the cumulative dose [4]. Several chemotherapeutic agents when given at high doses are recognised as toxic to young ovaries including alkylating agents, cisplatin procarbazine, and the nitrosoureas (CCNU and BCNU) [2, 36]. There is currently no data on threshold doses to cause ovarian failure, although it has been noted that exposures to procarbazine at any age or cyclophosphamide between 13 and 20 years are independent risk factors for AOF [34]. Additionally, cyclophosphamide and CCNU have been associated with a lower risk of pregnancy, with fertility rates decreasing with increasing doses of these agents [17]. Increasing alkylating agent score (based on the number of alkylating agents and cumulative doses) has also been identified as a risk factor for both nonsurgical premature menopause [34] and decreased fertility, with one study of 5149 survivors demonstrating that alkylating agent scores of three and four were associated with a lower observed risk of pregnancy compared to patients who had no alkylating agent exposure [17].

6. Combination Radiotherapy and Chemotherapy

A combination of radiation to the ovaries and chemotherapy poses the greatest risk of ovarian failure [25, 28, 37–39]. Amongst patients who are treated with alkylating agents plus abdominopelvic irradiation, 30–40% are estimated to develop non-surgical premature menopause [37, 39].

7. Assessment of Ovarian Reserve

7.1. Hormonal Markers. A slow and steady compensatory rise in early follicular phase follicle-stimulating hormone (FSH) has traditionally been used as marker of perimenopause and ovarian reserve [28, 40]. Unless significantly elevated however, early follicular phase FSH as an isolated test is not a sensitive early marker of diminished ovarian reserve [28]. Some women may experience transiently elevated FSH unrelated to their pool of follicles, which can add to potential erroneous presumptions of premature menopause. Inhibin-B is another hormonal marker which has been proposed to assess ovarian reserve. It is produced by follicles following recruitment during the early follicular phase and has been shown to decrease with age and during premature ovarian failure [40, 41]. However, it is a fairly late marker of reduced follicle reserve as levels do not decrease gradually with age [40].

Levels of antimullerian hormone (AMH) are more reflective of the number of preantral follicles and are thus a marker of oocyte pool [28, 40, 42, 43]. AMH levels in women of reproductive age appear to have a greater sensitivity and specificity for ovarian reserve over FSH and inhibin-B [40, 44]. Levels are independent of the phase of the ovarian cycle and should not vary significantly between menstrual cycles as levels are not dependent on the feedback mechanisms of the HPO axis [28, 40, 42–44]. Importantly, AMH levels decrease steadily over time and often fall before other markers of ovarian ageing occur [42]. There is a demonstrated age-dependent decrease after 30 years, with a decline in AMH levels below 0.086 μg/L signalling menopause [45]. Although a promising marker for ovarian reserve, its efficacy in assessing premature menopause and chance of pregnancy in young patients after cancer treatment is not well established, and more data are required.

7.2. Ultrasonographic Markers. Transvaginal ultrasound assessments of total ovarian volume and antral follicle count (AFC) are noninvasive and accurate tests of ovarian reserve as both exhibit an age-related decline [28, 40]. Mean premenopausal ovarian volumes of 4.9 cm^3 compared to mean postmenopausal volumes of 2.2 cm^3 have been determined [46]. The mean AFC is 15 at 25–34 years of age and decreases to 4 at 41–46 years of age [47]. AFC has also been shown to correlate tightly with plasma levels of AMH [48].

8. Options for Fertility Preservation

There are two main approaches to preserving fertility in female childhood cancer survivors, namely, cryopreservation of ovarian tissue, oocytes, and embryos, and interventions to minimise the effects of cancer therapies on the ovaries [49]. Within these approaches, there are established practices and experimental strategies.

8.1. Cryopreservation. Embryo cryopreservation is the main established method of fertility preservation, with delivery rates per embryo transfer ranging between 10–40% depending upon the age of the female partner and quality of oocyte [49, 50]. However, this option is of limited value in children as the patient must be postpubertal and have a partner or use donor sperm. This process also requires at least one

cycle of ovarian stimulation which may not be possible when chemotherapy needs to be commenced immediately or where stimulation is contraindicated due to hormone-sensitive tumours [49, 51, 52].

In contrast, oocyte cryopreservation may be utilised in some adolescent girls as it does not require partner or donor sperm. However, the method also requires the use of ovarian stimulation [53] and its success is dependent on the total number of oocytes retrieved (<10 oocytes is associated with minimal chance of pregnancy), which is often difficult in sexually immature patients [54, 55]. Ongoing advances in oocyte cryopreservation technique and the use of intracytoplasmic sperm injection (ICSI) appear to have improved success rates [53, 54]. In a prospective randomised controlled trial (RCT) conducted by Smith et al. [56], oocyte survival, fertilization, and establishment of pregnancy were significantly higher following vitrification/warming compared with freezing/thawing. In another RCT [57] fertilisation and embryo development rates using vitrified oocytes followed by ICSI approached that of fresh oocytes after ICSI. Cryopreservation of oocytes has also been described in association with ovarian tissue cryopreservation (OTC) in prepubertal girls, whereby any antral follicles observed on the ovarian surface at the time of biopsy are aspirated, matured in vitro and cryopreserved [55, 58]. Accordingly, Revel et al. [58] were able to cryopreserve 11 mature oocytes from three prepubertal girls aged 5, 8, and 10 years.

Ovarian tissue cryopreservation is the only means of preserving fertility in prepubertal girls [49, 51, 53, 59] and may also be utilised in girls who do not have enough time to undergo ovarian stimulation for oocyte and embryo cryopreservation [49, 51, 53, 57]. Ovarian cortical tissue is harvested laparoscopically without preparation, cryopreserved using standard slow-programmed freezing, and is reimplanted into the pelvic cavity (orthoptic site) or a heterotopic site once the patient is in remission [51]. The option has greater fertility potential in prepubertal girls due to a greater density of primordial follicles in the harvested tissue [54, 55]. This process has the added advantage of endogenous hormone production by the ovarian tissue, and avoidance of hormone therapy for bone health maintenance. To date, ten live births have been reported following orthoptic reimplantation of frozen-thawed ovarian cortex harvested from postpubertal girls [55], although the origins of these pregnancies are not definite as a vast majority of patients have demonstrated restoration of follicular growth and ovulation [55]. Risks of OTC include the surgical risks associated with the invasive procedure. An additional concern is reimplantation of the primary tumour and/or malignant transformation of reimplanted tissue, which is possible with leukaemias, neuroblastoma, and Burkitt's lymphoma which are common during childhood and metastasise to the ovaries [49, 53], although histological analysis of the cryopreserved ovarian cortex and further methods for monitoring minimal residual disease have been developed over recent years [55].

8.2. Interventions to Minimise Damage Caused by Cancer Therapies. Transposition of the ovaries (oophoropexy) outside of the field of radiation may be performed to reduce the ovarian radiation dose to 5–10% of that if the ovaries remained in situ [60]. The ovaries can either be relocated outside of the pelvis, in the case of pelvic irradiation, or, in the case of craniospinal irradiation, fixed laterally as far as possible from the spine [55]. Preserved ovarian function following oophoropexy outside of the pelvis has been reported between 16–90% [49, 54]; the wide variability is due to the inability to calculate and prevent scatter, combination chemotherapy and different radiation doses [49, 54]. A disadvantage of this technique is the invasive procedure which needs to occur at a time when the patient is planning cancer treatment. Moreover, ovarian failure may ensue if the ovaries are not transposed far enough or if they revert back to their original position or if the vascular supply of the ovary is affected by the surgical procedure [49]. Additional issues to consider prior to performing extrapelvic oophoropexy include problems achieving a spontaneous pregnancy and difficulties with oocyte retrieval for in vitro fertilisation (IVF) unless a second procedure is performed to relocate the ovaries back to the pelvis [49, 53]; this problem is often avoided in the case of lateral oophoropexy for cranial irradiation as the anatomic relations of the ovary with the uterus and fallopian tubes are maintained [55]. Commonly, ovarian biopsy and transposition is performed simultaneously for patients undergoing combination therapies.

GnRH (gonadotropin-releasing hormone) analogues have been suggested as chemoprotective agents. The exact mechanism remains unclear, although it is hypothesised that suppression of pituitary gonadotropin production with subsequent reductions in ovarian follicular cell division and growth render the follicles less vulnerable to cytotoxic agents [49]. Their use is limited in prepubertal girls who are hypogonadal. To date, the evidence for the use of GnRH analogues is controversial. There are several case series and small cohort studies that claim benefit [61–63], and a meta-analysis has shown benefit in this technique; however, when only high quality studies are included, the results are not significant [64]. A recent randomised study of 49 breast cancer patients with 30-month followup additionally found no difference in the incidence of ovarian failure [65]; however, a prospective multicentre study is currently underway with anticipated results.

9. Late Effects of Radiotherapy to the Uterus

Radiation to the uterus can impair uterine function, causing reduced uterine volume, decreased myometrial elasticity, and some uterine vascular damage [4, 25, 66–68]. Although data on threshold doses for uterine dysfunction is limited, earlier studies have reported reduced uterine length, poor endometrial thickness in response to oestradiol therapy, and absence of uterine artery blood flow detectable by Doppler ultrasound in patients treated with 14–30 Gy of radiation to the uterus [67, 69, 70]. The risk of uterine dysfunction increases with higher radiation doses and fields involving a greater uterine volume [4]. Radiation prior to puberty has also been associated with irreversible damage to the uterus, with prepubertal uterine morphology observed in postpubertal patients [68].

Reduced adult uterine volume and blood supply may restrict foetal growth and the ability to carry the foetus to term [22]. There is an increased risk of delivering low birth weight infants (<2.5 kg) amongst patients treated with abdominal/pelvic radiation [22, 23, 25, 71–73]. Patients who receive >5 Gy radiation to the uterus are also significantly more likely to deliver small for gestational age offspring (<10th percentile for gestational age) [25]. Higher frequencies of preterm birth (<37 weeks) following abdominal/pelvic radiation have additionally been reported [25, 71, 72, 74] with a more recent study observing a 2-fold elevated risk of preterm delivery in their cohort of 351 survivors who received abdominal radiation [22]. There is also an increased risk of miscarriage [22–24], with Reulen et al. reporting the risk to be particularly elevated during the second trimester [22].

In one study of 39 patients who received radiation to the pelvis a significantly increased risk of stillbirth at doses >10 Gy was observed [75]. Additionally, doses as low as 1.0–2.49 Gy were observed to significantly increase the risk in girls treated before menarche [75]. Although the exact mechanism of decreased uterine volume and blood supply on stillbirth is unknown, it is possible that these effects may increase the risk of placental or umbilical cord anomalies [75].

10. Conclusion

Although there is individual susceptibility, the late effects of childhood cancer therapies on the reproductive system can be anticipated amongst female childhood cancer survivors throughout their reproductive lives. Girls treated with radiation to the HPO axis are at risk of abnormal timing of menarche and pregnancy sequelae including miscarriage and implantation failure contributing to infertility. Survivors treated with chemotherapy and/or radiotherapy affecting ovarian reserve are also at risk of premature ovarian failure. Women with uterine dysfunction following radiotherapy are at risk of pregnancy complications including miscarriage, low-birth-weight and small-for-gestational-age infants, premature delivery, and stillbirth.

These findings have important implications on counseling and management. Girls and their families should be counseled regarding options for fertility preservation, the possibility of abnormal pubertal progression and menstrual dysfunction. Women who are at risk of premature ovarian failure should be advised to not delay their childbearing, have assessment of ovarian reserve with referral for specialist fertility consultation as required. Pregnant survivors who have had radiation to the uterus should be managed in a high-risk obstetric unit.

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Review Article

Stem Cell Interaction with Somatic Niche May Hold the Key to Fertility Restoration in Cancer Patients

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The spontaneous return of fertility after bone marrow transplantation or heterotopic grafting of cryopreserved ovarian cortical tissue has surprised many, and a possible link with stem cells has been proposed. We have reviewed the available literature on ovarian stem cells in adult mammalian ovaries and presented a model that proposes that the ovary harbors two distinct populations of stem cells, namely, pluripotent, quiescent, very small embryonic-like stem cells (VSELs), and slightly larger "progenitor" ovarian germ stem cells (OGSCs). Besides compromising the somatic niche, oncotherapy destroys OGSCs since, like tumor cells, they are actively dividing; however VSELs persist since they are relatively quiescent. BMT or transplanted ovarian cortical tissue may help rejuvenate the ovarian niche, which possibly supports differentiation of persisting VSELs resulting in neo-oogenesis and follicular development responsible for successful pregnancies. Postnatal oogenesis in mammalian ovary from VSELs may be exploited for fertility restoration in cancer survivors including those who were earlier deprived of gametes and/or gonadal tissue cryopreservation options.

1. Introduction

Stem cells hold tremendous potential and promise for regenerative medicine and have raised the hope of the public for a cure for several diseases. Reproductive biologists and infertile couples are further excited by the concept of deriving "synthetic gametes" from pluripotent stem cells, but one wonders whether generation of "synthetic gametes" is more of science fiction or a realistic option for healthy babies in the future. Hübner et al. [1] were the first to report spontaneous generation of oocytes enclosed within structures that resembled developing ovarian follicles by differentiation of mouse embryonic stem cells *in vitro*. Fetal pig skin stem cells [2] and rat pancreatic stem cells [3] cultured *in vitro* have also been shown to generate oocyte/follicle-like structures. Daley [4] summarized that the development of synthetic gametes from embryonic stem cells is fascinating basic research, but the clinical application is still a hypothetical possibility. The idea of producing gametes from induced pluripotent stem cells derived from skin fibroblasts has also been proposed [5]. Although interesting, the major concern that would limit translation of these research efforts into clinical applications is epigenetic and genetic stability of the gametes produced [6]. The other challenge involves establishing protocols to achieve robust and functional oocyte differentiation from embryonic stem cells, and at present this remains a highly inefficient process.

Another fast expanding area is the presence of stem cells in adult mammalian ovaries. The mammalian ovary harbors stem cells and possibly undergoes postnatal oogenesis during reproductive life rather than being endowed with a finite pool of primordial follicles at birth. Johnson et al. [7] provided evidence in support of postnatal oogenesis and challenged the six-decade-old paradigm by conducting simple experiments. The group demonstrated using mouse ovary the rate at which follicular atresia occurs, the ovary should be devoid of follicles by young adulthood, but this never happens. However, the idea of the ovary harboring stem cells is still not well accepted amongst reproductive biologists, and Notarianni [8] have recently reviewed available data in

support and against the presence of stem cells in the postnatal ovary. Research in the area of germ line stem cells in mice as well as human ovaries by various groups has recently been elaborately reviewed [9].

One of the markers to identify stem cells is OCT-4 (Pou5f1), an octamer binding nuclear transcription factor. It is normally used to define pluripotent state of a stem cell and is well studied in embryonic (ES) and carcinoma stem cells. It is also a germline-specific maternally expressed factor [10]. During embryonic development, OCT-4 is expressed by primordial germ cells (PGCs) and germ cells. Recently OCT-4 positive pluripotent very small embryonic-like stem cells (VSELs) have been reported in various adult somatic tissues including bone marrow and cord blood in mice as well as humans [11–13]. OCT-4 biology has indeed surprised and confused stem cell biologists due to the existence of its isoforms [14–16]. The pluripotent stem cell properties of OCT-4 are because of OCT-4A isoform localized in the nuclei as a transcription factor, whereas Oct-4B isoform is localized in the cytoplasm and has no known biological function [17, 18]. We recently reported nuclear OCT-4A positive VSELs in adult human and mice testis [19, 20]. These cells possibly undergo asymmetric cell division to give rise to slightly bigger A_{dark} spermatogonial stem cells (SSCs), which have cytoplasmic OCT-4B. OCT-4 expression is lost as the testicular germ cells undergo further differentiation and meiosis. Similar stem cell biology also exists in the adult mammalian ovary, which will be explained in the subsequent sections. Unlike testis, OCT-4 continues to be expressed in growing follicles, since it is a maternally inherited gene but this will not be elaborated further as it is beyond the scope of this paper.

2. Stem Cells in Ovaries

Mitotically active germ cells expressing mouse VASA homolog (MVH) and synaptonemal complex protein 3 (SCP3) were reported in the adult mouse ovarian surface epithelium (OSE) by Johnson et al. [7]. Niikura et al. [21] reported that aged mouse ovaries possess premeiotic germ cells that differentiate into oocytes on transfer into a young ovarian environment. Recently Zou and coworkers used MVH and FRAGILIS-based sorting method to isolate female germ line stem cells (FGSCs) from mouse ovaries [22, 23]. The MVH-sorted FGSCs of about 10–12 μm were cultured for more than 15 months and on transplantation in busulfan-treated mice resulted in live-births demonstrating postnatal oogenesis. Pacchiarotti et al. [24] have demonstrated the presence of FGSCs in postnatal mouse ovary using transgenic mice that express green fluorescent protein (GFP) under the control of Oct-4 promoter. They reported three different types of GFP-OCT-4 positive cells based on size—small (10–15 μm) sized cells in the ovarian surface epithelium, medium (20–30 μm) and big (50–60 μm) oocytes in the follicles. Ploidy analysis based on flow cytometry showed that 70% of these cells were tetraploid oocytes and 30% were diploid stem cells. Gong et al. [25] derived two pluripotent colony-forming cell lines from adult ovarian stromal cells, which also formed embryoid bodies and teratomas. They concluded that embryonic-like stem cells exist in either the ovarian stroma or the stromal cells, get reprogrammed *in vitro* to embryonic-like state. They have also reported that a small subgroup of the dissociated cells from adult ovary (unlike spleen and small intestine) is immunoreactive for both OCT-4 and NANOG (pluripotent marker). Reverse transcription-PCR (RT-PCR) results also demonstrate the presence of transcripts for both Oct-4 and Nanog in adult ovarian tissue.

Studies on human ovarian stem cells are relatively few in number because of scarcity of the ovarian tissue for research. Bukovsky et al. [26] were the first to show that scraped surface epithelium of postmenopausal human ovary develops into oocyte-like structures of about 180 μm in the presence of a medium with phenol red (estrogenic stimuli). Virant-Klun and her group [27–29] identified putative stem cells in ovarian sections and also in scraped ovarian surface epithelium (OSE) of postmenopausal women and those with premature ovarian failure. These stem cells express pluripotent transcripts Oct-4, Sox2, and Nanog, expressed cell surface antigen SSEA4, and differentiated into oocyte-like structures and parthenotes *in vitro*. We have recently shown the presence of VSELs in ovaries which can be easily isolated by gentle scraping of OSE in adult rabbit, sheep, monkey, and perimenopausal women. These stem cells spontaneously differentiate into oocyte-like structures and parthenotes *in vitro* [30] in agreement with published literature [28, 29, 31]. Besides VSELs with nuclear OCT-4, we have also shown slightly larger cells with cytoplasmic OCT-4, termed ovarian germ stem cells (OGSCs) similar to the terminology used by Pacchiarotti's group. Similar to testis, VSELs with nuclear OCT-4A are relatively less in numbers in ovary as compared to the progenitors (OGSCs) with cytoplasmic OCT-4B. Similarly, two distinct populations of stem cells were also detected in adult mouse ovaries by immunolocalization and quantitative PCR (Q-PCR) analysis (Figure 1). Nuclear Oct-4A transcripts are less abundant as compared to total Oct-4 transcripts that include both A and B isoforms. Thus probably a similar pluripotent stem cell network exists in the gonads of both sexes in mice as well as humans.

The VSELs are probably the PGCs persisting into adulthood as suggested by others as well [32, 33]. Ratajczak and his group were the first to report presence of VSELs in adult body tissues and have made significant contribution in the field, which was recently compiled [33]. It is believed that a common VSEL stem cell population exists in various body tissues, and depending on its immediate microenvironment, they differentiate into that particular lineage [11]. VSELs are highly mobile in nature, and whenever there is any damage or disease in any part of the body, they get mobilized into circulation from the bone marrow [34–37].

At this juncture, it becomes crucial to comprehend and consolidate the various published studies so that a strong and clear concept emerges. Table 1 is a list of various publications on ovarian stem cells and our attempt to explain the results in the context of VSELs biology. As evident, there is a general agreement in the location of ovarian stem cells in the OSE.

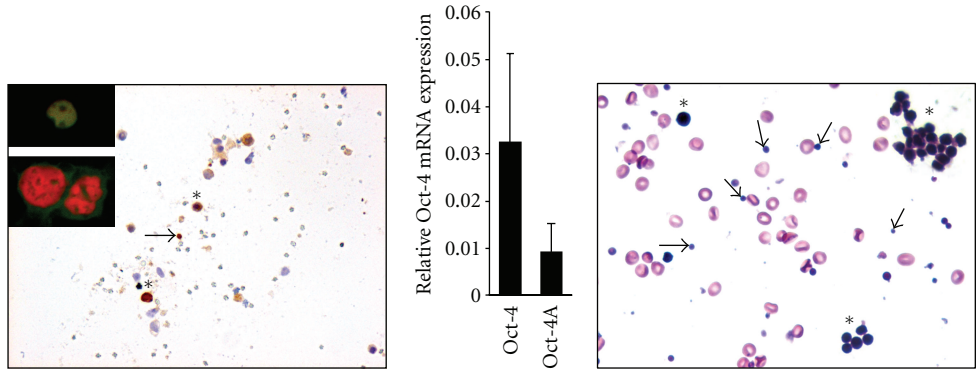

Figure 1: VSELs and OGSCs in adult mammalian ovary. (a) Immunolocalization of OCT-4, a stem cell marker on mouse ovarian cell smear using polyclonal antibody raised against C-terminal domain of OCT-4 (magnification 20x). Two distinct populations of stem cells were observed nuclear OCT-4 positive VSELs (arrow) and cytoplasmic OCT-4 positive OGSCs (asterisk). Inset is representative of the two stem cell populations by confocal microscopy using propidium iodide (PI) as a counterstain (magnification 63x with 5x optical zoom). VSEL has yellow stained nuclei as a result of co-localization of FITC labeled OCT-4 and PI whereas OGSC has distinct PI-stained red nuclei and cytoplasmic OCT-4. (b) Relative expression of Oct-4 and Oct-4A (transcript specific for pluripotent state) mRNA levels in normal mouse ovary by Q-PCR analysis. The levels of Oct-4A transcript in comparison to total Oct-4 were significantly lower suggesting that the VSELs positive for Oct-4A are less abundant compared to OGSCs. (c) H & E staining of human perimenopausal ovary surface epithelium smear showing the presence of RBCs, very small VSELs (arrow), and slightly bigger OGSCs (asterisk; present either as isolated cells or as clusters termed "germ cell nests" in developing ovary) (magnification 40x). Note the high nucleo-cytoplasmic ratio in stem cells with intense nuclear Hematoxylin staining.

3. Proposed Model for Oogenesis and Follicular Assembly in Adult Mammalian Ovary

Ovary harbors two distinct populations of stem cells, namely, VSELs and OGSCs (Figure 1). VSELs are quiescent stem cells whereas OGSCs are the progenitor stem cells, which proliferate, form germ cell nests, and differentiate into oocytes that get surrounded by somatic cells and assemble into primordial follicles. This model comprising two distinct stem cell populations in the gonads is in agreement with the concept put forth by Li and Clevers [38] in various adult body tissues like bone marrow, hair, and gut epithelium. Like the A_{dark} SSCs in the testis, OGSCs in the ovaries also have a relatively dark nucleus after Hematoxylin and Eosin (H & E) staining. This possibly reflects simple stem cell biology *in vivo* wherein the open euchromatin of pluripotent VSELs possibly gets compacted, appears dark, and undergoes remodeling and reprogramming for differentiation into a particular lineage. During "nuclear reprogramming" a dramatic change in facultative heterochromatin occurs [39]. Cells with pluripotent properties, that is, the nuclear Oct-4A positive cells, probably have abundant transcription permissive euchromatin, which becomes compacted due to stable association of histones with the chromatin in A_{dark} SSCs in testis and OGSCs in ovary, similar to that reported during ES cell differentiation [40]. Thus, because of intense "nuclear reprogramming" the early progenitor cells, namely, OGSCs and A_{dark} SSCs appear dark.

During three-week culture of the scraped OSE cells, the stem cells give rise to oocyte-like structures whereas the epithelial cells undergo epithelial-mesenchymal transition (EMT) to give rise to somatic granulosa-like cells [30]. The granulosa-like cells surround the developing oocyte resulting in follicular assembly *in vitro*. The differentiating oocyte undergoes meiosis and exhibits various germ cell markers, formation of Balbiani body-like structures, and characteristic cytoplasmic streaming *in vitro* (unpublished data). Similar views have been recently put forth by other groups as well [41, 42]. Bukovsky and group have proposed that possibly this EMT *in vivo* occurs in the tunica albuginea region of the ovary and may be involved in primordial follicle assembly. Figure 2 is a diagrammatic representation of the proposed model for postnatal oogenesis and follicular assembly from ovarian stem cells.

4. Stem Cells, Somatic Niche, and Menopause

Menopause implies exhausted ovarian follicle reserve and may be age related or induced prematurely by gonadotoxic insults including oncotherapy in the case of cancer survivors. But several groups have shown the presence of stem cells in the OSE of postmenopausal ovary [27–30] and in aged mouse ovary [21]. Why are these stem cells unable to differentiate and replenish the follicular pool? Why does menopause occur? The emerging literature supports the concept that it is most likely a compromised somatic niche (a cellular and molecular microenvironment that regulates stem cell function) that is unable to support stem cell differentiation [41, 43, 44] that causes menopause. Niikura et al. [21] demonstrated that stem cells exist in aged ovary, which is otherwise devoid of any oocytes. To demonstrate that the stem cells still retain the differentiation potential, they performed ovarian transplantation studies. Grafting of aged ovarian tissue of Oct4-GFP transgenic mice onto

Table 1: Consolidation of published literature on stem cells in adult mammalian ovary based on the concept of pluripotent (VSELs) and progenitor stem cell population (OGSCs).

Reference	Study highlights	Interpretation of published literature
Johnson et al. [7] Germline stem cells and follicular renewal in the postnatal mammalian ovary	Mitotically active SCP3$^+$ and MVH$^+$ germline stem cells in surface epithelium of adult mice ovary Chimeric follicles observed when wild type ovarian tissue is grafted onto ovary of GFP expressing transgenic mice	Several groups including our results also report the presence of stem cells in the ovary surface epithelium Probably they detected the bigger OGSCs since the cells were SCP3$^+$ and MVH$^+$ Chimeric follicles suggest that the oocyte and granulosa cells do not originate from a common bipotent progenitor stem cell as suggested by Bukovsky et al. [26, 61, 62]
Johnson et al. [63] Oocyte generation in adult mammalian ovaries by putative germ cells derived from bone marrow and peripheral blood	Extraovarian bone marrow (BM) origin of germ stem cells (GSCs) in adult mice and distribution by peripheral blood (PB) to the ovaries BM and PB express primordial germ cell markers Oct-4, Dazl, Mvh, Stella, Fragilis, and Nobox	Compromised ovaries in young mice (due to chemotherapy) possibly mobilized VSELs from the bone marrow to enter circulation In addition to the pluripotent markers (Oct-4 and Nanog), the mobilized VSELs expressed germ cell specific markers. On similar note during stroke the mobilized VSELs exhibit neural markers like GFAP, nestin, beta-III-tubulin, Olig1, Olig2, Sox-2, and Musashi [35] Possibly the VSELs sense the nature of damage—and thus proliferate and give rise to progenitor stem cells exhibiting specific markers Interestingly the mobilized cells reported were Lin$^-$ and Sca$^-$, implying that they neither were of hematopoietic origin nor were pluripotent. The authors probably detected GSCs which expressed germ cell specific markers
Lee et al. [47] Bone marrow transplantation generates immature oocytes and rescues long-term fertility in a preclinical mouse model of chemotherapy induced premature ovarian failure	Chemotherapy sterilized mice were transplanted BM cells from coat color mismatched donors All pups born were of recipient germ line	BMT possibly provides an endocrine/paracrine signal that improves the functionality of ovarian niche there by restoring function BM does not serve as a source of germ cells since all the pups are similar to the recipient
Bukovsky et al. [64] Bone-marrow-derived cells and alternative pathways of oogenesis in adult rodents	Suggested alternative pathway of oogenesis in adult rodents Explained that the rodent germ cells may, but do not necessarily originate from the OSE stem cells. Proposed alternative origin of putative germ cells from the medullary region Used neonatally estrogenized female rats which lack OSE but with normal stock of primordial follicles as study model Showed clusters of SSEA1$^+$ cells in the ovarian medulla-precursors of oocytes Proposed that female germ cells should receive an impulse from the immune system-related cells to become oocytes. Therefore, if triggered by BM derived cells, the germ cells in ovarian medulla may represent an alternative source of oocytes for renewal of primary follicles	We propose that there may not be any alternative pathway existing in rat ovaries Ovary after neonatal exposure to estradiol is compromised and its homeostasis is disturbed. This may mobilize VSELs from BM through PB. Mobilized VSELs possibly enter ovarian medulla through the blood vessels and then try to reach the cortex for follicular assembly Their results probably show that SSEA1$^+$ cells migrate from the BM into the medulla of the ovaries

Table 1: Continued.

Reference	Study highlights	Interpretation of published literature
Szotek et al. [65] Normal ovarian surface epithelial label-retaining cells exhibit stem/progenitor cell characteristics	Identified a label-retaining cell (LRCs) population in coelomic epithelium of adult H2B-GFP transgenic mouse ovary These cells exhibit quiescence, functional response to the estrus cycle, slow cycling, and may undergo asymmetric cell division, exhibit cytoprotective mechanisms by enrichment for side population, and show increased growth potential *in vitro*	The LRCs reported by them are possibly the VSELs which undergo asymmetric cell division Interestingly VSELs do not stain with DAPI possibly because they mostly comprise of euchromatin whereas DAPI binds preferentially to heterochromatin [30]
Zhang et al. [66] Expression of stem and germ cell markers within nonfollicle structures in adult mouse ovary	Used Oct-4-EGFP transgenic mouse model to study the expression of stem and germ cell markers in adult murine ovaries OCT3/4, MVH, SSEA-1, and SCF-R in specific cell aggregates of 50–200 cells (distinct from follicles) within the adult mouse ovary Aggregates have large round nuclei, intensely stain with Haematoxylin, positive for OCT-4, SSEA-1, SCF-R, and MVH; also have SCP3 and DMC1 (meiotic markers by RT-PCR); interestingly they lacked GDF-9 (a postmeiotic marker) Authors conclude a mixed population of committed stem cells as well as transitional stage germline cells that might retain the capacity of proliferation and differentiation	These aggregates possibly represent clonal expansion of OGSCs with cytoplasmic continuity described as germ cell nests in developing fetal ovary [67]. We have observed similar structures in adult mouse and human ovary (Figure 1). Like the OGSCs, cells comprising the germ cell nests have characteristic dark stained nuclei after H staining. OGSCs are immediate progenitors of VSELs and since this involves a shift from euchromatin to a committed genome of a germ cell—extensive chromatin compaction, remodeling occurs—giving dark appearance after H stain. OGSCs divide rapidly to form germ cell nests. This data directly supports postnatal oogenesis in adult mammalian ovary. However, the group have reported OGSCs and not VSELs
Zou et al. [22] Production of offspring from a germline stem cell line derived from neonatal ovaries	Proliferative MVH positive (10–12 μm) large FGSCs purified from neonatal and adult mouse ovaries and maintained *in vitro* for months These cells, after transplantation into ovaries of chemotherapy sterilized recipients, generate chimeric follicles that were fertilized and produced viable offspring	Such large VASA positive cells have been reported also by Zhang et al. [66] However, they immunosorted the initial cells for establishing the cultures based on MVH, a germ cell marker and not an early stem cell marker
Zou et al. [23] Improved efficiency of female germline stem cell purification using fragilis-based magnetic bead sorting	Use of Fragilis, an early germ cell marker, to enrich cells (10–12 μm) for initiating cultures—further enhanced isolation efficiency of mouse FGSCs	Possibly sorted OGSCs based on the size of the cells sorted by them
Pacchiarotti et al. [24] Differentiation potential of germ line stem cells derived from the postnatal mouse ovary	Demonstrated the presence of GSCs in adult mouse ovary using Oct-4-EGFP transgenic mouse model Detected three different types of GFP-OCT-4 positive cells based on size, namely, small (10–15 μm) sized in OSE; medium (20–30 μm) and big (50–60 μm) oocytes in the follicles by flow cytometry Ploidy analysis results showed that 70% of these cells were tetraploid (possibly oocytes) and 30% were diploid (stem cells). They further showed that $CD133^+$ cells exist in the ovary but do not co-localize with GFP-OCT-4 suggesting that germ line stem cells in ovary are distinct from the circulating $CD133^+$ cells	Flow Cytometry data shows that diploid stem cells exist in ovary and are further of two sizes in agreement with our data However, their approach of using GFP-OCT-4 mice did not allow them to differentiate between cytoplasmic and nuclear OCT-4 since GFP will be expressed by both the stem cells as both the transcripts are under the control of common Oct-4 promoter. $CD133^+$ cells are possibly the VSELs but it is intriguing that they did not co-express OCT-4 Thus whether nuclear OCT-4 positive VSELs express GFP or not needs further investigation

Table 1: Continued.

Reference	Study highlights	Interpretation of published literature
Gong et al. [25] Embryonic stem cell-like cells established by culture of adult ovarian cells in mice	Ovarian stromal cells (<40 μm) were subcultured on fibroblast monolayer and colony-forming cells were characterized Detected pluripotent stem cells in adult mice ovary which could be expanded in culture Two ES-like cell lines were established which expressed pluripotent markers and formed embryoid bodies and teratomas	The group was unable to provide information on the exact location on the pluripotent stem cells since they used all cells of size less than 40 μm to establish cultures. They mention stromal origin of stem cells but cells for initiating cultures were obtained by mincing whole ovary which will include the OSE also Ovarian smears used to demonstrate the presence of Oct-4 positive cells and also RNA was extracted from the whole ovary for RT-PCR to show the pluripotent transcripts—thus OSE as a source of pluripotent stem cells is not ruled out in their study
Bukovsky et al. [26, 61, 62] Immunohistochemical studies of the adult human ovary: possible contribution of immune and epithelial factors to folliculogenesis Origin of germ cells and formation of new primary follicles in adult human ovaries Oogenesis in cultures derived from adult human ovaries	Putative germ cells within the OSE of adult human ovary and originate from OSE stem cells which differentiate from mesenchymal progenitors in the ovarian tunica albuginea Scraped OSE cells from adult human ovary in culture form large oocyte-like cells and follicle-like structures Put forth the concept of bipotent progenitors capable of differentiating into oocytes and granulosa cells	The model of bipotent progenitors giving rise to germ and granulosa cells does not explain the chimeric follicles reported by other groups [22] Our results are in agreement with theirs that stem cells in OSE can generate oocyte-like structures *in vitro*
Virant-Klun et al. [27–29] Putative stem cells with an embryonic character isolated from the ovarian surface epithelium of women with no naturally present follicles and oocytes Parthenogenetic embryo-like structures in the human ovarian surface epithelium cell culture in postmenopausal women with no naturally present follicles and oocytes Stem cells in aged mammalian ovaries	Small (diameter 2–4 μm) round putative stem cells also able to forming oocyte-like cells *in vitro* isolated from human OSE These cells expressed mRNA for pluripotent markers like Oct-4, SSEA-4, Nanog, and Sox-2 After 20 days of culture formed oocyte-like cells expressing VASA, c-KIT and ZP2 transcripts Accompanying bubble-like putative stem cells growing in close contact with oocytes possibly acting like granulosa cells supplying essential cellular machinery to the developing germ cells Oocytes derived from these putative stem cells *in vitro* underwent parthenogenetic activation to form blastocyst-like structures Investigators concluded that they had discovered small cells with pluripotent characteristics comparable to VSELs found in other adult human tissues and organs	Surface epithelial location of the stem-like cells in postmenopausal ovaries reported by them matches initial reports of the location of presumptive GSC (MVH-BrdU double-positive cells) in juvenile and young adult mouse ovaries [7] The cells reported are probably the VSELs
Parte et al. [30] Detection, characterization, and spontaneous differentiation *in vitro* of very small embryonic-like putative stem cells in adult mammalian ovary	Two distinct populations of putative stem cells detected in scraped OSE of adult mammalian ovary, namely, VSELs (1–3 μm) and progenitor stem cells (4–7 μm) termed OGSCs VSELs express nuclear OCT-4 whereas the OGSCs show cytoplasmic OCT-4 Pluripotent markers Oct-4, Oct-4A, Nanog, Sox-2, TERT, and Stat-3 in human and sheep OSE c-KIT, DAZL, GDF-9, VASA, and ZP4 expressing oocyte-like cells spontaneously differentiate in three weeks cultures	VSELs are the quiescent stem cell population that undergo asymmetric cell division whereas the OGSCs are the progenitors similar to A_{dark} SSCs in testis, undergo extensive proliferation, and form aggregates just like cytoplasmic bridges in testis [20] VSELs are totipotent to pluripotent in nature and give rise to OGSCs which further differentiate into oocyte-like structures, parthenotes, neuronal-like cells, and so forth Observed close association of developing oocytes with mesenchymal cells *in vitro* formed by EMT of the OSE cells in initial cultures, similar to the results published recently [41]. We propose that granulosa-like cells are formed by EMT Thus VSELs differentiate to give rise to oocytes whereas the epithelial cells undergo EMT to form supporting granulosa-like cells—thus resulting in primordial follicle assembly

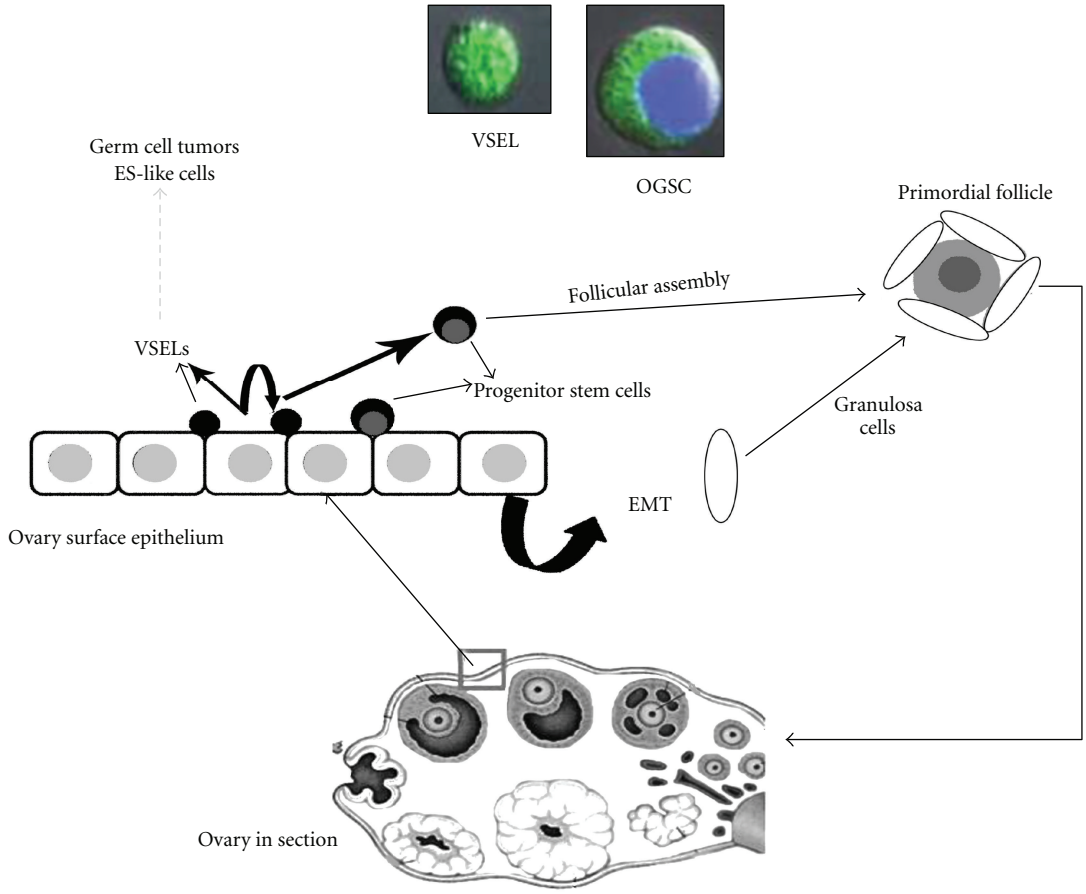

Figure 2: Proposed model for postnatal oogenesis in adult mammalian ovary. Pluripotent stem cells with nuclear OCT-4 (VSELs) being located in the ovary surface epithelium (OSE). These cells undergo asymmetric cell division and give rise to cells with cytoplasmic OCT-4 (OGSCs, which intensely stain with Haematoxylin). The OGSCs undergo further proliferation, meiosis, and differentiation to assemble into primordial follicles in the OSE. The granulosa cells are formed by the epithelial cells that undergo epithelial mesenchymal transition [30]. As the follicles grow and further mature, they shift into the ovarian medulla. Confocal images represent VSEL and OGSC isolated by scraping the surface epithelium of perimenopausal human ovary [30].

wild type young mouse ovary resulted in follicles containing GFP positive oocytes. In contrast, exposure of young ovarian tissue to aged environment resulted in reduced number of immature follicles. They proposed that failure of oocyte replenishment in the aged ovary was probably due to impairment of the somatic microenvironment rather than depletion/aging of the stem cells. In a similar study performed previously in male mice, SSC transplantation in irradiated testis was only able to support colonization and not differentiation. This has lead to a similar conclusion that the compromised somatic niche does not support stem cell differentiation [45].

We studied the presence of VSELs and OGSCs in chemosterilized mouse ovaries. We have observed that the quiescent VSELs persist and are resistant to therapy whereas the rapidly dividing OGSCs and mature follicles are lost resulting in premature ovarian failure (unpublished results). Similar resistance of VSELs has recently been demonstrated in mouse bone marrow after whole body irradiation [46].

It becomes pertinent to refer to two published studies here. Firstly Lee et al. [47] could rescue chemotherapy-induced premature ovarian failure in a mouse model by bone marrow (BM) transplantation. They were however intrigued by the fact that all the pregnancies were of recipient origin and not of donor BM. Their results can be explained, if we consider that the autologous VSELs that survived chemotherapy (because of their quiescent nature) underwent differentiation, folliculogenesis, and pregnancy in response to some signal provided by the transplanted BM. Secondly Fu et al. [48] transplanted bone-marrow-derived mesenchymal stem cells (MSCs) in ovaries of chemotherapy-induced ovarian damage and reported improved ovarian function. They showed that the MSCs secreted cytokines and inhibited chemotherapy-induced apoptosis of granulosa cells. They concluded that transplanted MSCs play an important role in ovarian microenvironment and protect ovary from chemotherapy-induced damage through secretion of cytoprotective proteins.

5. Clinical Evidence for Spontaneous Restoration of Fertility

The current available options offered to female cancer patients for fertility preservation include gonadal shielding, cryopreservation of egg/embryo, and/or ovarian cortical tissue prior to oncotherapy. The eggs or embryos are utilized to achieve parenthood by standard assisted reproductive techniques when required whereas the cryopreserved ovarian tissue fragments are transplanted at either orthotopic or heterotopic site to serve as a source of gametes [49]. To date thirteen pregnancies have been reported after orthotopic transplantation of ovarian cortical tissue on the surface of the atrophied ovary [50]. Interestingly, after heterotopic transplantation of cryopreserved ovarian cortical tissue or after allogeneic bone marrow transplantation, spontaneous recovery of intact, atrophied, and menopausal ovary has been reported resulting in spontaneous pregnancies [51–55]. Similarly a study has shown that bone marrow transplantation (BMT) in aged mice also helps sustain ovarian function [56].

Fertility restoration in these cases could be because of (i) restoration of lost germ stem cells or (ii) improved functionality of compromised niche in the atrophied ovary that is now able to support oogenesis and follicular assembly. Veitia et al. [57] provided evidence that spontaneous fertility after oncotherapy or allogeneic bone marrow transplantation was not because of donor bone-marrow-derived germline stem cells, as microsatellite analysis showed that the baby was of recipient origin. Thus it is becoming clear that the BMT or transplanted tissue somehow provides the necessary endocrine/paracrine signals to the compromised niche (rather than being a source of oocytes) and helps in restoration of ovarian function. The stem cell connection with spontaneous restoration of fertility has already been suggested by Oktay [58].

Research efforts must be intensified to identify the actual factors that are essential to restore functionality of the gonadal niche. Similar regenerative signals exist in young and aged male blood [59] which can also rejuvenate follicular dynamics in an aged ovary. Sönmezer et al. [60] have thrown open a discussion that low levels of androgens may have a role in the regenerative effect reported by Niikura et al. [59]. To support their view they gave the example of polycystic ovarian syndrome, where mildly increased androgens may be responsible for higher than average number of follicles observed and delayed menopause. Whether it is a reflection of increased stem cell activity needs to be demonstrated! Secondly treatment with dehydroepiandrosterone (a mild androgen) has been shown to improve ovarian response to fertility drugs [60].

6. Conclusion

This paper consolidates the published literature and discusses it in the context of the existence of two distinct stem cell populations in the ovary in an effort to bring more clarity in the field of adult mammalian oogenesis. It also discusses the possibility of restoring fertility by reconstructing the ovarian somatic niche. If true, various epigenetic and genetic concerns associated with long-term culture and differentiation of embryonic stem cells to make "synthetic gametes" or *in vitro* culture of OSE to generate autologous oocytes or maturation of primordial follicles *in vitro* may be overcome. This approach will open up new and novel, non-invasive avenues for fertility restoration, offer new means to treat female infertility, and delay menopause. Moreover, even patients who were deprived of fertility preservation options prior to oncotherapy stand to benefit by advances in this field.

Abbreviations

BM: Bone marrow
BMT: Bone marrow transplantation
EMT: Epithelial mesenchymal transition
ES: Embryonic stem cells
FGSCs: Female germline stem cells
GFP: Green fluorescent protein
H & E: Hematoxylin & eosin staining
LRCs: Label retaining cells
MSC: Mesenchymal stem cells
MVH: Mouse VASA homolog
Oct-4: Octamer binding protein 4
OGSC: Ovarian germ stem cells
OSE: Ovarian surface epithelium
PB: Peripheral blood
PGC: Primordial germ cells
Q-PCR: Quantitative polymerase chain reaction
RT-PCR: Reverse transcription polymerase chain reaction
SSCs: Spermatogonial stem cells
VSELs: Very small embryonic-like stem cells.

Key Messages

(i) Two distinct populations of stem cells exist in mammalian gonads including nuclear OCT-4 positive very small embryonic like stem cells (VSELs) and progenitor stem cells with cytoplasmic OCT-4 that is, A_{dark} spermatogonial stem cells (SSCs) in testis and ovarian germ stem cells (OGSCs) in ovary.

(ii) VSELs are relatively quiescent in nature and possibly undergo asymmetric cell division to give rise to progenitor stem cells which divide rapidly and maintain tissue homeostasis. The progenitor stem cells further differentiate and undergo meiosis to give rise to haploid gametes.

(iii) Oncotherapy being nonspecific by nature targets all rapidly dividing tissues in the body in addition to tumor cells. It destroys the progenitor stem cells in the gonads and also the haploid gametes, resulting in azoospermia in men and premature ovarian failure and menopause in women. Since the somatic microenvironment is compromised, it is unable to support differentiation of the persisting VSELs into functional gametes.

(iv) The existing challenge is to restore the somatic niche, thereby facilitating the differentiation of resident/persisting VSELs to form functional gametes leading to fertility restoration. Preliminary clinical evidence of spontaneous pregnancies after heterotopic transplantation of ovarian cortical tissue or allogeneic bone marrow transplantation lends support to this concept.

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Review Article

Cancer, Fertility Preservation, and Future Pregnancy: A Comprehensive Review

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Given the increases in 5-year cancer survival and recent advances in fertility preserving technologies, an increasing number of women with cancer are presenting for discussion of fertility preserving options. This review will summarize the risk of infertility secondary to cancer treatment, available treatment options for fertility preservation, and techniques to reduce future risks for patients. Concerns that will be addressed include the risk of the medications and procedures, the potential delay in cancer treatment, likelihood of pregnancy complications, as well as the impact of future pregnancy on the recurrence risk of cancer. Recent advances in oocyte cryopreservation and ovarian stimulation protocols will be discussed. Healthcare providers need to be informed of available treatment options including the risks, advantages, and disadvantages of fertility preserving options to properly counsel patients.

1. Introduction

An estimated 1 out of 47 women will be diagnosed with some type of invasive cancer by age 40 years, and approximately 774,370 women will be diagnosed with cancer in 2011 [1]. The most common cancers in reproductive age women are breast, melanoma, cervical cancer, non-Hodgkin's lymphoma, and leukemia [2]. Fortunately, the 5-year relative survival for all cancers is up from 50% (1975–1977) to 68% (1999–2006) reflecting improved diagnosis and treatment. The 5-year female cancer survival is dependent on stage at diagnosis but is currently 90% for breast, 91% for melanoma, 71% for cervical, 69% non-Hodgkin lymphoma, and 55% for leukemia [1]. Given the relatively high incidence of cancer in reproductive age women and improvements in 5-year survival, an increasing number of women are presenting for discussion of fertility preservation and pregnancy after cancer treatment.

Fertility preservation is a rapidly evolving field that includes medical and surgical treatments to decrease the impact of cancer treatments on future fertility. Traditional fertility preserving techniques for patients undergoing radiation treatment included pelvic shielding or surgical repositioning of the ovaries out of the pelvis. Medical treatments to suppress ovarian function during chemotherapy have also been reported to decrease the effect on cancer treatments on future ovarian function. These modalities still rely on residual ovarian function after cancer treatments to conceive. Newer techniques to preserve ovarian reserve, oocytes, and embryos prior to cancer treatments have been developed to provide an opportunity to conceive in the event that cancer treatments result in permanent loss of ovarian function.

This review will summarize available treatment options for fertility preservation in cancer patients. Concerns that will be addressed include the risk of the medications and procedures, the potential delay in cancer treatment, as well as

the impact of future pregnancy on the recurrence risk of cancer. Healthcare providers need to be aware of available treatment options including the risks, advantages, and disadvantages of fertility-preserving options to properly counsel patients.

2. Methods and Materials

We performed a MEDLINE search to July 2011 using the following terms: fertility preservation, cancer, in vitro fertilization, assisted reproduction, ovarian stimulation, oocyte vitrification, ovarian preservation, hereditary cancer, childhood cancer, preimplantation genetic diagnosis, ovarian reserve, pregnancy, and cancer. Studies included in this review include publications in peer-reviewed journals.

3. Results and Discussion

3.1. Counseling Patients on Future Fertility Prior to Cancer Therapy. Future fertility is a significant concern for patients undergoing cancer treatment. Studies have shown that the psychological impact of cancer-related infertility is substantial with 77% of patients reporting clinically significant levels of distress in relation to loss of fertility [3]. A survey of over 600 women with breast cancer indicated that 73% of women reported some degree of concern about the possibility of becoming infertile after treatment and 29% of patients indicated that their desire for future fertility impacted their cancer treatment decisions. In fact, many women indicated that they may choose a less toxic dose of chemotherapy to help preserve fertility even if it may increase the risk of cancer recurrence [4]. Cancer survivors also have higher depression and distress scores if they have unmet informational needs about future reproductive options [5]. Recognizing these concerns, the American Society of Clinical Oncology (ASCO) published recommendations in 2006 on fertility preservation in cancer patients. These guidelines state that oncologists should address the possibility of infertility with cancer patients and be prepared to discuss possible fertility preservation options or refer the patient to a reproductive specialist [6].

Despite the 2006 ASCO recommendations, a nationwide survey of oncologists in 2009 reported that less than 50% referred patients to a reproductive specialist [7]. A survey of academic medical centers reported similar results with less than 40% referring patients to a reproductive specialist although 95% reported that they routinely discussed the effect of cancer treatment on fertility [8]. Factors that were associated with a higher likelihood to refer in patients with breast cancer included patients with a family history of breast cancer, older age, early stage cancer, and receiving care at an academic center [9]. Interestingly, a review of NIH intramural clinical trials for pediatric cancer, gynecologic cancer, or for stem-cell transplantation found that only 47% of patient consents addressed future infertility risks after cancer treatment [10].

3.1.1. Risk of Infertility after Cancer Treatment. The adverse effects of chemotherapy and radiotherapy on female reproduction have long been recognized. Part of the difficulty in counseling patients regarding the risk of infertility and/or subsequent pregnancy complications is that the risks are dependent on several factors. These risks include the dose and duration of treatment, other risk factors for infertility, the age of the patient, and the patient's baseline ovarian reserve at the time of initiation of treatment.

Pelvic and/or abdominal radiation impacts future fertility by affecting both uterine and ovarian function. Radiation is typically administered as external beam therapy (teletherapy), intracavitary (brachytherapy), or total body irradiation as is utilized with stem-cell transplantation. Radiation is most commonly used in children for treatment of Wilm's tumor, abdominal rhabdomyosarcoma, and Ewing's sarcoma of the pelvis or spine. The effects of radiation therapy are dependent on the dose and the field applied. Radiation is typically targeted at the affected area; however, the impact of scattered radiation during treatments is also a consideration. Total body irradiation as used in stem-call transplantation has an over 80% risk of permanent amenorrhea. Limited field external beam radiation has a reduced risk depending on the location, dose, fractionation schedule, and age of the patient at the time of radiation treatment. In a study of 2000 women treated with pelvic radiotherapy, 95% had permanent ovarian failure following radiotherapy of 5–105 Gy [11]. It was reported that radiation doses over 5 Gy for women over 30 years results in permanent amenorrhea, however, it has been reported more recently that the lethal dose (LD50) of the human oocyte is actually less than 2 Gy [12].

Although the uterus is relatively resistant to the effect of radiotherapy, there is a concern that radiation may decrease uterine blood supply, volume, and endometrial thickness. Exposure to 20–30 Gy of abdominal or pelvic radiation has been shown to increase the future risk of miscarriage, preterm labor, and low birth weight [13]. The impact of radiation on future uterine function is dependent on the age at radiation in childhood cancers. The prepubertal uterus appears to be more vulnerable to the effects of radiation. Hormonal stimulation with estrogen and progesterone to improve endometrial thickness, and blood flow after radiation has been evaluated with variable effectiveness. The uterine volume increased significantly from 6.5 mL to 16.3 mL but was still less than controls after 3 months of hormonal replacement provided to patients with amenorrhea after radiation treatments. It was noted than patients exposed to prepubertal radiation had less improvement that patients exposed postpubertally [14]. Another study evaluating 3 childhood cancer survivors that received high-dose abdominal or pelvic radiation (30–54 Gy) found no increase in uterine volume, blood flow, or endometrial thickness with high-dose estrogen therapy [15]. These limited studies indicate that higher dosages of radiation affect uterine function and are most significant if administered prepubertally. Hormonal therapy may have limited benefit for improving endometrial development and patients that conceive should be considered at higher risk of preterm labor, delivery, and low-birth weight.

The primary impact of chemotherapy on fertility is related directly to the loss of ovarian function secondary to the gonadotoxicity of many chemotherapeutic agents. Cell-cycle nonspecific alkylating agents such as cyclophosphamide will destroy resting primordial oocytes while antimetabolite agents (methotrexate) have limited effect on ovarian function. The greatest risk is in women over age 40 years receiving alkylating agents with up to 80% of patients having permanent amenorrhea after treatment. However, in women under 30 years, the risk of permanent amenorrhea is substantially decreased to less than 20% [6]. The effect of chemotherapy will also depend on whether it is radical or adjuvant, single agent, or combination. Fortunately, the more recent ABVD regimen (Doxorubicin, Bleomycin, Vincristine, and Dacarbazine) used in the treatment of Hodgkin's disease is significantly less toxic to fertility than the older MOPP (Mechlorethamine, Vincristine, Procarbazine, and Prednisilone). The classical CMF (Cyclophosphamide, methotrexate, 5-fluoruracil) regimen for breast cancer will result in over 70% amenorrhea rates for women over 40 years [16]. The newer Taxanes are still being evaluated for their impact on fertility but hopefully will be less gonadotoxic than currently used regimens. Unfortunately, estimates in the impact on fertility vary widely dependent on various factors, and, therefore, there is no definitive predictor prior to treatment making counseling on future fertility challenging for health care providers.

3.1.2. Determining the Impact of Cancer Treatment on Ovarian Reserve. The peak number of oocytes is found in females at 20 weeks of fetal life, and this number declines until menopause. The number of primordial follicles is approximately 500,000 at menarche; menopause occurs once that pool is nearly depleted. Although chronologic age is the most important predictor of oocyte quality and quantity, there is variability in the rate of ovarian aging. The term "ovarian reserve" is used to describe remaining ovarian oocyte quantity. Although menstrual cycles do not start to become irregular until a mean age of 45 to 55 years, endocrinologic changes associated with ovarian aging have been demonstrated for women age 35 to 40 years and at earlier ages after cancer treatment. Several modalities have been evaluated as markers of ovarian reserve including cycle day 2-3 FSH, antimullerian hormone (AMH), and ovarian ultrasound of antral follicles. Assessment of a patient's ovarian reserve both before and after cancer treatment may provide valuable information for patients in discussion of fertility preserving option prior to treatment and future fertility after treatment.

Basal FSH values drawn during menstrual day 2-3 have been the routinely utilized as a marker of ovarian reserve. As FSH values increase, ovarian responsiveness decreases. An FSH value of 10–15 IU/L is generally considered borderline, and values over 15 IU/L are considered significantly elevated [17]. It is important when assessing FSH values to also evaluate basal estradiol as an elevation may suppress FSH and give a falsely reassuring value. A normal basal estradiol may vary between laboratories but typically is less than 60 pg/mL. FSH values may fluctuate widely between cycles particularly for patients with decreased ovarian reserve which limits its effectiveness as a marker of remaining ovarian function.

AMH is a member of the transforming growth factor B family and is produced by the granulosa cells of the secondary, prenatal, and antral follicles. AMH levels decrease progressively until it becomes undetectable at menopause. Theoretically, this may be a better marker of ovarian reserve as it represents the number of early and developing follicles and appears to have less intercycle variability than FSH [18]. One significant advantage of this test is that it does not require assessment on cycle day 2 or 3 since there is limited variability during the menstrual. However, this test may not be routinely available at all laboratories, and no international standard has been developed yet for this assay.

Ultrasound of ovarian follicle counts (AFC) has also been evaluated as a tool for predicting ovarian reserve. The number of antral follicles (<10 mm) present on a menstrual cycle day 2 to 3 by transvaginal ultrasound has also been correlated with other serum markers of ovarian function [19]. AFC is directly correlated with the number of oocytes retrieved during IVF and may prove to be the best predictor of ovarian reserve. Intercycle variability is present with all forms of ovarian reserve testing, and no single test has been consistently recommended. Basal FSH has been the mainstay of screening, but basal AFC and AMH may prove to be superior.

There is limited information on the impact of cancer treatment on markers of ovarian reserve. Small studies in young cancer patients have indicated that FSH, AMH and AFC have all been demonstrated to change in response to chemotherapy [20]. One study evaluated 42 premenopausal women receiving neoadjuvant chemotherapy that were followed over 5 years. Pretreatment FSH, AMH, and AFC and all were found to reflect future ovarian activity for women with menses after chemotherapy, but AMH was the most predictive by logistic regression [21, 22]. Further research is needed to determine the impact of cancer treatments on markers of ovarian reserve and any correlation with future fertility. It is important to consider that most research has evaluated these markers in relation to success of ovarian stimulation for IVF; therefore, caution must be used in counseling patients on the likelihood of spontaneous pregnancy or with other fertility treatments.

3.2. Options for Fertility Preservation

3.2.1. Ovarian Suppression during Chemotherapy. It has been well documented that chemotherapeutic agents, particularly alkylating agents, have high levels of ovarian toxicity. Oocytes are contained in ovarian primordial follicles, and it is estimated that hundreds to thousands of these follicles initiate the maturation process each month and are susceptible to the gonadotoxic effects of chemotherapy. Primordial follicles are stimulated to initiate maturation through a complex process that is initiated by follicle stimulating hormone (FSH) release from the pituitary in response to hypothalamic gonadotropin releasing hormone (GnRH). Suppression of ovarian function

through manipulation of GnRH has been evaluated as a mechanism to decrease the loss of primordial follicles.

Administration of GnRH analogs results in downregulation of pituitary receptors within 10–14 days of administration and subsequent suppression of FSH release. This has been studied in animal models with promising results but data regarding effectiveness in humans is limited to small retrospective reports. A recent systematic review [23] evaluating the utility of GnRH agonists in patients with breast cancer summarized data on the 5 available nonrandomized studies. The largest study of 100 women receiving 12 months of GnRH analogs during cancer treatment found that 67% of patients recovered normal menses with 100% return of menstrual function for women less than 40 years of age. However, only 3 pregnancies were reported [24]. Smaller studies have reported resumption of menses for 72–90% of patients with several pregnancies reported.

Unfortunately, it cannot be determined from these studies that the administration of GnRH agonists provided definitive ovarian protection. There are 4 reported ongoing prospective, randomized trials in women with hormone receptor-negative breast cancer to evaluate the effect on preserving fertility [25]. Outcome data from these studies will provide valuable information on the utility of this treatment in preserving ovarian function. It should be noted that there is some concerns regarding the use of GnRH agonists. It has been suggested that GnRH agonists may decrease the effect of tamoxifen if administered simultaneously and until more data is available, the ASCO recommends that women interested in this treatment receive it only as part of an approved clinical trial [6].

3.2.2. Embryo Cryopreservation. The basic principle of cryopreservation is to store cells or tissue for future use. Damage to cells during the cryopreservation process has been a barrier to the general use of this technology. Cryopreservation is typically performed by incubation in a low concentration of cryoprotectant to minimize ice crystal formation during freezing; however, cells with a high osmotic content such as oocytes are particularly vulnerable to damage. Embryos are composed of multiple blastomere cells and are more stable for cryopreservation. Due to the difficulties with oocyte cryopreservation, embryo cryopreservation has been the primary modality for fertility preservation and has been available since the 1980s. The most recent available data have indicated that over 21,000 embryo transfers occurred in USA in 2009 from frozen, thawed embryos resulting in per-cycle pregnancy rates of 35.6% 9 (<35 years), 30.9% (35–37 years), 26.8% (38–40 years), and 22.1% (40–42 years) with an average of approximately two embryos transferred per patient (http://www.sart.org/).

Embryo banking has several advantages for patients interested in preserving fertility. It provides reassurance to a patient that she will have some potential to conceive if the cancer treatments result in permanent amenorrhea. There is also over 20 years of outcome data for cryopreserved embryos showing no effect on miscarriage, implantation rates, or live birth [26]. A disadvantage of embryo banking is the need to administer ovarian stimulation medications to obtain oocytes for fertilization. Ovarian stimulation is a particular concern for patients with hormonal sensitive tumors such as breast cancer and will be addressed further in this review.

The American Society for Reproductive Medicine (ASRM) Ethics Committee published guidelines in 2005 on fertility preservation and reproduction in cancer patients. These guidelines state that the only established method of female fertility preservation is embryo cryopreservation and that experimental procedures such as oocyte or ovarian tissue cryopreservation should be offered only in a research setting with IRB oversight [27]. However, these recommendations may be revised with improvements in oocyte preservation technology and increasing numbers of live birth reported in the past several years.

3.2.3. Oocyte Cryopreservation. Recent advances in oocyte cryopreservation technology have expanded the use of this technology for fertility preservation. Disadvantages are similar to those of embryo banking including the risk of ovarian stimulation for patients with hormonally responsive cancers and the potential delay in starting cancer treatments. Oocyte banking is preferable over embryo banking for patients that do not have a partner and/or are not interested in utilizing donor sperm or have ethical concerns regarding cryopreservation of embryos.

Until recently, the primary disadvantage of oocyte banking has been the lower success rate compared to embryo cryopreservation. The first pregnancy from oocyte cryopreservation was reported in 1986 [28], but few pregnancies were subsequently reported due to poor survival rates for oocytes. The poor survival rates for oocytes that have been cryopreserved and thawed are attributable to several factors. Oocytes have a relatively high volume compared to other cells and are susceptible to intracellular ice crystal formation. Cryopreservation of oocytes has also been shown to result in chromosome and DNA abnormalities as the meiotic spindle of oocytes is very sensitive to chilling. Oocytes are also more susceptible to damage from reactive oxygen species than other cells. Many of these parameters improve after fertilization, making embryos less susceptible to damage than oocytes [29].

The more recent development of oocyte vitrification incorporates several modifications to traditional cryopreservation that result in less toxicity to oocytes. Oocyte vitrification exposes oocytes to higher concentrations of cryoprotectants for shorter durations of time followed by very rapid cooling. There have been over 500 pregnancies reported worldwide since 2005 with improvements in oocyte cryopreservation techniques [30]. A meta-analysis of randomized controlled trials assessing efficacy of oocyte vitrification reported similar fertilization, embryogenesis, and pregnancy from oocytes derived from vitrified oocytes compared to fresh oocytes. The authors state that increasing reports of successful cryopreservation of oocytes warrant reexamination of whether oocyte vitrification should still be considered an experimental technique [31].

3.2.4. Ovarian Tissue Cryopreservation. Ovarian tissue cryopreservation has also been evaluated as a modality to preserve future fertility. A portion of ovarian cortex is cryopreserved and then transplanted back to the pelvis, or other location (arm or abdominal wall has been reported) [32, 33]. The first report of an ovarian transplant operation occurred in 2000 [34] with the first pregnancy reported in 2004 [35] in a patient with non-Hodgkin's lymphoma. There have been fewer than 15 reported pregnancies worldwide with this technique; however, the first report of a woman that gave birth to a second child by natural conception after ovarian tissue transplantation has recently been reported [36].

Advantages of ovarian tissue transplantation include that it can be performed in prepubertal girls and adolescents, can be performed at any point in the menstrual cycle, has the potential to save large numbers of oocytes, and may allow for spontaneous pregnancy in the future without in vitro fertilization or ovarian stimulation. Disadvantages include the need for surgery (typically by laparoscopy) to remove the tissue and risk of graft failure. There is also some evidence that oocyte quality may be compromised with lower than expected fertility rates even with IVF. Another very significant concern which may limit its usefulness for cancer patients include the possibility of contamination of ovarian tissue by malignant cells which has been reported with hematologic cancers and Ewing's sarcoma [21, 22].

Patients undergoing ovarian tissue cryopreservation may still require future ovarian stimulation with gonadotropins and/or in vitro fertilization. Options that have been investigated to eliminate the risk of exposure to gonadotropins include in vitro maturation, (IVM) or in follicle maturation (IFM) of oocytes. These techniques require surgical removal of immature oocytes followed by in vitro exposure to gonadotropins to mature oocytes outside the body. There has been limited success with this approach utilizing immature oocytes aspirated during either the follicular or luteal phase of the menstrual cycle and matured in vitro. Although the survival rate is lower than with oocytes matured in vivo and vitrified, survival rates of 67.5% and clinical pregnancy rates of 20% have recently been reported [37]. Further data is needed to determine if this will be an effective treatment option for patients.

3.2.5. Recent Developments in IVF for Cancer Patients. Until recently, preserving oocytes or embryos have required a delay in cancer treatment of up to 4–6 weeks to complete the IVF cycle. Traditional ovarian preparation for IVF required 10–14 days of ovarian stimulation with exogenous gonadotropins preceded by ovarian suppression with GnRH agonists for approximately 2 weeks to prevent premature ovulation. Medications were initiated in the luteal phase of the cycle which may add up to 3 additional weeks to the process depending on when the patient presents for treatment.

Recent advances that include the development of GnRH antagonists have significantly decreased the interval from patient presentation to gamete cryopreservation. In contrast to GnRH agonists, GnRH antagonists immediately suppress pituitary release of FSH and LH and do not require the 10–14 days of administration prior to gonadotropin initiation. GnRH antagonists are initiated at approximately day 6 of gonadotropin stimulation which begins on day 2-3 of a menstrual cycle. This approach still requires awaiting menses prior to initiating gonadotropins but decreases the interval to oocyte retrieval compared to traditional IVF stimulation protocols.

A recent report of 3 patients initiating "random start IVF" evaluated the effectiveness of initiating GnRH antagonists at the time of patient presentation (menstrual cycle day 11, 14, and 17) rather than waiting for menses. This was then followed by the standard 10–14 days of ovarian stimulation and subsequent oocyte retrieval. The goal was to decrease the time to oocyte retrieval for breast cancer patients and resulted in a reasonable ovarian response with 7–10 embryos cryopreserved per patient [38]. This approach provides a significant advantage by decreasing total time for the IVF cycle, but further data is needed to determine its effectiveness compared to traditional IVF stimulation regimens.

In addition to the delay in cancer treatment, ovarian stimulation for IVF poses another theoretical risk to patients with hormonally responsive cancers. Ovarian stimulation with gonadotropins for IVF often results in supraphysiologic estradiol levels of over 2000 pg/mL compared to normal physiologic peak estradiol levels of 200–350 pg/mL. The high estradiol levels sustained during IVF treatment are a particular concern in women with estrogen receptor positive breast cancer. In the initial nonrandomized studies, stimulation protocols that include the selective estrogen receptor modulator tamoxifen or aromatase inhibitors such as letrozole administered during gonadotropin treatment have been shown to decrease estradiol level production while not decreasing overall oocyte numbers. Initial reassuring data indicates that this approach has not been shown to increase short-term cancer recurrences for breast cancer patients [39, 40].

Additionally, estradiol levels may be reduced after oocyte retrieval by the use of GnRH agonists to trigger ovulation instead of hCG. This has been shown to substantially reduce the risk of ovarian hyperstimulation in patients undergoing IVF by decreasing ovarian stimulation after retrieval. This has been evaluated in oocyte donors undergoing oocyte vitrification and has been shown in a retrospective study to result in similar numbers of oocytes retrieved. There was also no significant difference in the percentage of oocytes surviving thawing, oocyte fertilization, and pregnancy rates [41]. Further research is needed to determine if this will be beneficial in cancer patients undergoing oocyte or embryo banking but holds promise to further decrease any theoretical risks of breast cancer progression or recurrence as a result of ovarian stimulation.

3.2.6. Additional Considerations. Counseling of patients for future fertility should also include a discussion of alternative options including third-party reproduction. Third-party reproduction includes the use of either oocytes donated by another individual (either known or anonymously) as well as gestational carriers ("surrogates") to carry a pregnancy.

Oocyte donation may be utilized for patients without residual ovarian function after cancer treatment. Oocyte donation enables a patient to conceive and carry a pregnancy if she is unable to conceive with her own oocytes. Gestational carriers are most commonly used for patients that do not have a functional uterus to carry a pregnancy. They may also be considered for patients that are concerned about the recurrence of hormonally responsive tumors during pregnancy or any increased risks of pregnancy complications after cancer treatment. Adoption is also a consideration for family building but may be more difficult for cancer survivors than patients without a history of cancer [42].

Unfortunately, a significant barrier for many cancer patients is the cost of fertility-preserving treatments. Insurance coverage is often not provided for these treatments that they are often considered "elective." It has been argued that insurance companies should provide coverage for iatrogenic infertility as a result of cancer treatments similarly to coverage provided for other iatrogenic postcancer treatment conditions such as breast reconstruction after mastectomy and wigs for alopecia [43]. The average cost of fertility preservation for female cancer patients pursuing either embryo or oocyte cryopreservation is $8655 [44] and remains a barrier to access. Resources such as Fertile Hope's Sharing Hope Program can help patients and clinicians find centers with fertility preservation services as well as programs to provide financial assistance (http://www.fertilehope.org/).

Despite the concerns for patients including costs and potential risks, a followup survey of 28 cancer survivors who attempted fertility preservation found that 92.3% felt positively about their decision to undergo fertility preservation with only one patient, diagnosed with metastatic cancer shortly after oocyte retrieval, expressing regret [45]. The fact that patients with cancer recurrence may die and leave a minor child with one parent is an ethical concern. It has been suggested that it may be unethical to enable a woman to reproduce if she is expected to have a shortened lifespan. A review of fertility preservation and reproduction in cancer patients by the Ethics Committee of the Society for Reproductive Medicine stated that this concern may not be persuasive given that the risk of recurrence for many patients may not be excessively high, and the child may have a meaningful life despite the death of a parent [27].

3.3. Pregnancy after Cancer

3.3.1. Conceiving after Cancer and the Risk of Pregnancy Complications. The likelihood of conceiving after cancer treatments is dependent on the type of cancer, age at diagnosis, treatments with gonadotoxic agents including type and duration, and various other fertility factors. The chance for conception at best can only be estimated based on individual patient history and characteristics. It also appears that future fertility may also be influenced by gender. Overall, the likelihood of future children was found to be lower for female cancer survivors than male survivors either spontaneously or with fertility treatments [46]. When stratifying for age at diagnosis and estimating from probability charts, men with a cancer diagnosis prior to age 30 years had the highest overall chance of future parenthood (50%), followed by women diagnosed at age 30 years or younger (32%), then males diagnosed after age 30 years (12%), and then females diagnosed after age 30 years (<5%). For female patients the likelihood of pregnancy was dependent on the type of cancer and was highest for patients after uterine choriocarcinoma (65%), followed by lymphoma (23%) and malignant melanoma (22%), all other cancers (<5%).

If pregnancy is established, there are several potential risks to a fetus conceived after cancer treatment. Both radiation and chemotherapy may induce chromosomal aberrations in oocytes that may theoretically increase the risk of birth defects and genetic disease in offspring. A review of studies evaluating the risk of malformations in offspring of breast cancer survivors did not report an increased risk of birth defects compared with controls [47]. It may be that any remaining pool of primordial follicles after treatments is unaffected by the prior treatment, and/or those oocytes that fertilize and develop into ongoing pregnancies are from a cohort of oocytes that do not demonstrate any carcinogenic effect. However, when considering the half-life of treatments and the duration of time for oocyte maturation, it has been recommended to delay pregnancy for at least 6 months [47] after treatment with chemotherapy and 12 months following completion of radiotherapy to minimize risks to offspring [48].

Pregnancy complications and the subsequent risk to the fetus are another concern for cancer patients. A review of pregnancies in patients previously treated for breast cancer reported variable outcomes [47]. This meta-analysis evaluated 6 studies reporting birth outcome data after breast cancer compared to women without breast cancer. Four studies found no increased risk of any pregnancy complications; however, one study reported a higher risk of miscarriage and another reported no higher risk of miscarriage but a higher risk of cesarean section, preterm birth, low birth weight, delivery complications, and congenital abnormalities. The authors of the review suggest that although the large majority of births from women previously treated for breast cancer had no adverse effects, these women are at higher risk and may need careful monitoring until additional studies resolve the discrepancy in the data.

A recent report of birth outcomes obtained from a childhood and adolescent cancer registry from 4 US regions has reported that infants born to female childhood cancer survivors were more likely to be preterm (RR 1.54), and to weigh less than 2500 g (RR 1.31). Although there appeared to be a higher risk during the pregnancy, there were no increased risks to the offspring of malformations, infant death, or altered sex ratio indicating no increased risk of germ cell mutagenicity [49]. A review of pregnancies postcancer diagnosis in adults indicated that subsequent pregnancy did not represent a major health risk for the mothers or children. In 678 pregnancies there were no increased risk of congenital malformations (OR 0.6) though pregnancies more often resulted in preterm delivery (OR 2.8), low birth weight (adds ratio 2.5), and cesarean section (OR 2.3) and were delivered on average 6 days earlier even after controlling

for multiple births from patients utilizing fertility treatments to conceive [46].

It is not clear whether the increased risks in pregnancy are related to the malignancy itself or the result of treatments such as radiation or chemotherapy. Several considerations exist in cancer patients that may affect the risk to a developing fetus including altered metabolism, nutrition deficiencies from malabsorption of nutrients, increased stress, and general overall decreased health. It is also possible that these patients might be subjectively viewed as higher risk by their physicians and are electively delivered earlier. In summary, it does appear that there may be an increased risk of preterm birth and associated neonatal complications for female cancer survivors, but the outcomes of the majority of pregnancies appear similar to noncancer patients.

3.3.2. Risk of Transmission of Genetically Linked Cancers to Offspring. Although there does not appear to be a definitive increased risk of congenital abnormalities for the offspring of female cancer patients, there is a concern over the transmission of genetically linked cancers. Hereditary cancers account for about 5% of all malignancies [50]. Most hereditary cancers follow an autosomal dominant mode of inheritance with the most common being hereditary nonpolyposis colorectal cancer, familial breast and ovarian cancer, neurofibromatosis type 1, familial retinoblastoma, multiple endocrine neoplasia type 2, and familial adenomatous polyposis. Fewer hereditary cancers have an autosomal recessive inheritance such as ataxia teleangiectatica and Fanconi anemia [51].

Preimplantation genetic diagnosis (PGD) is a technique to screen embryos for genetically transmissible diseases prior to implantation. PGD involves removing one or more cells from an embryo after IVF and testing for predisposing mutations. PGD may be performed for genetic diseases where the gene has been identified and tested. PGD has been performed for all of the cancer predisposition syndromes mentioned previously in additional to several other less common susceptibility syndromes [52].

Significant controversy exists over the ethical aspects of screening embryos for disease. A survey of 4,834 Americans in 2004 found that approximately 52% of women and 62% of men reported that they approved of PGD for screening embryos that had a tendency to develop a disease such as adult onset cancer [53]. A more recent survey of attendees at a national conference for individuals and families affected by hereditary breast and ovarian cancer reported that only 32% of participants had ever heard of PGD; however, 57% believed that it was an acceptable option for high-risk individuals and that patients should be given this information by their health-care provider [54]. The ASRM Ethics committee guidelines state that the concerns about the welfare of resulting offspring should not be cause for denying cancer patient's assistance and that preimplantation genetic diagnosis to avoid the birth of offspring with a high risk of inherited cancer is ethically acceptable. However, selection to avoid a genetic disease may not always be appropriate and factors such as the severity of the disease, the probability of its occurrence, and the age at onset should be considered.

3.3.3. Pregnancy and Cancer Recurrence. Cancer is diagnosed in one of every 118 pregnant women each year. There are several concerns for patients pursuing pregnancy after cancer treatment that may be dependent on the type of cancer and treatments. Concerns include the risk of cancer recurrence either during or after treatment, the possible increased risk of cancer recurrence secondary to pregnancy itself (breast cancer, endometrial cancer, and malignant melanoma), and the difficulty in detecting cancer during pregnancy (breast cancer and endometrial cancer).

For most cancers, future pregnancy does not negatively impact the likelihood of recurrence. However, concern exists for several hormonally mediated cancers due to the consideration that the hormonal milieu of pregnancy may increase the risk of recurrence. The most common female tumors in reproductive age women that have been associated with hormonal mediators include breast cancer, endometrial cancer, and malignant melanoma. The most common cancer in women of childbearing age is breast cancer and is particularly concerning due to its clear association with hormonal markers. Patients with estrogen and/or progesterone receptor-positive tumors pose a particular challenge in counseling patients regarding recurrence risks during pregnancy and long-term overall recurrence risk.

Approximately 2% of all breast cancers occur in women between 20 and 34 years of age and 11% in women between 35 and 45 years. Given the relatively young age at diagnosis and initial treatment, there is a risk of recurrence during the reproductive years. The overall risk of recurrence and timing of recurrence in the context of pregnancy is difficult to evaluate due to the complex associations with other predisposing factors such as age at diagnosis, prior pregnancy history, age at menarche, and family history. Additional considerations include whether or not pregnancy itself affects the long term survival for patients with breast cancer, and whether or not the timing of pregnancy affects any risk of recurrence.

Initial studies in the 1980s and early 1990s indicated that there did not appear to be a difference in survival for time intervals from diagnosis to pregnancy [55–57]. In contrast, followup data indicated that patients that become pregnant in the first 3 months [58] or the first 6 months [59] of the initial breast cancer diagnosis may have an increased mortality. Clark et al. compared women that conceived within 6 months after a diagnosis of breast cancer to those patients that became pregnant between 6 and 24 months and more than 5 years after a diagnosis and found 5-year survival rates of 54%, 78%, and 100%, respectively. Another population-based study in 2006 showed a statistically nonsignificant increased mortality risk (RR 2.20, $P = 0.58$) for women diagnosed with breast cancer less than 6 months before pregnancy. However, if the interval was more than 2 years, the risk of death was reduced significantly (RR 0.48, $P = 0.009$) [60].

A recent 2011 meta-analysis by Azim et al. has addressed the optimal timing of pregnancy for breast cancer patients. Five studies compared 353 patients who became pregnant within 6–24 months and after 2 years of a breast cancer diagnosis and found that pregnancy within 6–24 months or

beyond 2 years did not have an effect on overall outcome. In summary, the data is controversial but it would appear prudent to advise waiting a minimum of 6 months after diagnosis to attempt pregnancy but more than 2 years is perhaps advisable and will depend on individual patient characteristics. For patients at higher risk of recurrence, a delay of 5 years or more may also be recommended.

Interestingly, several studies have suggested that pregnancy is actually associated with a better long-term prognosis for breast cancer patients. The Azim et al. meta-analysis reviewed 14 studies of women who became pregnant after breast cancer and reported that 8 studies demonstrated a significant survival advantage while the remaining 6 showed a trend-favoring pregnancy but did not reach statistically significance [61], Once criticism of studies reporting an improved survival for patients with breast cancer is that they may have included selection bias referred to as the "healthy mother effect."

The "healthy mother effect" infers that women who become pregnant represent an overall healthier group of patients with perhaps a lower risk of disease relapse. The Azim et al. meta-analysis incorporated several sensitivity analyses to attempt to control for the "healthy mother effect" but still reported that women who got pregnant following breast cancer diagnosis had a 41% reduced risk of death compared to women who did not get pregnant and was most notable in patients with a history of node-negative disease. In a subgroup analysis, they compared the outcome of women with a history of breast cancer that became pregnant to breast cancer patients who did not get pregnant and did not find a difference in survival between the groups. A separate meta-analysis also controlling for the "healthy mother effect" also found similar results with a survival that was higher among early stage breast cancer patients compared to control (hazard ratio 0.51) for pregnancy that occurred at least 10 months after the diagnosis [62].

The mechanism by which pregnancy may provide a protective effect is not clearly understood. It has been found that parous women have changes in expression of markers of disease recurrence including estrogen receptor alpha and beta (ERα, ERβ) and human epidermal growth factor receptor 2 (HER2) for up to 10 years after pregnancy, which may provide protection from cancer recurrence [63]. Patterns of breast cancer recurrence have been evaluated with regard to estrogen receptor status in two randomized trials with 25 years of median followup. It was reported that most breast cancer recurrence occurred within the first 5–7 years in ER-negative after randomization while ER-positive patients had events spread through 10 years. Patients with ER-positive breast cancer generally receive 5 years of adjuvant hormonal therapy and are recommended to delay childbearing although some women may elect to interrupt hormonal therapy to conceive. Even for patients that may not be receiving adjuvant hormonal therapy, it is recommended that patients wait a minimum of 2 years following diagnosis to conceive due to a generally higher incidence of recurrence in the first 2 years after diagnosis [64]. Overall, available data support that pregnancy after breast cancer are safe for women at low risk of recurrence but the timing of pregnancy will depend on individual patient characteristics and estrogen receptor status.

Although less common than breast cancer, malignant melanoma is a cancer with a peak incidence in the 30s and 40s resulting in a substantial number of women in their reproductive years interested in pursuing pregnancy after treatment. In contrast to breast cancer, there is limited evidence that hormonal mediators significantly influence this cancer. It has been noted that patients diagnosed during pregnancy often have a more rapid progression of their cancer, and estrogen-receptor proteins have been detected in tumor specimens. However, most studies have not found a statistical difference in 5-year survival rate for pregnant or nonpregnant patients. A study investigating endocrine ablation with procedures such as oophorectomy on patients with melanoma showed no benefit [65]. However, this author does suggest advising against future pregnancies for patients with nodal metastases or those who experienced tumor activation during a prior pregnancy. Others have recommended that all women with a history of melanoma avoid pregnancy for 3–5 years after treatment [66].

Endometrial cancer is another hormonally mediated cancer as evidence by the fact that exposure to unopposed estrogen is a significant predisposing factor. Progesterone offers a protective effect on the endometrium but both estrogen and progesterone are elevated during gestation. Limited data is available regarding absolute risk of cancer progression or recurrence for patients with a history of endometrial cancer as most patients are treated with a hysterectomy. A small case series and literature review of 50 women reported data on women with early stage endometrial cancer treated with conservative hormonal treatment in lieu to a hysterectomy. There were 65 deliveries reported with 77 live births. No neonatal morbidity was noted but one of the 50 women died of her disease after delivery [67]. Another study found that 40% of patients treated with conservative treatment of progestin therapy conceived but had a 36% relapse rate of their cancer [68].

4. Conclusions

Given the relatively high incidence of cancer in reproductive age women and improvements in 5-year survival, an increasing number of women are presenting for discussion of fertility preservation and pregnancy after cancer treatment. The ASCO published recommendations in 2006 on fertility preservation in cancer patients. These guidelines state that oncologists should address the possibility of infertility with cancer patients and be prepared to discuss possible fertility preservation options or refer the patient to a reproductive specialist.

Part of the difficulty in counseling patients regarding the risk of infertility and/or subsequent pregnancy complications is that the risks are dependent on several factors. These risks include the dose and duration of treatment, other risk factors for infertility, the age of the patient, and the patient's baseline ovarian reserve at the time of initiation of treatment. Advancements in ovarian reserve testing may help

counsel patients about the impact of their cancer treatments on fertility and chances for future pregnancy.

Fertility preservation is a rapidly evolving field that includes medical and surgical treatments to decrease the impact of cancer treatments on future fertility. Ongoing trials will address the effectiveness of GnRH agonists in protecting ovarian reserve. Several technologies exist to help preserve future fertility including embryo cryopreservation, oocyte, and ovarian tissue cryopreservation. Embryo cryopreservation is currently the only recommended method of gamete preservation, but recent advances in oocyte vitrification may increase the utility of this treatment for cancer patients. Additionally, PGD may decrease the risk of disease transmission of hereditary cancer syndromes. The risk to the patient of IVF may also be decreased with recent advances in IVF stimulation protocols.

There may be an increased risk of preterm birth and associated neonatal complications for female cancer survivors, but the outcomes of the majority of pregnancies appear similar to noncancer patients. It is not clear whether the increased risks in pregnancy are related to the malignancy itself or the result of treatments such as radiation or chemotherapy. Also, the risk of disease recurrence will depend on several factors, but for most cancers the risk of recurrence is not increased secondary to pregnancy. Overall, pregnancy appears safe for most patients after cancer treatment but will depend on individual patient characteristics.

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Review Article

Fertility Preservation in Girls

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Children that undergo treatment for cancer are at risk of suffering from subfertility or hormonal dysfunction due to the detrimental effects of radiotherapy and chemotherapeutic agents on the gonads. Cryopreservation of ovarian tissue prior to treatment offers the possibility of restoring gonadal function after resumption of therapy. Effective counseling and management of pediatric patients is crucial for preserving their future reproductive potential. The purpose of this article is to review recent literature and to revise recommendations we made in a 2007 article. Pediatric hemato-oncology, reproductive endocrinology, surgery, anesthesia and bioethics perspectives are discussed and integrated to propose guidelines for offering ovarian cryopreservation to premenarcheal girls with cancer.

1. Introduction

Ovarian cryopreservation has been offered to patients for more than a decade and, to date, more than 15 babies have been born worldwide after successful transplantation of ovarian tissue [1, 2]. The success of ovarian cryopreservation in adult women at risk for infertility secondary to exposure to chemotherapy and radiation therapy has led pediatric oncologists to consider ovarian tissue cryopreservation in prepubertal girls undergoing potentially gonadotoxic therapy. However, offering ovarian cryopreservation to young girls raises major medical, ethical, and legal issues unique to this age group that must be addressed. Firstly, the efficacy of the procedure in this setting is unclear, and it remains to be shown that ovarian tissue harvested from prepubertal girls can yield a successful pregnancy when retransplanted after puberty. Secondly, identifying the patients most likely to benefit from the procedure is complex, because of the difficulty in evaluating the risk for fertility impairment in young patients, especially when the time interval between administration of chemotherapy and clinical presentation of premature ovarian insufficiency is measured in years [3]. Lastly, since preservation procedures must be performed soon after diagnosis, while the patient herself is not old enough to provide informed consent, there are bioethical concerns regarding the validity of the process. The purpose of this article is to review recent literature and to revise our recommendations from the article published in 2007 [4].

2. Pediatric Hematology-Oncology Perspective

In 2010, a total of 10,700 children and adolescents under the age of 14 were diagnosed with cancer in the US alone. More than 80% will survive the disease [5]. For some

common pediatric cancers such as Wilms' tumor, Hodgkin's disease (HD), and B-cell non-Hodgkin lymphoma (B-NHL) cure rates approach 90% [6]. These high cure rates result in a growing number of children who will become long-term cancer survivors [7]. However, survivors often face long-term sequelae of their treatment because of irreversible tissue damage including to the reproductive organs that may harm pubertal development. Alternatively, they may present in adulthood with infertility or premature menopause, defined as cessation of ovarian function below the age of 40 [3]. Clearly, removal of the reproductive organs during treatment of a malignancy will prevent patients from conceiving with their own gametes. However, chemotherapy and radiotherapy may also eliminate or severely compromise hormonal production, as well as a patient's reproductive potential, by damaging steroid-producing cells and gametes.

2.1. Effect of Chemotherapy on Reproductive Potential. Both female and male gonadal tissues are very sensitive to chem otherapy [8, 9]. The degree of damage is determined by the patient's sex and age, as well as the drug used and the dose administered [10, 11].

Some pediatric chemotherapy regimens such as MOPP (mustragen, oncovin, procarbazine, and prednisone) for HD, and high-dose cyclophosphamide and busulfan for bone marrow transplantation, cause sterility in a significant number of patients [12–16]. Other regimens such as high-dose cyclophosphamide for B-NHL and Ewing sarcoma are associated with a significant risk for fertility impairment. No comprehensive data exists on the exact rates of fertility impairment associated with current pediatric oncology therapeutic regimens.

All chemotherapeutic drugs affect gonadal function although some are considered more harmful than others [17]. For example, alkylating agents such as cyclophosphamide and procarbazine are categorised as high-risk drugs [8, 18], whereas vincristine and methotrexate are considered to have lower risks. The problem, however, when trying to advise patients and their parents is that most patients are treated with multiagent protocols, and the relative contribution of individual drugs is difficult to determine. Hence, it is virtually impossible to give the patient or his/her parents an accurate assessment of the risk to fertility, but rather the patients are considered to be at a low, intermediate, or high risk of infertility [19, 20].

2.2. Effect of Radiation on Reproductive Potential. Total-body irradiation (TBI) or radiation therapy delivered to the pelvis/abdomen may cause irreversible damage to the gonads. In both sexes, the degree of damage depends on the radiation dose and field, fractionation schedule, and the patient's age. In females, it has been shown that for a given dose of radiation, the younger the patient at the time of treatment, the later the onset of premature menopause is [21]. A radiation dose of 2 Gy is estimated to damage 50% of ovarian follicles irreversibly; doses ranging from 5 to 20 Gy cause complete loss of ovarian function resulting in sterility [22].

2.3. Reduction of Treatment-Related Gonadotoxicity. Several approaches have been developed to reduce treatment-related gonadotoxicity. The ideal approach is to design treatment regimens that will maintain high cure rates while decreasing or eliminating agents with significant tissue toxicity. However, despite a growing understanding of the biology of cancer, and the identification of molecular targets specific to the malignant cell, the concept of targeted therapy, affecting only the malignant clone and sparing normal tissues, is still, in clinical practice, the exception rather than the rule and most survivors of childhood cancer are still at risk for significant long-term side effects including effects on fertility.

Another approach to decreasing the risk to fertility is the use of gender-tailored therapy. Boys with HD, who are more susceptible to the sterilizing effects of chemotherapy, are treated with lower doses of chemotherapy combined with radiation. Conversely, girls, who have a prohibitively high risk of radiation-induced secondary malignancies (especially breast cancer) and are less prone to chemotherapy-induced gonadal damage, are treated with more intensive chemotherapy but without radiation when feasible [23, 24].

Reduced chemotherapy and/or radiation therapy in diseases with high cure rates such as HD is conceptually appealing and may be efficacious. This approach, however, is not feasible in many pediatric malignancies. For example, high-risk acute lymphoblastic leukemia, neuroblastoma, Ewing sarcoma, high-risk rhabdomyosarcoma, and high-grade B-cell lymphoma are all treated with dose-intensive schedules that rely heavily on the use of alkylating agents [25–28]. Treatment regimens for these cancers have not changed significantly in the last decade and most patients treated with these regimens are expected to become long-term survivors, and to experience significant gonadal damage. Female patients with stage III Wilms' tumor who receive whole-abdomen radiation therapy are also at a high risk for fertility impairment [29]. Unfortunately, it is unlikely that these regimens will be supplanted in the foreseeable future by equally effective but less gonadotoxic regimens. The same applies to pediatric patients undergoing bone marrow transplantation, the overwhelming majority of whom will develop gonadal failure [30]. Therefore, it is vitally important to develop effective approaches to fertility preservation that may be offered to preadolescent children who are about to receive cancer treatment that is associated with a high risk of gonadal damage.

3. Reproductive Endocrinologists' Perspective

In adult patients that have a partner, cryopreservation of embryos remains the most reliable method to preserve fertility [20]. Another efficient option is vitrification of mature unfertilized oocytes [31]. Both these methods require hormonal stimulation and delay of treatment, thus cannot be offered to young girls or patients that must receive urgent treatment. Cryopreservation of gonadal tissue can now be offered to patients with malignancies that require gonadotoxic therapy.

3.1. Indications for Ovarian Tissue Cryopreservation. As the extent of the insult to gonadal function varies across different diseases and treatment protocols, it is important to counsel patients effectively about their risk of infertility. A classification system has therefore been developed, which lists most malignant diseases and their associated treatments [32]. However, the precise course of any disease is never completely predictable despite the best attempts to estimate prognosis prior to treatment. For example, in a series of 58 girls <16 years old, Jadoul et al. showed that 14%, who initially received treatment that placed them at low- to-medium risk of premature ovarian failure, needed more aggressive gonadotoxic treatment in the months following cryopreservation [33].

Benign indications for fertility preservation procedures include haematological or autoimmune diseases, as well as certain genetic conditions such as Fragile-X and Turner syndrome which predispose women to premature ovarian failure. In addition, repeated surgery due to ovarian cysts or ovarian torsion may result in decreased ovarian reserve [33].

3.2. Ovarian Tissue Cryopreservation. The procedure of ovarian tissue cryopreservation consists of several steps following the harvesting of the tissue. After harvesting, ovarian tissue is promptly delivered to the laboratory. Aspiration of any follicles present should be performed before cryopreserving ovarian tissue. Immature oocytes obtained from premenarchal girls can be matured *in vitro* and cryopreserved for future fertilization [34]. Ovarian tissue is traditionally cryopreserved using a "slow freezing" method [35]. First, ovarian cortex is separated from the medulla. The cortex is then dissected into small fragments, to maximize permeation of cryoprotective agents into the cells; these must be used to protect the oocyte and surrounding stromal cells from freezing injuries [36]. Ovarian fragment size ranges between 5 mm [1] and 350 μm in thickness [2]. The exact composition of the cryoprotective solution and the freezing protocol vary between institutions [37, 38]. Most commonly, the solutions contain permeating cryoprotectants such as dimethyl sulfoxide (DMSO), 1,2-propanediol, or ethylene glycol, in combination with nonpermeating substances such as sucrose and human serum albumin. Tubes containing immersed ovarian tissue fragments are gradually cooled by a programmable freezer that allows slow and stepwise decreases in temperature. When the temperature reaches -140°C, the tubes can be plunged into liquid nitrogen at -196°C for storage.

3.3. Experimental Techniques for Ovarian Tissue Storing. A recent and promising technique for storing ovarian tissue is "rapid freezing," termed vitrification. Small ovarian cortical fragments are immersed for a short period in a highly concentrated cryoprotective solution. Without a slow cooling delay, the ovarian tissue is plunged directly into liquid nitrogen. This induces a glass-like state that avoids the formation of ice crystals, which may harm the oocyte and stromal cells. The efficiency and safety of the technique still need to be fully investigated before it becomes standard practice. It has been suggested that vitrification is superior to slow freezing in terms of follicular survival and tissue preservation in general [39, 40]. Others have reported that conventional freezing is a more suitable method for ovarian tissue cryopreservation than vitrification [41, 42]. Currently, new vitrification protocols are being developed and may achieve better results [43]. Cryopreservation of an intact ovary is challenging because cryoprotective agents cannot penetrate all cells equally. The vascular pedicle of the ovary must be harvested and carefully dissected, but it can be difficult to avoid damaging the ovarian vessels. The ovarian artery is usually perfused, via a catheter, with a heparinised physiological solution so as to drain all the blood from the ovary. Thereafter, the ovary is perfused by, and immersed in, a cryoprotective solution followed by a cooling process, using a slow freezing protocol, as described above [44].

3.4. Transplantation of Ovarian Tissue. Autotransplantation of ovarian tissue once the patient is well enough can take place in several anatomical sites: the normal ovarian (orthotopic site) or another (heterotopic site) location. Regardless of the site, transplantation of ovarian tissue fragments is performed without any vascular anastomosis as the tissue is sutured directly to the recipient site. Orthotopic transplantation is performed in or onto the remaining ovary or ovarian stump, or by transplanting the tissue into a peritoneal pocket created by the surgeon in the broad ligament or pelvic peritoneum of the ovarian fossa. Transplantation can be performed at laparoscopy [45] or laparotomy [46]. In the presence of intact and patent fallopian tubes, spontaneous conception has been reported after orthotopic transplantation [45]. Alternatively, oocytes can be aspirated from the transplanted tissue for IVF [47]. Successful transplantation of ovarian tissue has not yet been described in paediatric patients, mainly because the cryopreservation and transplantation techniques are relatively new. However, we can assume that significant numbers of patients will undergo transplantation in the near future given that harvesting and cryopreservation of ovarian tissue has been performed in children for more than a decade. In adults, 14 healthy babies have been born after autotransplantation of cryopreserved ovarian tissue (see review by [1, 2]). These results suggest that the harvesting, cryopreservation, storage, and transplantation procedures are both feasible and safe. Ovarian tissue in children is rich in primordial follicles that appear to survive the cryopreservation and transplantation insults well. Abir et al. showed that ovarian biopsy from a 5-year-old patient contained viable follicles after cryopreservation that were suitable for transplantation [48]. The tissue harvested from young children is therefore expected to yield positive results after transplantation. A work in mice that analysed ovarian function after transplantation of immature ovarian tissue demonstrated that immature ovarian grafting can restore spontaneous puberty and fertility [49]. All live births that have resulted from ovarian tissue transplantation have arisen from orthotopic sites. Heterotopic transplantation can be to any site in the body other than the ovary or the adjacent peritoneum, for example, the subcutaneous space of the forearm or the abdominal wall [50, 51]. Other sites that

have been proposed include the uterus, rectus abdominal muscle, and the space between the breast and superficial fascia of the pectoralis muscle [52]. Clearly, spontaneous conception is impossible at such sites, and IVF treatment is required. The advantages of using heterotopic sites are that the transplantation procedure is easier and the oocytes are more accessible for aspiration during IVF treatment. However, no clinical pregnancies have been achieved from heterotopic sites even though ovarian function has been restored [51]. Whole ovary can be transplanted through microsurgical anastomosis of the ovarian vessels to a recipient site, either heterotopic or orthotopic. Although successful transplantation of a frozen-thawed whole ovary has not yet been described in humans, encouraging data have been published in sheep [53]. In humans, a successful pregnancy was achieved following a microsurgical anastomosis of an intact fresh ovary [54]. Several sites for transplantation have been proposed, including the deep inferior epigastric, and the deep circumflex iliac, pedicles [55]. The optimal site for transplantation remains to be determined in further studies.

3.5. Risks in Ovarian Tissue Transplantation. The biggest drawback to ovarian tissue transplantation, carried out without a vascular anastomosis, is that the graft may not survive. In the immediate period after transplantation there may be ischaemic damage to the tissue, resulting in massive follicular death; however, most primordial follicles survive this ischaemic insult [36, 56]. Surgical manipulations have been described to encourage prompt neovascularisation of the transplanted tissue, for example, a two-step procedure, involving the creation of granulation tissue one week before orthoptic transplantation, is believed to decrease the ischaemic damage [45]. Transplantation of a whole ovary with its vascular pedicle clearly avoids such an ischaemic period, as immediate reperfusion of the ovary should occur: in sheep, hormonal function is reported to have continued for 6 years following transplantation of whole ovaries [57]. The only other major risk of transplantation is the possibility of seeding malignant cells by reintroducing ovarian tissue containing micrometastases, as recently shown through quantitative reverse-transcribed polymerase chain reaction (RT-PCR) studies. In cryopreserved ovarian tissue from leukaemia patients, RT-PCR, and long-term xenotransplantation detected malignant cells, which had been missed histologically [58]. This risk is more evident in patients with hematological malignancies [59], but cannot be excluded in patients with solid tumors as well. For this reason, molecular studies are recommended prior to transplanting the tissue; long-term followup is also advisable to monitor for disease recurrence.

Success in molecular detection of malignant cells depends on the precise diagnosis and presence of genetic markers. In chronic myeloid leukemia (CML) the presence of BCR-ABL gene is characteristic and always allows for detection of leukemic cell contamination in ovarian tissue. In B- and T-cell lymphomas, as well as in acute leukemia (ALL), PCR for immunoglobulin and T-cell receptor gene rearrangements can be performed. Unfortunately, not all cases display a specific genetic marker that can be detected on PCR [59]. Histology and immunohistochemistry were unable to locate malignant cells within the cryopreserved ovarian tissue, and thus cannot be considered sufficient testing prior to transplantation [58, 60].

3.6. Function of Ovarian Tissue Graft. Ovarian activity usually returns approximately 4 months after transplantation [1]. This period corresponds to the time it takes for primordial follicles, which are the ones that principally survive freezing and the insult of transplantation, to mature into antral follicles. Ovarian activity is confirmed by tracking follicular development with ultrasound, detecting ovulation and measuring circulating sex hormones.

4. Pediatric Surgery Perspective

Ovarian cryopreservation involves the surgical harvesting of ovarian tissue. An important prerequisite for performing this procedure is a critical appraisal of the feasibility and safety of ovarian surgery in children. Pathological conditions of the ovary are encountered in infancy and childhood. Ovarian cysts, torsion, or masses, can be treated surgically in infancy and even the in neonatal period when indicated [61, 62].

4.1. Minimally Invasive Surgery. Over the last decade operative endoscopy and the concept of minimally invasive surgery has changed the practice of surgery. Following improvement and miniaturization of the required equipment, pediatric surgeons adopted laparoscopic and thoracoscopic surgery [63–65]. Laparoscopy has the advantage of exploring the abdominal cavity through a small incision, evaluating both ovaries before resection for fertility preservation. Laparoscopic oophorectomy is performed by isolating the fallopian tube from the ovary and gaining control of the ovarian blood supply. The ovary can be removed in a special bag through one of the trocar sites or a small lower abdominal incision. It is important to avoid using electrocoagulation on the ovarian surface to preserve the cortical tissue that contains follicles. The reported rate of complications is very low [66]. When performed by experienced surgical and anesthetic teams, oophorectomy for fertility preservation either by laparotomy or by laparoscopy, can be done with minimal complications.

The emerging technique of single port-laparoscopy requires a single umbilical incision rather than the standard 3-4 incisions and thus may improve cosmetic outcome [67, 68]. Due to the novelty of the technique, there is limited experience in children. Therefore, we believe that this surgical technique can be offered in selected cases [69].

4.2. Experience in Ovarian Tissue Cryopreservation in Children. We estimate that several thousands of women worldwide have undergone ovarian tissue cryopreservation. A recent publication from Denmark's registry, reports 18% of patients younger than 14 years of age [37]. Donnez and Dolmans reported successfully performed ovarian tissue cryopreservation in 59 girls under 16 years of age without complications [70]. In our program, about 15% of the patients who have undergone ovarian cryopreservation were

under the age of 15 (see Table 1). We report no surgical complications in pediatric patients. None of the procedures had to be converted to open laparotomy.

In the future, laparoscopic harvesting of the whole ovary including its vascular pedicle may be performed with the prospect of subsequent microvascular anastomoses of the ovarian vessels [44, 54]. It is important to take special care to resect the full length of the infundibulopelvic ligament, as it is crucial for the cryopreservation and transplantation procedures.

5. Pediatric Anesthesiology Perspective

Any anesthetic is associated with a risk of complications. Anesthetic complications may increase in the presence of additional risk factors due to either the subject's condition or the surgical procedure to be performed. These need to be offset against the benefit that is conferred upon the patient by the surgery or the risks to the patient of withholding surgery.

5.1. Benefit to the Patient. Although fertility preservation is not a life-saving procedure, fertility is in many patients' eyes the very essence of life and many patients are prepared to take significant risks in order to become pregnant. The discussion in this case is more complicated as the patients are children, the surgery is still experimental (albeit with extremely promising results) and fertility is not guaranteed. These factors will be discussed in greater depth in the next section; but if joint discussion between parents and the multidisciplinary medical team suggest that there is a genuine potential for fertility preservation, we feel that this justifies anesthetic risk in most cases. Sadly, this is usually one of many anaesthetics that these children will need and the offer of hope of fertility is an important component in their holistic care. Our primary focus in this section is on assessing anesthetic risk factors.

5.2. The Effect of Age on Anesthetic Risk. Anesthesia-related complications remain more common in pediatric patients than in adults. Currently, there are no clear guidelines regarding the appropriate age to harvest ovarian tissue. We previously suggested that harvesting should not be performed in girls under the age of 3 because of anaesthetic considerations [4]. However, Poirot et al. have reported a large series of paediatric patients who underwent ovarian tissue harvesting: 13 out of 47 were <3 years old, the youngest of them being 10 months old at the time of the surgery [71].

The majority of intraoperative anesthetic complications in children are respiratory complications, and intraoperative laryngospasm is among the most common and important of these. In a retrospective study of 130 cases of intraoperative laryngospasm over 10 years in one large institution, 57%, 68%, and 83% of laryngospasm were recorded in children younger than 3, 5, and 10 years old, respectively [72]. In a prospective study of 24165 anesthetics over a two year period, the incidence of adverse respiratory events per 1000 anesthetics increased with reducing age (<1 year: 36.1; 1–7 years: 15.3; 8–15 years: 8.6) [73]. A prospective study of 9297 pediatric anesthetics in a large single medical center also reported that older children were less likely to have laryngospasm and other perioperative respiratory adverse events (cough, desaturation, or airway obstruction) than were younger children [74]. However, there was no particular age cutoff that was associated with a step-wise change in anesthetic risk. Furthermore, that study showed only very modest effects of age on the risk for perioperative laryngospasm (RR 0.9; 95%CI 0.88–0.91; $P < 0.0001$); the relative risk decreased by 11% for each yearly increase in age. However, as the baseline probability for perioperative laryngospasm was only 2% (and only 8% in the highest risk patients), the impact on the incidence of perioperative laryngospasm is actually very small. The impact of age on perioperative desaturation (RR 0.95; 95%CI 0.94–0.96; $P < 0.0001$) was even smaller and there was no significant effect of age on perioperative bronchospasm (RR 0.99; 95%CI 0.96–1.02; $P = 0.33$). By contrast, the risk for perioperative laryngospasm increased markedly if a nonspecialist anaesthesiologist was managing the case (RR 3.85; 95%CI 2.47–5.98; $P < 0.0001$), if inhalation (rather than intravenous) induction was used (RR 2.38; 95%CI 1.79–3.17; $P < 0.0001$) and if there was anesthesia change-over during the case (RR 4.09; 95%CI 2.65–6.34; $P < 0.0001$).

From the balance of data currently available we feel that the 3-year-age cutoff is excessively conservative. Although this was appropriate when the procedure was more experimental, we would not now withhold anesthesia for laparoscopic oophorectomy for any child over the age of 1 year based solely on age without any other anesthetic risk factors. Below 1 year of age, each case should be assessed individually and should warrant careful multidisciplinary discussion, but here too, age alone should not be the overriding consideration.

5.3. Other Anesthetic Risk Factors. Operative laparoscopy in children [65, 75] involves the insufflation of carbon dioxide into the peritoneal cavity to a preset pressure, typically 13 cm H_2O. This is associated with several physiological challenges, including impaired venous return, hypotension, impaired respiratory compliance and hypercarbia. The effects on respiratory compliance are accentuated by the usual steep head-down position. However, these effects are limited to the duration of gas insufflation, which is typically brief and which may be curtailed or applied intermittently if necessary.

Clearly this procedure is not offered in healthy children and a wide range of haematological and oncological diagnoses will be present, each with their own spectrum of infectious, metabolic, and respiratory complications. Where possible these all require optimization prior to surgery. In cases of marked respiratory compromise (including pneumonia, pleural effusions, tense ascites, or hepatosplenomegaly) the added physiological burden of operative laparoscopy may be hazardous.

5.4. Balancing Risk and Benefit. It is advisable to combine ovarian tissue harvesting with other imaging or surgical procedures that require anaesthesia, such as bone marrow aspiration, lumbar puncture, or central line insertion [4].

Table 1: Ovarian cryopreservation in pediatric patients, Hadassah Medical Center (1997–2011).

Patient	Age at cryo (years)	Diagnosis	No. oocytes retrieved	No. oocytes frozen
1	15	Ewing sarcoma	0	0
2	15	Thalassemia major	0	0
3	15	Osteosarcoma, cervical cancer	18	2
4	15	AML	5	0
5	14	Ewing sarcoma	16	6
6	14	Rhabdomyosarcoma	5	3
7	13	Hodgkin's disease	0	0
8	13	Non-Hodgkin's lymphoma	0	0
9	13	Osteosarcoma	0	0
10	13	Hodgkin's disease	2	2
11	13	Ewing sarcoma	9	5
12	13	Hodgkin's disease	13	5
13	12	Ewing sarcoma	23	9
14	10	Sarcoma	0	0
15	10	Osteosarcoma	17	8
16	9	Osteosarcoma	6	2
17	8	Ewing sarcoma	8	2
18	5	Wilms' tumor	7	1
19	3	Neuroblastoma	4	1
20	3	Rhabdomyosarcoma	0	0

Nevertheless, laparoscopy imposes additional physiological challenges on the patient, and it is strongly advised to correct hematological, infectious, and metabolic derangements where possible prior to surgery and to optimize the child's respiratory and volume status. We would recommend considering withholding the procedure from children with markedly compromised respiratory function. Laparoscopic oophorectomy should be performed by a surgical team with appropriate training and equipment [76] and that includes an anesthesiologist with specialist training in pediatric anesthesia.

6. Bioethics Perspective

Fertility preservation in young girls through ovarian-tissue cryopreservation is still considered experimental and requires a procedure that involves certain risks. At the same time, with a number of pregnancies and deliveries already obtained by this method, it does provide young girls with a real potential benefit. A bioethical analysis should, therefore, address the ethical obligation of clinicians to offer this alternative and to discuss it with their patients as well as the ethical prerogative of parents to make this decision on behalf of their daughter.

The ethical principles of beneficence and respect for patient autonomy are usually interpreted in the bioethics literature as entailing an obligation to disclose any medical information that is pertinent to the patient's ability to make informed decisions, particularly when such information is directly related to a potential benefit [77]. Applying these principles can be relatively uncomplicated regarding medical procedures that are already accepted as the standard of care. It becomes more complicated regarding procedures that are still considered by most to be experimental and when the risk-benefit ratio is unclear.

While ovarian-tissue cryopreservation is still considered experimental, it can be argued that even at present, clinicians have an ethical obligation to inform their patients of its existence and discuss it with them. First, in terms of risk-benefit analysis, the risks are small while the benefits are significant. The procedure does not require delaying cancer treatment and the risk of general anesthesia can be mitigated by performing it during another medically indicated anesthetic session. On the other hand, it offers a tremendous potential benefit considering how devastating infertility can be later in life. Second, the bioethics literature encourages clinicians to acknowledge the "specific informational needs" of patients [77]. Considering this is currently the only option to preserve fertility in prepubertal girls and considering the irreversibility of the situation, it can be argued that patients have a specific need to be informed.

Do parents (or other legal guardians) have an ethical prerogative to make a decision regarding ovarian-tissue cryopreservation on behalf of their daughter? Parents have an ethical obligation to make decisions that are in the best interest of their child and they lose their parental liberty if they do not. In this case, to evaluate their girl's best interest they need to consider her present interest in minimizing risk against her future interest in fertility preservation. Since the risk involved in the procedure is small, the crucial factor is the nature of the girl's interest in fertility preservation.

A child's right to fertility preservation has been acknowledged in the bioethics literature as a "right in trust," a unique type of right that ought to be safeguarded until the child

reaches adulthood but can be violated before the child is even in a position to exercise it [78]. Davis claims that "therefore the child now has the right not to be sterilized, so that she may exercise the right to have children in the future" [79]. Since parents are authorizing cancer treatment that may impact future fertility, it is clear that they have the ethical prerogative to consent to ovarian-tissue cryopreservation which is the only way of protecting this "right in trust." When the patient has already reached an age at which it is possible to explain to her the procedure's purpose, it is of course preferable to obtain her own assent as well.

7. Synthesis and Summary

The majority of children diagnosed with cancer are expected to be cured and become long-term survivors. A substantial number of these survivors are expected to face impaired fertility secondary to the gonadotoxic effects of chemotherapy and radiation. Pretreatment fertility counselling and fertility preservation have great impact on quality of life of cancer survivors [80].

Cryopreservation of female gametes is considered efficacious using vitrification techniques [31]. However, this requires hormonal stimulation and transvaginal aspiration of oocytes—both are not applicable in young girls.

During the last years, most medical centres have opened programs that offer various fertility preservation strategies. The American Society of Clinical Oncology (ASCO) has established practice guidelines—however, these are not specific for children [81].

We currently recommend that all girls planned for radiotherapy or chemotherapy be referred for consultation by physicians specializing in fertility preservation**.** Fertility preservation procedures should be offered only after a detailed discussion and informed consent process with the parents, and, where age appropriate, with the child herself. The experimental nature of the procedure should be clearly stated along with presentation of potential benefits. A multidisciplinary team including pediatric oncologists, reproductive endocrinologists, pediatric surgeons, and anesthetists, as well as social workers, must work closely together to provide optimal counselling for patients and their parents**.** The decision on whether to perform oophorectomy or biopsies of ovarian tissue depend on the patient's age, treatment gonadotoxicity, and physician's preferences. The approach should be discussed with the patient/parents prior to surgery.

Although we previously [4] recommended a lower limit of age: 3 years old, we currently consider that ovary cryopreservation can be safely offered even to younger girls since the potential benefits are currently more evident and the risks of anesthesia appear not to be increased. To provide an additional margin of safety, we propose that if another necessary medical or surgical procedure is planned (e.g., insertion of an indwelling venous catheter, bone marrow aspiration, or harvest), then ovarian cryopreservation should be performed during the same anesthetic session.

A summary of our revised guidelines for offering ovarian cryopreservation to girls with cancer is presented in Table 2.

Table 2: Revised guidelines for offering ovarian cryopreservation to premenarcheal girls with cancer.

The treating team will conduct a detailed discussion of the risks and potential benefits of ovarian cryopreservation with the parents/guardians, and, where age-appropriate, with the patient.
Ovarian cryopreservation will be offered in cases where necessary medical treatments pose a high-risk of ovarian damage (e.g, bone marrow transplantation, whole-abdomen radiationtherapy, and alkylator-intensive chemotherapy).
Ovarian cryopreservation will be offered to girls over 1 years of age. Below 1 year of age, each case should be assessed individually in a multidisciplinary discussion (may be modified with increasing experience).
Ovarian cryopreservation should preferably be performed during a medically indicated anesthetic session (insertion of an indwelling venous catheter, bone marrow biopsy, and autologous bone marrow harvest).
Medical centers providing pediatric oncology care should form multidisciplinary teams to offer this treatment.

The field of fertility preservation is constantly evolving, as new experience is acquired and new lessons are learned. Expertise in performing ovarian tissue cryopreservation and transplantation is increasing rapidly. We believe that all considerations and guidelines should be reevaluated and modified according to new data, in order to provide the best benefit-to-risk ratio for the patients.

We strongly believe that there is an ethical obligation of clinicians to offer fertility preservation and to discuss fertility issues with cancer patients or their parents, in order to provide the opportunity for future parenthood.

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Research Article

Cryopreservation of Ovarian Tissue in Pediatric Patients

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Cancer treatments improve the survival rate of children and adolescents; however chemo- and radiotherapy result in gonadal damage leading to acute ovarian failure and sterility. Ovarian tissue cryopreservation allows long-term storage of primordial follicles and represents the only possibility of preserving the potential fertility in prepubertal girls. The aim of the present study is to describe our experience in ovarian tissue cryopreservation in 45 pediatric patients. The number of follicles per square millimeter of the overall section area and follicle quality were evaluated histologically. A strong negative correlation was found between age and follicular density in patients both prior to and after chemotherapy ($P < 0.0001$). Damage in follicular quality, that is, increased oocyte vacuolization and detachment of the oocyte from granulosa cells, was found after chemotherapy. Ovarian tissue cryopreservation, preferably performed before initiation of chemotherapy, should be offered to pediatric patients, including prepubertal girls, at risk of sterility.

1. Introduction

The survival rate of children and adolescents with cancer has increased markedly over the past 30 years as a result of advances in supportive care and changes in cancer therapies. The use of cancer multimodal therapy (e.g., surgery, radiotherapy, and intensive multiagent chemotherapy) is now routine in the treatment of children with neoplastic disease. However many children treated for cancer suffer from side-effects of curative chemo- and radiotherapy, such as the risk of Acute Ovarian Failure (AOF) or early menopause, then sterility [1]. Gonadal damage results in a development arrest of secondary sexual characteristics that prevent the attainment of sexual maturity. As the majority of women who survive cancer in childhood expect a normal reproductive life span, an early loss of fertility can seriously harm their quality of life.

The level of ovarian damage varies according to the cancer treatment protocol: ovarian integrity is affected by the type of chemotherapeutic agent, the cumulative dose of chemotherapeutic agents, treatment duration, and total dosage effect. In general alkylating agents such as cyclophosphamide are associated with the highest risk of infertility [2–5]. Ovarian failure has also been reported in patients treated with other agents such as cisplatin and vinca alkaloids. Of the multiagent treatment protocols, the adriamycin (doxorubicin), bleomycin, vinblastine, and dacarbazine (ABVD) combination is considered less cytotoxic than other protocols. Furthermore, the prebone marrow transplantation (BMT) conditioning protocol of busulfan and cyclophosphamide has been found to lead to high rates of sterilization, whereas high-dose melfalan seems to be safer [2].

Previous studies indicate that radiation affects the ovaries and the degree of impairment depends on the radiation dose, fractionation schedule, and radiation field. Doses as low as 400–600 cGy in adults and 1000–2000 cGy in children have been noted to decrease ovarian function [6]. The risk is higher with increasing doses of radiation. Radiotoxicity appears to be higher when radiation is given as a single dose as opposed to fractionated. Irradiation to abdominal, pelvic, or spinal regions is associated with increased risk for AOF [7]. However, even scattered radiation, not directed

specifically to the pelvis or abdomen, can cause ovarian damage. Patients treated with total body irradiation before BMT together with high doses of chemotherapeutic agents are very likely to have severe ovarian dysfunction [2].

The most well-established options for female adult fertility preservation are embryo and oocyte cryopreservation. Unfortunately, in the pediatric setting embryo cryopreservation is inappropriate and ovarian tissue cryopreservation maybe an alternative option for fertility preservation in these patients: with ovarian tissue freezing, no ovarian stimulation is needed and ovarian tissue can be harvested laparoscopically without any preparation. Furthermore ovarian tissue cryopreservation has the greatest fertility potential in children since they have a high number of primordial follicles [4, 7–15].

After thawing, cryopreserved ovarian tissue can be grafted into its normal anatomical position or into a heterotopic site. Alternatively the thawed tissue can be placed in culture with the aim to achieve antral follicle development and to avoid the risk of reimplantation of malignant cells [16]. However this option has not so far progressed sufficiently to be a therapeutic possibility [12].

Until today all pregnancies obtained after transplantation of frozen-thawed ovarian cortex were observed in adult patients at the time of harvesting; however there is no reason to doubt the capacity of prepubertal ovarian cortex to develop and function correctly after reimplantation [14]. Animal studies have demonstrated that puberty and cyclic hormonal activity can be restored by reimplantation of fresh and frozen–thawed prepubertal ovarian tissue in both prepubertal and adult mice [17, 18]. In humans only one experience has been reported by Andersen (data shown to the ESHRE Campus Symposium, Fertility Preservation in Cancer, Bologna, 2010). The patient was affected by Ewing Sarcoma at the age of 9 years, and her ovarian tissue was cryopreserved before receiving chemo- and radiotherapy. After cancer treatment, the patient presented clinical and biological Premature Ovarian Failure (POF) and, at the age of 13, she underwent reimplantation of cryopreserved ovarian tissue. One year later, the patient had her first menstrual cycle and evident signs of pubertal development. However, the uterus of the patient did not grow to adult size, perhaps because the pelvis had received high doses of irradiation. It is well known that pelvic radiotherapy decreases uterine blood flow and reduces uterine volume and endometrial thickness [3, 4, 19].

The purpose of this paper is to describe our experience in ovarian tissue cryopreservation in pediatric patients focusing on the clinical characteristics of the patients, the technical procedures, and strategies used to harvest and preserve the tissue. In adolescent patients, the opportunity to preserve their fertility enabling a future normal life is a psychological support allowing the patients to overcome the difficulties that the disease entails.

2. Materials and Methods

2.1. Subjects. From January 1999 to August 2011, 51 pediatric patients with malignant and benign diseases approached the Gynecology and Reproductive Medicine Unit, Department of Obstetrics and Gynecology, S. Orsola-Malpighi Hospital, Bologna, for consultation about the possibility of ovarian tissue cryopreservation. The ovarian tissue of 45 pediatric patients, including pre-and postmenarcheal girls, was collected and cryopreserved. Parents of the patients gave their consent for both laparoscopy and cryopreservation of ovarian tissue. Ovarian tissue cryopreservation was not carried out for six patients: in two cases patients did not fit the inclusion criteria; in two cases parents refused because the procedure was too new or they did not want their daughter to be subject to further surgery; in the last two cases the patients suffered from Turner Syndrome with very small ovaries and a menopausal value of FSH, so, given the possibility of cryopreservation of few follicles, their parents refused consent.

At our centre, the exclusion criteria for ovarian tissue cryopreservation included patients positive for Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), Human Immunodeficiency Virus (HIV), or Treponema Pallidum (TP); patients with ovarian cancers; and patients who had contraindications for surgery. Regarding patients with leukaemia could be removed and cryopreserved in the hope that in vitro maturation of the oocytes will be possible in the future [3].

As listed in Table 1, the majority of patients sent to our centre were cancer patients (38 of 45); others were affected by primary or genetic bone marrow disorders (6 of 45) and autoimmune disorders (one of 45). Within the cancer patients, more than half (23 of 38) had completed one or more cycles of chemotherapy before the collection of ovarian tissue (Table 1). According to the Karnofsky Performance Status Scale Index, the patients enrolled in the study reached over 90% in the criteria scale.

Ovarian tissue cryopreservation was carried out within a maximum of 20–30 days from the last chemotherapy cycle, while within 10–15 days in patients not undergoing chemotherapy before ovarian tissue recovery. The ovarian preservation procedure was not associated with any delay in the commencement of anticancer treatment.

2.2. Surgical Procedure. Ovarian tissue was collected at our department by laparoscopy. In all cases laparoscopy was carried out as a day surgery procedure under general anesthesia. A carbon dioxide pneumoperitoneum at a pressure of 10 mmHg was created via a Verres needle inserted into the peritoneal cavity through an umbilical incision. A three-port laparoscopy was performed. The primary 5 mm port was inserted through the umbilicus for the telescope; two secondary 3 mm or 5 mm ports were inserted to the left and the right, respectively, lower quadrants at the same level lateral to the rectus sheath. Ovarian tissue was removed with scissors proceeding along the connecting line between two atraumatic graspers positioned in the ovary just above the infundibulopelvic and utero-ovarian ligaments first on the left and then on the right. Each ovarian fragment was placed in an Endobag (Cook Medical) and retrieved through the umbilical 5 mm port to avoid tissue damage. The harvested tissue was then transferred to the laboratory for immediate cryopreservation. In the majority of cases,

Table 1: Subjects.

Pathology	No. patients[a]	No. patients underwent chemotherapy[b]	Pathology	No. patients[a]	No. patients underwent chemotherapy[b]
Acute lymphoblastic leukaemia	5	5	Synovial sarcoma	1	1
Acute myeloide leukaemia	2	2	Ewing sarcoma	7	1
Hodgkin lymphoma	16	10	Myelodisplasia	1	
Non-Hodgkin lymphoma	1		Bone marrow aplasia	2	
Wilms tumor	3	2	Systemic lupus erythematosus	1	
Peripheral neuroectodermal tumor (PNET)	1	1	Thalassemia major	2	
Mixoide tumor	1		Medulloblastoma	1	1
Shwachman syndrome	1				

[a]Total subject number enrolled in the study.
[b]Subject number underwent chemotherapy beforesurgery.

there was negligible bleeding of the ovaries; haemostasis was carried out by bipolar current. The port sites were closed using 4–0 resorbable (Biosyn) sutures. The median duration of this technique was 30 minutes (range: 15–45 min). All patients were discharged on the day of surgery or at most the day after, with the discomfort experienced being no greater than after diagnostic laparoscopy. No complications occurred due to anesthesia or surgery in any patient.

Forty-three patients underwent laparoscopic harvesting of ovarian tissue from both ovaries, while in two patients the ovarian tissue was harvested from only one ovary because the other ovary was too small (patient with Wilms Tumor) and in the other case unilateral ovariectomy was performed (patient with Hodgkin Lymphoma). The amount of ovarian tissue removed was subordinated to the anticancer treatment protocol and to the size of the ovaries: in premenarcheal patients, due to the small size of the ovary and a high density of primordial follicles, the biopsy was smaller compared to the postmenarcheal ones.

2.3. Ovarian Transposition. When pelvic radiotherapy is indicated, ovarian transposition can be performed in order to displace the ovaries away from the radiation field [3–5, 20]. In our experience, we reported a combined approach of ovarian tissue cryopreservation and ovarian transposition in four patients ($n = 19$, Table 2; $n = 9$, 10 and 17, Table 3). In these cases the laparoscopy was carried out as a standard procedure. After ovarian tissue removal for immediate cryopreservation, the residual ovaries were temporarily transposed to the lower anterolateral abdominal wall without transection of the ovarian ligament and mesovarium. In particular, they were fixed intracorporeally to the peritoneum close to the ipsilateral round ligament of the uterus, using one 2–0 resorbable braided (Vicryl) continuous suture [21]. Ovarian transposition induces no particular injuries on the ovary per se, or to the blood supply vessels, with no adhesions and disruption of anatomical relationships, indicating that it may be a valid strategy for the preservation of ovarian function [21].

2.4. Cryopreservation Procedure of Ovarian Tissue. The cryopreservation protocol used by our group was approved by the Ethics Committee of S. Orsola-Malpighi Hospital (Clinical trial 74/2001/0 approved on 13/02/2002).

After biopsy, the ovarian tissue was placed in Dulbecco's phosphate-buffered solution (PBS) (Gibco, Life Technologies LTD, Paisley, Scotland) with 10% human serum added (HS was provided by the Transfusion Centre of S. Orsola-Malpighi Hospital) and cut into pieces (mean size $1 \times 0.3 \times 0.1$ cm) using a scalpel. The mean number of pieces obtained was 23.1 ± 6.8 (mean $\pm$ SE) in premenarcheal patients and 27.6 ± 2.9 (mean $\pm$ SE) in postmenarcheal patients. One or two pieces (1–3 mm) from each ovarian sample were immediately fixed for histological analysis. The remaining tissue was maintained in PBS + 10% HS solution until cryopreservation. Samples were cryopreserved using a slow-freezing/rapid-thawing protocol, according to Fabbri et al. [22]. At the end of the cooling program, the cryovials were transferred into liquid nitrogen and stored until thawing.

For each patient, a cryopreserved sample from each ovary was thawed after one month and fixed for histological analysis in order to evaluate the preservation of cortical tissue.

2.5. Histological Analysis. Fresh and frozen/thawed tissue samples were fixed in a freshly prepared solution of 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4, overnight at 4°C. After osmium tetroxide postfixation and alcohol dehydration, the samples were embedded in Araldite epoxy resin (Fluka, Buchs, Switzerland) and then sectioned with an ultramicrotome (Ultracut; Reichert, Wien, Austria). For each sample one 0.5 μm thick section out of every 50 was collected and stained with toluidine blue for light microscopy (LM) examination (Leitz Diaplan light microscope equipped with a CCD JVC video camera). Semithin sections were examined, double blind by two trained pathologists, to (i) identify and count follicles, (ii) establish the number of damaged follicles, and (iii) assess follicular density, expressed as the number of follicles per

Table 2: Characteristics of the pre-chemotherapy patients who had ovarian tissue cryopreservation.

No.	Pathology	Age (months)	D/mm^2 (RO)	D/mm^2 (LO)	Cryopreserved fragments No.
1	Wilms tumor	53	49.89	—	63
2	Shwachmann syndrome	118	58.83	24.05	10
3	Ewing sarcoma	78	36.92	39.04	10
4	Ewing sarcoma	120	64.02	102.77	15
5	Hodgkin lymphoma	161	11.51	16.53	23
6	Hodgkin lymphoma	177	31.8	13.59	44
7	Hodgkin lymphoma	182	11.26	1.00	14
8	Hodgkin lymphoma	195	90.80	44.28	10
9	Non-Hodgkin lymphoma	205	1.92	1.74	9
10	Hodgkin lymphoma	206	12.18	15.64	14
11	Ewing sarcoma	144	159.24	26.30	11
12	Ewing sarcoma	173	0.69	1.58	16
13	Ewing sarcoma	184	18.41	15.45	23
14	Ewing sarcoma	190	3.20	11.84	21
15	Bone marrow aplasia	152	14.68	15.12	45
16	Bone marrow aplasia	160	14.47	10.37	38
17	Myelodisplasia	152	4.07	11.26	17
18	Mixoide tumor	203	15.47	8.01	16
19	Thalassemia major	180	9.98	17.80	22
20	Thalassemia major	193	2.58	6.96	33
21	Hodgkin lymphoma	214	18.24	13.87	12
22	Systemic lupus Erythematosus	198	1.87	1.66	33

No. 1–4 premenarcheal patients, No. 5–22 postmenarcheal patients.
D/mm^2: follicle density per mm^2.
RO: right ovary; LO: left ovary.

square millimeter of the overall section area. Measurements were performed on digitalized images using ImageProPlus software.

Semithin stained sections were initially observed at 10x to determine whether tissue was appropriate for histological examination: artefacts, erroneous sampling, presence or absence of follicles, and pathological involvement of ovarian tissue were recorded. Sections were then observed at medium (25x) and high magnification (40x). Follicles were counted and classified as resting type (primordial, intermediary primordial follicles, and small primary), growing type (large primary, secondary, preantral, antral), and atretic type, according to Gougeon classification [23]. At 40x the quality of follicles was determined; follicles containing oocytes with empty appearance, large cytoplasmic vacuoles, dark and granular cytoplasm, hyperchromatic nuclear staining, and detachment of the oocyte from the granulosa cells were considered degenerated.

2.6. Hormone Assays. Blood samples were analyzed for FSH, LH, Estradiol, Estrone, Prolactin, Testosterone, and 17-Hydroxyprogesterone one or two days before laparoscopy.

2.7. Follow-Up. Follow-up was performed six months after the end of therapy, and then every year, using ultrasound and hormonal tests.

2.8. Statistical Analysis. We studied the follicular density of the pre- and post-chemotherapy groups and, within each group, the follicular density of pre and postmenarcheal patients. The relationship between age and follicular density was evaluated using a linear regression model. Follicular density value used for statistical analysis was obtained by averaging the follicular densities of the left and right ovary. Results were statistically analysed by unpaired t-test. A value of $P < 0.05$ was accepted as significantly different.

3. Results and Discussion

Ovarian tissue was collected and cryopreserved from 45 pediatric patients aged 160.9 ± 6.9 months (mean ± SE). Patients were grouped into two categories: pre-chemotherapy patients ($n = 22$) aged 53–214 months (161.4 ± 8.9, mean ± SE) (Table 2) and post-chemotherapy patients ($n = 23$) aged 19–215 months (156.4 ± 10.6, mean ± SE) (Table 3). The specific chemotherapy regimen for each patient in relationship to the individual disease is given in Table 3.

In both groups, menarcheal age was 150 ± 3.8 months (mean ± SE). In the pre-chemotherapy group 80% of patients had regular menstrual cycles and normal flow, whereas 20% had slightly irregular menstrual cycles of up to 35 days. In post-chemotherapy patients, it was not possible

Table 3: Characteristics of the post-chemotherapy patients who had ovarian tissue cryopreservation.

No.	Pathology	Chemotherapy	Age (months)	D/mm^2 (RO)	D/mm^2(LO)	Cryopreserved fragments No.
1	Wilms tumor	Vincristine, AMD	19	268.63	49.69	4
2	Wilms tumor	Vincristine, AMD	48	—	—	1
3	Acute myeloide leukaemia	ICE, AVE, HAM, ARA-C	70	112.84	56.23	19
4	Acute lymphoblastic leukaemia	Vincristina, cyclophosphamide, methotrexate	91	40.61	92.09	24
5	Acute lymphoblastic leukaemia	AIEOP 9502	170	12.03	22.62	67
6	Hodgkin lymphoma	COPP/ABV	81	59.38	—	10
7	Hodgkin lymphoma	COPP/ABV + IEP-OPPA	134	9.96	11.05	31
8	Hodgkin lymphoma	COPP/ABV	131	7.18	13.90	42
9	Hodgkin Lymphoma	ABVD	150	12.64	29.98	13
10	Hodgkin Lymphoma	COPP/ABV	165	5.66	8.87	55
11	Hodgkin lymphoma	COPP/ABV	166	19.19	43.34	14
12	Hodgkin lymphoma	COPP/ABV	175	5.55	10.81	13
13	Hodgkin lymphoma	IEP/IOPPA	177	30.33	31.57	21
14	Hodgkin lymphoma	COPP/ABV	197	6.80	11.99	8
15	Hodgkin lymphoma	COPP/ABV	215	5.08	7.67	49
16	Synovial sarcoma	IFO, AMD	154	15.12	14.95	47
17	Ewing sarcoma	ISG/SSG IV	170	8.04	7.82	71
18	Acute myeloide leukaemia	Idarubicin, etoposide, citrabina	180	3.67	4.56	20
19	Acute lymphoblastic leukaemia	Vincristine, daunomycin, predmisone	183	9.61	10.45	12
20	Acute lymphoblastic leukaemia	Unspecified	192	19.37	12.18	33
21	Acute lymphoblastic leukaemia	Vincristine, cyclophosphamide, methotrexate	209	12.02	11.36	53
22	Medulloblastoma	Vincristine	199	9.85	10.19	25
23	Peripheral neuroectodermal tumor	IFO, etoposide, thiotepa, adriamycin melphalan	212	0	0	61

No. 1–7 premenarcheal patients; No. 8–23 postmenarcheal patients.
D/mm^2: follicle density per mm^2.
RO: right ovary; LO: left ovary.
AMD: Actinomycin D; ICE: Idarubicin, Etoposide, Cytarabin; HAM: Mitoxantrone; ARA-C: Citosinarabinoside; AIEOP9502. Protocol: Vincristine, Daunomycin, Predmisone, Asparaginase, Cyclophosphamide, 6-Mercaptopurine, Methotrexate, Adriamycin, Dexamethasone, Cyclophosphamide, Citosinarabinoside, 6-Thioguanina; COPP/ABV: Vincristine, Procarbazine, Cyclophosphamide, Prednisone, Adriamycin, Vinblastine, Bleomycin; IEP-OPPA: Ifofosfamide, Etoposide, Prednisone, Vincristine, Procarbazine, Prednisone, Adriamycin; ABVD: Adriamycin, Bleomycin, Vincristine, Doxorubicin; IFO: Ifofosfamide; ISG/SSG IV. Protocol: Vincristine, Adriamycin, Ifosfamide, Cyclophosphamide, Etoposide.

to evaluate menstrual cycles because they had been treated with pills or GnRH analogues.

A total of 5749 follicles from pre-chemotherapy patients and 2684 follicles from post-chemotherapy patients were analyzed; in both series LM showed that 99% of follicles were of the resting type and 1% distributed among primary, secondary, and preantral follicles. In one patient ($n = 2$, Table 3), the ovarian biopsy was too small and the entire cortex was cryopreserved. In all patients, LM on serial sections did not identify any neoplastic cells. Damaged follicles were observed in 26.1 ± 4.2% (mean ± SE) of the pre-chemotherapy group and in 34.3 ± 5.3% (mean ± SE) of the post-chemotherapy group (NS). The main alterations of damaged follicles observed were oocyte vacuolization and detachment of the oocyte from the granulosa cells. The high percentage of follicle damage in the pre-chemotherapy group could be attributed to the large number of patients with Hodgkin Lymphoma enrolled in this study. As shown in our study (Fabbri et al., 2011) [24], Hodgkin Lymphoma patients have a high frequency of vacuolization in resting oocytes (73.7%) before chemotherapy that positively correlates with disease stage, whereas in the control group only the 5.7%

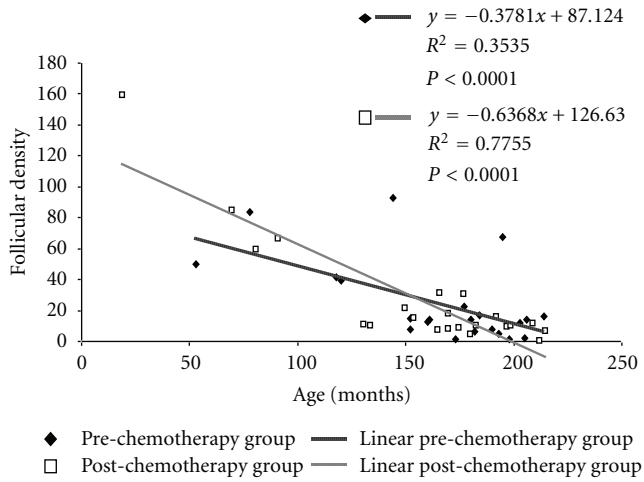

Figure 1: Correlation between age and follicular density in pre-chemotherapy and post-chemotherapy groups.

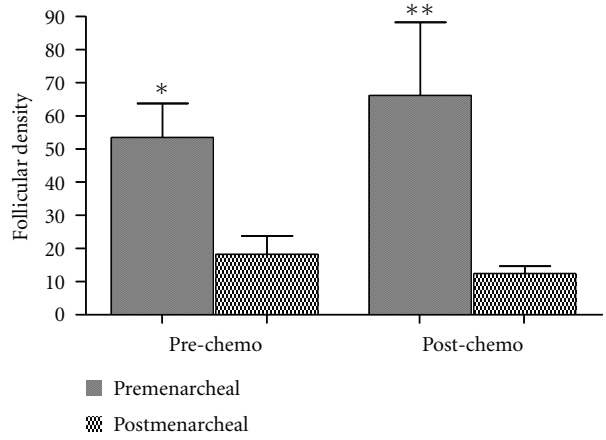

Figure 2: Comparison of follicular densities between premenarcheal and postmenarcheal patients in pre- and post-chemotherapy groups. $^{*}P < 0.05$; $^{**}P < 0.001$.

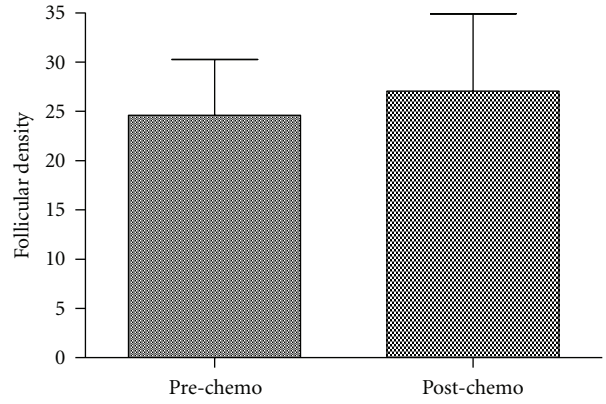

Figure 3: Comparison of follicular densities between pre-chemotherapy and post-chemotherapy groups. The difference is not significant.

of the oocytes showed microvacuolar changes and large-sized vacuoles were never seen. In the post-chemotherapy group, the highest percentage of follicular damage confirmed a previous study demonstrating the gonadotoxic effect of chemotherapy on follicular quality [2, 25]. However the lack of significant difference in the percentage of damaged follicles between pre- and post-chemotherapy groups could be due to the great variability of chemotherapic protocol and to the low number of cycles performed in this group of patients. It is therefore possible that chemotherapy has not yet fully demonstrated its real injury to the ovary.

In agreement with literature [10, 26], we found an age-dependent decline in follicular density in pediatric patients. In the pre-chemotherapy group as well as in the post-chemotherapy group, the follicular density showed a statistically negative correlation dependent on patient age ($y = -0.378x + 87.124$, $R^2 = 0.354$, $P < 0.0001$; $y = -0.637x + 126.63$, $R^2 = 0.776$; $P < 0.0001$) (Figure 1). Follicular density results were higher in premenarcheal patients than in the postmenarcheal in the pre-chemotherapy group (53.4 ± 10.3 versus 18.2 ± 5.6, $P < 0.05$; mean $\pm$ SE) as well as in the post-chemotherapy group (66.1 ± 22 versus 12.4 ± 2.1, $P < 0.001$; mean $\pm$ SE) (Figure 2).

Follicular density results were slightly higher in the post-chemotherapy group than in the pre-chemotherapy group (24.6 ± 5.7 versus 27.1 ± 7.8, NS; mean $\pm$ SE) (Figure 3). This finding may be due to the inclusion of the youngest patient ($n = 1$, Table 3 19 months) with the highest follicular density (159.16) in the post-chemotherapy group.

Hormone levels of pre-chemotherapy patients were within the reference range of our laboratory for age and phase of menstrual cycle. Hormone levels of post-chemotherapy patients were not considerable because these patients were receiving pills or GnRH analogue treatment to protect the ovarian function.

Median follow-up time was 30 months (range 10–50 months). Thirty-six of the girls are still alive. Nine patients died of the primary pathology during the study period: three of these patients suffered from Acute Leukaemia, two from Hodgkin's Lymphoma, one from Wilms Tumor, one from PNET, one from Thalassemia Major, and one from Synovial Sarcoma. To date we have not received any request for ovarian tissue reimplantation because most of the patients are very young, many are still undergoing treatments, and others do not currently need or desire motherhood.

Ovarian tissue cryopreservation offers great promise for fertility preservation among young patients affected by cancer and represents the only option for prepubertal girls. The human ovary has a fixed number of primordial follicles which progressively decrease with increasing age in a biexponential fashion, culminating in the menopause at around 50 years of age. Both chemotherapy and radiotherapy quicken follicle depletion leading to premature menopause [27]. Ovarian tissue cryopreservation is particularly important in children because they have a high number of primordial and primary follicles that are particularly resistant to freezing and thawing protocols. A further benefit for pediatric patients is the possibility of using the cryopreserved ovarian

tissue after a longer period than with adult patients. The probability of restoring fertility is related to the number and quality of primordial follicles in the ovarian cortical transplanted. Ovarian tissue cryopreservation also allows the restoration of steroidogenic function and promotes physiological development, puberty induction, and consequently the uterus growth. The puberty induction should be the main aim of ovarian tissue cryopreservation in prepubertal patients; alternatively these patients may undergo hormone replacement therapy and use the frozen ovarian tissue only if there is desire for motherhood. The final decision can only be made together by the oncologists, specialists in reproductive medicine and the parents who must make decisions based on the assumed future wishes of the girl.

To date many authors have written about availability, feasibility, safety, and efficiency of ovarian tissue cryopreservation in pediatric patients. Jadoul et al. [14] performed ovarian cryopreservation in 58 girls under 16 years of age in order to validate the technique, concluding that ovarian cortex cryopreservation is feasible and as safe as comparable operative procedure in children. Poirot et al. [10] conducted a study to describe their experience of ovarian tissue cryopreservation in 49 prepubertal females before chemotherapy, focusing on the feasibility of the technique and specific features of the procedure for prepubertal children. They concluded that this procedure could be systematically offered to prepubertal girls at risk of sterility due to gonadotoxic treatment. Revel et al. [13] performed a study in which all patients undergoing ovarian cryopreservation were subject to integrated oocyte aspiration from antral follicle of the tissue, followed by In Vitro Maturation and oocyte cryopreservation as an additional fertility preservation strategy. They investigated the success of this procedure in 19 patients, aged 5–20 years, and retrieved oocytes from even the youngest patients (5- and 8-year-old girls). They concluded that patients undergoing ovarian cryopreservation could benefit from supplementary oocyte aspiration from the cortex. However being an experimental approach, it should be cautiously presented to young patients. Oktay and Oktem [7] reported the long-term follow-up and their experience with ovarian tissue cryopreservation in a series of 26 patients aged between 4 and 21 years of age, between 1999 and 2008. Their analysis suggested that tissue freezing in patients with different diseases and ages could be performed without significant risk. The study also revealed that the utilization rate of banked tissue is low, because the technology is only a decade old and because most patients undergoing ovarian cryopreservation are young and many are still undergoing treatment. Weintraub et al. [11] reported their experience on an ovarian cortex bank and presented a multidisciplinary discussion of the risks and benefits associated with ovarian tissue cryopreservation, suggesting that all girls about to receive treatment with a high risk of infertility be offered consultation on the possibility of performing ovarian cryopreservation. Anderson et al. [12] reviewed the advantages and disadvantages of ovarian tissue cryopreservation in the context of their experience of 36 women, 15 of whom were aged 16 or less. They highlighted the uncertainties surrounding the development of criteria for patient selection, the effects of chemotherapy on fertility, the most appropriate surgical techniques, and how many women will return to use their stored tissue. According to Borgstrom et al. [28], even patients with Turner Syndrome may benefit from cryopreservation as long as they exhibit essential characteristics for the recovering of follicles in the ovaries. In this study the authors involved 57 girls aged 8–19,8 with Turner Syndrome in order to evaluate which girls might benefit from ovarian tissue freezing for fertility preservation. They established five characteristics as being important for finding follicles in the ovaries of girls with Turner Syndrome: karyotype, low FSH levels, high AMH, spontaneous menarche, and spontaneous onset of puberty.

4. Conclusions

This study reports on a large number of pediatric girls undergoing ovarian tissue cryopreservation to preserve fertility. Our data show a high correlation between follicular density and age, and a decrease in follicular quality after chemotherapy. In light of these results and those reported in literature, the orientation of our group is in favour of ovarian tissue cryopreservation in pediatric patients because the procedure has a high potential of maintaining endocrine function and preserving fertility. It is well known that the optimal timing of ovarian cryopreservation is before sterilizing treatment. However, the live births obtained in adults after transplantation of ovarian tissue cryopreserved after chemotherapy and the high number of healthy follicles found in histological sections of tissue after chemotherapy in pediatric patients indicate that ovarian tissue cryopreservation could be offered after chemotherapy to young girls. In addition, the indication for fertility preservation in children can be extended to patients genetically predisposed to premature ovarian failure (e.g., Turner Syndrome), with acceptable results. Although no births have yet resulted from freeze-thawing of prepubertal ovarian cortex, expectations of this approach are encouraging, considering the high follicular pools of these patients.

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Review Article

Oocyte Cryostorage to Preserve Fertility in Oncological Patients

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Thanks to the progress in oncostatic treatments, young women affected by cancer have a fairly good chance of surviving the disease and leading a normal post-cancer life. Quite often, however, polychemiotherapy and/or radiotherapy can induce ovarian damage and significantly reduce the content of follicles and oocytes inside the ovary, thus predisposing the patient to menstrual disorders, infertility, and precocious menopause. Several techniques have been proposed to preserve fertility in these patients; among them oocyte collection and cryopreservation prior to the oncostatic treatment has been widely applied in the last decade. The proper indications, the permitting conditions, the available hormonal stimulation protocols, as well as the effectiveness and limits of this option will be discussed herein, with a comprehensive and up-to-date review of the two techniques commonly used to cryostore oocytes, the slow-freezing technique and the vitrification technique.

1. Introduction

During the past decade, fertility preservation has become an important issue in cancer patients' management. Survival rates after malignancy treatment have improved markedly, especially for young women affected by melanoma, onco-haematological diseases, and breast cancer, leading to the generation of long-term cancer survivors [1, 2]; nonetheless, this population is quite frequently affected by iatrogenic infertility and/or premature ovarian insufficiency (POI) [3]. Recent studies, in fact, showed that abdominal radiotherapy may lead to ovarian damage in a dose-dependent fashion; similarly, total body irradiation may result in POI in about 97% of cases [4]. Chemotherapy regimens, especially those involving alkylating agents, may cause acute loss of follicles within the ovary, leading to hormone deficiency and permanent infertility [5]. Psychological distress induced by the loss of reproductive perspective as well as factors related to a premature menopause (osteoporosis, cardiovascular diseases, depression, etc.) may dramatically affect survived women's quality of life. Although disease remission obviously remains the first goal of cancer treatment, patient's awareness toward safeguarding future fertility is increasing [6].

Many approaches have been considered to preserve fertility and avoid POI. Embryo cryostorage has been considered for years the only valid option, albeit this procedure is applicable only to stable couples and not to singles, and still raises a lot of ethical, legal, and practical concerns. Ovarian cortex ablation and cryostorage, with subsequent autografting, is the only strategy which can be proposed to prepubertal girls, though it is still considered an experimental approach with limited results.

Nowadays, oocyte cryostorage is considered an important tool for fertility preservation worldwide, as no surgery is required and minimally invasive ovarian stimulation protocols are available. Moreover, storing oocytes implies no concerns regarding possible cancer cells contamination, a problem that affects ovarian cortex retransplantation strategy [7]. As a result of oocyte cryostorage and *in vitro* fertilization (IVF), over 2000 babies were born in nononcological, routine IVF patients.

2. History of Oocyte Cryostorage

Since oocyte cryostorage was introduced in the mid 1980s, general interest rose around the possibility of increasing

pregnancy rates using frozen eggs, overcoming the ethical and legal concerns related to embryo freezing.

Since the very beginning oocyte freezing was quite problematic, with survival and fertility rates below 50%, and pregnancy rates as low as 1-2% [8–10]. Chen and Van Uem et al. reported the first pregnancies obtained after oocyte freezing/thawing, raising many expectations in scientific community [11, 12]. Unfortunately, many other attempts failed to reach the same result [13–15]: oocyte freezing strategy was dramatically less effective than zygote or embryo cryopreservation. Before intracytoplasmatic sperm injection (ICSI) was introduced in most IVF laboratories, the premature release of cortical granules by the frozen oocyte with the consequent irreversible thickening of the zona pellucida was able to halt sperm penetration and impair fertilization [16, 17].

Cryobiologists encountered several problems in freezing oocytes, including ice crystal formation, osmotic stress, and cryoprotectant agents (CPAs) toxicity [8, 18, 19]. Compared to other mammalian cells, human mature oocytes are constituted by a very high amount of water and have a small surface-to-volume ratio, which strongly affects cells dehydration that is essential for survival after thawing. Zona pellucida cracking, mitochondria shrinkage, and microfilaments alteration were also addressed as cryostorage-induced injuries on the human oocyte [20]. Moreover, meiotic spindle (MS) disassembly induced by cooling was clearly shown after the introduction of polarized light microscopy analysis. Many authors reported that when oocytes are exposed to low temperatures, the MS disappears from their oocytes, and reappears as a consequence of repolymerization after a few hours of incubation at thawing temperature [21, 22]. The survival of an oocyte after warming can be assessed when a bright cytoplasm surrounded by an intact zona pellucida is observed; anyway, chilling female gametes to subzero temperatures provides damages to their ultrastructure, as observed by several authors who performed electron microscope analysis: the main consequences of freezing/thawing procedures involve organelle displacement, mitochondrial disruption, vacuolization of the ooplasm, and loss of spindle polarity predisposing to an altered chromosomal alignment [23–25].

Cryobiology aims at minimizing these harmful effects on the human oocyte, and by now, two well-established laboratory protocols have been proposed and are widely diffused in the clinical practice: the slow freezing protocol and the vitrification procedure.

3. Candidates for Oocyte Cryopreservation

Fertility preservation should be discussed with all young women at high risk of POI. The most common cause of POI are ovarotoxic anticancer therapies, and cancer patients are by far the main candidates to fertility preservation. However, benign diseases like some genetic syndromes (Turner's syndrome, X-fragile carrier condition, etc.), ovarian diseases (severe endometriosis or ovarian cysts requiring ablative surgery), or autoimmune disorders requiring immunosuppressive therapy may determine POI as well.

Cancer in women in reproductive age is an increasing problem. The American Cancer Society estimates that 1/47 women will develop a cancer before the age of 39 (American Cancer Society, Inc., Surveillance Research 2011). Every year, about 200.000 new cases of breast cancer are diagnosed in USA, 15% of which occur in women under the age of 45. Most of these patients will receive adjuvant chemotherapy with alkylating drugs 4 to 6 weeks after surgery, developing a high risk of POI [26] (National Institute of Health Consensus Development Panel, 2001). Many breast cancer cases, however, are hormone-sensitive, and appropriate ovarian stimulation regimens are needed to keep low circulating estrogen levels while stimulating the ovary to cryostore oocytes [27–30]. Haematological malignancies such as lymphoma and leukemia show now quite good survival rates, and the attention is shifting toward the prevention of side effects like infertility [31].

Even nonneoplastic diseases (autoimmune disorders or benign haematologic diseases being treated with chemo- or radiotherapy) should routinely implicate the offer of oocyte cryopreservation [32, 33].

The selection of candidates for fertility preservation is crucial in order to offer the best suitable technique for each patient. Oocyte cryopreservation is probably the best technique to preserve fertility of women without an established partner or wishing to avoid ethical and legal problems associated with embryo cryostorage. When feasible, oocyte cryostorage may be preferable to ovarian tissue freezing because it does not require surgery and has already resulted in many live births [9].

The most important limiting factor for oocyte cryopreservation is age: storing oocytes in women after 40 years of age will probably result in a very poor chance to get a pregnancy in the future. Indeed, patients with a poor ovarian reserve have to be screened carefully, since they may not benefit from the treatment. Ovarian reserve assessment is crucial for patients requiring oocyte cryostorage: besides age, the antral follicle count and hormonal parameters such as FSH and anti-Mullerian hormone (AMH) have to be carefully considered. On the other side, oocyte cryopreservation is unavailable for prepubertal girls because the ovarian stimulation used to obtain oocytes needs the full maturity of the hypothalamus-pituitary-ovarian axis.

Another limiting issue is the timing of the procedure: oocyte cryopreservation requires an average of 12 days for ovarian stimulation and ovum pickup (OPU) to occur [34]. Women starting oncostatic therapy in a very short time from diagnosis or having already started chemotherapy may not benefit from oocyte cryostorage.

Oocyte cryopreservation implies a controlled ovarian hyperstimulation with exogenous gonadotropins that leads to largely supraphysiological levels of serum estradiol. Type and stage of the neoplastic disease and the patient's overall health influence the feasibility of an ovarian stimulation, and stimulation protocols must be individualized for every woman. In this perspective, the use of conventional ovarian stimulation protocols is possible only in women with estrogen-insensitive tumours, while hormone-responsive diseases require appropriate regimens [35].

4. Ovarian Stimulation Protocol

Each protocol that aims at obtaining oocytes for cryostorage should be (i) safe, with limited risk of stimulating the growth of a pre-existing neoplasia, (ii) fast, with very limited delay in starting cancer therapy, (iii) effective, with good chances of oocyte retrieval.

Ovarian stimulation requires approximately 10–14 days to achieve mature oocytes at OPU. In case of estrogen-sensitive diseases, the protocol with letrozole (5 mg/day from the second day of menstrual cycle for 5–7 days) plus gonadotropins (150–300 IU of recombinant FSH) and GnRH-antagonists [30] is one of the most recommendable: this regimen allows an acceptable oocyte yield and keeps the circulating estradiol levels rather low [36], a GnRH-agonist may be used to trigger the follicle final maturation, minimizing the risk of ovarian hyperstimulation syndrome (OHSS); moreover, letrozole or GnRH-antagonist can be readministered from the day of OPU until blood estradiol concentration falls below 50 pg/mL. In case of shortage of time, alternative regimens include to start stimulation in the luteal phase. Some women, in fact, need an urgent cancer treatment and cannot delay the beginning of the oncostatic therapies until the onset of menstruation; in this case, a GnRH-antagonist is administered to induce an abrupt luteolysis and then gonadotropins are started [37]. The egg retrieval rate is similar to the one observed using longer, conventional stimulation regimens.

A growing literature showing encouraging results of oocyte *in vitro* maturation (IVM) followed by vitrification for cryostorage is now available [38, 39]. This option consists in the possibility to retrieve immature oocytes from unstimulated preantral follicles, which are arrested in the prophase of the first meiotic division. Immature oocyte retrieval followed by *in vitro* maturation (IVM) resulted in several live births [40] and it is claimed that live births could be achieved combining oocyte IVM and vitrification. This technique is safe and theorically effective for all oncological patients, as no hormonal stimulation is needed, and it can be performed with no time restrictions [39]. The effectiveness of the procedure appears to be higher when immature oocytes are first matured *in vitro* and then frozen [41]. The potential of oocyte maturation is, in fact, reduced after vitrification [42, 43].

Overall, some data suggest that immature oocytes could be less sensible to cryodamage than mature oocytes because their nuclear apparatus is still not fully developed, and after thawing, they can be matured *in vitro* to metaphase II [56, 57]. Cryopreservation of immature oocytes should be considered in oncological patients who cannot undergo hormonal stimulation with high peak estradiol concentrations, for example, patients with breast cancer [58].

5. Slow Freezing Method

The slow freezing/rapid thawing method was the first cryostorage protocol adopted for oocytes in IVF laboratories. It was originally introduced with the aim to preserve supernumerary embryos obtained from assisted reproduction procedures [59, 60].

Oocyte freezing was initially a damping technique: rates of survival and cleavage after thawing were significantly lower than those obtained using zygote or cleavage stage embryos. The major burden of mammalian egg cryopreservation was found to be membrane permeability to cryoprotectants: after fertilization, in the zygote and in the cleavage-stage embryo, water permeability kinetics change, rendering the cells more prone to freezing [61].

The original protocol introduced for mouse embryo cryopreservation was slightly modified and adapted to human cells [62]. Small permeating molecules, like dimethylsulfoxide (DMSO) or propandiol (PROH), were adopted to allow water substitution in the intracellular compartment and were found to be useful to avoid ice crystal formation within the oocyte's cytoplasm. Sucrose-supplemented media were effective in reducing the shrinking/swelling phenomenon occurring when osmotic imbalance between the intracellular compartment and the extracellular environment is generated. Some authors [63] observed that increasing sucrose concentration from 0.1 to 0.2 M increased oocyte survival and fertilization after thawing; further raising sucrose concentration up to 0.3 M yielded even better results [64]. Sodium replacement with choline in the cryopreservation medium also obtained satisfactory results [65, 66].

Another crucial point of the oocyte freezing technique is the rate of freezing, which has to be performed under strictly controlled conditions: room temperature, as well as equilibration temperature of cryopreservation media, is able to interfere with membrane permeability to cryoprotectants, possibly affecting the oocyte survival chance [19]. Moreover, since slow freezing technique slowly dehydrates oocyte cytoplasm, a programmable freezer is required in the laboratory. The cooling rate must reach, starting from room temperature (20°C), a temperature of −7/8°C with a speed of −2°C/min. In order to prevent spontaneous ice crystal formation, at this stage the operator must perform manual seeding by touching the device where oocytes have been previously loaded (usually a plastic straw) with a nitrogen-cooled object. Subsequently, samples are cooled to −30°C at a speed rate of −0,3°C/min and then definitively frozen to −150°C at a speed rate of −50°C/min. Differently, the warming rate must be very rapid in order to prevent the recrystallization of intracellular water.

The slow freezing protocol has been considered the gold standard technique for oocyte cryopreservation for years; it is a well-established procedure with survival rates usually as high as 60–80% (Table 1) [45, 47, 48, 67, 68]. Nevertheless, some authors emphasized the detrimental effects of high sucrose concentration on oocyte cytoplasm organelles and proposed alternative freezing techniques and timing schedules [23, 52, 69].

Clinical reports on slow freezing show a pregnancy rate ranging between 13 and 20% (pregnancy/embryo transfer) (Table 1) and implantation rates still low in comparison to those observed in fresh cycles [44]. Grifo and Noyes compared slow freezing to vitrification on sibling oocytes, showing similar results in terms of survival, but higher

Table 1: Results from different oocyte cryopreservation protocols: slow freezing (high-sucrose concentration) and vitrification.

	Vitrification (VIT)	Slow freezing (SF)	Survival	Fertilization	Pregnancy	Miscarriage	Egg donation program
Chen et al., 2005 [44]	—	Yes	75% (119)	67% (80)	33% (7)	0%	Partially
Li et al., 2005 [45]	—	Yes	90% (73/81)	82% (60/73)	47% (7/15)	28% (2/7)	Partially
Kuwayama et al., 2005 [46]	Yes	—	91% (58/64)	81% (52/64)	41% (12/29)	17% (2/12)	No
Borini et al., 2006 [47]	—	Yes	43,4% (306/705)	51,6% (158/306)	19,2% (14/73)	28,6% (4/14)	No
Barritt et al., 2007 [48]	—	Yes	86,1% (68/79)	89,7% (61/68)	75% (3/4)	NS	Yes
Lucena et al., 2006 [49]	Yes	—	96,7% (143)	87,2% (105)	56,5% (13)	NS	Yes
Antinori et al., 2007 [50]	Yes	—	99,4% (328/330)	92,9% (305/328)	32,5% (39/120)	20,5% (8/39)	No
Cobo et al., 2008 [51]	Yes	—	96,9% (224/231)	76,3% (171/224)	65,2% (15/23)	20% (3/15)	Yes
Parmegiani et al., 2008 [52]	—	Yes	75,1% (328/437)	80% (227/328)	19% (16/83)	31,2% (5/16)	No
Cao et al., 2009 [42]	Yes	Yes	SF 61% (75/123)	SF 61,3% (46/75)	ND	ND	No
			VIT 91,8% (268/292)	VIT 67,9% (182/268)	ND	ND	No
Smith et al., 2010 [53]	Yes	Yes	SF 65% (155/238)	SF 67% (104/155)	SF 13% (4/30)	SF 25% (1/4)	No
			VIT 75% (260/349)	VIT 77% (200/260)	VIT 38% (18/48)	VIT 18% (4/18)	No
Rienzi et al., 2010 [54]	Yes	—	97% (120/124)	79,2% (95/120)	30,8% (15/39)	20% (3/15)	No
Cobo et al., 2010 [55]	Yes	—	92,5% (3039)	73,3% (NS)	55,4% (148)	NS	Yes

NS = Data not reported.
ND = Data not calculated, not a study endpoint.

fertilization and blastocyst formation rates using the former [67].

In standard IVF procedures, cryopreserving oocytes combines the chances to achieve a pregnancy by both fresh and thawing cycles, thus yielding a rather high cumulative pregnancy rate [47].

6. Vitrification Method

Early studies on oocyte vitrification were performed at the end of 1980s, when the first attempts on mouse or hamster eggs were reported [70, 71]. The introduction of oocyte vitrification in human IVF is a relatively recent phenomenon [46, 54, 72].

The scientific basis of vitrification consists in the ultrarapid freezing of cells, whose intra- and extracellular aqueous environment turns into a glassy-like state. Vitrification combines two different biophysical processes: a preliminary equilibration step, in which oocytes are exposed to low concentrations of cryoprotectants to allow water outflow, and a subsequent vitrification phase in which cells undergo a high osmotic gradient that completes cells dehydration. Under these conditions, oocytes can be directly merged into liquid nitrogen and then stored. Similarly, warming of oocytes must be rapid in order to avoid recrystallization of water.

The cryoprotectants used during vitrification are the same employed for slow freezing, but they are three-to-four-folds more concentrated in vitrificaton than in slow freezing. DMSO, PROH, and ethylene glycol (EG) (5-6 M) as well as sucrose (1 M) are currently used, though their toxicity is still under evaluation [73].

Appropriate carriers for freezing oocytes are also very important. Successful vitrification occurs when samples are loaded in a minimal fluid volume and then frozen/thawed at an extremely fast rate (1500–2000°C/min). Open systems guarantee direct contact with liquid nitrogen [74, 75]: open-pulled straws, cryo-tops and cryo-loops, cryo-leafs, electron microscopy grids, and many other devices were tested in the last years [39, 46, 76, 77]. All open systems raise some concerns about the possible viral contamination of stored material, either from nitrogen or from cross-contamination among samples [73]: strategies to avoid this risk include the formulation of high-security closed devices, exposure to nitrogen vapors, and nitrogen ultraviolet (UV) sterilization [55, 78].

Although no cross-contamination between liquid nitrogen and stored oocytes has been signalled to date, closed systems may provide the safer and probably most effective vitrification procedure. In particular, many carriers have been approved by FDA in the last years, and several of them are now commercially available: Cryotip (Irvine Scientific, CA, USA), high-security vitrification (HSV) straw

(Cryo BioSystem, Paris, France), VitriSafe (VitriMed, Austria), Cryopette (Origio, Denmark), and Rapid-i (Vitrolife Sweden AB) [79]. DNA integrity assessed on warmed mouse oocytes is comparable in open versus closed vitrification systems; anyway, there is still an ongoing debate whether closed or open vitrification carriers provide the best results in terms of fertilization and cleavage rates [80]. On the other hand, there is wide agreement in considering vitrification an operator-dependent procedure.

Oocyte survival after vitrification reaches 90% in several reports (Table 1) [38, 42, 46, 49, 50, 54, 81]. Oocyte quality seems to be poorly affected by chilling injury: spindle repolymerization occurs within one hour after warming, suggesting that the ultrastructure of these gametes is better preserved by vitrification rather than slow freezing [82]; moreover, the metabolomic profiling of vitrified oocytes was found to be comparable to the one of fresh eggs [83, 84].

Data on the clinical use of vitrified eggs in routine IVF show that pregnancy rates can be comparable to those achieved with fresh oocytes (Table 1) [9, 54, 85]. Studies aimed to compare vitrification and slow freezing reported implantation and pregnancy rates trendly higher with vitrification, although the number of observed cases overall is still too low to draw final conclusions [42, 51, 53, 81, 86].

7. Conclusions

Cryostoring oocytes is an effective method to preserve fertility in postpubertal young women at risk of POI. In the last years, significant improvements in the clinical effectiveness of oocyte freezing/thawing techniques have been achieved using both slow freezing method and vitrification. The available trials comparing these two different approaches are still insufficient to establish the superiority of one over the other, but the growing interest of scientist and the increasing awareness of women about the possibility of storing oocytes will likely lead to the development of an optimal protocol for oocyte storage in the next few years.

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Review Article

Multiple Approaches for Individualized Fertility Protective Therapy in Cancer Patients

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In the last decade, fertility preservation has risen as a major field of interest, creating new interactions between oncologists and gynecologists. Various options, such as cryopreservation of ovarian tissue, have been developed and are currently routinely proposed in many centers. However, many of the options remain experimental and should be offered to patients only after adequate counseling. This paper addresses the efficiency and the potential of the different fertility preservation approaches.

1. Introduction

Concurrent with the progress over the last few decades in oncology, the concept of fertility preservation has gained attention, driving new research endeavors. In the UK, approximately one out of every 10 cancers occurs in adults aged 15 to 49 years (Cancer Research UK statistics) and currently, one out of every 715 adults is a survivor of childhood cancer [1].

The new approaches to fertility preservation include cryopreservation and transplantation of ovarian tissue, follicular culture, pharmacological protection, or adaptation of an ovarian stimulation protocol for in vitro fertilization (IVF) treatment. The choice of an appropriate method is based mainly on whether a delay in treatment is required, the type of the disease, and the treatment the patient has. Importantly, chemotherapy regimens differ widely in their effects on the gonads. For example, chemotherapy regimens such as vincristine and fluorouracil carry a small risk, whereas other regimens, such as conditioning of the bone marrow for transplantation with busulfan, induced premature ovarian failure in more than 90% of the cases studied, even during childhood [2, 3].

To address the question of fertility preservation management, oncology centers must build a close collaboration with fertility units. In turn, the fertility units themselves must be able to manage these patients with minimal delay and offer the optimal option for each case. Although many centers throughout the world have developed strategies to propose fertility preservation to their patients, increased efforts must be made to inform and to offer adequate advice to the patients regarding their risk of future infertility and the option of fertility preservation. A recent review reported that from 34% to 72% of cancer survivors recalled being counseled by a health provider regarding the impact of cancer treatments on fertility [4]. In addition to the fear of losing their fertility, cancer evokes the possibility of death leading to emotional and psychological distress. While addressing the issue of fertility preservation, patients are encouraged to think about their future, which may help them focus their energies in a positive direction.

This paper aims to discuss the major concerns regarding the risk of premature ovarian failure after cancer therapy and the options for preserving fertility in these patients.

2. Effect of Cancer Treatment on Fertility

Human females are born with a fixed and nonrenewable number of primordial oocytes that represent the so-called ovarian reserve. Histological studies and mathematical models have determined that approximately 1,000,000 oocytes are present at birth. At menarche, the number of primordial oocytes has decreased to approximately 180,000 and this number continues to decline over time with fewer than 1000 oocytes remaining at menopause [5]. Oocyte growth, differentiation, and apoptosis are strictly regulated through autocrine and endocrine loops, with molecular mechanisms that have recently been elucidated in the Nos3-knockout mouse model [6, 7]. The pituitary gland produces FSH and LH in response to LHRH released from the hypothalamus, whereas estrogen and progesterone produced by the granulosa and theca cells, respectively, induce a negative feedback on LH and FSH production.

Through the partial or total destruction of the ovarian reserve, cancer treatments may temporarily or definitively affect the ovarian function. The most frequent neoplasms of the reproductive age are breast cancer, malignant lymphomas, malignant melanomas, and gynecological cancers, with an overall incidence of 82.7 cases per 100,000 [8]. Surgery, radiotherapy, and chemotherapy, together with recently isolated target-oriented molecules, have significantly improved the prognosis of young cancer patients. Thus, quality-of-life issues have become a high priority on the patients' agenda. Premature menopause and irreversible sterility are the most dramatic outcomes of ovarian dysfunction; however, infertility and low estrogen levels are associated with an impaired quality of life and severe psychological consequences. Thus, infertility and premature menopause are relevant issues for young women with cancer and may also influence their treatment compliance [9].

The mechanism via which chemotherapy impairs ovarian function has not been completely elucidated. It has been established that drugs have varying effects on ovarian function, with alkylating agents being the most toxic. Table 1 represents the degree of ovarian toxicity of specific drugs used for the treatment of cancer during reproductive ages. As expected, the total dose is directly correlated to the ovarian dysfunction [10]. Genetic polymorphism within the metabolic pathway of cyclophosphamide activation accounts in part for the different toxicities observed in different individuals [11]. The patients' age is another variable that accounts for the probability of ovarian dysfunction after chemotherapy. Young patients have a higher absolute number of primordial oocytes and have a lower rate of ovarian toxicity after chemotherapy. Nonetheless, young patients face a sharp reduction of their ovarian reserve after toxic chemotherapy, and it has been demonstrated that menopause occurs earlier in cancer survivors who have received chemotherapy in early adulthood [12, 13]. Table 2 summarizes the rate of chemotherapy-induced amenorrhea, segregated by different regimens used in breast cancer, according to the patients' age.

Table 1: Risk of ovarian toxicity of antineoplastic drugs.

High risk	Cyclophosphamide, ifosfamide, busulfan, mechlorethamine, melphalan
Intermediate risk	Doxorubicin, epirubicin, cisplatin, docetaxel, paclitaxel
Low risk	Vincristine, methotrexate, 5-fluorouracil, trastuzumab

Table 2: Rate of chemotherapy-induced amenorrhea, according to regimen and age.

Regimen	Age <30 yrs	Age 30–40 yrs	Age >40 yrs
No treatment	1%	< 5%	20–25%
Ac x4	—	13%	60%
CMF x6	19%	35%	85%
CAF/CEF x6	30%	—	85%
AC x4->Dtax	—	55%	—

AC: doxorubicin/cyclophosphamide,
CMF: cyclophosphamide/methotrexate/5-fluorouracil,
CAF: cyclophosphamide/doxorubicin/5-fluorouracil,
CEF: cyclophosphamide/epirubicin/5-fluorouracil,
Dtax: docetaxel.

The targets of chemotherapy-induced ovarian toxicity include primordial oocytes, granulosa cells, and ovarian stroma. Familiari et al. demonstrated the direct destruction of primordial oocytes and follicular depletion in ovaries of young patients treated with regimens containing alkylating agents for Hodgkin's lymphoma [14]. Meirow et al. described a similar situation in women who were exposed to nonsterilizing doses of chemotherapy [15]. Oktem and Oktay used an ovarian xenograft model to characterize the impact of chemotherapy on human primordial follicles. They reported apoptosis of primordial oocytes and growing follicles 12 and 24 hours after cyclophosphamide exposure, with a >90% reduction of follicle density 48 hours after treatment [16].

Meirow et al. also reported significant vascular damage in histological sections of human ovaries exposed to chemotherapy. They described thickening and hyalinization of cortical vessels, cortical proliferation of small vessels, and focal cortical fibrosis with segmental collagen deposition [15]. These alterations of the normal ovarian anatomy could be a consequence of drug-induced endothelial damage, followed by subsequent neovascularization. Another potential mechanism of primordial oocyte loss is explained by the follicular burnout theory. When exposed to chemotherapy, growing follicles are destroyed, and therefore the amount of inhibitory paracrine factors, in particular anti-Müllerian hormone (AMH), is reduced. These changes induce continuous and sustained recruitment of primordial follicles, thus, "burning out" the ovarian reserve. To date, there is no proof of this theory in humans, although AMH-knockout mice have an increased activation of primordial follicles with a greater number of atretic large follicles and a reduced ovarian reserve [17].

Little information regarding the ovarian toxicity of newly available drugs for the treatment of young women with neoplasms is available. For example, trastuzumab is a humanized IgG1 monoclonal antibody that recognizes the extracellular domain of c-erbB2 receptor. Although c-erbB2 is involved in many different cellular processes, no ovarian toxicity has been reported even after prolonged use [18]. Conversely, monoclonal antibodies targeting VEGF are able to impair the normal ovulation process in which angiogenesis plays an important role; however, the data regarding this affect in humans are not available. Imatinib is a small molecule that specifically targets c-Abl and c-Kit in chronic myeloid leukemia (CML) and gastrointestinal stromal tumors (GISTs), respectively. This drug has substantially improved the prognosis of patients affected by CML and GIST and has been proposed as a gonad protector for patients exposed to chemotherapy. In experimental models, DNA damage induces TAp63 phosphorylation via c-Abl, with subsequent cell death. Imatinib inhibits TAp63 phosphorylation in primordial oocytes, thus the oocytes are protected from apoptosis [19]. Inhibitors of m-TOR (mammalian target of rapamycin) have been used for the treatment of kidney cancer and more recently for the treatment of neuroendocrine tumors. In men, m-TOR inhibitors induce severe but reversible azoospermia; however, no data are available for the effects of m-TOR inhibitors on women [20].

In parallel with the development of a fertility preservation program, the evaluation of the gonadotoxicity of all new therapeutic regimens should be systematically taken into consideration to adequately inform and manage young patients who are particularly concerned regarding their future fertility.

3. Obstetrical Outcome of Cancer Survivors

In addition to difficulties with conception due to damage of the ovaries, long-term female cancer survivors may also face specific obstetrical risks. Additionally, concerns have emerged regarding possible health problems in the offspring.

Adverse events such as miscarriages, preterm delivery, neonatal low-birth weight, and postpartum hemorrhage may occur more frequently in cancer survivors than in the general population, depending on the age at treatment and of the type, dosage, and schedule of the anticancer treatment [21–23].

While chemotherapy has a major impact on ovarian function, it rarely directly affects pregnancy outcomes [22]. However, the long-term side effects of some chemotherapy regimens may increase the risk of adverse obstetrical and perinatal outcomes. For example, patients treated with anthracycline or >30 Gy of mediastinal radiation may suffer from peripartum cardiomyopathy and should be monitored for cardiac status before pregnancy. However, in a cohort of 53 childhood cancer survivors, none developed anthracycline-induced clinical heart failure, thus the risk of this complication appears to be low [24].

Although chemotherapy does not affect uterine function, radiotherapy results in different degrees of uterine damage depending on the total dose and the site of irradiation. When administered in children, pelvic or total body irradiation (TBI) may result in impaired uterine development. The data from the Childhood Cancer Survivor Study, based on more than 2,000 children born from cancer survivors, showed an overall increased risks for preterm births (21.1% versus 12.6% for the children born from a random sample of the cancer survivors' nearest-age full sibling included in the study as controls; OR = 1.9; 95% CI = 1.4 to 2.4; $P < 0.0001$). In this study, the risk differed depending on the type and dose of anticancer treatment (i.e., alkylating, nonalkylating, or radiotherapy treatment) [21]. The obstetrical risk is particularly high for patients subjected to pelvic radiation of >500 cGy; preterm delivery approaches 50% compared with 19.6% for those who did not receive any radiation treatment (OR 3.5, 95% CI = 1.5 to 8, $P < 0.003$). Additionally, these babies had lower birth weights (36.2% versus 7,6%, OR = 6.8, 95% CI = 2.1 to 22.2: $P < 0.0001$) and a higher percentage were small for gestational age (18,2% versus 7,8% OR = 4, 95% CI = 2.1 to 22.2, $P < 0.001$). Furthermore, even patients exposed to lower doses of uterine radiotherapy have a risk for preterm delivery (from 50 cGy) and low birth weight (from 250 cGy). These data were corroborated by Green (2009) on more than 4,000 pregnancies from the same database after inclusion of a larger number of cancer survivors, underlying the lack of evidence of increased risks of simple malformations, cytogenetic syndromes, or single-gene defects in the offspring [22].

Additionally, an increased risk of hypertension complicating pregnancy, fetal malposition [25], and post-partum hemorrhage [23] has been reported in patients receiving direct uterine radiotherapy, as observed with Wilm's tumor patients.

Treatment via estrogen supplements may be helpful, as it has been demonstrated that in women treated with total body irradiation, hormone replacement therapy significantly increases uterine volume and endometrial thickness as well as reestablishing uterine blood flow [26]. Unfortunately, established hormonal replacement therapy is not as effective for prepubertal childhood cancer survivors in rescuing their uterine function.

Thus, when counseling a patient who desires to conceive after anticancer therapy, evaluation of the type and total dose of treatment received and the age at treatment should be considered to estimate the obstetrical risks, with special concern for patients treated with radiation.

4. Cryopreservation of Ovarian Tissue

Cryopreservation of the ovarian cortex is one of the principal techniques utilized for preserving fertility. Indeed, this established technique has recently been identified as highly promising for this indication.

The first studies on cryopreservation of the ovarian cortex were performed on rats in the 1950s [27]. In 1960, Parrott was the first to obtain live young from a mouse after ovary removal, cryopreservation, and reimplantation. However, she noted a large decrease in the number of viable oocytes upon necropsy. This work involved the first pregnancies and live births following the freezing of oocytes

[28]. Paradoxically, despite this positive result, this work also marked the end of research on this subject for a considerable period. It was not until 1990 that interest in the freezing of ovarian tissue reemerged, with the work of Carroll on the freezing of primary follicles from mice [29]. The protocol used by Carroll, based on a gradual decrease in temperature, was similar to the protocols used for the freezing of embryos, with dimethylsulfoxide used as a cryoprotective agent. In mice, this technique allowed the survival of oocytes (80%) and of the surrounding somatic cells (65%). Offspring were obtained after freezing isolated follicles, thawing, and transplantation to the ovarian bursa of host animals [30].

The same freezing protocol has been used for fragments of ovary from ewes, leading to the birth of a lamb after an autologous transplantation [31].

The first work on cryopreservation of the human ovarian cortex was published in 1996. The various freezing protocols tested were compared with that described by Carroll et al. [29]. Hovatta provided the first demonstration that the freezing of human ovarian cortex was feasible [32]. After thawing and histological analysis, freezing and thawing were found to have had no effect on the number of follicles, follicle diameter, or oocyte diameter, regardless of the cryoprotectant agent used (DMSO- or PROH-sucrose). In the same year, Newton et al. compared the effects of four cryoprotectant agents (DMSO, ethylene glycol (EG), PROH, and glycerol) on the survival of human ovarian tissue after freezing. Follicular survival rates were the highest with EG (84%) and the lowest with glycerol (10%), with those for DMSO (74%) and for PROH (44%) falling between these values [33]. These studies also showed that freezing protocols similar to those used to obtain a live lamb from an ewe—a large mammal in which ovary organization resembles that in women—through freezing/thawing and the autologous transplantation of ovarian tissues were transposable to humans.

Thus, in the mid-1990s the idea first emerged from preserving the fertility of patients or from creating oocyte banks through freezing ovarian tissue to treat infertility [34]. It became clear that cryopreservation of the ovarian cortex was potentially useful for patients having to undergo highly gonadotoxic treatments because it would be possible to preserve a large number of primordial follicles. Of note, the first reported case of ovarian cortex cryopreservation in a patient was published in 1996 [35].

Cryopreservation of the ovarian cortex has since become an integral part of fertility preservation, and results from several cohort studies have been published [36–39].

Indications. Ovarian cryopreservation involves the removal of all or part of one of the two ovaries and thus, the excision of a substantial proportion of the follicular capital of the patient. Therefore, cryopreservation should only be offered to patients undergoing highly gonadotoxic treatments. The various treatments have been classified according to their toxicity to the ovaries [40, 41]. The age of the patient must also be taken into account, as the impact on the ovarian reserve is lower in younger patients. It would therefore be justified to offer ovarian tissue cryopreservation in three situations in which it is almost certain that ovarian function will be altered at the end of treatment: chemotherapy with high doses of alkylating agents (such as busulfan or cyclophosphamide); total body irradiation or abdominal radiation; bilateral ovariectomy or unilateral ovariectomy of a single-remaining ovary. Ovary cryopreservation may also be offered in cases of disease associated with premature ovary failure, such as Turner syndrome [42, 43].

This technique has many advantages. In addition to allowing the preservation of a large number of immature oocytes within primordial follicles, this technique does not require ovarian stimulation and can be organized very rapidly without the need to defer treatment of the disease. It is available to patients regardless of their marital status and is the only possible technique for fertility preservation in prepubescent girls [2, 36]. By contrast, an upper age limit of 35 to 37 years is recommended for the cryopreservation of ovarian tissue [44]. The risks of ovary tissue harvesting appear to be small: only a single patient was reported to have required surgical revision among the 500 cases of ovary cryopreservation described by the FertiPROTEKT network [44].

In practice, ovarian tissue is usually obtained via laparoscopy. However, it may be collected via laparotomy, particularly from patients treated for neuroblastoma. In these cases, the ovary can be harvested during resection of the residual tumor, a procedure scheduled prior to high-dose chemotherapy and the autologous transplantation of hematopoietic stem cells. The quantity of tissue removed is variable; part or all of an ovary may be removed or ovarian tissue may be excised via biopsy [45].

The operating theater is rarely adjacent to the laboratory in which the ovarian tissue is frozen and stored. Therefore, the ovary must be transported in optimal conditions to the site of freezing immediately after its removal. Most authors recommend that transport times should be kept as short as possible. However, a series of five pregnancies following the autologous transplantation of frozen and thawed ovarian tissue has been reported, including two cases in which the fragments of ovarian tissue were frozen only after transport periods of four to five hours [46].

The penetration of cryoprotectant agents into the tissues is limited. Isolation of the ovarian cortex through the removal of the medulla is therefore important, to reduce the thickness of the ovarian tissue, thereby optimizing follicular survival [47]. After a period of equilibration with a cryoprotective agent, the ovarian fragments are frozen in a slow-freezing protocol. Indeed, all the pregnancies achieved to date have involved the slow freezing of ovarian tissue.

Two major developments are currently the subjects of a heated debate: the vitrification of ovarian tissue fragments [48, 49] and the freezing of the entire ovary [50, 51]. It has recently been shown that the ultrastructure of human follicles is conserved during vitrification in a closed system based on cryotubes [52]. Freezing of the whole ovary has been proposed as a solution for limiting the phenomenon of follicular loss due to ischemia during ovarian cortex

fragment revascularization following autologous transplantation. Most relevant studies have been performed in the ewe, despite the ovary being smaller in lambs and differences in the anatomy of the vascular pedicle. The technique involves the perfusion of ovarian vessels with freezing solution, then placing the ovary in a tube containing a similar solution. The efficacy of this technique was demonstrated for the first time in 2004. The transplantation of an entire ovary that had been frozen in DMSO allowed the revascularization of the ovary using microsurgical reanastomosis in five of eight ewes (62.5%). Normal estrus cycles were reestablished in three of the ewes, as demonstrated via progesterone levels [51]. Oocytes have been obtained from frozen whole ovaries in ewes [53], although this procedure is inefficient (after autologous transplantation only, 25% of the ovaries were functional), and a single birth has been achieved using this method [54].

Only few studies have reported the freezing of an entire human ovary. No significant difference in follicular viability between whole frozen ovaries and frozen ovary fragments was observed [50]. These results have since been confirmed by Martinez-Madrid et al. [55].

Finally, ovary harvesting could also make it possible to develop additional techniques for preserving fertility. Indeed, the freezing of ovarian tissue may be combined with the removal, via puncture, of small antrum follicles that may be present in the ovary at the time of harvesting. This makes it possible (1) to freeze ovary tissue and isolated immature oocytes [42]; (2) to freeze isolated oocytes present in the dissection medium when ovary fragments are generated [56]; or (3) to freeze pre-antral follicles present in the medulla, where they may be highly abundant [57].

5. Transplantation of Ovarian Tissue

At present, ovarian tissue grafting is the only option for potentially restoring ovarian function and fertility after cryopreservation and storage of ovarian tissue. Different groups throughout the world have published the results of successful transplantation procedures leading to the reactivation of the ovarian endocrine activity and ovulation [58].

Despite these promising results, the technique should not be considered a routine clinical procedure, as there have been fewer than 20 babies born through this method in the last decade [59]. It is difficult to perform statistical analysis on the efficiency of the procedure because a limited number of patients underwent ovarian autotransplantation compared with the number of patients who cryopreserved their ovarian tissue. A pregnancy rate per transplantation ranging from 20% to 30% appears to be realistic, although the delivery rate may be lower [59–61].

Different protocols have been applied to perform the grafting, with a wide variability concerning the site of the transplantation [62]. The ovarian tissue is able to restart folliculogenesis up to ovulation when grafted either to an orthotopic site (ovarian fossa, remaining ovary) or to a heterotopic site (e.g., peritoneal abdominal wall, uterine serosa, subcutaneously on the abdominal wall or in the forearm). Despite the fact that ovulation, egg retrieval, in vitro fertilization, and early-stage embryo development can occur after heterotopic transplantation and lead to biochemical pregnancy, ongoing pregnancy and delivery have been obtained only after orthotopic graft at the ovarian site [63–66]. After the transplant, variability has been observed in the time needed to restore ovarian activity and in the lifespan of the graft. Follicular development generally occurs in 4 to 5 months because at least 120 days are necessary to initiate follicular growth, but it has been observed to occur in a wide window between 8 and 26 weeks [60]. The graft can be functional from 3 to 4 months up to more than five years (personal data). Several factors may explain these important clinical variations, such as the original follicle density in the ovarian tissue, the amount of tissue transplanted, the age of the patient at the time of cryopreservation, the freezing/thawing technique, the ischemic injury at the time of the graft before neoangiogenesis occurs, and the hormonal environment [62].

Based on these observations, various strategies have been suggested to optimize the function of the transplant, such as graft of isolated follicles, extremely thin ovarian fragments [67, 68], or pretreatment of the host and graft with hormones, vitamins, and growth factors [69, 70]. Moreover, orthotopic and heterotopic transplant of the whole ovary with vascular anastomosis of the pedicle vessels was performed successfully in humans with fresh organs in cases of pelvic irradiation or allotransplantation of monozygotic twins discordant for ovarian failure [71–74], but further investigations on cryopreservation of the entire ovary are needed in both humans and animal models.

A major concern when transplanting ovarian tissue to a patient treated for oncological disease is the possible risk of reintroduction of cancer cells within the graft. This risk is different depending on the type and stage of the original cancer. Different authors have classified pathology as low, intermediate, and high risks of recurrence in cases of transplant to drive their decision; for example considering Hodgkin's lymphoma as low risk, breast cancer as intermediate risk, and leukemia as high risk [75].

As transplantation is the only option available at present to restore fertility using cryopreserved ovarian tissue, each patient should be clearly informed regarding the safety issue and the limitations of ovarian tissue transplantation procedures at the time of the fertility preservation counseling.

6. In Vitro Maturation and Oocyte Vitrification

Vitrification of oocyte and embryos is a well-established procedure and is currently performed in many IVF laboratories with a high success rate. This procedure should be considered as a first option for fertility preservation, before gonadotoxic treatment, when feasible. However, stimulation protocols for oocyte or embryo freezing in cancer patients present two main safety issues.

The first concern is the exposure to superphysiological estrogen levels, which may be 10–20 times more than those observed in natural cycles [76, 77]. Although the effect of

a temporary increase in estradiol levels on the risk of the recurrence of cancer is uncertain, it remains a concern in cases of hormone-dependent tumors [78, 79].

The second concern is that the duration of the treatment may delay the start of the chemotherapy. Due to the risks inherent in the malignant disease, fertility preservation cannot postpone the treatment course.

Recently, emergency procedure of random start-controlled ovarian stimulation in the late follicular phase or in the luteal phase have been reported with success, significantly shortening the time required to undergone oocyte or embryo cryopreservation before chemotherapy [80]. Furthermore, this treatment can be associated with letrozole administration to reduce the effect of temporary increase of the oestradiol level in the case of hormone sensitive tumor [81].

Another strategy to avoid those safety issues is the use of in vitro maturation (IVM) of oocytes and vitrification.

6.1. Cryopreservation of Oocytes. During cryopreservation procedure, three major mechanisms may potentially damage the cell, including chilling injury (+15 to −5°C), ice crystal formation (−5 to −80°C), and fracture damage occurring from solidified fluid within the cell (−50 to −150°C). During warming stages, the cell is subjected to the same injuries in reverse order [82]. Two techniques are used in assisted reproduction laboratories for cryofreezing; controlled slow freezing (SF) and, more recently, an ultrarapid cooling method also known as vitrification. Both involve the use of cryoprotectants to minimize ice-crystal formation [82].

Specific characteristics unique to oocytes make them more susceptible to injuries induced by slow freezing; these characteristics include their size, shape, and water content. These injuries result in spindle and cytoskeleton damage, crystal formation, and zonal hardening. Those changes explain the lower fertilization and implantation rates observed with frozen-thawed oocytes [83].

Vitrification was introduced as a means to protect against the ice-crystal formation that has been observed with slow cooling. The tissue is submerged directly into liquid nitrogen after being treated with high concentration of cryoprotectant, and freezing occurs without allowing crystal formation [84]. Recently, the use of low concentrations of the cryoprotectants offsets their observed toxicity towards oocytes [85]. Furthermore, ICSI and improvements in culture media have helped overcome other side effects of vitrification, such as zonal cracks and hardening. Thus, a steady increase in oocyte survival, fertilization, and pregnancy rates has been observed. Pregnancy rates after oocyte vitrification have been reported in the 10–30% range [86, 87]. In a meta-analysis by Cobo and Diaz in 2011, vitrification methods demonstrated a higher fertilization rate, cleavage rate, and optimal quality rate of the embryo compared with slow freezing [88]. At the MUHC Reproductive Center, we reported a mean oocyte survival rate of 81% after thawing, a 76% fertilization rate per oocyte, a clinical-pregnancy rate per cycle of 45%, a live-birth rate of 40%, and the birth of 22 healthy babies [89]. By 2009, nearly 1,000 live births were reported from oocyte cryopreservation with an incidence of congenital abnormalities in these children of 1.3%, similar to that observed in the general ART population [90].

6.2. In Vitro Maturation of Oocytes. Due to a concern regarding hormonal sensitivity, gonadotropin treatment could be avoided via the collection of immature oocytes in unstimulated cycles for fertility preservation [91, 92]. This procedure results in the successful preservation of fertility with no delay in the administration of chemotherapy, no surgery, and a lack of the necessity of gonadotropin stimulation.

The oocyte collection is performed 38 hours after hCG priming, ideally when the largest follicle has reached 10–12 mm [93, 94]. If there is no sufficient time prior to chemotherapy for conventional follicular phase oocyte retrieval in a stimulated or unstimulated cycle, retrieval in the luteal phase could be considered [95, 96]. After collection, the oocyte maturation status is assessed; immature oocytes are cultured in IVM medium and periodically assessed for maturation status as previously described [93]. When the female patient does not have a stable partner and does not wish to use donor sperm to create embryos, she can opt for oocyte cryopreservation. However, vitrification of oocytes matured in vitro remains in the experimental phase, as only a 20% live-birth rate per cycle from vitrified of IVM oocytes has been reported [89] due to the significantly lower survival and fertilization rates compared with oocytes collected following ovarian stimulation [94]. Notably, these studies demonstrated that vitrification of in vitro matured oocytes collected from unstimulated cycles followed by later thawing and fertilization can result in successful pregnancies and live births. In 2009, we published a report of the first 4 live births achieved after vitrification and thawing of in vitro matured oocytes [86].

Although it was expected that smaller immature oocytes would have an increased survival rate when undergoing the cryopreservation procedure, the potential of oocyte maturation is reduced by the vitrification at the GV stage [97]. Therefore, immature oocytes are currently matured in vitro prior to vitrification.

After thawing, ICSI was routinely performed 2–4 hours after polar body extrusion due to a theoretical risk of zonal hardening during the in vitro culture period [98].

The main disadvantage of IVM concerns lower pregnancy rates when compared with conventional IVF due to lower implantation rates [99]. This has been overcome through transferring one embryo more than in IVF, which allows comparable pregnancy rates to those of IVF to be attained (McGill, unpublished data).

Safety issues of IVM have been evaluated in several studies. Buckett et al. compared congenital anomalies in 432 patients undergoing IVF (217), IVM (55), and ICSI (160) with non-ART-conceived children [100]. There was no increase in congenital anomalies after IVM when compared with spontaneously conceived children. A recent retrospective study also observed no increase in chromosomal abnormalities in 6 children born after IVM when compared with 30 children born after classical IVF [101]. Those initial reports are encouraging, though few in numbers. Thus, the safety of IVM must be confirmed with larger-scale studies.

To date, the MUHC Reproductive Center has provided fertility preservation to 183 patients with breast, hematological, brain, soft-tissue, colorectal, and gynecological cancers. Of these, 128 patients underwent oocyte retrieval without ovarian stimulation followed by IVM.

7. Pharmacological Protection

Pharmacological protection of the gonads during chemotherapy constitutes an attractive option to preserve fertility. It may allow the restoration of normal ovarian function and natural fertility after treatment and may therefore save the patients from adverse events related to premature ovarian failure.

The gonadotropin-releasing hormone agonist (GnRHa) is a decapeptide derived from the native hormone; however, this synthetic hormone binds to specific pituitary receptors with a higher affinity. The sustained-release of GnRHa initially stimulates the release of gonadotropins, inducing a brief ovarian hyperstimulation, commonly known as the flare-up response. After 10–15 days, pituitary GnRH receptors are downregulated and the inhibitory effect of the synthetic hormone on ovarian function is observed. During treatment, the hormonal profile is characterized by low gonadotropin and estradiol levels. This inhibitory effect may protect the ovarian function during chemotherapy through different mechanisms, such as maintaining the follicles at the primordial/primary stages, decreasing sensitivity to gonadotoxic treatment, or through the reduction of the blood supply.

The effectiveness of the treatment was first reported in rats and monkeys in the 1980s. Administration of GnRha during chemotherapy has proven to reduce follicular depletion in rats treated with cyclophosphamide [102, 103]. Experiments using monkeys demonstrated that 65% of the primordial follicular pool is destroyed after cyclophosphamide treatment compared with 29% with Gn-Rha cotreatment [104]. Although this protective effect in animals has been supported by another study [105], this effect has been reported as limited or dose dependent [106] and not sufficient to protect fecundity by others [107]. The efficiency of GnRh antagonist in inducing an immediate ovarian suppression through competitively blocking GnRh receptor in the pituitary has also been investigated with divergent conclusions according to the published reports [108–110].

In humans, the efficiency of GnRha in preventing premature ovarian failure was controversial for years and is still debated. Nonrandomized studies suggested a reduction of premature ovarian failure rate when GnRha was administered concomitantly to the chemotherapy [111, 112]. However, the methodology of these studies was criticized, thus calling the results into question [113, 114]. Waxman et al. first reported the results of a prospective, randomized study on a cohort of 18 patients and failed to demonstrate any significant protective effect of GnRHa treatment after two years of followup [115]. In a cohort of 80 randomized patients treated for breast cancer, Badawy et al. reported a reduction in premature ovarian failure rate with GnRha [116]. However, mean FSH values were lower than 20 IU/L in both groups and FSH value defining premature ovarian failure was not indicated. Furthermore, the data were collected after less than 1 year of followup.

Recently, GnRha cotreatment failed to prevent premature ovarian failure in a small cohort of patients with Hodgkin's lymphoma treated with a regimen of escalated BEACOPP (bleomycin, etoposide, adriamycin, cyclophosphamide, vincristine, procarbazine, and prednisolone) [117]. The ZORO study reported no significant differences in the restoration of the spontaneous cycle and hormonal profile after GnRha co-treatment compared with control in patients up to 45 years of age treated for breast cancer [118]. However, possible biases were highlighted regarding the results of this study [119]. A recent meta-analysis suggests a potential benefit of GnRha treatment during chemotherapy on spontaneous resumption of menses and ovulation, but not on pregnancy rate. The authors of this meta-analysis also moderated their conclusion by listing the risk of bias in the published trials assessing this issue [120]. The largest randomized study of 281 early breast cancer patients was recently published by del Mastro et al. (2011), showing a reduction in the occurrence of early menopause in the group of patients with breast cancer treated with GnRha during chemotherapy after 1 year of follow-up [121]. In contrast, the efficiency of triptorelin to prevent premature ovarian failure after 1 year of follow-up was not observed in a cohort of 80 randomized young patients treated for lymphoma [122].

Thus, despite the potential efficiency of GnRha to prevent premature ovarian failure suggested by some of the studies, the results must be confirmed in the future. Additionally, the effect of GnRha on the fertility of the patients must be further investigated. GnRha treatment may also have additional advantages that should be taken into consideration, such as reducing the risk of thrombocytopenia-associated menorrhagia during chemotherapy [123]. GnRha treatment during chemotherapy is actually proposed as an option to preserve fertility in many centers worldwide. However, this option should be offered as part of a clinical trial after careful counseling regarding the other existing available options to preserve fertility.

Globally, a new concept of "fertoprotective adjuvant therapy" has arisen. This concept supports that molecules could be developed to directly prevent DNA damage caused by chemotherapeutic agents on gonads without interfering with their efficiency. A more thorough understanding of the mechanism of oocyte damage and DNA repair during chemotherapy is essential to address this question. In mice, oocytes lacking the gene for acid sphingomyelinase or wild-type oocytes treated with sphingosine-1 phosphate-resisted apoptosis induced through anticancer therapy [124]. Sphingosine 1-phosphate also led to the preservation of fertility in irradiated female mice without propagating genomic damage to the offspring [125]. Other proteins involved in apoptosis, such as Bax and Rad51, play a critical role in oocyte death after doxorubicin treatment or during age-related physiological oocyte depletion [126]. Experiments in mice show that AS101, a nontoxic immunomodulator, can specifically protect against cyclophosphamide-induced damage in the testis [127].

Recently, a new potential protective drug, imatinib (trade name Gleevec), was also investigated in cisplatin-treated mice [19]. These results offer a promising approach for the use of chemotherapy without harming the resting germ cell [128].

This important ongoing experimental research may contribute to the development of new protective candidate therapies targeting individualized chemotherapeutic agents.

8. Conclusion

The option to preserve fertility must be chosen after multidisciplinary counseling for each patient. Each option presents advantages or contraindications that must be taken into consideration; however, none of them guarantees the success of the procedure. Thus, the combination of different techniques may offer the best chance to restore the fertility of the patients in the future.

The combination of ovarian tissue cryobanking and immature oocyte collection from the tissue followed by IVM and vitrification of the matured oocytes (or embryos) represents a promising approach for fertility preservation. Furthermore, these immature oocytes can be collected at any time of the cycle. Recently, preantral follicle isolation from the medulla during ovarian tissue cryopreservation was also reported, offering additional materials for storage. When feasible, a classical IVF protocol with prior gonadotropin stimulation should be proposed, as it remains the only established procedure for storing gametes.

Finally, reducing the gonadotoxicity of the treatment by adapting the chemotherapy regimen, protecting ovaries from irradiation, or using pharmacological protective therapy can also be proposed associated with a cryopreservation procedure.

9. Disclosure

The co-authors are listed alphabetically and equally contributed to the paper.

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Review Article

Safety of Ovarian Tissue Autotransplantation for Cancer Patients

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Cancer treatments can induce premature ovarian failure in almost half of young women suffering from invasive neoplasia. Cryopreservation of ovarian cortex and subsequent autotransplantation of frozen-thawed tissue have emerged as promising alternatives to conventional fertility preservation technologies. However, human ovarian tissue is generally harvested before the administration of gonadotoxic treatment and could be contaminated with malignant cells. The safety of autotransplantation of ovarian cortex remains a major concern for fertility preservation units worldwide. This paper discusses the main tools for detecting disseminated cancer cells currently available, their limitations, and clinical relevance.

1. Introduction

Thanks to progress made in the field of cancer treatments, long-term survival of patients has increased considerably over the last decade. Unfortunately, some of these treatments, such as chemo- and radiotherapy, can induce premature ovarian failure. It has been determined that nearly half of women diagnosed with invasive cancer will face premature ovarian failure [1]. Consequently, fertility preservation in reproductive-age women has become a major concern in oncology units during the last decades. However, many patients cannot benefit from classic fertility preservation technologies for medical and/or personal reasons. Cryopreservation of ovarian tissue by slow-freezing followed by autotransplantation of thawed tissue provides an alternative method for fertility preservation in young women and even prepubertal girls [2]. While the exact number of ovarian tissue autotransplantations performed worldwide is unknown, this procedure has resulted in 13 reported births of healthy children [3], including two in our centre [4, 5].

Despite these encouraging results, human ovarian cortex autografts still present some major limitations. A major concern is the possibility of reintroducing malignant cells into the patient. Indeed, as the ovarian biopsy and cryopreservation procedures are ideally performed prior to the administration of chemo- or radiotherapy, there is a risk of ovarian involvement and subsequent retransmission of the disease after autotransplantation. In a Japanese retrospective study performed on autopsy specimens, 22,4% of cancer patients under the age of 40 had ovarian metastases [6]. Most of the metastases affecting ovaries are derived from the gastrointestinal tract, breast cancer, or endometrial cancer [7–9]. It can be argued that these individuals are in advanced stages of the disease compared to women benefiting from ovarian tissue cryopreservation, but this underlines the fact that metastases can be found inside the ovarian tissue of young women. In fact, little is known about the presence of malignant cells inside the graft and the risk of neoplasia retransmission after autotransplantation of cryopreserved ovarian tissue. Of the 13 live births reported in the literature, the autotransplanted frozen-thawed ovarian tissue was derived from 8 cancer patients and two patients treated for benign disease [3]. Among these cancer patients, 4 had Hodgkin's lymphoma (HL), one had breast cancer, one had

non-Hodgkin's lymphoma (NHL), one had Ewing sarcoma, and one had neuroectodermic tumour. To date, there are no reports of disease recurrence following the procedure. However, using a mouse lymphoma model, it has been established that lymphoma can be transmitted through the graft even after cryopreservation and thawing of the ovarian tissue if cancer cells are present in the ovary [10]. Lymphoma can be contracted even with one small piece of ovarian tissue ($\sim$1 mm^3) containing cancer cells [10]. The same results have been observed for leukaemia in a rat testis model [11]. In this study, fresh or frozen-thawed testicular cells from leukemic rats were injected in the testis of recipient rats. All of the recipient animals developed signs of leukaemia although a 3–6 day delay was observed in the appearance of symptoms in the frozen-thawed cell transplantation group [11]. Interestingly, it was also demonstrated that only 20 leukemic cells were sufficient to cause leukaemia after 3 weeks in 60% of the animals [11]. These two studies illustrate that malignant cells from haematological cancer can induce relapse in cured patients if these cells are present in the ovarian tissue. Additionally, these results provide evidence that cancer cells are resistant to freezing-thawing process.

The safety of ovarian tissue transplantation in cancer patients should thus be addressed systematically for malignancies with low-to-moderate risk of ovarian implication. Herein, we will discuss the main tools that are currently available for the detection of disseminated cancer cells, specifically classic histology and immunohistochemistry and PCR and xenograft experiments.

2. Analysis of Ovarian Tissue by Histology and Immunohistochemistry

In our centre, more than 30% of indications for ovarian tissue cryopreservation concern young patients affected by breast neoplasia. Others have also reported breast cancer as the main indication for fertility preservation [12, 13]. In these cases, the risk of ovarian metastases is considered low to moderate (0.2% to 11%) [14]. Unfortunately, there is no established method for the detection of cancer cells in ovarian tissue. Only a few recent studies have analysed the incidence of ovarian metastasis in breast cancer patients who underwent cryopreservation procedure [15–17]. The authors investigated the presence of breast cancer cells by histology and immunohistochemistry in more than 160 ovarian cortex biopsies originating from 133 women entering the fertility preservation program. One of these studies [17] focused on gross cystic disease fluid protein-15 (GCDFP15) and mammaglobin-1 (MGB-1), two specific markers of breast epithelium that are not normally expressed in ovarian tissue [18–20]. In two additional studies, the authors used broad spectrum cytokeratin (CK) antibodies [15] or CK-7, CK-aecam and markers of ovarian epithelium [16]. In these studies, there was no evidence of malignant infiltration of the ovarian tissue, even in patients with local lymph-node involvement, which were 44% of the cases in the Rosendahl et al. study. The authors prudently concluded that ovarian tissue preservation seems to be a safe procedure in women with early stages of breast cancer, but "*new methods of cancer screening may change their perception of this procedure*" [17]. Similarly, a series of ovarian cortex originating from 28 breast cancer patients benefiting from the fertility preservation program in our centre were analysed by immunohistochemistry. The histology and CK-19 staining revealed no invasion of ovarian tissue by metastatic cells of mammary origin (personal data).

Haematologic malignancies are also reported as a frequent indication for ovarian tissue cryopreservation [21]. In lymphoma cases, the risk of residual disease in the ovary was particularly highlighted by the Shaw et al. study, which reported that ovarian tissue collected from mice with lymphoma could transfer the disease to healthy recipient animals [10]. Many authors have thus addressed the safety of ovarian tissue autotransplantation in lymphoma patients. In addition to ovarian biopsies from patients benefiting from ovarian tissue autotransplantation, ovarian tissues from 79 patients with HL were analysed and demonstrated no histological evidence of malignant contamination [22–25]. The same analysis was performed on the ovarian tissue of NHL patients and did not reveal any ovarian involvement either [22, 24]. Moreover, in xenograft experiments of ovarian tissue from both HL and NHL patients, none of the grafted animals developed the disease [22]. As a consequence of these analyses, the autotransplantation of frozen-thawed ovarian tissue originating from lymphoma patients is currently considered safe [26–29]. In the case of leukaemia, the risk of disease retransmission is much more significant because malignant cells may be present in the patient bloodstream at the time of tissue retrieval. Surprisingly, histological examination of ovarian biopsies from leukaemia patients reveals no invasion of the tissue. In contrast, more sensitive methods of detection have determined that this tissue is, in fact, contaminated by leukaemia cells [21, 24, 30]. This illustrates the limitations of histology and immunohistochemistry in terms of sensitivity of detection. Moreover, these methodologies only examine a small part of the tissue, and, therefore, are necessary but not sufficient to establish the safety of ovarian autotransplantation in cancer patients. As a result of these limitations, complementary approaches to improve the detection of metastatic disease in ovarian tissue are being developed. As discussed below, polymerase chain reaction (PCR) offers several advantages in this context when used in state of the art.

3. Molecular Analysis of Ovarian Tissue

Numerous studies detailed above were carried out using only classic histology and immunohistochemistry. However, several recent studies used a complementary molecular approach to improve the sensitivity of detection for disseminated cancer cells. Indeed, quantitative reverse transcription-polymerase chain reaction (RT-PCR) has a high sensitivity and specificity of disseminated cancer cell detection, one cancer cell in up to 10^7 normal cells, and can be applied to virtually all types of cancer if adequate tissue or cancer-specific molecular markers are available. In clonal diseases,

such as leukaemia and NHLs, molecular markers for assessing minimal residual disease can often be identified. In general, these markers are an immunoglobulin gene rearrangement in B-cell lymphoma, a T-cell receptor gene rearrangement in T-cell lymphoma, the BCR-ABL (breakpoint cluster region-Abelson) translocation in chronic myeloid leukaemia (CML), or translocations and mutations in acute lymphoblastic leukaemia (ALL). For these diseases, markers are often, but not always, available, and this approach has been used by several teams on ovarian tissue for the detection of leukaemia and lymphoma cell contamination [21, 24, 30]. In the Dolmans et al. study, no malignant cells were detected by histology in the ovarian tissue of six patients with CML and 12 patients with ALL, whereas ovarian tissue in 33% of CML patients and 70% of ALL patients were found to be positive by quantitative RT-PCR [21]. Moreover, xenograft experiments showed leukemic invasion of grafts originating from 5/12 ALL patients [21]. The same conclusions were made in another study in which histology and multimarker immunohistochemical analyses were both negative for the presence of malignant cells, whereas disease-specific genetic markers were detected by quantitative RT-PCR in 6 of 8 patients with CML or ALL [30]. These results demonstrate the presence of residual disease in a high percentage of the ovarian biopsies from patients with leukaemia, which suggests that autotransplantation of frozen-thawed ovarian tissue in these patients is not safe.

Surprisingly, although breast cancer constitutes the major indication for ovarian tissue cryopreservation in several centres, no molecular analyses of the presence of disseminated breast cancer cells in ovarian tissue have been reported to date. As the safety of ovarian autotransplantation in breast cancer women remains a major concern, we have initiated the validation of quantitative RT-PCR markers specific to breast epithelium using the same method employed for the detection of metastatic cells in the sentinel node. We attempted to evaluate ovarian tissue contamination by metastases of mammary origin and the presence of circulating mammary tumour cells (CTCs) in the peripheral blood of patients retrieved at the time of the cryopreservation procedure. Indeed, the presence and number of CTCs are a poor prognostic factor in terms of relapse, survival, and the presence of micrometastases in several solid neoplasias including breast cancer [31]. Several molecular markers permitting the detection of mammary CTCs by RT-PCR have been described in the literature [32, 33], and we have evaluated the sensitivity and specificity of five of them for the detection of CTCs in peripheral blood and disseminated tumour cells (DTCs) in ovarian tissue. Unfortunately, while the majority of these markers are useful for the detection CTCs, none of them are useful for the detection of mammary micrometastases inside the ovarian cortex due to their strong basal expression in normal tissue (personal data). Additional investigations are necessary to evaluate whether these positive signals are the result of illegitimate transcription or normal expression in the ovarian epithelium. To identify new mammary molecular markers that could be used in ovarian tissue, it would be interesting to explore a group of small noncoding ribonucleic acids (RNAs), called micro-RNA (mi-RNAs). Indeed, some mi-RNAs are expressed in a tissue-specific manner and can be differentially expressed between tumours and normal tissues [34]. These characteristics could be very useful for the detection of disseminated breast cancer cells and also in other neoplasias.

Despite the sensitivity and the fact that this molecular tool can theoretically be applied to all malignancies, quantitative RT-PCR detection of disseminated cancer cells inside ovarian tissue is not devoid of limitations. Indeed, extracting RNA from this dense and fibrous tissue is challenging and, the results obtained from this analysis are highly dependent on the quality of RNA extracted and the efficiency of cDNA synthesis. We have also noticed that genetic markers are not always available for all patients, particularly for ALL patients. In addition, illegitimate transcription has been well described and could lead to false positives. Finally, the clinical relevance of a positive signal inside ovarian tissue has not been established yet. Therefore, further studies are required to evaluate if cancer cells detected in thawed ovarian tissue are viable and the transplantability threshold of these cells. As discussed below, xenograft experiments can partially address these last issues.

In conclusion, it is now clear that the detection sensitivity of disseminated cancer cells can be increased by the use of molecular detection tools, such as quantitative RT-PCR. However, this technique should not be used alone in this context, but only in combination with other detection tools, like immunohistochemistry and/or xenografting.

4. Xenotransplantation, a Tool to Evaluate the Safety of Ovarian Tissue Autotransplantation in Cancer Patients

The first clinical attempts at xenotransplantation date back to the 17th century when blood from animals was used to transfuse humans in France and England [35]. In the 19th century, tissues (mainly the skin) and during the 20th century, vascularised organs were attempted to be grafted into humans without success [35]. The reason for these failures was the acute rejection of the transplanted tissue by the immune system [36]. The development of transgenic immunotolerant animal models allowed xenotransplantation to have new insights in the research field. Today, some mice strains have mutations that make them sufficiently immunodeficient to permit xenotransplantation [36]. Among them, nude mice that are athymic, and thus T-cell deficient, [37] and severe combined immunodeficient mice (SCID) carrying an autosomal recessive mutation that severely affect lymphopoiesis, which makes mice that are homozygous for this mutation deficient in B and T lymphocytes [38], are the most frequently used for ovarian tissue xenografts. Many sites have been used for ovarian cortex xenografts, including subcutaneous sites, the bursal cavity, under the kidney capsule, or in the muscle [39–47].

As a risk of reintroducing cancer into remission patients is theoretically possible following autotransplantation of ovarian tissue, xenotransplantation of frozen-thawed ovarian

cortical tissue to immunodeficient animal hosts has been suggested as an alternative to assess the safety of the procedure. Xenotransplantation models have initially focussed on studying ovarian follicular development, in which primordial follicles are activated in an immunocompromised animal model and, after initial growth, are transferred to an in vitro culture system [36]. This approach eliminates the risk of cancer cell reintroduction, and, additionally, the problematic unaccomplished phase of primordial follicle culture is bypassed [36]. Unfortunately, the use of xenotransplantation to mature follicles "in vivo" is still not ready to be used in clinical applications, as its safety and ethical issues have yet to be discussed [48].

The use of xenotransplantation to evaluate the risk of reintroducing malignant cells is quite recent [22]. The first in vivo evaluation of residual disease using xenograft models was studied in HL and NHL [22]. No clinical sign of the disease and no microscopic evidence of residual disease were found in animals xenografted with ovarian tissue from patients diagnosed with HL or NHL [22]. Although these results are quite reassuring, they cannot be interpreted as absolute evidence of safety. Some years later, ovarian tissue xenotransplantation to immunodeficient mice was used to evaluate the risk of reintroducing leukaemia, in parallel with histology and quantitative RT-PCR [21]. In fact, as leukaemia is considered as a systemic cancer, malignant cells may be present in the bloodstream and can thus easily migrate to the ovary. Furthermore, a retrospective analysis of an autopsy study demonstrated leukemic invasion of the ovaries in 8,4% of patients [6]. After long-term xenografting (6 months) of frozen-thawed ovarian tissue from patients with CML and ALL into SCID mice, one third of the mice grafted with tissue from ALL patients showed massive macroscopic peritoneal invasion [21]. No malignant cells were microscopically identified in grafts retrieved from mice transplanted with ovarian tissue from CML patients; however, obvious invasion of lymphoblasts was observed in 5 of the 12 mice grafted with ovarian tissue from ALL patients [21]. These results are quite alarming considering all ovarian tissues that were determined be healthy and disease-free following histological analysis preceding the xenograft. Moreover, only histological, and eventually immunohistochemical, analysis is routinely performed prior to autotransplantation of ovarian tissue in a cured patient.

More recently, the safety of ovarian tissue autotransplantation from patients with ovarian tumours was investigated using xenografting to SCID mice [49]. After 24 weeks, no sign of malignancy was detected either macroscopically or histologically [49]. Despite these reassuring results, the risk of reintroducing malignant cells in cases of ovarian cancer is considered high, as bilateral carcinoma is found in approximately 25% of all ovarian cancers [50].

Regarding breast cancer, no study using a xenograft model has been published to date. The first evaluation by histology and immunohistochemical analysis showed no sign of metastases [16, 17]; however, further investigations, using xenograft models, for instance, are still required to confirm the safety of the procedure in breast cancer patients.

5. Conclusion

It is now clear that cryopreserved ovarian tissue may harbour malignant cells that could provoke relapse following grafting. Currently available data suggest that autotransplantation of frozen-thawed ovarian tissue is a safe procedure for patients with Hodgkin's disease at the time of ovarian biopsy [26–29]. Indeed, there is no evidence of ovarian involvement in the ovarian cortex of patients undergoing cryopreservation. Moreover, no relapse has been reported in patients after ovarian autotransplantation and spreading to the ovary has only been described in extremely rare cases [51]. However, even though these results are reassuring, it does not mean that there are no risks associated with autotransplantations. Moreover, the safety of the procedure has not yet been established for all other malignancies. If it is clear that autotransplantation of ovarian tissue cannot be proposed for leukemic patients, precautionary decisions have to be taken for all other patients [21, 24, 30]. In the case of other neoplasias, and in particular for breast cancer patients, additional strategies must be developed to determine the safety of this procedure. This implies the identification of (breast) cancer molecular markers that are usable in ovarian tissue and xenograft experiments. We believe that quantitative RT-PCR, despite its limitations, is a promising tool for the detection of micrometastases inside ovarian tissue. Additionally, xenotransplantation studies in immunotolerant mice provide additional information concerning putative ovarian involvement.

However, these tools are not perfect for the detection of micrometastases as they also have some disadvantages. A major limitation of RT-PCR is the interpretation of positive results. Indeed, it has been shown that BCR-ABL mRNA can be detected at very low levels in healthy patients [52, 53]. Similarly, a recent study showed that leukemic markers can be detected in ovarian tissue from ALL patients; however, some mice xenografted with this tissue did not develop neoplasia [21]. As expected, none of the mice grafted with PCR-negative tissue developed leukemic progression either [21]. A potential limitation of xenotransplantation experiments is that a lack of tumour growth in recipient mice could be explained simply by the absence of malignant cells in the very small piece, usually 1 mm^3 or less, of ovarian cortex typically used for the graft, whereas malignant cells could be present in the rest of the cryopreserved tissue. Indeed, the distribution of malignant cells in the tissue is not homogeneous or uniform. It is therefore of major importance to standardise detection techniques of residual malignant cells and to assess their clinical relevance. To accomplish this, it would be interesting to develop a multicentric and multidisciplinary approach combining molecular and xenograft analyses for pathologies with low-to-moderate risk of ovarian invasion. For cancer patients who cannot benefit from ovarian tissue autotransplantation due to the risk of disease retransmission, alternatives such as in vitro follicle culture [54] and isolated follicles transplantation [55] are promising approaches. However, these alternatives are still in the early stages, and huge research efforts need to be conducted prior to clinical implementation.

In conclusion, the decision to graft a patient or not must involve a multidisciplinary discussion involving oncologists, gynaecologists, anatomopathologists, and molecular biologists. It is essential to balance the risks and benefits for each patient and remain extremely cautious regarding ovarian cortex autotransplantation. We should keep in mind that although recovering fertility is very important to some patients, reimplantation of contaminated ovarian tissue could be a life-threatening event.

Author' Contribution

L. Bockstaele and S. Tsepelidis have contributed equally to this paper.

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