Fertility preservation in women

Jacques Donnez and Marie-Madeleine Dolmans

Abstract | In women, ~10% of cancers occur in those <45 years old. Chemotherapy, radiotherapy and bone marrow transplantation can cure >90% of girls and young women with diseases that require such treatments. However, these treatments can result in premature ovarian failure, depending on the follicular reserve, the age of the patient and the type and dose of drugs used. This article discusses the different fertility preservation strategies: medical therapy before chemotherapy; ovarian transposition; embryo cryopreservation; oocyte vitrification; and ovarian tissue cryopreservation. The indications, results and risks of these options are discussed. Whether medical therapy should be used to protect the gonads during chemotherapy remains a source of debate. Fertility preservation needs to be completed before chemotherapy and/or irradiation is started and might take 2–3 weeks with established techniques such as embryo or oocyte cryopreservation. Further studies are needed in patients with cancer to confirm the excellent outcomes obtained in patients without cancer or in egg donation programmes. For prepubertal girls or cases where immediate therapy is required, cryopreservation of ovarian tissue is the only available option. Finally, possible future approaches are reviewed, including *in vitro* maturation of nonantral follicles, the artificial ovary, oogonial stem cells and drugs to prevent follicle loss.

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Introduction

Many cancers are no longer considered to be incurable, and quality of life after cancer now needs to be addressed.[1] In the USA, the number of new cases of invasive cancer in women in 2012 was in the region of 790,740, and ~10% of these cases were in women <45 years old.[2] Advances in cancer therapy over the past two decades have led to a remarkable improvement in survival rates. Indeed, during the past 5 years, overall rates of death attributable to cancer in women have fallen by >1.6% per year.[3] Unfortunately, treatments such as chemotherapy, radiotherapy and/or surgery can induce premature ovarian failure (POF) in some circumstances.[4–6]

Non-oncological systemic diseases such as autoimmune and haematological conditions sometimes require chemotherapy or radiotherapy, as well as bone marrow transplantation.[4,5,7] This treatment combination has the greatest risk of POF, estimated to be 92–100%.[8,9] Impairment of ovarian function after bone marrow transplantation is mostly related to increased age at the time of treatment and use of total body irradiation before the transplant.[10,11]

The ovaries are very sensitive to cytotoxic drugs, especially alkylating agents, which are likely to cause gonadal dysfunction.[3–6,11–14] Cyclophosphamide is the alkylating agent that causes the most damage to oocytes and granulosa cells in a dose-dependent manner.[6–8] The combination of abdominal ionizing radiation and alkylating agents induces POF, leaving almost 100% of patients infertile.[15,16]

Pelvic radiation therapy is also known to cause POF, as exposure to 5–10 Gy is toxic to oocytes.[15] The human oocyte is very sensitive to radiation, with a dose of <2 Gy estimated to be sufficient to destroy 50% of primordial follicles.[15]

Giving a patient or her parents an accurate assessment of the risk to fertility is difficult, as how a disease will develop cannot be predicted.[17] Therefore, evaluating the likelihood of POF after chemotherapy or radiotherapy is often extremely problematic. Moreover, it should be stressed that the ovarian reserve varies enormously from one woman to the next (Figure 1),[18] which also affects the probability of developing POF. Fertility preservation counselling should, therefore, be offered to all girls and women with reproductive potential. Mental health professionals, reproductive endocrinologists and paediatricians should be able to coordinate with oncologists to optimize patient care and evaluate quality of life after therapy.

Only a small fraction of patients at risk of POF is referred to specialists to discuss fertility preservation options; of this group, only a few women actually undergo fertility preservation owing to social, economic or technical hurdles.[19] In addition, women are increasingly postponing childbearing to later in life for social or financial reasons, and the incidence of most cancers increases with age (Box 1).

This Review analyses the options that are currently available to preserve fertility in female patients with cancer and in women at risk of POF.

Competing interests
J. Donnez declares associations with the following companies: Ferring, MSD, Organon, PregLem SA, Serono. See the article online for full details of the relationships. M.-M. Dolmans declares no competing interests.

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Key points

- Approximately 10% of cancers in women occur in those <45 years old, and in the USA, the number of new cases of invasive cancer diagnosed in women during 2012 was almost 800,000
- Chemotherapy, radiotherapy, surgery and bone marrow transplantation have led to a considerable improvement in survival rates, but these treatments can result in premature ovarian failure (POF)
- The risk of POF depends on the follicular reserve, the age of the patient and the type and dose of drugs used
- Different options exist to preserve fertility in women at risk of POF, including ovarian transposition, embryo and oocyte cryopreservation, and ovarian tissue cryopreservation
- In prepubertal girls or patients requiring immediate chemotherapy, ovarian tissue cryopreservation is the only method currently available
- Medical therapy (with a gonadotropin-releasing hormone agonist) before chemotherapy is still controversial

The ovarian reserve

The term ovarian reserve is typically used to refer to the population of primordial follicles, which has been called the 'true' ovarian reserve.[14,20,21] In the field of assisted reproduction, this term refers to the population of small growing follicles (that is, small antral follicles that are detected by vaginal ultrasonography).

Initiation of the resting primordial follicle reserve begins in the fetus, when some 100–2,000 primordial germ cells colonize the genital ridges and enter a massive proliferation process that results in 7×10^6 potential oocytes at mid-gestation. In the human ovary, around 85% of these potential oocytes are lost before birth.[14,18,22] The apoptosis-inhibiting *BCL2* gene and apoptosis-inducing *BAX* gene might act as rheostats to determine the survival or death of germ cells.[23,24] The decline in the number of follicles continues throughout reproductive life, during which time only ~450 monthly ovulatory cycles occur, with the majority of follicles undergoing atresia (follicles degenerate and are reabsorbed) during the growth phase of the follicles. Cyclic folliculogenesis and ovulation, with massive follicular atresia and ageing-induced apoptosis, lead to ovarian atrophy and reduced fertility.[18,20–22]

Numerous mechanisms have been proposed to explain decreased fertility in women >40 years of age, including poor oocyte quality, which are characterized by abnormalities in the meiotic spindle, shortened telomeres and chromosome misalignment.[25,26] At menopause (occurring at an average age of 50–51 years), ~1,000 primordial follicles remain (Figure 1). Although mitotically active germ cells have been reported in mouse and human ovaries,[27,28] their presence and capacity for neo-oogenesis remain controversial.[29] The isolation and characteristics of mitotically active germ cells from human ovaries have

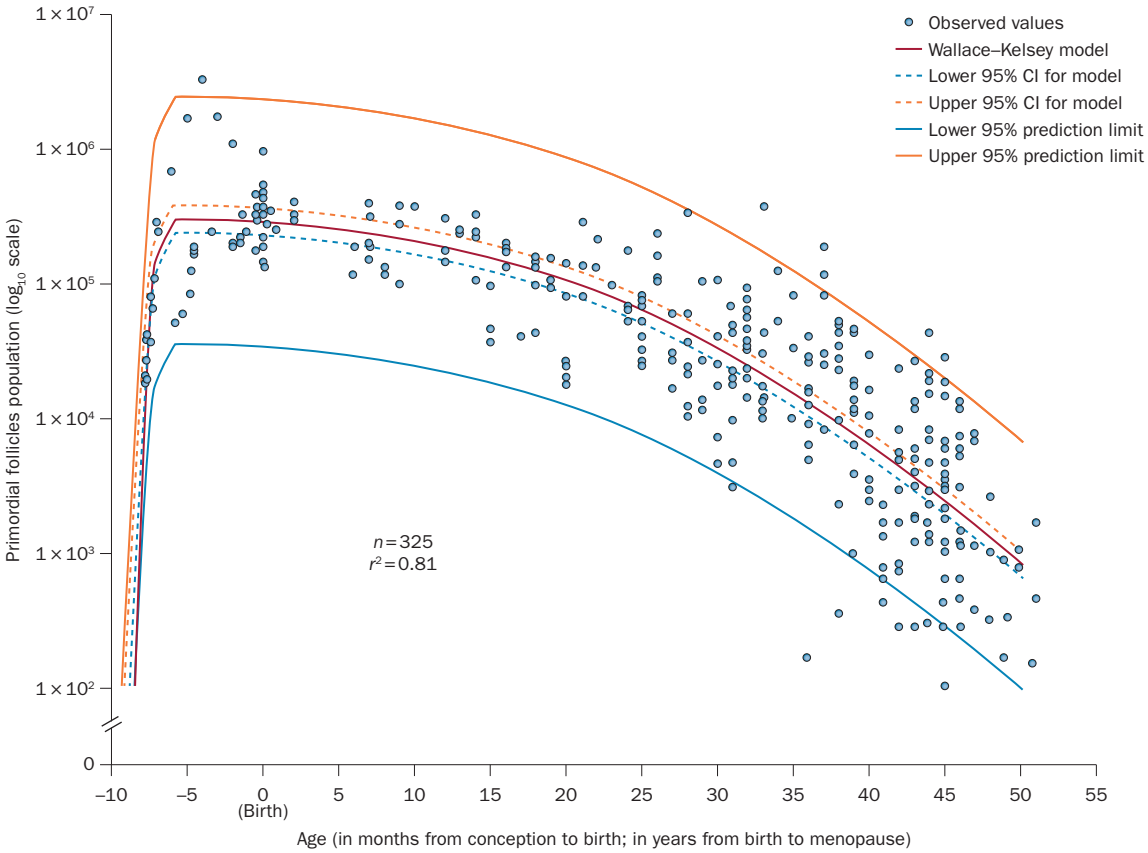

Figure 1 | The ovarian reserve: the model that best fits histological data according to Wallace and Kelsey. The ovarian reserve is depicted throughout a woman's life, from conception to the age of 55 years. The Y axis illustrates the number of primordial follicles constituting the ovarian reserve. After an initial increase (germ-cell formation), the ovarian reserve starts to diminish even before birth, resulting in only ~1,000 follicles present at menopause. This figure is adapted from Wallace, W. H. & Kelsey, T. W. from *PLoS ONE* **27**, e8772 (2010), published under an open-access license by PLoS.

been described, and their oocyte-forming potential will soon be tested *in vivo*.[30]

A model described by Wallace and colleagues enables the number of primordial follicles present in the ovary to be estimated at any given age (Figure 1).[18] They demonstrated that 95% of variation in the population of primordial follicles is attributable to age alone up to the age of 25 years.[18,21]

It is impossible to evaluate the 'true' ovarian reserve to estimate the length of the reproductive lifespan using *in vivo* imaging, as the resolution of such techniques is insufficient. Anti-Müllerian hormone (AMH), which is correlated with the number of primordial follicles, can be used to estimate reproductive lifespan, even though this hormone is not a direct product of primordial follicles.[14,18,31] In addition, AMH is detectable in girls of all ages, which suggests that AMH levels might be of value in the assessment of ovarian function in prepubertal girls and as a marker of gonadotoxicity in girls treated for cancer.[23,31]

Levels of AMH decline steadily over the course of repeated chemotherapy—to which extent depends on the dose and type of drug used—and recovery of these levels is variable. Indeed, some women will show no AMH recovery following treatment, indicating POF, whereas others undergoing lower-risk treatment will show recovery of AMH levels and ovarian function.[14,15,21] On the basis of their large study, Anderson and Wallace are in favour of the potential value of AMH assessment before cancer therapy to determine ovarian reserve, but they also recognize that other more focused prospective trials are needed to address this challenging issue in the field of paediatric oncology.[14,18] In adult patients, the level of AMH before chemotherapy has also been demonstrated to be of use in predicting recovery of AMH levels after treatment.[32,33] Indeed, in patients with breast cancer, high pretreatment serum levels of AMH have been shown to predict long-term ovarian function after chemotherapy. Nevertheless, levels of AMH are not predictive of reduced fertility in healthy young women and might be of limited value in childhood survivors of cancer.[14,32–36]

Risk factors for fertility impairment have been described (Box 1),[6,12,18] but only a few studies have evaluated the ovarian reserve of patients with cancer.[37] Ovarian reserve determined by levels of AMH was found to be considerably reduced in patients with lymphoma before chemotherapy compared with healthy individuals.[37] One study reported a notably reduced antral follicle count in female patients with cancer between the ages of 25 and 40 years, which could be explained either by accelerated follicle loss as a result of the disease or a defect in the recruitment of antral follicles attributable to the disease state.[38]

Box 1 | Risk of infertility after cancer treatment

Low risk (<20%)
- Leukaemia
- Cerebral tumour <24 Gy
- Wilms' tumour
- Germinal cell tumour (no radiotherapy)

Medium risk (20–80%)
- Leukaemia
- Cerebral tumour >24 Gy
- Non-Hodgkin lymphoma
- Hodgkin lymphoma
- Ewing sarcoma, no metastases
- Osteosarcoma
- Hepatoblastoma
- Neuroblastoma

High risk (>80%)
- Total body irradiation
- Pelvic irradiation
- Bone marrow transplantation
- Hodgkin lymphoma, alkylating agent

Fertility preservation

Fertility preservation, in the context of treating cancer or benign diseases or for social reasons (Box 2), will be a major challenge over the next 5 years.[39,40] Medical therapy (particularly with gonadotropin-releasing hormone [GnRH] agonists) to protect the gonads during chemotherapy remains a source of debate and will be discussed later in the article.

Women with cancer have several options to preserve their fertility and enable them to conceive when they have recovered: ovarian transposition; embryo cryopreservation; immature or mature oocyte cryopreservation; and ovarian tissue cryopreservation.[4–6,39,40] Currently, embryo and mature oocyte cryopreservation following *in vitro* fertilization (IVF) are the only methods endorsed by the American Society of Reproductive Medicine (ASRM). However, in the opinion of many pioneers, enough evidence now exists to support the technique of ovarian tissue cryopreservation and stop considering it an experimental and investigational approach.[40–47]

GnRH agonist for fertility preservation

The possibility of administering adjuvant therapy that could minimize damage to the gonads by gonadotoxic drugs is clearly an attractive option and was first tested more than three decades ago.[48] As ovarian function is preserved in most long-term survivors of lymphoma treated before puberty, but only in a minority of similarly treated adult patients, researchers attempted to temporarily recreate prepubertal conditions in postpubertal women during gonadotoxic chemotherapy.[49] Indeed, Blumenfeld and von Wolff speculated that by reducing levels of FSH, GnRH agonists could positively influence the vicious cycle of chemotherapy-induced depletion of the ovarian reserve, increase levels of FSH and accelerate recruitment of further follicles.[49] However, a meta-analysis of published clinical studies led them to conclude that, statistically, GnRH analogue co-treatment does not reduce gonadotoxicity.[49]

A separate systematic review and meta-analysis on the possible benefits of GnRH analogue co-treatment during chemotherapy identified 28 randomized controlled trials (only six met the inclusion criteria). The analysis suggested that although evidence indicates a potential

Box 2 | Indications for cryopreservation of ovarian tissue

Malignant pathology

Systemic diseases
- Hodgkin lymphoma
- Non-Hodgkin lymphoma
- Leukaemia
- Medulloblastoma

Extrapelvic diseases
- Bone cancer (osteosarcoma, Ewing sarcoma)
- Breast cancer
- Melanoma
- Neuroblastoma
- Bowel malignancy

Pelvic diseases
- Pelvic sarcoma
- Rhabdomyosarcoma
- Sacral tumour
- Rectosigmoid tumour
- Early cervical carcinoma
- Early vaginal carcinoma
- Early vulvar carcinoma
- Selected cases of ovarian carcinoma (stage IA)
- Borderline ovarian tumour

Non-malignant pathology

Unilateral or bilateral oophorectomy
- Benign ovarian tumour
- Severe and recurrent endometriosis
- *BRCA1* or *BRCA2* mutation carrier

Risk of premature menopause
- Turner syndrome
- Family history
- Benign diseases requiring chemotherapy: autoimmune diseases (systemic lupus erythematosus, rheumatoid arthritis, Behçet's disease, Wegener's granulomatosis)

Bone marrow transplantation
- Benign haematological diseases: sickle cell anaemia, thalassaemia major, aplastic anaemia
- Autoimmune diseases unresponsive to immunosuppressive therapy

Social reasons
- Age of the patient
- Childbearing postponed to later in life for social or financial reasons

benefit of GnRH agonist co-treatment in premenopausal women, with increased rates of resumption of menses and ovulation, pregnancy rates do not improve.[50] Moreover, the analysis revealed possible selected reporting of trial data.[50] A later study demonstrated no benefit of using GnRH agonists in patients with breast cancer receiving cyclophosphamide-based chemotherapy.[51] No differences in menstruation resumption rates or hormonal or ultrasonographic markers of fertility were identified between patients receiving a GnRH agonist and the control group.

A randomized study evaluated the incidence of early menopause (defined as no resumption of menstrual activity or levels of FSH usually seen in postmenopausal women) 1 year after the last cycle of chemotherapy in 281 patients with breast cancer (only 21% of whom were <34 years old).[52] The rate of early menopause was 25.9% in the chemotherapy-alone group and 8.9% in the chemotherapy and GnRH agonist combination group.[52] Some weaknesses were nevertheless noted in this study, such as lack of data on long-term maintenance of ovarian function. Moreover, the use of different types of chemotherapy and absence of FSH and estradiol evaluation in almost 30% of patients might have led to some bias in the conclusions on fertility preservation.

A multicentre prospective randomized trial published in 2013 that involved patients with lymphoma found that ~20% exhibited POF after 1 year of follow-up, in women receiving a GnRH agonist plus norethisterone, and those receiving norethisterone alone.[53] The researchers concluded that the GnRH agonist was not associated with a significantly decreased risk of POF.[53]

The ASCO 2013 recommendations for fertility preservation in patients with cancer have now been published.[54] They state that evidence regarding the effectiveness of a GnRH agonist in fertility preservation is currently insufficient, but that GnRH agonists might yield other medical benefits, such as reduced vaginal bleeding when patients have low platelet counts as a result of chemotherapy. Of note, complete ovarian suppression is not achieved for several weeks after administration of a GnRH agonist, and adverse effects such as hot flushes and loss of BMD are induced by this class of drugs.[53,54] In conclusion, use of GnRH agonists for fertility preservation remains controversial and, according to the ASCO 2013 recommendations, GnRH agonists should not be relied on to preserve fertility.[54]

Ovarian transposition

Preservation of ovarian function by ovarian transposition before pelvic radiation has been suggested for all women of reproductive age requiring this treatment.[54,55] Ovarian transposition has been offered to patients with advanced cervical cancer,[56,57] but has been found to be somewhat underused, with only 28% of patients <40 years of age undergoing ovarian transposition before radiotherapy.[58]

Ovarian transposition can be easily performed by laparoscopy. The ovaries are usually fixed to the anterolateral abdominal wall, 3–5 cm above the umbilicus. Lateral transposition is preferable to medial transposition. The ovarian vessels (arteries and veins) should be carefully mobilized to make sure the blood supply to the ovary is not compromised. Usually, 5 mm metallic clips are placed at the base of the transposed ovary.[55,56] It is strongly advised to take large biopsy samples of ovarian tissue (for cryopreservation) during the same procedure, as the risk of ovarian failure following pelvic irradiation in women who have undergone ovarian transposition varies from 15% to 40%.[56,59]

In a systematic review conducted in 2003, it was concluded that laparoscopic ovarian transposition in women <40 years old is associated with preservation of ovarian function in 88.6% of cases.[59] This rate was confirmed in a retrospective analysis published in 2013, in which 90% of patients who had undergone ovarian transposition before radiotherapy had normal levels of FSH and estradiol.[60]

In a study that included 107 women treated for cervical cancer, bilateral ovarian transposition was achieved in 104 women.[56] Rates of ovarian preservation were 100% for patients treated exclusively with surgery, 90%

for patients treated with vaginal brachytherapy after surgery and 60% for patients treated with external radiation therapy and vaginal brachytherapy after surgery. The investigators, therefore, concluded that ovarian transposition is a safe and effective procedure. Of note, the main risk of ovarian failure was found in patients treated with external radiation therapy,[56] and this risk was increased if chemotherapy was also administered.[6,15] The risk of ovarian involvement should be taken into account if pelvic malignancy is also present.[61] To avoid this risk, ovarian transposition should be restricted to women <40 years of age with low-grade cervical cancer.[61]

Embryo cryopreservation

Embryo cryopreservation is a well-established procedure that can be used to preserve fertility in women of reproductive age with an available partner (or women using donor sperm). Until 2012, embryo cryopreservation was the only method of fertility preservation endorsed by the ASRM. The technique has proven to be safe and effective in patients undergoing IVF treatment and has been proposed for different clinical reasons, including storage of supernumerary embryos, risk of ovarian hyperstimulation syndrome (OHSS), impaired endometrial development and impractical embryo transfer.[3,62,63]

Slow-freezing and vitrification techniques, the main cryobiology strategies, offer good results in experienced hands and their use is becoming more widespread, especially for blastocyst cryopreservation.[62–64] A growing number of centres are incorporating vitrification as a simpler and less expensive alternative, as the slow-freezing procedure requires use of controlled-rate freezers and large quantities of liquid nitrogen.

Embryo cryopreservation has reliable success rates, and a meta-analysis suggests that frozen embryo transfer is more successful than fresh embryo transfer, probably because of improved embryo–endometrium synchrony.[64] Although no randomized controlled trials have been conducted to evaluate the effect of cryopreservation on the health of children born via this method, outcome data after frozen embryo transfer are generally reassuring.[65–68]

When feasible (that is, if enough time is available before initiation of chemotherapy) and indicated (the woman has gone through puberty and sperm is available), embryo cryopreservation should be offered as the primary method of fertility preservation as, despite the increasing success rates of oocyte vitrification, it seems to offer better results than other methods.[3,69,70] Nevertheless, it should be pointed out that cryopreserved embryos are the joint property of the woman and her male partner in most countries, which might be an issue when they come to be used a number of years later.

Oocyte preservation

The introduction of vitrification into assisted reproduction technology has yielded female gamete (oocyte) cryopreservation outcomes similar to those obtained with fresh oocytes.[69,71–78] Among the main cryobiology strategies (slow-freezing and vitrification), vitrification was found to be very effective at avoiding crystallization, thus reducing damage to cells caused by formation of ice crystals and chilling injury during the freezing process.[73–78]

In the 1980s, possible deleterious effects of cryopreservation on the meiotic spindle were suggested.[79,80] However, evidence in the past 5 years demonstrates that the meiotic spindle is able to restore itself, ensure proper chromosome segregation and generate embryos from either slow-frozen or vitrified oocytes.[73–81] Moreover, the incidence of congenital anomalies was not found to be increased in children born after oocyte cryopreservation compared with those conceived naturally.[79,80]

Two reviews have concluded that slow-freezing of mature oocytes yields low survival rates compared with vitrification and that resultant embryo development seems to be impaired, with outcomes favouring vitrification.[69,82] Direct contact with liquid nitrogen is controversial, but open systems (direct contact) seem to be more efficient than currently available closed systems (indirect contact), at least for oocytes.[69]

Studies of oocyte vitrification in egg donation programmes have shown outcomes (such as survival rates and ongoing pregnancy rates [that is, confirmed sustained pregnancies rather than live birth rates]) equivalent to those using vitrified and fresh oocytes.[71,75,81,83,84] A study published in 2010 showed an overall survival rate of 92.5% and an ongoing pregnancy rate of 43.7%, confirming the efficacy of oocyte vitrification.[75]

These results after vitrification of mature oocytes in egg donation programmes are very promising, but cannot be extrapolated to outcomes after treatment for cancer. Indeed, information on outcomes after oocyte vitrification in patients with cancer is lacking, essentially for two main reasons. First, this option has only recently become available and second, participants are patients with cancer, and complete disease remission must first be established as a priority. Only two live births have been reported in patients with cancer who chose oocyte vitrification for fertility preservation before being treated for malignant disease.[85,86]

If chemotherapy can be delayed, oocyte vitrification should be proposed to patients with cancer, but further studies are needed to confirm the excellent results obtained in egg donation programmes.[75] Moreover, patients should be aware that around 20 vitrified oocytes are required to achieve a live birth, as the live birth rate per vitrified oocyte (in egg donation programmes) is 5.7% in the most experienced teams in the world.[69,75,77,87]

A combination of oocyte cryopreservation and ovarian tissue cryopreservation could increase the efficacy of the fertility preservation procedure and give young patients with cancer a good chance of future fertility.[88] However, cryopreservation of ovarian tissue on the day of oocyte retrieval is difficult and might not be effective. This procedure is, therefore, not recommended after the ovaries have been stimulated by human menopausal gonadotropin or recombinant FSH, following administration of human chorionic gonadotropin (hCG) or after oocyte retrieval.[4,13,40,89] We strongly suggest first cryopreserving ovarian tissue and then starting controlled ovarian stimulation (COS) with a view to vitrifying mature oocytes.[70]

To avoid any delay due to COS, immature oocyte retrieval (after oophorectomy or at the time of ovarian tissue cryobanking) for future *in vitro* maturation and vitrification of mature oocytes might be proposed.[40] Nevertheless, to our knowledge, no live births have yet been reported in patients with cancer using this strategy.[40]

COS protocols

Special attention should be paid to ovarian stimulation for fertility preservation purposes. Use of a GnRH antagonist is associated with a reduced risk of OHSS, and this risk could be further reduced by triggering final oocyte maturation with a GnRH agonist.[3,62,63]

The choice of COS protocol depends on the disease the patient is being treated for, not only because the patient often has only a single opportunity because of the time available until initiation of radiotherapy and/or chemotherapy, but also due to the specificity of some estrogen-sensitive cancers.[3,62]

Conventional COS for IVF requires 9–14 days of ovarian stimulation with gonadotropins, which is preceded by ovarian suppression with a GnRH antagonist. Fortunately, use of GnRH antagonists enables immediate suppression of pituitary release of FSH and luteinizing hormone, which shortens the interval from patient presentation to embryo cryopreservation.[90,91]

Random-start stimulation (during the late follicular or luteal phases rather than the early follicular phase) could be proposed in emergency cases when waiting for the next menstruation cycle is not an option, as cancer treatment needs to begin as soon as possible. Late follicular phase-start or luteal phase-start antagonist IVF cycles have been shown to be as effective as early follicular phase-start antagonist IVF cycles in patients with cancer.[62,92,93] Whilst oocyte quality does not seem to be compromised by random-start stimulation, the efficacy of this strategy in terms of ongoing pregnancy rates and live births needs to be confirmed.[62]

Patients with estrogen-sensitive cancer are at potential risk of reaching high estradiol levels during COS that might influence growth of estrogen receptor-positive breast cancer. Stimulation protocols with aromatase inhibitors have been proposed to reduce estrogen production, proportional to the number of growing follicles, as natural-cycle IVF is fairly ineffective. Since 2006, some clinicians have advocated the use of aromatase inhibitors such as letrozole, which markedly suppress plasma levels of estrogen by competitively inhibiting the activity of the aromatase enzyme.[94–97] In patients with estrogen-sensitive cancers, the main advantage of adding daily letrozole to gonadotropins in ovarian stimulation protocols is to obtain estradiol levels no higher than 1,835 pmol/l, resulting in a similar number of mature oocytes and comparable fertilization rates to those of the normal population of the same age.[19,62,94–98] To date, no increased risk of breast cancer recurrence has been observed in patients with breast cancer treated with the combined protocol (namely letrozole and gonadotropin).[96,97] This protocol could, therefore, be routinely recommended for fertility preservation in patients with estrogen-sensitive cancers.[3,19,62] However, although the number of mature oocytes looks encouraging, live birth rates are not yet available to confirm the efficacy of this approach.

In patients with cancer undergoing fertility preservation, OHSS should be avoided as it can delay initiation of cancer therapy. Even if the goal is to obtain sufficient numbers of oocytes or embryos to increase the chances of pregnancy in the future, all efforts should be made to reduce this risk, which mainly results from triggering final oocyte maturation with hCG. Several studies have demonstrated that using a GnRH agonist as the ovarian stimulation trigger yields at least similar numbers of mature oocytes and cryopreserved embryos at a lower risk of OHSS than hCG.[62,63,95,99,100] Nevertheless, some cases of trigger failure with GnRH agonists have been observed.[62,93] It was, therefore, suggested that either the dose of GnRH agonist be increased or hCG supplementation (≤1,500 IU) be used at the time of trigger. The other option is to use 4 mg of leuprolide acetate as the trigger, but this approach is only advisable in patients at high risk of OHSS, in whom hCG administration could cause OHSS.[62]

A review published in 2013 analysed different protocols for COS, including random-start stimulation.[62] The specific malignancy and the patient's multisystemic condition were both found to have a possible effect on the response to ovarian stimulation. A meta-analysis of seven retrospective studies revealed that women with malignancies had lower numbers of both total and mature oocytes after COS (for fertility preservation) than did healthy, age-matched control individuals undergoing COS for IVF.[101] Candidates for fertility preservation in the context of malignancy, especially carriers of *BRCA1* mutations, should be informed that the expected number of oocytes retrieved after COS might be lower than that obtained from healthy patients of a similar age.

Ovarian tissue cryopreservation

Cryopreservation of ovarian tissue is the only fertility preservation option available for prepubertal girls and for women who cannot delay the start of chemotherapy (Box 2). As the follicular reserve of the ovary is age-dependent, the age of the patient should be considered before offering this treatment. Many teams worldwide have set the upper limit for undergoing this treatment at 35 years.[4,5,13,40–44,89] Ovarian tissue cryopreservation in children is a specific issue. Currently, no consensus or directive exists on the age at which reproductive potential is actually reached, which makes it unclear how recommendations can be effectively applied to patients with cancer <18 years old.[6,11,17]

A number of investigators have addressed the question of ovarian tissue cryopreservation during childhood,[6,11,14,17,102–105] but only a few series performing this procedure in children have been documented.[17] How much ovarian cortex should be harvested for cryopreservation is an important issue. This decision is influenced mainly by the estimated risk of ovarian failure relative to the planned treatment and existing ovarian volume. Oophorectomy should be performed in patients

undergoing pelvic irradiation or total body irradiation and in those receiving high doses of alkylating agents. This procedure should also be performed in very young girls (that is, prepubertal girls) because of the small size of their ovaries.[17] Otherwise, in adults, 4–5 ovarian cortical biopsy samples of ~1 cm in length, 4–5 mm in width and 1.0–1.5 mm in depth are taken in most departments around the world.[4,5,40] However, left oophorectomy is carried out almost systematically in some countries.[41] At least one entire ovary should nevertheless be left in place to enable subsequent orthotopic reimplantation.[40,47]

Two techniques of orthotopic reimplantation (that is, in the pelvic cavity) are available; which one is used depends on whether or not the patient still has an ovary.[40] If an ovary is present, pieces of thawed ovarian cortex are fixed to the medulla after decortication of the ovary. If no ovary is present, the ovarian pieces are placed in a peritoneal window in an area where some small retroperitoneal vessels are visible.[40,106] In our opinion, the pelvic cavity (orthotopic site) provides the optimal environment for follicular development compared with heterotopic sites, as temperature, pressure, paracrine factors and blood supply are more similar to those observed in a physiological situation. Even if transplanting ovarian tissue to heterotopic sites has some advantages,[107–109] only one pregnancy has been reported following this procedure,[110] making this approach questionable.[40]

So far, 30 live births have been described after orthotopic reimplantation of cryopreserved ovarian tissue,[40,84] six of which have been recorded since May 2013.[40,111,112] Unfortunately, the number of reimplantations performed worldwide (the denominator) is not known. Results from three centres (in Denmark, Spain and Belgium) were collected to evaluate a series of 60 cases of orthotopic reimplantation.[40] Of the 60 patients, 51 had a follow-up of >6 months. Of these women, 11 (21%) became pregnant and six have already delivered 12 healthy babies (follow-up 1–10 years).[40] Restoration of ovarian activity was observed in 93% of the patients. In three patients, the absence of primordial follicles in their cryopreserved grafted tissue explained why ovarian function was not restored, highlighting the importance of evaluating follicular density before taking the decision to reimplant ovarian tissue. Restoration of ovarian activity occurred between 3.5 months and 6.5 months after grafting.[4,5,40,113] This finding is consistent with follicle growth from the primordial to the antral stage, although some variations were observed that were mainly attributable to differences in follicular reserve at the time of cryopreservation (Figure 2).

The mean duration of ovarian function after transplantation is ~4–5 years if follicular density is well-preserved,[10,40,113] but can persist for up to 7 years.[108] Markers of ovarian function are of limited value, as AMH remains undetectable and levels of inhibin β were found to be very low.[36] Among the different factors influencing ovarian graft longevity, ovarian reserve (that is, follicular density, which is age-dependent) and absence of chemotherapy before cryopreservation are the most important.[47]

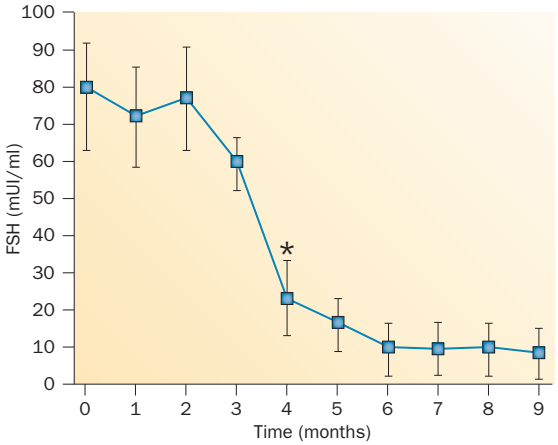

Figure 2 | FSH values in the months after orthotopic transplantation of frozen–thawed ovarian tissue. The significant decrease in levels of FSH (median ± SEM) 4 months after transplantation demonstrates restoration of ovarian activity after reimplantation. Asterisk indicates $P<0.05$. Permission obtained from Informa Healthcare © Donnez, J. *et al. Ann. Med.* **43**, 437–450 (2011).

Variable outcomes of transplantation

Not all women who have received an ovarian transplant go on to become pregnant. A study reporting an empty follicle rate as high as 29–35% during IVF when this procedure was required after transplantation (women with no fallopian tubes or no pregnancy despite restoration of ovarian function) has attempted to explain this variation.[114] Dysfunctional folliculogenesis was attributed to asynchrony between granulosa cells and oocyte maturation,[115] reduced ovarian reserve after grafting and the delay that occurs before efficient revascularization of the graft. Indeed, >50% of primordial follicles are lost following ovarian transplantation, mainly as a result of tissue ischaemia after transplantation while awaiting angiogenesis.[116–118] The graft takes 4–5 days to be reoxygenated and this delay could lead not only to loss of follicles, but also to dysregulation of communication between granulosa cells and the oocyte.[115] In transplanted tissue, the oocyte frequently shows ultrastructral alterations such as cytoplasm vacuolization and reduced numbers of cortical granules and microvilli.[114] Ischaemia and oxidative stress could be reduced by using drugs to stimulate revascularization of the graft (such as VEGF or sphingosine-1-phosphate [S1P]) or some inhibitory hormones (for example, AMH) that normally operate in an intact ovary.[40]

Indeed, if ischaemia is responsible for loss of follicles, another mechanism should also be pointed out: the remaining follicles are overactivated due to lack of AMH.[40,118,119] To understand how freezing and grafting might affect follicular development, expression of kit ligand and AMH, two key factors that activate and inhibit follicle growth, were assessed after long-term grafting of human ovarian cortex to severe combined immunodeficient (SCID) mice. AMH present in growing follicles after transplantation is able to act as a 'brake' on initial follicular activation (occurring

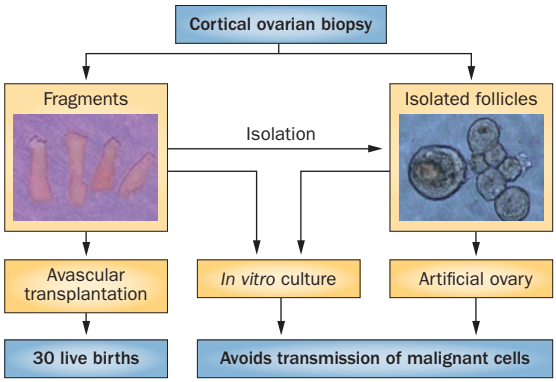

Figure 3 | Options for cryopreservation of ovarian tissue and reimplantation. The ovarian cortical biopsy sample can be cut into fragments (strips of about 10×3×1 mm) before cryopreservation. After thawing, the fragments are reimplanted without vascular reanastomosis. This technique (orthotopic transplantation) has yielded 30 live births so far. Another option would be to isolate ovarian follicles from the thawed biopsy sample, either for *in vitro* culture and *in vitro* maturation, or for grafting (through the concept of the artificial ovary). This technique is still experimental but would avoid transmission of malignant cells. Permission obtained from Informa Healthcare © Donnez, J. *et al. Ann. Med.* **43**, 437–450 (2011).

immediately after transplantation)[118] and protects the pool of residual primordial follicles.[119]

Optimizing outcomes of transplantation

Vitrification of ovarian tissue might be one way of improving outcomes after freezing and reimplantation.[120–124] Indeed, excellent results have been obtained in baboons after reimplantation of vitrified ovarian tissue in terms of follicular density and viability, as well as development of antral follicles.[125] Moreover, damaged granulosa cells in secondary follicles have been observed more frequently after slow-freezing than after vitrification.[124]

All these encouraging results are nevertheless not enough to enable us to propose vitrification of human tissue, as all live births in humans to date have been achieved after slow-freezing.[40] However, we believe live births in baboons (after reimplantation of vitrified ovarian tissue) would constitute strong proof that the quality of both granulosa cells and oocytes is well preserved using vitrification.[40,125]

The other aspect that needs to be improved is graft revascularization. Some publications suggest that angiogenesis favouring vascularization of the graft might be stimulated by delivery of both angiogenic factors and antiapoptotic factors.[126] The concept of the vascular bed,[40] which involves preparing the host (vascular bed) prior to grafting by encapsulated VEGF[127] or stromal cells enriched in CD34 cells, represents one important way of improving graft revascularization.[128]

Risk of reimplanting malignant cells

One serious concern that remains is the risk of reimplanting malignant cells together with the grafted tissue. A review published in 2013 examined all available evidence of this risk, particularly in patients with leukaemia, which is the most common haematological cancer in women <20 years old, followed by Hodgkin lymphoma and non-Hodgkin lymphoma.[129] These haematological malignancies are the most common indications (37.5%) for ovarian tissue cryopreservation.[16,130]

Leukaemia

Molecular biology has been used to evaluate the presence of leukaemic cells in ovarian tissue from patients with chronic myeloid leukaemia, acute myeloid leukaemia and acute lymphoblastic leukaemia.[130] PCR was used to show that ovarian tissue from patients with leukaemia was positive for malignant cells in >50% of patients, which led the researchers to conclude that reimplantation in these patients is unsafe.[130]

However, ovaries from patients with leukaemia in complete remission have not been found to contain malignant cells.[131] It should be stressed that these results, even if reassuring, should be interpreted with caution and remain to be confirmed.[132] By contrast, to avoid compromising the pool of primordial follicles by exposure to chemotherapy before cryopreservation, alternative methods such as *in vitro* maturation or isolated follicle transplantation (artificial ovary) should be further investigated and could be proposed to patients with leukaemia (Figure 3).[132,133]

In conclusion, each type of leukaemia might represent a different risk scenario depending on how the first course of chemotherapy is performed, how long the patient has been in remission and other factors, such as the number of viable malignant cells present that could cause relapse.[129]

Hodgkin lymphoma

The findings of several studies testing the safety of grafting cryopreserved human ovarian tissue from patients with Hodgkin lymphoma suggest that ovarian tissue transplantation might be considered safe in these patients.[134–137] One case report showed ovarian involvement in stage III Hodgkin lymphoma.[138] In another study reporting analysis of 5,571 autopsy findings,[139] the ovaries were found to be affected by Hodgkin lymphoma in 4.3% of autopsies; as disease stage was unknown, this prevalence should be interpreted with caution.

Among 16 patients with Hodgkin lymphoma who underwent autotransplantation, none experienced disease recurrence after their ovarian transplantation.[13,47,113,140–143] The maximum follow-up after autotransplantation in a patient with Hodgkin lymphoma is now >10 years.[46]

Non-Hodgkin lymphoma

Six autotransplantations of frozen–thawed ovarian tissue from patients with non-Hodgkin lymphoma have been reported, with no signs of disease recurrence.[40,47] Although ovarian involvement was observed in 9.8% of non-Hodgkin lymphomas,[139] histology did not reveal the presence of malignant cells in the ovarian cortex in a number of studies.[134,136,144,145] In addition, no evidence of metastasis was found after xenografting tissue to SCID mice for 16 weeks (13 patients with Hodgkin lymphoma and five with non-Hodgkin lymphoma).[136]

However, malignant cells were detected by histological evaluation in 2 of 32 (6%) patients with non-Hodgkin lymphoma.[129] Immunohistochemical analysis found non-Hodgkin lymphoma cells in the medulla (1 of 32) and the cortex (1 of 32) of the ovary.[129] Whilst the risk is low, it nevertheless exists and thus warrants further investigation.[129]

Gynaecological cancer

Breast cancer

In the female population of the USA, ~230,000 new cases of invasive breast cancer were reported in 2011, ~5% of which were in women <40 years old at the time of diagnosis.[146] The incidence of ovarian metastasis in patients with breast cancer can vary between 13.2% and 37.8%[139,147] but is more commonly observed in women with advanced-stage breast cancer.[148,149]

Two studies have analysed frozen–thawed ovarian cortical biopsy samples from patients with breast cancer.[150,151] Neither study revealed any evidence of malignant cell infiltration of the cryopreserved ovarian tissue by histology or immunohistochemistry. Moreover, autotransplantation of frozen–thawed ovarian fragments seems to be safe in patients with early-stage breast cancers.[40]

In conclusion, although these studies are so far reassuring, they nevertheless demonstrate that further procedures, such as PCR and long-term xenografting, are necessary to prove the safety of frozen–thawed ovarian tissue transplantation in women with breast cancer.[152]

Cervical carcinoma

Ovarian involvement is known to be more frequent in adenocarcinoma than in squamous cell carcinoma. Ovarian metastasis has been reported in 0.7–2.5% of patients with squamous cell carcinoma, and in up to 6.8% of patients with adenocarcinoma of the cervix.[13,40,61,153] Five cases of ovarian tissue autotransplantation after cervical carcinoma have been published and no sign of relapse from the grafted tissue has so far been noted.[13,40,108]

Endometrial carcinoma

According to histological studies, the risk of ovarian metastasis with endometrial cancer ranges from 1.9% (Federation of Obstetricians and Gynaecologists (FIGO) stage I tumours)[154] to 41.7% (FIGO stage I–III tumours).[155] Thus, the risk of metastasis is very low in the early stages of the disease.

Central nervous system tumours

Neuroblastoma has been classified together with leukaemia in the high-risk category for ovarian involvement.[156] Primitive neurectodermal tumours, including medulloblastoma (the most common) and neuroblastoma, generally occur in children and are classified into two types: peripheral primitive neurectodermal tumours (that is, Ewing sarcoma) and central nervous system primitive neurectodermal tumours.

A study has reported reimplantation of frozen–thawed ovarian tissue in a patient cured of a metastatic neurectodermal tumour of the orbit, who had her ovarian tissue cryopreserved before undergoing bone marrow transplantation.[10] Histological evaluation of an ovarian sample before transplantation did not reveal ovarian metastasis and the patient has now delivered her third child. Another patient treated for Ewing sarcoma who underwent transplantation of her cryopreserved ovarian tissue has also given birth to healthy babies twice.[13,157]

Two studies failed to detect the presence of malignant cells,[45,145] whereas in another,[69] real-time PCR proved positive for Ewing sarcoma. Real-time PCR analysis of specific markers is thus required before proposing reimplantation to women with this disease.

The risk of ovarian involvement, divided into three categories (low, intermediate and high) according to the type of malignant disease, was first published by Oktay.[156] This classification was subsequently slightly modified to recategorize Ewing sarcoma and non-Hodgkin lymphoma from low to moderate risk (Box 3).[129]

Box 3 | Risk of ovarian metastasis according to cancer

High risk (>11%)
- Leukaemia
- Neuroblastoma
- Burkitt lymphoma

Moderate risk (0.2–11%)
- Breast cancer (stage IV infiltrating lobular subtype)
- Colon cancer
- Adenocarcinoma of the cervix
- Non-Hodgkin lymphoma
- Ewing sarcoma

Low risk (<0.2%)
- Breast cancer (stage I–II infiltrating ductal subtype)
- Squamous cell carcinoma of the cervix
- Hodgkin lymphoma
- Osteogenic carcinoma
- Non-genital rhabdomyosarcoma
- Wilms' tumour

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The future

Follicle culture and *in vitro* development

Initiation of primordial follicle development and early growth, and optimization of development from the preantral to the antral stage with completion of oocyte growth are important steps in folliculogenesis.[29]

A dynamic multistep culture system is required to support each of the transitional stages of follicles.[29,158] The multistep approach for follicles grown *in vitro* needs to support the changing requirements of the developing oocyte and its surrounding somatic (granulosa) cells to maintain interactions between oocytes and somatic cells.[29,158,159]

The development of culture conditions for immature germ cells is one of the greatest challenges in reproductive technology. Indeed, mechanisms regulating activation of primordial follicles require inhibitory, stimulatory and maintenance factors.[29] The final goal of this multistep approach is to obtain developmentally competent oocytes that are capable of producing

embryos and yielding live births as the ultimate clinical end points.

Clinical application of follicle culture is directed towards fertility preservation when there is a risk of reimplanting malignant cells.[132] In macaques, continued growth of secondary follicles (in 3D systems) from vitrified ovarian tissue to the antral stage, accompanied by steroidogenesis *in vitro*, has been reported.[123,124]

The artificial ovary

Another alternative is to obtain mature oocytes with the help of the so-called artificial ovary. Isolation of primordial follicles and their transfer onto a scaffold to create an artificial ovary will, of course, eliminate the risk of transmission of malignant cells. Human preantral follicles can be successfully cryopreserved before and after isolation without impairing their ability to survive and grow *in vitro*.[133,160] Indeed, survival and growth of preantral follicles from vitrified human ovarian tissue has been demonstrated, as has survival and growth of isolated follicles enclosed in biomatrices.[133,161]

The first step in developing an artificial ovary, namely a biodegradable scaffold (consisting of an alginate matrigel matrix) onto which isolated preantral follicles and ovarian cells can be grafted, was accomplished in 2012.[133] Transplanted beads were able to degrade, enabled vascularization and elicited a low inflammatory response.[133]

Ovarian stem cells

The discovery of ovarian stem cells has challenged the theory that production of germ cells in female mammals ceases before birth.[6,22,23] In fact, the debate started in 2004, when a paper reported the presence of mitotically active or oogonial stem cells (OSCs, also known as germline stem cells) in the mouse ovary, which might maintain oocyte and follicle production after birth according to the authors.[162] Since then, studies have demonstrated that mouse OSCs can be isolated from adult ovaries for long-term propagation *in vitro* and generation of fertilization-competent eggs *in vivo* following intraovarian transplantation.[27]

One team was able to isolate, culture and form early follicle structures after injection of both mouse and human OSCs into ovarian tissue that was xenotransplanted to NOD–SCID mice.[28] Establishing a close parallel between human ovary-derived DDX4-positive cells and mouse OSCs, this team concluded that the rare cells showing DDX4 expression on their cell surface that are present in human adult ovaries represent OSCs and might offer new opportunities to enhance fertility preservation strategies.[28] It should be noted that OSCs are extremely scarce. Indeed, they constitute just 0.014% of all cells in mouse ovaries[28] and become even scarcer with increasing age.[163]

Other stem cells, such as embryonic stem cells and induced pluripotent stem cells, can be turned into primordial germ cell-like cells. These cells can then generate germinal vesicle-stage oocytes after transplantation into mice, with gonadic somatic cells.[164]

Of course, obtaining early follicle-like structures after xenotransplantation of human OSCs is ethically unacceptable in clinical practice, but development of a numbered-step culture system could be an interesting alternative to produce mature oocytes from OSCs completely *in vitro*.[164,165]

However, some controversial issues persist despite the success of achieving live offspring from OSC cultures in mice and generating early follicle-like structures in human tissue.[29,164] Criticisms have been levelled at the experimental techniques and their interpretation.[164] Concerns also exist that *in vitro*-derived oocytes might interfere with the complex genomic imprinting and epigenetic mechanisms required for the development of fully competent oocytes.[166] We can thus conclude that rare cells with germline characteristics can be obtained from human ovaries, but their potential use remains unclear.

Other novel and original approaches in fertility therapy have been proposed in the past few years. New perspectives have been described in reproduction, including autologous germline mitochondrial energy transfer and use of OSCs as a source of mitochondria for the rejuvenation of eggs.[24,28,167] Given the abundant literature on the topic, new treatments in infertility therapy and fertility preservation will no doubt result from ongoing OSC research.[167]

Are there new avenues of research?

AS101

Cyclophosphamide has been shown to activate growth of quiescent primordial follicles in mice, leading to loss of the ovarian reserve.[168] This accelerated follicle activation, which results in a 'burn-out' effect and follicle depletion, was prevented by administration of the AS101 immunomodulator. Further studies are required to investigate whether AS101 could have a role as an ovarian protective agent in clinical practice to preserve fertility in female patients with cancer.

S1P

S1P is a sphingolipid metabolite that inhibits cell apoptosis induced by radiation and chemotherapy in mice.[169] In a xenograft model, S1P promoted neoangiogenesis and reduced the severity of ischaemia–reperfusion injury. In this model, the mice received S1P through an osmotic pump for the first 4 days after xenografting.[170]

In another model, mouse ovaries were pretreated with S1P for 1 h and then vitrified.[171] The ovaries were removed 10 days after autotransplantation. Levels of AMH and the number of primordial follicles were higher in the group pretreated with S1P than in those who only underwent vitrification. Other studies are clearly needed to explore the possible potential of S1P in enhancing angiogenesis in human ovarian cortical grafts.

Conclusions

Aggressive chemotherapy and/or radiotherapy and bone marrow transplantation can cure >90% of girls and young women affected by diseases requiring such

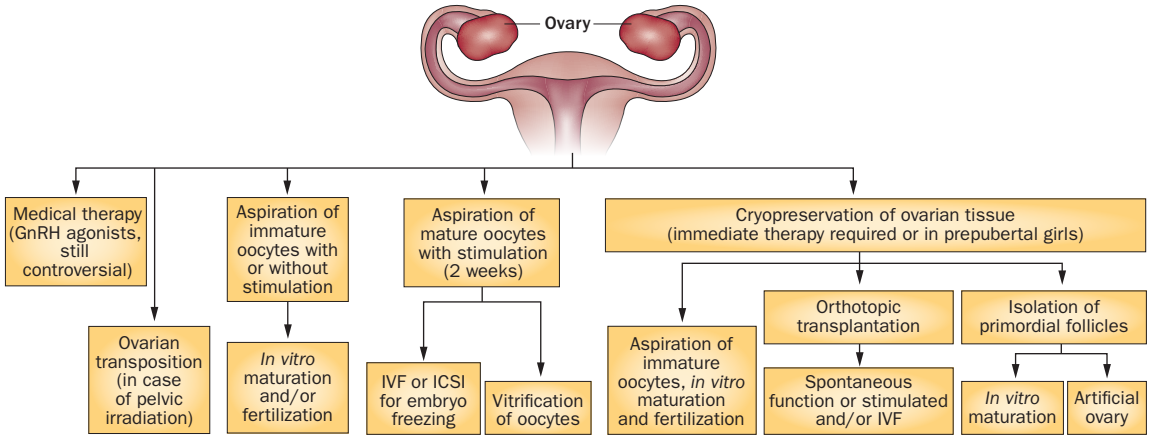

Figure 4 | Fertility preservation methods in women at risk of premature ovarian failure. Depending on the patient's age and the possible delay before starting chemotherapy, options include; medical therapy to protect the ovaries from chemotherapy (still controversial); ovarian transposition before pelvic radiotherapy; immature oocyte retrieval followed by *in vitro* maturation; mature oocyte retrieval followed by fertilization and embryo freezing or oocyte vitrification; or ovarian tissue cryopreservation. At the time of ovarian tissue cryopreservation, immature oocytes can also be retrieved. After thawing of ovarian tissue, orthotopic transplantation is currently the goal in clinical practice. Indeed, isolation of primordial follicles is still at the research stage. Abbreviations: GnRH, gonadotropin-releasing hormone; ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization. Permission obtained from Elsevier © Donnez, J. *et al. Fertil. Steril.* **99**, 1503–1513 (2013).

treatment. Nevertheless, the risk of impairing gonadal function is high, particularly when gonadotoxic drugs (especially alkylating agents) are used.

Embryo and oocyte cryopreservation are the options of choice (and are endorsed by the ASRM) if chemotherapy can be delayed, giving patients with cancer the hope of a successful pregnancy when they have overcome their disease. In prepubertal girls and patients who require immediate treatment, ovarian tissue cryopreservation is the only available method.

In the future, cryopreservation of ovarian tissue might be combined with the removal of small antral follicles (by puncture), making it possible to freeze both ovarian tissue and isolated immature oocytes, including those present in the dissection medium. Indeed, they might be highly abundant in the medulla, especially in very young patients (Figure 4).

In July 2013, the American Society of Clinical Oncology (ASCO)[54] added some clarifications to the 2006 ASCO recommendations, with the goal of updating guidance for health-care providers. Health-care providers (including gynaecologic oncologists, paediatric oncologists and haematologists) should address the possibility of infertility with patients requiring treatment with gonadotoxic drugs and/or irradiation as part of the informed consent procedure and advise them as to available methods of fertility preservation, referring them to appropriate reproductive specialists.[54] In the ASCO recommendations, it is stressed that no patients should be excluded from discussion of fertility preservation options for any reason (such as age, prognosis or parity), even if financial or insurance barriers might be present.[54]

Optimizing techniques and minimizing the risks of fertility preservation strategies represent the challenges of the next decade. In the near future, indications for benign diseases (such as recurrent endometriosis) and social reasons will also become increasingly frequent, even if the most immediate concern is helping women affected by cancer.[172]

Review criteria

We searched the PubMed database for eligible articles. Search terms covered medical subject headings and/or text words relating to "fertility preservation", "ovarian follicles", "embryo cryopreservation" and "oocytes". Search conditions were limited to publications in English until June 2013. Reference lists of the retrieved papers and reviews were searched for additional relevant articles. In case of duplicate publications or overlapping data, only those published most recently or with the largest samples were included. Only the most recent and appropriate reviews were selected, according to each specific topic.

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Author contributions
Both authors contributed equally to all aspects of the manuscript.