Improving female fertility preservation care

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For reasons of consistency, terminology may be changed throughout this thesis when compared to the original publications.

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PART A
Safety

PART B
Efficacy and efficiency

PART C
Patient-centeredness, timeliness, and equitability

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General introduction

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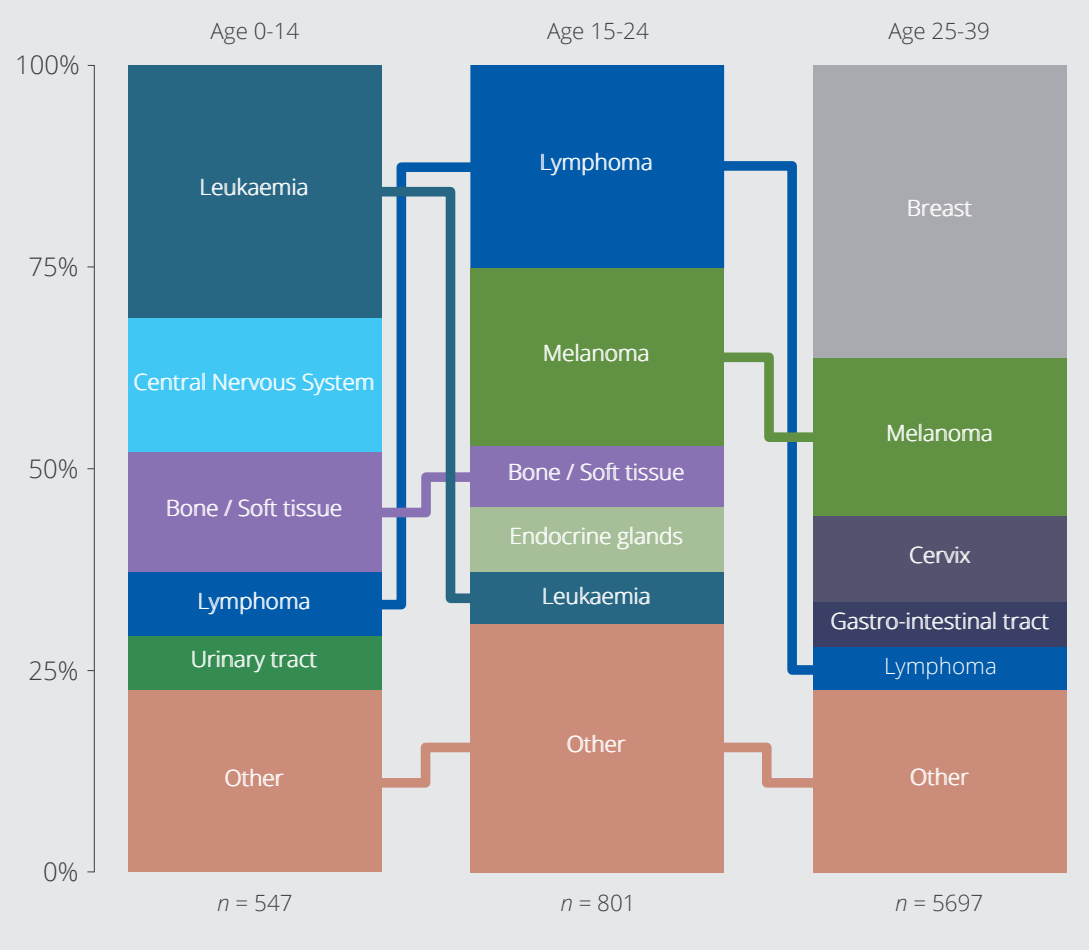
General introduction

During the past decades, the survival of paediatric, adolescent, and young adult cancer patients has improved considerably.[1-3] For this reason, issues relating to the quality of life after cancer have gained increased attention in oncological care. One of the most pivotal issues related to the quality of life of cancer survivors concerns their fertility, as various forms of oncological therapy pose a serious threat to the reproductive capacity.[4-6] Preserving the possibility to have biological children is very important for many girls and young women affected by cancer.[7-9] In this thesis, several aspects concerning fertility preservation for female cancer patients are considered.

Figure I. The 5 most commonly diagnosed cancers in females aged 0 - 39 years in the Netherlands, 2009-2011.



Average percentages of new cases for 2009-2011 based on data from the Dutch Cancer Registry.
Source: www.cijfersoverkanker.nl

Cancer in girls and young women

In the Netherlands, 2300 to 2400 girls and women of reproductive age (0-39 years) are diagnosed with cancer each year.[10] Of these patients, 150 to 200 are girls under the age of 15, whereas 250 to 300 are women aged 15 to 24 years.[10] A wide range of oncological diseases has been shown to occur in girls and young women, with incidences varying with age (Figure I).[1,10,11]

Cancer therapy and fertility problems

The ovarian function

Chemotherapy or radiotherapy may result in a premature depletion of ovarian follicles and oocytes, thereby decreasing the chance of a spontaneous pregnancy.[4-6] A low ovarian follicle reserve will eventually become clinically apparent as an impaired reproductive capacity, climacteric symptoms, and/or amenorrhoea (absent menstrual periods for at least 6 months). When cessation of menstrual periods occurs before the age of 40 years, this is called "premature ovarian insufficiency" (POI).[6]

The uterine function

In addition to the effects of cancer therapy on the ovaries, oncological treatment may also affect the uterine function.[12-14] After radiotherapy, histopathological changes such as uterine vascular damage and uterine contraction with fibrosis and loss of distensibility have been described.[15,16] Female patients who received an allogeneic haematopoietic stem cell transplantation (HSCT) with total body irradiation as pre-transplant conditioning appeared to have a higher risk of preterm delivery, low birth weight babies,[13,14,17] and possibly also of miscarriage than healthy women.[13,14,16] Higher risks of intrauterine growth restriction and premature birth were not observed in patients who received HSCT conditioning with chemotherapy only.[13]

Benign diseases and fertility problems

HSCT, chemotherapy, and ovarian surgery are also used in the treatment of some *benign* conditions. For severe autoimmune diseases, including aplastic anaemia and systemic lupus erythematosus (SLE), HSCT is a treatment option.[4,18-22] In addition, extensive surgery required for benign ovarian tumours or cysts[23,24] could impair the reproductive capacity. Apart from treatment-related fertility problems, POI could also result from benign (genetic or autoimmune) diseases themselves, including galactosaemia[25] or a fragile X pre-mutation.[26]

Estimating the risk of infertility

In a study comparing female cancer survivors with their siblings, cancer survivors had a significantly higher risk (relative risk of 1.48) of infertility (more than one year of attempts at conception without success) after adjustment for socio-demographic and behavioural risk factors for fertility problems.[27] Relative to their siblings, younger patients had a higher relative risk of infertility than older women, with relative risks of infertility of 2.92 in participants aged ≤ 24 years and 1.61 in participants aged 25-29 years.[27] The exact risk of an individual patient to develop POI after gonadotoxic treatment depends on the cancer treatment regimen and the ovarian reserve at the start of treatment.

Ovarian reserve

As ovarian reserve relates to age,[6,28] relatively older women with an already decreased number of primordial follicles have a higher risk of developing POI as a result of oncological therapy when compared to young women.[17,29] In addition, it has been shown that a low pre-treatment level of anti-Müllerian hormone (AMH) – a granulosa-cell derived growth factor reflecting the ovarian reserve – is predictive for a higher risk of amenorrhoea at 2 years after cancer treatment.[30] However, AMH levels do not only correlate with the number of non-growing follicles, but also with other factors including the presence of oncological disease and a cancer patient's general health status.[31] Long-term follow-up studies are required to assess the exact value of pre-treatment serum AMH levels in estimating the risk to developing POI after oncological treatment.[32]

Treatment characteristics

Types of treatment associated with a high risk of POI are pelvic irradiation, alkylating chemotherapeutic agents, and HSCT combined with total body irradiation or alkylating agents.[4-6,29,33,34] Platinum agents and taxanes have also been associated with an evident loss of primordial follicles.[29,34] As cancer patients usually undergo combinations of chemotherapeutics or combinations of radiotherapy and chemotherapy, several studies have focussed on the effects of regularly used combined regimens in patients of certain ages.[29] For instance, a treatment combining doxorubicin, bleomycin, vinblastine, and decarbazine (ABVD) that is used for Hodgkin's lymphoma, results only in a low risk of POI.[29,34] The results of studies on the risk of POI with combined regimens have been translated to an online risk calculator.[35]

Fertility preservation

To adequately address the reproductive concerns of young female (cancer) patients, specialised care on both the psychological and biological aspects of the field of fertility preservation has been established. Fertility preservation includes all techniques aimed

at helping patients to retain their ability to have children. Several surgical and laboratory techniques – all with their own pros and cons – are currently being used in clinical practice. These techniques include the transposition of the ovaries outside the radiation field, the cryopreservation of embryos, the vitrification of oocytes, and the cryopreservation of ovarian tissue. Obviously, fertility preservation techniques should ideally be performed before the start of gonadotoxic treatment.

Ovarian transposition

Laparoscopic transposition of the ovaries outside the field of irradiation may be used to prevent damage to the ovaries as a result of radiotherapy to the pelvic region. The main indications for this technique are cervical and rectal cancers in adults and rhabdomyosarcomas or pelvic bone sarcomas in children.[36] After mobilisation, the ovaries can be fixated at various locations using resorbable or non-resorbable suture material. Common locations for the transposition include the paracolic gutters, the retrouterine position, or a lateral position near the inguinal ring, depending on tumour location.[36] The effectiveness of a second surgical procedure to return the gonads to their original position remains controversial. Apart from ovarian cysts being associated with transposition, the possibility of malignant cells being present in the transposed ovary should be kept in mind.[36,37] Using ovarian transposition in adults, the ovarian function is preserved in 33 to 92% of the cases.[36]

Embryo cryopreservation and vitrification of oocytes

Since the updated guideline of the American Society of Clinical Oncology in 2013,[38] not only the cryopreservation of embryos, but also the vitrification of oocytes is considered to be an established fertility preservation option. Both techniques require the collection of mature oocytes after ovarian hormonal stimulation. Hormonal stimulation takes approximately two weeks and can be started at various moments of the menstrual cycle[38] or overlap with the use of oral contraceptives. After the aspiration of mature oocytes under transvaginal ultrasound control,[39] unfertilised oocytes are either rapidly frozen (vitrified) or fertilised to create embryos that can be cryopreserved. As the mature oocyte is a large cell with an intrinsically unstable chromatin arrangement, the oocyte is much more vulnerable to cryodamage than an embryo that consists of small blastomeres and displays a more stable chromosome arrangement.[39] However, for patients without cancer, survival rates of vitrified oocytes after warming of 75 – 97% have been reported, accompanied with fertilisation and implantation rates similar to those of fresh oocytes.[40] Live birth rates reported for vitrification of oocytes in a subfertile population were 5.4% per vitrified oocyte[41] and more than 1000 live births have been reported.[42-44] For slow-frozen embryos transferred at a cleavage stage, post-thaw survival rates of 73 - 93% and implantation rates of 10 – 32% have been demonstrated.[45] There are only limited published data on pregnancy rates after embryo cryopreservation or oocyte vitrification

carried out as an emergency procedure in oncological patients.[39,46] Contradictory findings have been published with respect to the impact of oncological disease on the ovarian response to hormonal treatment, with some reporting a similar and others a reduced response in oncological patients compared to healthy women.[47]

The storage of embryos or oocytes cannot be offered to all oncological patients and comes with various issues of concern. As oocyte vitrification and embryo cryopreservation require hormonal stimulation, these two techniques are not suitable for pre-pubertal patients or women who need immediate oncological treatment. Although the vitrification of oocytes may be considered in young post-pubertal patients, low doses of gonadotrophins must be administered to reduce the risk of ovarian hyperstimulation syndrome (OHSS).[48] Furthermore, transvaginal oocyte retrieval may be difficult in sexually immature teenagers.[48] For the cryopreservation of embryos, either a stable relationship with a male partner is required, or the use of donor sperm. Permission of both parties is required for the use and storage of the resulting embryos while withdrawal of consent will result in the destruction of the embryos.[39] With a mean number of 8.5 metaphase II oocytes retrieved per cycle,[46] the vitrification of oocytes and the cryopreservation of embryos do not guarantee a pregnancy after cancer survival. Due to the need for gonadotoxic therapy, most patients do not have the chance to receive an additional cycle of ovarian stimulation to yield a higher number of embryos or oocytes. Ovarian stimulation protocols using aromatase inhibitors are applied to induce follicle development while minimizing oestrogen production in patients with hormone-dependent cancer, such as oestrogen receptor positive breast cancer.[39] However, it is currently unknown whether or not these protocols result in a lower breast cancer recurrence rate on the long-term.[39,49,50]

Ovarian tissue cryopreservation and autotransplantation

The cryopreservation and autotransplantation of ovarian tissue is still considered an experimental technique to preserve fertility.[38] With this technique, ovarian tissue is cryopreserved preferentially before the start of gonadotoxic treatment. The cryopreserved tissue can be retransplanted when a patient is cured from her disease and wishes to conceive, but experiences POI due to her treatment. After laparoscopic removal of (part of) one ovary, the tissue is transported to the laboratory of a specialised centre. Next, the ovarian medulla is separated from the millimetre thin cortex and small cortex fragments are prepared. Cortex fragments, containing most of the ovarian follicles, are cryopreserved in the presence of a cryoprotectant to prevent the formation of ice crystals that may damage the tissue. At the time the cortex fragments are needed for autotransplantation, (part of) the tissue is thawed. During the thawing of ovarian tissue, the cryoprotectant – toxic for the ovarian tissue at ambient temperature – is washed out. Subsequently, the cortex fragments are autotransplanted at the operation theatre, either to the contralateral ovary (orthotopic) or to a heterotopic site (i.e. the forearm or abdominal wall).[51] In 2001, the first successful orthotopic autotransplantation,

leading to a resumption of menses, was reported.[52] This was followed by the first live birth in 2004.[53] At present, 37 live births have been reported after autotransplantation of cryopreserved-thawed ovarian cortex fragments.[54] These live births resulted from spontaneous conceptions as well as in vitro fertilisation (IVF) or Intracytosplasmic Sperm Injection (ICSI).[55]

The technique of ovarian tissue cryopreservation and autotransplantation comes with both advantages and challenges. An important advantage of the cryopreservation of ovarian tissue is the possibility to store a large number of primordial follicles. Moreover, the technique requires no hormonal ovarian stimulation and can thus be rapidly used in both pre-pubertal as well as post-pubertal patients.[56] After the autotransplantation of ovarian tissue, the ovarian graft restores the premenopausal hormonal status, eliminating the need for hormone replacement therapy during the graft's lifespan. The cryopreservation and autotransplantation of ovarian tissue have recently also been described as a technique to induce puberty after gonadotoxic therapy during childhood for a benign disease.[57,58] Despite the advantages of cryopreservation of ovarian tissue, there is concern about the efficacy.[59-61] As there is no international register of all autotransplantation procedures that have been performed,[61] the success rate of cryopreservation and autotransplantation is still largely unknown. Moreover, success rates may differ from one clinic to another as a variety of laboratory protocols are being used.[59] Ovarian tissue may sustain damage during the various steps of the cryopreservation and thawing procedures, or more importantly, from warm ischaemia after autotransplantation.[56] As it may take up to weeks before the graft is revascularised, the graft's follicular pool is seriously affected due to lack of sufficient oxygen and nutrients, thereby reducing the life span of the graft and reproductive chances after autotransplantation.[5,56] A matter of great concern is the potential presence of metastatic cancer cells in the ovary at the time of tissue cryopreservation. This cryopreserved tissue will not be exposed to oncological therapy and cancer cells present in the graft may lead to a renewed oncological disease after autotransplantation.[62]

Counselling and decision-making in female fertility preservation

When confronted with a need for gonadotoxic treatment, (parents of) girls and young women will have to make a decision regarding fertility preservation. This decision is irreversible and has to be made in a short and burdensome period of life. Patients indicated a wish to be informed about fertility-related issues related to cancer and counselling on fertility-preservation.[7] Nevertheless, only 34-72% of the oncological patients recall counselling about the impact of cancer therapy on their fertility.[7] In the United States of America, financial reasons to refrain from fertility preservation or even the consultation of a specialist for counselling have been identified.[63,64] The costs of fertility preservation also played a key role in patients' difficulties during decision-

making regarding fertility preservation.[64,65] However, little is known about the current referral practices and patients' experiences with decision making with respect to fertility preservation in countries where fertility preservation is reimbursed.

Table I. Overview of clinically used fertility preservation techniques for female patients

	Aims to preserve the ovarian function from the effects of:	Requires	Applicable for	Established or experimental
Ovarian transposition	Radiotherapy	Ability to undergo surgery	Pre- and post-pubertal patients	Established
Cryopreservation of embryos	Radiotherapy and chemotherapy	Time for hormonal stimulation Male partner or semen donor	Post-pubertal patients	Established
Oocyte vitrification	Radiotherapy and chemotherapy	Time for hormonal stimulation	Post-pubertal patients	Established
Cryopreservation of ovarian tissue	Radiotherapy and chemotherapy	Ability to undergo surgery	Pre- and post-pubertal patients	Experimental

The aim of this thesis: Improving the quality of fertility preservation care

The aim of this thesis is to improve the quality of fertility preservation care for female patients. The Institute of Medicine argues that high quality healthcare should achieve major gains in six areas.[66] Healthcare should be:

» ***Safe***: avoiding injuries to patients from the care that is intended to help them.
» ***Effective***: providing services based on scientific knowledge to all who could benefit, and refraining from providing services to those not likely to benefit.
» ***Patient-centered***: providing care that is respectful of and responsive to individual patient preferences, needs, and values, and ensuring that patient values guide all clinical decisions.
» ***Timely***: reducing waits and sometimes harmful delays for both those who receive and those who give care.
» ***Efficient***: avoiding waste, including waste of equipment, supplies, ideas, and energy.
» ***Equitable***: providing care that does not vary in quality because of personal characteristics such as gender, ethnicity, geographic location, and socioeconomic status.

Outline

In this thesis, which is divided in three parts, we address various questions concerning the quality of fertility preservation care for female patients. Part 1 covers the safety of cryopreservation and autotransplantation of ovarian tissue. Part 2 of this thesis zooms in on the efficacy and efficiency of various techniques for ovarian tissue cryopreservation and thawing. In part 3, the patient-centeredness, timeliness, and equitability of female fertility preservation care are evaluated. The main questions leading to this thesis were:

Part 1: Safety

» Why is there concern about the safety of ovarian tissue autotransplantation in cancer survivors? (Chapter 2)
» How should we assess the risk of oncological relapse due to the reintroduction of tumour cells via an ovarian tissue transplant? (Chapter 2)
» What is the risk of reintroducing malignant disease via ovarian tissue autotransplantation in survivors of various malignant diseases? (Chapter 3)
» Is it possible to mimic the growth of (metastatic) cancer cells in ovarian tissue to develop techniques for tumour cell purging protocols that would be useful to apply in clinical practice? (Chapter 4)

Part 2: Efficacy and efficiency

» What is the efficacy of ovarian tissue cryopreservation and thawing using the protocols from a major European centre? (Chapter 5)
» What is the difference in the efficacy and efficiency of two very different protocols for cryopreservation and thawing on the viability of ovarian cortex tissue? (Chapter 6)

Part 3: Patient-centeredness, timeliness, and equitability

» Are there any changes in the numbers and characteristics of Dutch female cancer patients receiving fertility preservation consultation during time? (Chapter 7)
» What are the current referral rates of girls and young women with cancer for fertility preservation consultation in a setting with reimbursement of fertility preservation services? (Chapter 7)
» Which determinants influence the referral of women for fertility preservation? (Chapter 7)
» How do Dutch female patients experience consultation and decision-making with respect to fertility preservation? (Chapter 8)
» Are patients' experiences with fertility preservation consultation associated with decisional conflict during decision-making? (Chapter 8)
» Does decisional conflict during decision-making with respect to fertility preservation relate to decision regret? (Chapter 8)

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PART A

Safety

2

Cryopreservation and autotransplantation of ovarian tissue in cancer patients: is it safe?

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Oncological therapy may severely compromise the future fertility of girls and young women with cancer and thereby limit their quality of life. Various strategies to preserve fertility before the start of gonadotoxic treatment have been proposed, such as *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), vitrification of oocytes, and cryopreservation of ovarian tissue.[1] Unfortunately, not all of these techniques are suitable for pre-pubertal girls and adolescents. Not only do IVF and ICSI require a male partner or donor sperm for fertilization, procedures using gonadotrophin administration and oocyte retrieval are considered inappropriate for sexually immature patients.[2] In the case of ovarian tissue cryopreservation, neither hormonal stimulation nor a stable relationship is necessary. These characteristics make ovarian tissue cryopreservation especially suitable for adolescents and the only option available for fertility preservation in pre-pubertal girls.[1,2] Furthermore, as ovarian tissue can be obtained directly after diagnosis, there is only minimal interference with cancer treatment.[1]

The main aim of cryopreservation of ovarian tissue is to restore reproductive potential by retransplanting the tissue. This can be performed once the patient has overcome her disease and wishes to conceive, but experiences premature ovarian insufficiency due to the cancer treatment.[1,3] Although the technique of ovarian tissue autotransplantation and subsequent autografting is still considered experimental, 16 live births have already been reported; 12 of these children were born to 9 women who survived cancer.[3,4]

As no central registration for ovarian tissue cryopreservation or autotransplantation exists, the total number of procedures carried out worldwide is unknown.[5] Nevertheless, a considerable number of cryopreservation and autotransplantation procedures have been reported,[6-14] indicating that cryopreservation of ovarian tissue may have been performed on a large scale in the past decade. Therefore, the number of cancer survivors requesting autotransplantation of their ovarian tissue is anticipated to increase considerably in the near future.[5]

Although autotransplantation procedures are already being performed in oncological patients worldwide, the risks of recurrent malignancy due to transmission of cancer cells via the ovarian graft still remains largely unknown. This information is critical for proper counselling and clinical decision-making for cancer survivors considering autotransplantation, as well as for newly diagnosed oncological patients.[1,5] Here we discuss ovarian tissue autotransplantation-related safety issues and make recommendations for future research and patient counselling.

Why is there concern about the safety of ovarian tissue autotransplantation?

The alarm was raised on the safety of ovarian tissue autotransplantation in cancer survivors for the first time in 1996. At that time, healthy mice transplanted with fresh or cryopreserved ovarian tissue from mice with lymphoma developed the disease and died, though one mouse that received cryopreserved tissue remained healthy.[15] A

later xenotransplantation study also showed that acute lymphoblastic leukaemia was transmitted to recipient animals via human ovarian grafts.[16]

By using histology or polymerase chain reaction (PCR), cancer cells have been detected in the ovaries from patients with leukaemia and Ewing sarcoma.[16-18] Apart from these findings, it is known from clinical experience that different types of oncological diseases have the potential to metastasise to the ovaries. For example, breast cancer, a common indication for ovarian tissue cryopreservation,[9,11-13] has been repeatedly demonstrated to metastasise to the ovaries.[19,20] In contrast to these alarming results, other studies report more reassuring findings when it comes to the safety of ovarian tissue autotransplantation. Several studies failed to show the presence of malignant cells in ovarian cortical tissue from patients with breast cancer,[21,22] lymphoma[23,24] and various other oncological diseases.[25]

Several authors have tried to provide guidance for clinical decision-making and counselling by classifying different oncological diseases as having a low-, intermediate-, or high risk of ovarian involvement based on the literature available.[26-28] However, the absolute magnitude of the risk of ovarian metastasis remains unclear, with some diseases being classified in different risk categories in various publications.

How to assess the risk of oncological relapse due to the reintroduction of tumour cells via transplantation?

One of the pivotal issues when assessing this risk of relapse is the chance that malignant cells derived from a certain tumour type and stage are present in the ovaries at the time ovarian tissue is cryopreserved. Therefore, an overview of epidemiological data on ovarian metastasis in different primary tumour types, as well as the use of valid diagnostic tools with which the involvement of the ovaries can be assessed in each individual cancer patient, would be useful.

Histology and Polymerase Chain Reaction

Minimal residual disease in ovarian tissue from cancer patients can be detected using histology and/or PCR.[16-18,22-25] During histological examination, the presence of cancer cells may go unnoticed.[18] PCR is much more sensitive than histology, but tumour-specific PCRs are available for only a limited number of oncological diseases. In addition, a positive PCR signal does not provide information about the viability of tumour cells present in the positive tissue, nor about their ability to cause relapse after transplantation.

Cortex strips from the same ovary analysed by PCR may give different results with respect to the detection of cancer cells, indicative of sampling bias.[18] As the ovarian strips that are analysed by histology or PCR are no longer available for transplantation purposes, it remains uncertain whether the strips that are actually used for transplantation purposes are indeed devoid of cancer cells.

In conclusion, histology and PCR may provide information about the incidence of minimal residual oncological disease in the ovarian tissue, but do not guarantee that transplantation is safe.

Xenotransplantation

Prior to performing an autotransplantation of ovarian tissue to the human recipient, one or a few cortex fragments may be xenografted to a suitable immunodeficient host animal.[15,16,23] Should the recipient animal develop malignant disease, the tissue obviously contains tumour cells, as with the mice that developed intraperitoneal leukaemic masses after being grafted with frozen-thawed ovarian tissue from patients with leukaemia.[16] However, if the animals remain healthy, this still offers no guarantee for safe autotransplantation, as there is again inevitable sample bias.[29] In addition, growth of the cancer cells might be different in the humans and immunocompromised animals.

Follow-up after autotransplantation of ovarian tissue

The duration of follow-up after autotransplantation of ovarian tissue is still relatively short and transplantations have not yet been performed on a large scale.[3,5,8] Therefore, the safety of autotransplantation cannot be ensured by the follow-up data currently available. Finally, one should consider that the available follow-up data might be biased by selective reporting, as no central registration exists.[5]

Epidemiological data on the incidence of ovarian metastases

Follow-up of patients with a certain type of tumour or reports from prophylactic oophorectomy during tumour resection may provide information about the incidence of ovarian involvement. However, cancer patient follow-up studies investigating the incidence of ovarian involvement do not always clearly describe how they assessed the presence of ovarian involvement; tumour stage, prior treatment, and other important prognostic variables may also not be described. With such a large number of confounding factors left unspecified, it is quite difficult to extract useful information from this type of report. Therefore, studies should provide clear information about tumour types, stages, patient characteristics, and other relevant prognostic factors, as well as details of the research methods used. Studies meeting these strict criteria may be scarce, and a systematic literature search for these reports is strongly advisable.

One of the difficulties in interpreting epidemiological data is that these reports include patients who did not have their ovarian tissue cryopreserved. This means that for a large number of cases, their ovaries have been exposed to radio- or chemotherapy that may have eliminated minimal residual disease in this organ. This is generally not the case in patients who did opt for ovarian tissue cryopreservation. In addition, although clinical studies may provide information about the incidence of clinically relevant ovarian metastases during the follow-up period, they do not inform about the percentage of

patients who have minimal ovarian involvement at the time of cancer diagnosis. As a result, it is difficult to estimate the significance of a single tumour cell in cryopreserved ovarian tissue, as its capacity to develop into a metastatic lesion after autotransplantation is unknown.

Conclusion and recommendations

In conclusion, the available information for counselling cancer survivors with regard to their risk of oncological relapse after ovarian tissue autotransplantation is insufficient. In addition, data from different types of studies cannot be interpreted unambiguously. It is clear that data on the risk of reintroducing tumour cells with ovarian transplantation are necessary, as a growing number of patients are expected to request cryopreservation and autotransplantation in the near future. We would therefore like to make the following recommendations.

First, we propose the creation of a database in which all cancer-related cryopreservation and autotransplantation cases are registered, including follow-up data. Registration should preferably take place on an international level. The European Society of Human Reproduction and Embryology (ESHRE; Task Force Fertility Preservation), the International Society for Fertility Preservation (ISFP), or the American Society for Reproductive Medicine (ASRM) may provide the necessary infrastructure for such a registry, and would be logical candidates for setting up this initiative. The anonymous data from the resulting database should be available to all clinicians who counsel patients on fertility preservation, ensuring that unbiased follow-up data will be available as soon as possible.

Next, we suggest performing additional research to improve knowledge and introduce new options into clinical practice. Studies focussing on the diagnosis and clinical significance of minimal residual disease in the ovary will be pivotal. In addition, further research is needed on techniques that circumvent the risk of reintroducing cancer cells via autotransplantation, such as xenotransplantation of ovarian tissue followed by IVF, *in vitro maturation* (IVM)[1,30] and oocyte formation from stem cells.[31]

To date, autotransplantation of ovarian tissue is the only option clinically available to restore fertility after ovarian tissue cryopreservation.[1] For current patient counselling, a systematic review of the available data on ovarian involvement in neoplastic disease is therefore urgently needed. Together with the follow-up data from autotransplantations, this review will facilitate patient counselling for today's patients who are confronted with fertility preservation choices.

These recommendations should lead to improvements in our ability to select the most suitable fertility preservation option for each individual patient. Until then, we strongly feel that both clinicians and patients should be aware of the uncertainties regarding autotransplantation safety.

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3

Autotransplantation of cryopreserved ovarian tissue in cancer survivors and the risk of reintroducing malignancy: a systematic review

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Abstract

Background: The risk of recurrent oncological disease due to the reintroduction of cancer cells via autotransplantation of cryopreserved ovarian tissue is unknown.
Methods: A systematic review of literature derived from MEDLINE, EMBASE and the Cochrane Library was conducted. Studies on follow-up after autotransplantation; detection of cancer cells in ovarian tissue from oncological patients by histology, PCR or xenotransplantation; and epidemiological data on ovarian metastases, were included.
Results: A total of 289 studies were included. Metastases were repeatedly detected in ovarian tissue obtained for cryopreservation purposes from patients with leukaemia, as well as in one patient with Ewing sarcoma. No metastases were detected in ovarian tissue from lymphoma and breast cancer patients who had their ovarian tissue cryopreserved. Clinical studies indicated that one should be concerned about autotransplantation safety in patients with colorectal, gastric and endometrial cancer. For patients with low stage cervical carcinoma, clinical data were relatively reassuring, but studies focussed on the detection of metastases were scarce. Oncological recurrence has been described in one survivor of cervical cancer and one survivor of breast cancer who had their ovarian tissue autotransplanted, although these recurrences may not be related to the transplantation.
Conclusions: It is advisable to refrain from ovarian tissue autotransplantation in survivors of leukaemia. With survivors of all other malignancies, current knowledge regarding autotransplantation safety should be discussed. The most reassuring data regarding autotransplantation safety were found for lymphoma patients.

Introduction

The past decade has seen a rapid increase in the number of ovarian tissue cryopreservation and autotransplantation procedures performed worldwide, resulting in 21 live births being reported from several countries.[1] As infertility due to premature ovarian failure (POF) may arise as a consequence of chemo- or radiotherapy,[2-4] oncological diseases are the leading indications for ovarian tissue cryopreservation.[5-12] Ovarian tissue harvesting is preferentially performed before the start of gonadotoxic treatment. As neither ovarian stimulation nor a partner is required, this specific fertility preservation strategy especially holds promise for adolescent and even pre-pubertal girls, as well as for women who cannot delay oncological therapy.[6,13]

Despite its clinical success, the procedure of autotransplantation after ovarian tissue cryopreservation comes with a significant issue of concern. Namely, ovarian grafts from oncological patients may harbour cancer cells and autotransplantation of such grafts could theoretically lead to recurrence of oncological disease.[14-26] As the ovarian strips that are analysed by histology or Polymerase Chain Reaction (PCR) are no longer available for transplantation purposes, it remains unclear whether the strips that are actually transplanted are also devoid of cancer cells.[27]

The magnitude of the risk of reintroduction of a malignancy in specific situations is currently unknown, although it has been hypothesised to be influenced by cancer type and stage, the mass of malignant cells transferred, and the time of ovarian tissue harvesting in relation to oncological treatment.[15,18,28-30] Malignant diseases have been classified into three categories representing a low, intermediate or high risk of ovarian involvement.[28,29,31] Unfortunately, the exact magnitude of the risk of ovarian metastasis for these different categories remains unspecified as is the selection of relevant data supporting these classifications.

The recent development in the field of ovarian tissue autotransplantation has fuelled the urgency for reliable information on the safety of the procedure. With cryopreservation and autotransplantation procedures already being performed on a large scale during the last decade,[1,5,7-9,28,32,33] the number of cancer survivors requesting autotransplantation is expected to increase considerably in the near future. Exact figures on the number of cryopreservation procedures performed are unknown, as there is no international registry to which these procedures should be reported. Nevertheless, over 2500 cryopreservation procedures have been performed.[5,10-13,23,33-37] Other factors possibly stimulating the demand for ovarian tissue autotransplantation are the increasing cancer incidence and improving cancer survival in the adolescent and young adult population.[38] Finally, postponement of parenthood in Western countries[39] may enlarge the group of newly diagnosed cancer patients with an interest in fertility preservation options.

Ideally, patients requesting autotransplantation, as is newly diagnosed cancer patients who consider cryopreservation of their ovarian tissue, should be comprehensively

counselled on the risks of recurrent disease after autotransplantation as compared to their risk of oncological relapse when no autotransplantation would be performed.[40] The current study aims to systematically review all articles containing relevant information on the risk of reintroducing malignancy via ovarian transplants. In addition, this study will reveal gaps in the current knowledge.

Data from autotransplantation procedures performed thus far seem the most appropriate when it comes to assessing autotransplantation safety. These studies, however, are relatively scarce and suffer from a short follow-up and low numbers of patients. Therefore, other parameters that can serve as a proxy for the risk of recurrent malignancy after autotransplantation will be taken into account. These include studies aimed at the detection of cancer cells in ovarian tissue from oncological patients by means of histology, polymerase chain reaction (PCR), or xenotransplantation, as well as clinical or autopsy studies assessing the frequency of ovarian metastases in different oncological diseases.

Methods

Study design

We aimed to identify peer-reviewed studies meeting one of the following designs:

» Studies describing the follow-up of cancer survivors after autotransplantation of cryopreserved ovarian tissue.
» Studies focussing on detection of residual cancer cells in the ovarian tissue of oncological patients who had ovarian tissue cryopreservation by histology, PCR or xenotransplantation.
» Studies in which ovarian involvement is reported for a group of cancer patients. The study populations from these studies consisted of cancer patients who have not had ovarian tissue cryopreservation. Both clinical studies and autopsy studies were included.
» Case reports and case series reporting on ovarian metastases in cancer patients. These studies were included only when no ovarian involvement was described for the specific tumour type and stage from clinical studies or studies focussing on the detection of residual malignant cells in ovarian tissue from cancer patients.

Study population

Studies focussing on detection of residual disease in ovarian tissue

Study populations of these studies consisted of women who were part of a fertility preservation programme. All patients were pre-menopausal oncological patients who applied for ovarian tissue cryopreservation.

Clinical studies and case reports

Regarding clinical studies, we aimed to identify studies describing patients whose clinical situation and pattern of tumour spread and metastasis would best represent patients applying for ovarian tissue cryopreservation. Studies were excluded if:

» their patient population included women with a pre-malignancy, primary ovarian cancer, widespread intraperitoneal malignant disease, or a tumour directly adhering to the ovary.
» they only reported on ovarian metastases as the first site of recurrence.
» their patient population included women with a hereditary cancer syndrome associated with an increased risk of ovarian cancer, such as a BRCA1 or BRCA2 mutation, Lynch type II, or Peutz Jeghers syndrome.

Studies including patients with tumours that already showed spread or metastases to other sites than the ovary were included only when a homogeneous study group was described.

Age also seems to be a relevant factor when it comes to a pattern of tumour spread, as it has been shown that patients with breast, intestinal or gastric cancer with ovarian spread are significantly younger than patients without ovarian involvement.[41] In multivariate analyses of two studies concerning cervical and gastric cancer, age proved to be a risk factor for ovarian metastasis.[42,43] For this reason, studies including patients who were post-menopausal at the time of oncological diagnosis were not taken into account. When no menstrual status was reported, female patients less than 51 years old at the time of oncological diagnosis were included, based on the observation that the median age of onset of menopause in Europe and Northern America ranged from 50.1 to 52.8 years.[44]

Autopsy studies

Data on the prevalence of ovarian involvement obtained from deceased patients' autopsy reports presumably represent an upper extreme. For this reason, autopsy studies reporting on post-mortem examination of female cancer patients, including the investigation of the ovaries, were included. Studies describing populations containing the following types of patients were excluded:

» Patients with a hereditary cancer syndrome associated with an increased risk of ovarian cancer, such as a BRCA1 or BRCA2 mutation, Lynch type II, or Peutz Jeghers syndrome.
» Patients with primary ovarian cancer.
» Patients who were post-menopausal or, if no menstrual status was given, older than 51 years of age at diagnosis.

Subgroups
When only part of a study population met the inclusion criteria, relevant subgroup(s) were analysed if possible. For instance, from studies describing both pre- and post-menopausal patients, only data regarding pre-menopausal women were extracted.

Search strategy

Relevant studies were identified from MEDLINE (using the PubMed database), EMBASE, and the Cochrane Library, without any restrictions on the date of publication. A combination of Medical Subject Headings (MeSH) or Emtree terms and free text words, formulated after consultation of an information specialist from the Radboud University Nijmegen Library, was used to generate a list of citations. The search was restricted to articles written in the English language and was last updated mid-June 2012. Details on the search strategy for PubMed are displayed in Table I. This strategy was modified for EMBASE and the Cochrane Library. We complemented our electronic search with a manual search of bibliographies from relevant articles, aiming to identify additional relevant studies not captured by our electronic search.

Study selection

The selection of relevant studies was independently conducted by two reviewers (L.B. and R.P.). First, titles and abstracts were examined to decide whether the study might fulfil the predefined selection criteria. Secondly, full texts from selected articles were read to make a final in- or exclusion decision. When one or both reviewers were not sure about this final decision, consensus was resolved by discussing the article together or by arbitration by a third reviewer (C.C.B.).

Data collection

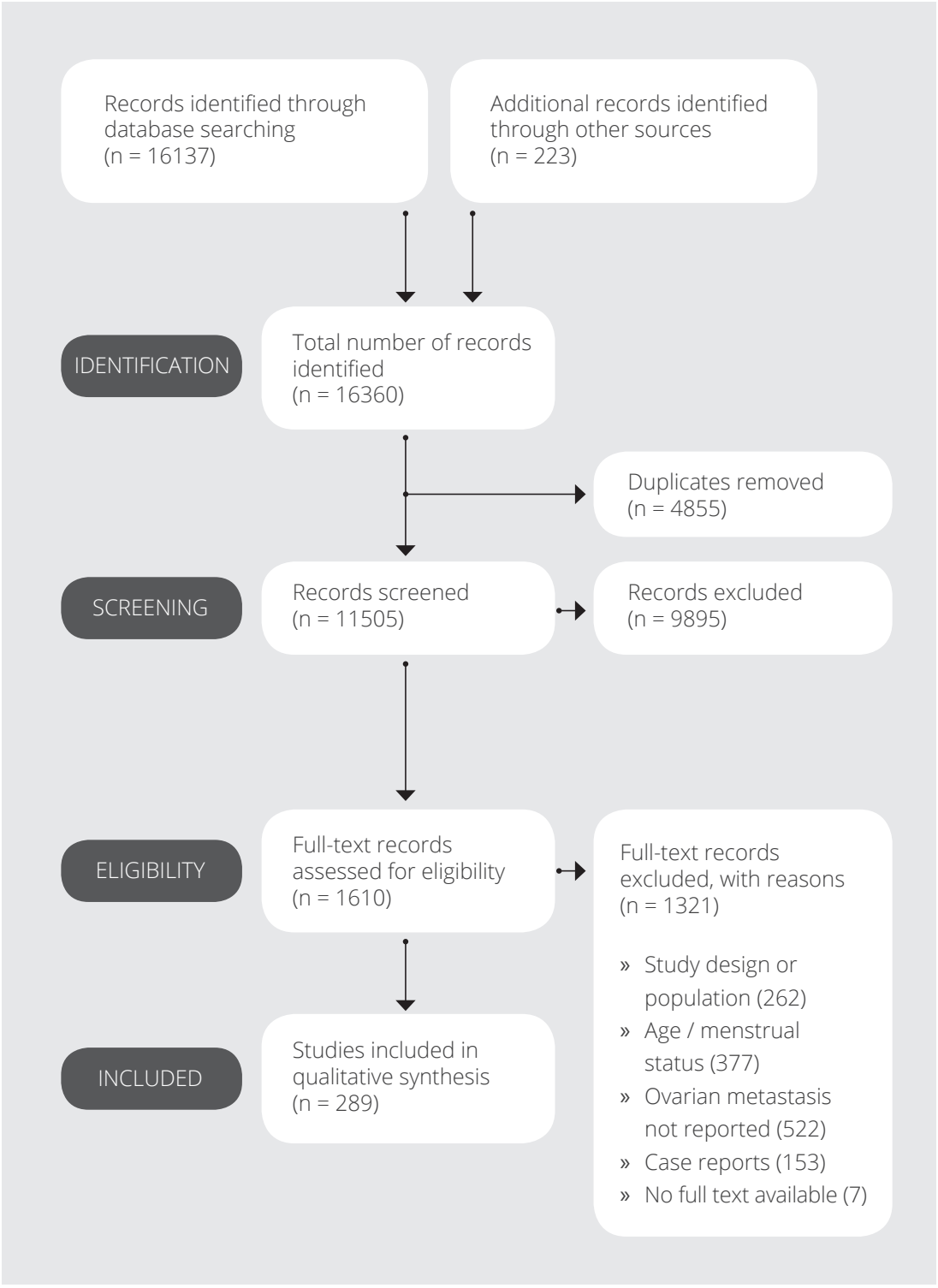
Data extraction was performed by two authors independently (L.B. and R.P) and disagreement was resolved by consensus or arbitration by a third reviewer (C.C.B.) or by discussing the paper with a medical oncologist (S.E.K.) or gynaecological oncologist (L.F.M.). The following information was recorded from the included studies: author's names, publication year, study design, patient and tumour characteristics, oncological treatment, and outcome of ovarian involvement. Additionally, duration of follow-up was recorded in follow-up studies after autotransplantation and in xenotransplantation studies. In studies reporting on analysis of ovarian tissue, diagnostic tools were also recorded.

Table I. Search strategy for identification of studies in PubMed.

Type I studies Follow-up autotransplantation	**Type II studies Histology, PCR and xenotransplantation**	**Type III and IV studies Clinical studies**
(Ovary[mesh] OR ovar*[tiab]) AND (Ovary/ transplantation[mesh] OR transplantation, autologous[mesh] OR autologous transplant*[tiab] OR autotransplant*[tiab] OR autograft*[tiab] OR ovarian graft[tiab] OR reimplant*[tiab]) AND (Cryopreservation[mesh] OR cryopreserv*[tiab] OR cryofixat*[tiab] OR cryonic suspension* OR frozen-thawed[tiab] OR frozen[tiab] OR (fertility[tiab] AND preserv*[tiab]))	(Ovary[mesh] OR ovar*[tiab]) AND ((Histology[mesh] OR Polymerase chain reaction[mesh] OR histolog*[tiab] OR immunohistochemi*[tiab] OR histocytochemi*[tiab] OR polymerase chain reaction[tiab] OR polymerase chain reactions[tiab] OR PCR[tiab] OR pathology[mesh] OR ovary/ pathology[mesh] OR patholog*[tiab]) OR (Transplantation,heterologous [mesh] OR xenotransplant*[tiab] OR xenograft*[tiab] OR heterograft*[tiab] OR heterologous transplant*[tiab] OR reimplant*[tiab] OR ovary/transplantation[mesh] OR transplant*[tiab])) AND (Neoplasms[mesh] OR neoplasm*[tiab] OR cancer[tiab] OR cancers[tiab] OR cancerous[tiab] OR malignancy[tiab] OR malignant[tiab] OR tumor[tiab] OR tumour[tiab] OR tumors[tiab] OR tumours[tiab] OR carcinoma[tiab] OR lymphoma[tiab] OR leukemia[tiab] OR leukaemia[tiab]) AND (Ovarian neoplasms/secondary[mesh] OR neoplasm, residual[mesh] OR minimal residual disease[tiab] OR ((tumor[tiab] OR tumour[tiab] OR tumors[tiab] OR tumours[tiab] OR disease[tiab] OR neoplasm[tiab] ORcancer[tiab] OR cancers[tiab]OR malignan*[tiab]) AND (residual[tiab] OR reseeding[tiab] OR contamination)))	(Ovary[mesh] OR ovar*[tiab]) AND (Ovarian neoplasms/ secondary[mesh] OR Neoplasm metastasis[mesh] OR metasta*[tiab]) AND ('Risk Assessment'[Mesh] OR 'Risk Factors'[Mesh] OR risk[tiab] OR ovarian neoplasms/ epidemiology[mesh] OR epidemiology[mesh] OR epidemiolog*[tiab] OR incidence[mesh] OR inciden*[tiab] OR prevalence[mesh] OR prevalen*[tiab] OR patients[tiab] OR cases[tiab] OR population[tiab] OR 'Population'[Mesh] OR 'Patients'[Mesh])

The search terms displayed in the three columns were combined with OR in the definitive search.
Terms with [tiab] reflect free text terms appearing in title or abstract.

Figure I. PRISMA flow chart of the systematic review process



Presentation of results

For each tumour type, all relevant publications were discussed. Tumour stages (AJCC TNM classification, FIGO stages, Duke's stages and Bormann stages) as described in the results may not reflect current classifications, as these are subjected to regular revisions. When tumour stages were not reported for the relevant subgroup of a particular study, tumour characteristics will be presented for the study population as a whole. Data were not pooled due to the large methodological and clinical heterogeneity of the included studies, both due to different study designs and large differences in patient populations regarding tumour stages and other characteristics.

Results

Study selection

Our electronic search yielded 16137 hits. A flow scheme of our selection process is outlined in Figure I, following the PRISMA Statement (Preferred Reporting Items of Systematic Reviews and Meta-Analyses).[45] After exclusion of 4855 duplicates and 9895 articles on title or abstract basis, the full texts of the remaining 1610 articles were screened.

A total of 1321 studies did not meet our eligibility criteria and were therefore excluded. This group consisted of 262 studies that did not provide original data or that did not meet one of the study types mentioned in our eligibility criteria. Although describing (the follow-up of) a group of cancer patients, 522 clinical and autopsy studies were excluded since they did not report on ovarian metastases or even excluded patients with ovarian involvement. A total of 377 studies did not meet the criteria for age or menstrual status. There were 153 case reports excluded as they indicated ovarian involvement in a malignancy on which information was already available from clinical studies. Finally, from a total of 7 studies, no full text version could be obtained by contacting the authors or by consulting international libraries. The remaining 289 articles were included in this review.

Tumours of the breast

Several cases of ovarian tissue autotransplantation in breast cancer patients have been described. Of these women, one had a local breast cancer recurrence.[46] Obviously, this relapse may not have any relation to the autotransplantation of the ovarian tissue, as oncological recurrences do also occur spontaneously. Unfortunately, the authors did not explicitly state whether this 'local recurrence' referred to a recurrence in the breast

or a recurrence near the ovarian transplant.

An additional breast cancer survivor was reported to be free of disease 18 months after transplantation.[24] Other reports did not explicitly state the health status of their patients during follow-up, although these patients are likely to be free of disease since most of them were pursuing pregnancy.[8,9,47-51] The maximum duration of follow-up after autotransplantation was 19 months.[50]

Histological examination and xenotransplantation of ovarian tissue from breast cancer patients provided reassuring results (Table II). Two clinical studies indicated ovarian metastases in breast cancer patients, although the study with the largest population reported a very low percentage. However, results from autopsy studies suggested that ovarian metastases are fairly common in advanced breast cancer. As no explicit information on BRCA testing was reported in the clinical and autopsy studies, it remains unclear whether BRCA patients were part of the studies.

Tumours of the genital tract

Cervical carcinoma

Kim et al. reported a total of 4 procedures of ovarian tissue autotransplantation in cervical cancer survivors.[48,51,56] Histological analysis showed no ovarian involvement in these patients.[48,56] One patient had an oncological relapse and deceased shortly after autotransplantation. Although specific information about the nature of this oncological recurrence cannot be found in the publication, the authors do not suspect the relapse to be a result from the autotransplantation, but consider it to be arisen spontaneously.[51;57] Despite a maximum period of 7.5 years after ovarian tissue autotransplantation, the health status of the other patients was not described.[51]

Apart from these autotransplantations, results were available from histological examination for only a small group of patients, as well as from clinical studies (Table III). Whereas most clinical studies reported low percentages of ovarian involvement in their populations, 2 studies reported metastases in more than 4% of the patients in their (sub)population: one study with only 14 patients and another study with a subgroup of 146 premenopausal adenocarcinoma patients.[58,59]

Endometrial carcinoma

One can hypothesise that patients suffering from endometrial cancer may not prefer cryopreservation of ovarian tissue, especially when facing hysterectomy or pelvic irradiation as cancer treatment. Nevertheless, these patients may wish to fulfil their child wish with help of a surrogate mother in the future. Presumably due to the nature of the disease and its treatment, ovarian tissue autotransplantation has not been reported in endometrial cancer patients. Data from histological examination are scarce, while clinical studies with relatively small sample sizes are available (Table IV). These studies revealed that ovarian metastases could occur in patients with different tumour stages.

One larger study including only FIGO I patients reported a relatively low percentage of ovarian involvement.[60]

Other tumours of the female genital organs

No autotransplantation procedures have been reported in patients with other types of gynaecological malignancies. One study reported on the incidence of ovarian metastases in a group of patients who died after a diagnosis of uterine cancer, without specifying cancer type or stage.[54] Included in this study were 4 patients of 20 years or younger who had had uterine cancer without ovarian involvement. Amongst patients aged 21 to 30 years, 12.8% had ovarian metastases, while from the patients aged 31 to 40, 13.3% had metastases.

Although the incidence of ovarian involvement in patients with other gynaecological malignancies cannot be obtained from studies with larger populations, case reports indicated ovarian involvement in various malignancies. Ovarian metastases have been found in women with an epithelioid or placental site trophoblastic tumour,[61-65] leiomyosarcoma,[66,67] endometrial stromal sarcoma,[67-69] and other tumours.[70]

Table II. Epidemiological data from breast cancer studies

First author, year	Sample size	Assessment	Menstrual status or age (years)	Characteristics study group	Characteristics patients with ovarian metastasis	Oncological therapy study group	Ovarian metastasis
Histology or PCR (OTC patients)							
Azem, 2010 [17]	13	Histology / Histochemistry Fresh ovarian tissue	Pre-menopausal	OTC patients	-	No CT or RT	0%
Rosendahl, 2011 [23]	51	Histology / immunohistochemistry Cryopreserved and thawed ovarian tissue	Pre-menopausal	OTC patients Median tumour size 18 mm, (5-75) (Data available for N=47) N1: 44% (Data available for N=44)	-	NR	0%
Sanchez-Serrano, 2009 [24]	69	Histology / immunohistochemistry Fresh ovarian tissue N=63; Cryopreserved and thawed tissue N=6	Pre-menopausal	OTC patients Exclusion: BRCA1/2 or HER2neu mutation carriers ER+: 76.2%; PR+: 69.7% N0: 49.2%; N1: 50.8%	-	17% received CT before OTC	0%
Xenotransplantation							
Rosendahl, 2011[46]	9	Xenotransplantation of cryopreserved and thawed ovarian cortex into immunodeficient nude mice. Histology 4 weeks after xenotransplantation	Pre-menopausal	OTC patients	-	NR	0%

Clinical studies							
Lecca, 1980 [55]	15	Histology after therapeutic oophorectomy	Pre-menopausal	NR	NR	Radical mastectomy (all patients), RT, CT, hormonal therapy	46.7% (7/15)
Lee, 2010 [52, 192]	406	Clinical follow-up (mean 74 ± 48.19 months)	≤35	Patients with IDC (Otherwise NR for subgroup)	NR	NR for subgroup	IDC: 0-0.2% (max 1/406)*
Autopsy							
Bumpers, 1993 [53]	15	Autopsy; evaluation of medical records	<50	Died of disseminated ILC	NR	NR	46.7% (7/15)
Kyono, 2010 [54]	648	Autopsy	<41	NR	NR	NR	24.2% (157/648) Age 11-20: 0% (0/3) Age 21-30: 19.4% (14/72) Age 31-40: 25.0% (143/573)

OTC = Ovarian tissue cryopreservation; ILC = Infiltrating lobular carcinoma of the breast; IDC = infiltrating ductal carcinoma of the breast; LN = lymph node, CT = chemotherapy, RT = radiotherapy; max = maximum; NR = not reported.
** An exact percentage of ovarian involvement could not be derived from these studies, as age or menopausal status was not provided for the women with ovarian metastases.*

Table III. Epidemiological data from cervical cancer studies

First author, year	Sample size	Assessment	Menstrual status or age (years)	Characteristics study group	Characteristics patients with ovarian metastasis	Oncological therapy study group	Ovarian metastasis
Histology or PCR (OTC patients)							
Azem, 2010 [17]	2	Histology / Histochemistry Fresh ovarian tissue	Pre-menopausal	OTC patients	-	No prior CT or RT	0%
Huser, 2007 [78]	1	Histology Fresh ovarian tissue	Pre-menopausal	OTC patient Tumour stage NR	-	Prior treatment NR	0%
Xenotransplantation							
-							
Clinical studies							
Kim, 2008 [71]	156	Histology	<45	SCC or Non-SCC; FIGO Stage IA-IIB	NR	RH + PLND + BSO with or without appendectomy No prior CT	3.2% (5/156)
Kodama, 2007 [72]	109	NR Follow-up 1-143 months	<50	SCC, AC or ADSC; FIGO Stage IB-IIB	NR	RH + PLND (all patients) EPI, CT, chemoradiation	0 - 3.7% (max 4/109)*
Landoni, 2007 [43]	807	Histology	<45	SCC, AC, or ADSC; FIGO stage IA2, IB or IIA	NR	RH + PLND + BSO	0.2% (2/807)
Morice, 2000 and 2001[73,74]	95	Clinical follow-up (14-15 years)	<43	AC (N = 15) or SCC (N = 80) FIGO Stage IB1, IB2 or IIA LN+: 20%	Both cases: Age 34; SCC; Stage IB1; LN- RH + PLND + brachytherapy	RH + PLND VB (N = 84); EPI (N = 25)	2.1% (2/95) SCC: 2.5% (2/80) AC: 0% (0/15)

Nakanishi, 2001[58]	SCC: 556 AC: 146	Histology	Pre-menopausal	All SCC and AC patients who underwent BSO or USO and hysterectomy and PLND	SCC (N = 4); AC (N = 10)	Hysterectomy + PLND + BSO or USO	2.0% (14/702) SCC: 0.7% (4/556) AC: 6.8% (10/146)
Natsume, 1999 [59]	14	Histology	≤40	SCC or AC FIGO stage IB, IIA and IIB	Case 1: Age 29, Stage IB AC Case 2: Age 27, Stage IIB, AC	RH + PLND + BSO	14.3% (2/14)
Pahisa, 2008 [75]	28	Clinical follow-up (mean 44.3 ±23.1 months; N=4 lost to follow-up)	Pre-menopausal	AC (N = 6) or SCC (N = 22); FIGO Stage IB1 Patients who underwent oophoropexy	-	RH + PLND + BSO VB (N = 12); EPI (N = 5)	0% (0/28)
Parente, 1964 [76]	88	Histology	≤50	Stage I epidermoid carcinoma	-	RH + PLND + BSO	0% (0/88)
Yamazawa, 2003 [77]	69	Clinical follow-up (4-137 months)	<50	FIGO Stage IB1 - II	NR	RH or simple hysterectomy, PLND, postoperative CT or RT	0 - 1.4% (max 1/69)*
Autopsy							
-							

OTC = Ovarian tissue cryopreservation; AC = Adenocarcinoma; SCC = Squamous cell carcinoma; Non-SCC = Non-squamous cell carcinoma; ADSC = adenosquamous cell carcinoma; RH = radical hysterectomy; PLND = pelvic lymph node dissection; CT = chemotherapy; RT = radiotherapy; EPI = External pelvic irradiation; VB = vaginal brachytherapy; BSO = bilateral salpingo-oophorectomy; USO = unilateral salpingo-oophorectomy; LN+/-: Lymph node positive/negative; NR = Not Reported. An exact percentage of ovarian involvement could not be derived from these studies, as age or menopausal status was not provided for the women who had ovarian metastasis. The minimum and maximum percentages of ovarian involvement that could possibly result from the data given are shown in the table.

Table IV. Epidemiological data from endometrial cancer studies

First author, year	Sample size	Assessment	Menstrual status or age (years)	Characteristics study group	Characteristics patients with ovarian metastasis	Oncological therapy study group	Ovarian metastasis
Histology or PCR (OTC patients)							
Azem, 2010 [17]	1	Histology / Histochemistry Fresh ovarian tissue	Pre-menopausal	OTC patient	-	No CT or RT	0%
Xenotransplantation							
-							
Clinical studies							
Dundar, 2002 [79]	24	Histology	<50	FIGO stage I-III EC	EC	Hysterectomy, partial omentectomy, LND, RT	41.7% (10/24)
Evans-Metcalf, 1998 [80]	37	Histology	≤45	FIGO stage I-IV	NR	RH, BSO, RT	2.6% (1/39)
Farhi, 1986 [81]	10	Clinical follow-up (3 months – 10 years)	<25	Grade I-II AA: n=6; AC: N=3; ADSC: N=1	Grade II ADSQ	Hysterectomy, BSO, RT, progestogens	10% (1/10)
Gitsch, 1995 [82]	17	Clinical follow-up (12 months – 78 years; N=2 lost to follow-up)	Pre-menopausal	FIGO stage I-IV AC	AC FIGO stage IIIa: N = 1; stage IV: N = 2	RH, BSO, LND	17.6% (3/17)
Hachisuga, 2000 [83]	81	Histology	<50	Grade I-III EC	EC	Hysterectomy, BSO, RT, CT	7.4% (6/81)
Hanprasertpong, 2008 [84]	51	Histology	<45	FIGO stage I-III EC: n=50; AA: n=1	NR	Surgery, CT, RT	5.9% (3/51)
Kaku, 1993 [85]	17	Clinical follow-up (4 months – 11 years)	≤40	FIGO stage Ia-IIIc EC: N=14; AA: N=3; UC: N=1 Grade I-III	FIGO stage IIIa AC Grade I	Hysterectomy, BSO, LND, RT, CT	5.9% (1/17)

Lee, 2007 [86]	79	Histology	≤45	FIGO stage I-IV	EC: N=3; mixed undifferentiated and EC: N=1 LN+: N=1	Hysterectomy, LND	5.1% (4/79)
Niwa , 2000 [87]	14	Clinical follow-up (7–144 months)	<40	FIGO stage Ia-IVb: LN+: N = 5 EC with or without squamous differentiation	FIGO stage IIIb and IVb LN+ (N=1) EC	CT, RT, surgery	14.3% (2/14)
Pan, 2011[60]	160	Histology	≤45	FIGO stage I	FIGO stage I (n=3)	Primary total hysterectomy, BSO, LND No CT before surgery	1.9% (3/160)
Quinn, 1985 [88]	32	Clinical follow-up (from <5 to >15 years)	Pre-menopausal	Stage I-IV	NR	Hysterectomy, PLN, BSO, RT	3.1% (1/32)
Walsh, 2005 [89]	102	Histology Clinical follow-up after ovarian preservation (N=16): 1-50 months.	<45	FIGO stage I-III Grade I-III EC: N=98 ADSC: N=4	FIGO stage IIIa1: N=1; IIIa2: N=2	Hysterectomy, BSO (N=86), CT, hormonal treatment	2.9% (3/102)
Yamazawa, 2000 [90]	20	Clinical follow-up (7 - 126 months)	Pre-menopausal	FIGO stage Ia-IIIb EC	EC	RH, CT, PLND, BSO	5% (1/20)
Zhou, 2005 [91]	11	Histology	≤40	FIGO stage I-IV	FIGO stage I (n=3)	hysterectomy, BSO, LND, CT, RT, progesterone	27.3% (3/11)
Autopsy							
-							

OTC = Ovarian tissue cryopreservation; ADSC = adenosquamous cell carcinoma; AA = adenoacanthoma; AC = adenocarcinoma; EC= endometrioid carcinoma; UC = undifferentiated carcinoma; RH = Radical hysterectomy; LND = lymph node dissection; PLND = pelvic lymph node dissection; CT = chemotherapy; RT = radiotherapy; BSO = bilateral salpingo-oophorectomy; LN+/-: Lymph node positive/negative; NR = Not Reported.

Tumours of the gastrointestinal tract

Gastric cancer

Autotransplantation has not been performed in patients suffering from gastric cancer. Five clinical and autopsy studies were identified that presented data on ovarian involvement in gastric cancer, (Table V) all from Asian countries. The incidences reported for ovarian metastases varied considerably. The lowest incidence (7.4%) was reported in a clinical follow up study amongst 380 patients with gastric cancer.[92] Two relatively small autopsy studies described metastases in all premenopausal patients studied, although one of these studies focussed on gastric cancer patients who already had cervical metastases.[93,94]

Colorectal, appendiceal and anal cancer

Two reports were retrieved describing ovarian tissue autotransplantation in a patient with anal cancer. However, no information was provided on the analysis of the ovarian tissue for malignant cells or on the health status of the patient after transplantation.[95,96]

Data on histological examination of ovarian tissue from colon cancer patients undergoing ovarian tissue cryopreservation were scarce, while several clinical and autopsy studies indicated ovarian metastases to be present in colon carcinoma patients (Table VI). Unfortunately, study populations were relatively small for 4 out of 5 clinical studies and populations consisted of patients with varying tumour stages.

A single report on the frequency of ovarian metastases from appendiceal cancer reported an incidence of 28.6% and indicated that patients with advanced cancer stages were most at risk for having ovarian metastases.[97] Additional case reports described ovarian involvement in various histological types of appendiceal cancer.[98-115]

Other tumours of the gastrointestinal tract

For tumours of other parts of the gastrointestinal tract, neither autotransplantation reports nor epidemiologic data were available. Nevertheless, ovarian involvement has been indicated in case reports describing patients with hepatocellular carcinoma,[116-122] hepatoblastoma,[123] and bile duct or gallbladder carcinomas.[124-137] Tumours of the small bowel and pancreatic tumours were also shown to have the capacity to metastasise to the ovaries.[103,138-145]

Lymphomas

The follow-up from lymphoma survivors who received ovarian tissue autotransplantation has not been extensively described when it comes to disease status. Nevertheless, numerous autotransplantation procedures have been performed in lymphoma survivors and no recurrent cancer has been reported following transplantation.[8,9,46,47,51,146-160] In

accordance with these findings, histological assessment and xenotransplantation of ovarian cortex fragments obtained for cryopreservation purposes failed to reveal any tumour components (table VII).

In a clinical study focussing on patients with lymphoma in the gynaecological organs, as well as an autopsy study regarding lymphoma patients, ovarian involvement has been described. (Table VII). Unfortunately, these studies did not provide insight in the risk of ovarian involvement in *different types* of lymphoma. Case studies indicated that ovarian metastases could occur in patients suffering from Hodgkin's and non-Hodgkin's lymphoma, Burkitt's lymphoma, large and small cell lymphoma, mixed lymphocytic histiocytic and lymphocytic lymphoma, lymphosarcoma, and follicular lymphoblastoma.[161-178]

Leukaemia

Autotransplantation of ovarian tissue has never been reported for patients with leukaemia, presumably due to the alarming results from PCR analysis and xenotransplantation experiments. Indeed, ovarian involvement has been repeatedly indicated in different types of leukaemia by means of xenotransplantation or PCR-analysis using a disease specific molecular marker. In addition, ovarian involvement in leukaemia has been reported in autopsy studies (Table VIII).

Tumours of the urinary tract

Autotransplantation of ovarian tissue has not been performed in patients suffering from urinary tract tumours. Histological analysis of ovarian tissue from 2 patients with nephroblastoma showed no evidence of ovarian involvement,[12] although case reports *did* demonstrate ovarian involvement in nephroblastoma.[170,188]

Only one clinical study has reported on the incidence of ovarian metastases in a group of pre-menopausal female patients with a tumour of the urinary tract, namely carcinoma of the bilharzial urinary bladder.[189] In this study, no evidence of ovarian metastases was found in 103 patients. Despite these findings, several case reports have indicated ovarian involvement in bladder cancer patients.[190-194] Renal cell carcinoma also has the potential to metastasise to the ovaries,[195-201] as well as transitional cell carcinoma of the urinary tract[202-204] and other urinary tract tumours.[205]

Table V. Epidemiological data from gastric cancer studies

First author, year	Sample size	Assessment	Menstrual status or age (years)	Characteristics study group	Characteristics patients with ovarian metastasis	Oncological therapy study group	Ovarian metastasis
Histology or PCR (OTC patients)							
-							
Xenotransplantation							
-							
Clinical studies							
Kim, 1999 [42]	238	Clinical follow-up (2-108 months)	Pre-menopausal	Papillar and tubular, mucinous or SRCC AJCC stage Ia-IV Borrmann type I-IV	NR	Gastrectomy, LND	9.7% (23/238)
Yook, 2007 [92]	380	Histology	<50	Moderately or poorly differentiated, mucinous, or SRCC Early carcinoma - Borrmann type IV	Borrmann type II-IV	NR	7.4% (28/380)
Autopsy							
Hirono, 1983 [93]	8	Autopsy	Pre-menopausal	NR for subgroup	NR	Treated but not further specified	100 % (8/8)

Imachi, 1993 [94]	13	Autopsy	Pre-menopausal	Poorly differentiated AC or SRCC metastatic to the cervix Early carcinoma - Borrmann type IV	NR	(sub)total gastrectomy	100% (13/13)
Kyono, 2010 [54]	1095	Autopsy	<40	NR	NR	NR	55.7% (611/1095) Age 0-10: 0% (0/1) Age 11-20: 78.3% (18/23) Age 21-30: 60.4% (125/207) Age 31-40: 54.2% (468/864)

OTC = Ovarian tissue cryopreservation; SRCC = Signet ring cell carcinoma; AC = Adenocarcinoma; LND = lymph node dissection; NR = Not Reported.
Stages in this table are according to the AJCC TNM or Bormann classification.

Table VI. Epidemiological data from colorectal cancer studies

First author, year	Sample size	Assessment	Menstrual status or age (years)	Characteristics study group	Characteristics patients with ovarian metastasis	Oncological therapy study group	Ovarian metastasis
Histology or PCR (OTC patients)							
Azem, 2010 [17]	1	Histology / Histochemistry Fresh ovarian tissue	Pre-menopausal	OTC patient with colon cancer	-	No CT or RT	0%
Xenotransplantation							
-							
Clinical studies							
Blamey, 1981[179]	201	Clinical follow-up (5 - 96 months)	≤49	Patients undergoing resection of a primary AC of the colon or rectum Duke's stage B-D	Duke's stage B and C	Resection	2.5% (5/201)
Cutait, 1983 [180]	14	Clinical follow-up (NR)	Pre-menopausal	Adenocarcinoma of the colon Duke's stage A-C	Duke's stage C	Resection	7.1% (1/14)
Domergue, 1988 [181]	38	Clinical follow-up (from 3 to >15 years)	<40	Patients treated for colorectal (mucinous) AC Duke's stage A - D	NR	Resection, CT, RT	7.9% (3/38)
MacKeigan, 1979 [182]	18	Histology	Pre-menopausal	Patients who received prophylactic oophorectomy for colorectal AC	Duke's stage B: N=1; C: N=4; D: N=1	Resection	33.3% (6/18)

Pitluk, 1983 [183]	17	Clinical follow-up	≤40	AC of colon or rectum Duke's stage B-D	NR	Resection	23.5% (4/17)
Autopsy							
Kyono, 2010 [54]	256	Autopsy	<40	NR	NR	NR	26.6% (68/256) Age 11-20: 16.7% (2/12) Age 21-30: 31.1% (14/45) Age 31-40: 26.1%(52/199)

OTC = Ovarian tissue cryopreservation; CT = chemotherapy, RT = radiotherapy; AC = Adenocarcinoma; NR = not reported.

Table VII. Epidemiological data from lymphoma studies

First author, year	Sample size	Assessment	Menstrual status or age (years)	Characteristics study group	Characteristics patients with ovarian metastasis	Oncological therapy study group	Ovarian metastasis
Histology or PCR (OTC patients)							
Huser, 2007 [78]	4	Histology Fresh ovarian tissue	Pre-menopausal	OTC patients HL: N=3 NHL: N=1 Tumour stage NR	-	Prior treatment NR	0%
Kim, 2001[19]	18	Histology Fresh ovarian tissue	Pre-menopausal	OTC patients HL: N=13 (3 primary disease, 10 recurrence) NHL: N=5 (2 primary disease; 3 recurrence) Tumour stage NR	-	Prior treatment NR	0%
Meirow, 1998 [20]	7	Histology Fresh ovarian tissue	Pre-menopausal	OTC patients HL Stage 2B: N=3 Stage 3A: N=1 Stage 4: N=3	-	At least 1 patient had CT prior to OTC	0%
Meirow, 2008 [21]	47	Histology / PCR / immunohistochemical staining to detect Reed-Sternberg cells (HL) or molecular markers (NHL)	Pre-menopausal	OTC patients HL: N=33 NHL: N=14	-	CT before OTC: HL: N=16 NHL: N=9	0%

Oktay, 2010 [8]	18 or 19	Histology Fresh ovarian tissue	Pre-menopausal	OTC patients HL: N=12 or 13 patients NHL: N=6	-	NR	0%
Poirot, 2007 [12]	3	Histology Fresh ovarian tissue	Pre-pubertal	OTC patients HL: N=1 Lymphoma (NOS): N=2	-	All patient underwent several courses of CT before OTC	0%
Seshadri, 2006 [25]	26	Histology / immunohistochemistry	Pre-menopausal	OTC patients Stage III or IV: N=9 Disease below diaphragm: N=7	-	Prior CT: N=9: -ABVD: N=7 -Stanford V: N=1 -ChlVPP: N=1	0%
Xenotransplantation							
Kim, 2001[19]	18	Xenotransplantation into 20 NOD/LtSz-SCID mice. Animal autopsy 16 weeks after transplantation	Pre-menopausal	OTC patients HL: N=13 (3 primary disease, 10 recurrence) NHL: N=5 (2 primary disease; 3 recurrence) Tumour stage NR	-	NR	0%
Rosendahl, 2011[46]	9	Xenotransplantation of cryopreserved and thawed ovarian cortex into immunodeficient nude mice. Histology 4 weeks after xenotransplantation	Pre-menopausal	OTC patients with HL and NHL	-	NR	0%
Schmidt, 2004 [184]	1	Histological examination of tissue after xenotransplantation into SCID mouse Follow-up: 4 weeks	Pre-menopausal	OTC patient with B-cell lymphoma stage III of the mediastinum	-	No prior treatment	0%

Table VII. Continued

First author, year	Sample size	Assessment	Menstrual status or age (years)	Characteristics study group	Characteristics patients with ovarian metastasis	Oncological therapy study group	Ovarian metastasis
Clinical studies							
Harris, 1984 [185]	19	Histological examination of resection specimens or biopsies	≤50	Patients with lymphoma of the uterine endometrium (N=1), cervix (N=16) or vagina (N=2), retrospectively collected from consultation files. FIGO stages cervical lymphoma: Stage 1: N=9; stage 2: N=6; Stage 3: N=2 FIGO stages vaginal lymphoma: Stage I: N=1; Stage IV: N=1 FIGO stage endometrial lymphoma: Stage III	Case 1: 48 year old woman with FIGO stage I cervical lymphoma Case 2: 34-year old woman with FIGO stage III endometrial lymphoma	NR	10.5% (2/19)
Autopsy							
Kyono, 2010 [54]	736	Autopsy	<40	NR	NR	NR	13.3% (98/736) Age 0-10: 10.5% (8/76) Age 11-20: 10.7% (15/140) Age 21-30: 13.9% (27/194) Age 31-40: 14.7% (48/326)

OTC = Ovarian tissue cryopreservation; HL = Hodgkin's lymphoma; NHL = non-Hodgkin's lymphoma; CT = chemotherapy; NOS = not otherwise specified; NR = not reported.

Table VIII. Epidemiological data from leukeamia studies

First author, year	Sample size	Assessment	Menstrual status or age (years)	Characteristics study group	Characteristics patients with ovarian metastasis	Oncological therapy study group	Ovarian metastasis
Histology or PCR (OTC patients)							
Courbiere, 2010 [186]	1	Histology and RQ-PCR	Pre-menopausal	OTC patient with chronic phase CML	Chronic phase CML	Imatinib	100% (1/1)
Dolmans, 2010 [14]	18	Histology and RT-PCR (N=16)	Pre-menopausal or pre-pubertal	OTC patients with: CML: N=6 ALL: N=12	CML patients: aged 31 and 30 at OTC. No prior CT. ALL patients: 11-20 years old at OTC. Prior CT: N=3	8 patients received chemotherapy before OTC	50% (9/18) CML: 33.3% (2/6) ALL: 58.3% (7/12)
Greve, 2012 [18]	25	Histology and PCR (N=7)	Pre-menopausal or Pre-pubertal	OTC patients with: ALL: N=11 AML: N=10 CML: N=3 JMML: N=1	4 patients with positive PCR but negative histology: CML, chronic phase: N=2 ALL, complete remission: N=1 AML, complete remission: N=1	18 patients received chemotherapy before OTC	16% (4/25)
Meirow, 2008 [21]	9	Histology Fresh ovarian tissue: N=9 PCR and histology on cryopreserved-thawed tissue: N=2	Pre-menopausal	OTC patients with: AML: N=5 Myelodysplastic syndrome: N=1 CML: N=3	20 year old CML patient with positive RT-PCR signal in thawed tissue	All CML and AML patients had CT prior to OTC The MDS patient did not have prior CT.	11.1% (1/9) CML: 33% (1/3)
Poirot, 2007 [12]	6	Histology Fresh ovarian tissue	Pre-pubertal	OTC patients with leukaemia (NOS)	-	All underwent several courses of CT before OTC	0%

Table VIII. Continued

First author, year	Sample size	Assessment	Menstrual status or age (years)	Characteristics study group	Characteristics patients with ovarian metastasis	Oncological therapy study group	Ovarian metastasis
Histology or PCR (OTC patients) - *continued*							
Rosendahl, 2010 [15]	26	Histology / immunohistochemistry and PCR (N=8)	Pre-menopausal or pre-pubertal	OTC patients with: ALL: N=13 (PCR possible: N=2) AML: N=7 (PCR possible: N=1) CML: N=5 (PCR possible: N=5) JMML: N=1	6 patients with positive PCR results but negative histology: CML in complete remission: N=1 (Age: 13 years) CML chronic phase: N=4 (Age: 7, 17, 24, 26 years) ALL in complete remission: N=2 (Age: 4, 9 years) AML in complete remission: N=1 (Age: 21 years)	NR	23.1% (6/26) CML: 100% (5/5) ALL: 15.4% (2/13) AML: 14.3% (1/7)
Xenotransplantation							
Dolmans, 2010 [14]	18	Xenotransplantation to immunodeficient mice	Pre-menopausal or pre-pubertal	OTC patients with: CML: N=6 ALL: N=12	ALL patients aged 11-21 at OTC. Prior CT: N=2; PCR (possible in 4 patients): all positive	8 patients received chemotherapy before OTC	27.8% (5/18) CML: 0% (0/6) AML: 41.7% (5/12)
Greve, 2012 [18]	25	Histology and PCR (N=7) of ovarian cortex after 20 weeks of xenotransplantation in immunodeficient mice	Pre-menopausal or Pre-pubertal	OTC patients with: ALL: N=11 AML: N=10 CML: N=3 JMML: N=1	-	18 patients received chemotherapy before OTC	0% (0/25)
Rosendahl, 2011[46]	7	Xenotransplantation of cryopreserved and thawed ovarian cortex into immunodeficient nude mice. Histology 4 weeks after xenotransplantation	Pre-menopausal	OTC patients with ALL AML or CML.	-	NR	0%

Clinical studies							
Turial, 2009 [187]	>300	Clinical follow-up (retrospective design)	Pre-pubertal and pre-menopausal	Girls treated for ALL	Case 1: Diagnosis pre-B-ALL at age of 3 years. Treatment: CoALL 82-protocol. Bone marrow relapse at 7 and 9 years of age, treated with CT. Age 11: ovarian metastases. Case 2: Diagnosis pre-B-ALL at age of 14 years. Treatment: CoALL 06-97 protocol. After 18 months of remission: ovarian metastases.	NR	<0.7% (2/>300)
Autopsy							
Reid, 1980 [22]	27	Autopsy	<9	Girls with leukaemia: ALL: N=12 AML: N=9 AMML: N=3 Lym → L: N=3	ALL: N=6 AML: N=6 AMML: N=3 Lym → L: N=2	NR	62.3% (17/27)
Kyono, 2010 [54]	2027	Autopsy	<40	NR	NR	NR	8.4% (171/2027) Age 0-10: 7.9% (31/392) Age 11-20: 10.2% (52/511) Age 21-30: 7.8% (34/438) Age 31-40: 7.9% (54/686)

OTC = Ovarian tissue cryopreservation; CML = Chronic myeloid leukaemia; AML = Acute myeloid leukaemia; ALL = Acute lymphoblastic leukaemia; JMML = Juvenile myelomonocytic leukaemia; AMML = acute myelomonocytic leukaemia; LYM → L = Leukaemic conversion of lymphoma; NOS = Not otherwise specified.

Tumours of the respiratory tract

No studies on ovarian tissue autotransplantation, xenotransplantation or histological analysis of ovarian tissue from lung cancer patients were retrieved. However, one autopsy study revealed a percentage of 20.9% to 24.8% of ovarian metastases in patients with pulmonary carcinoma, depending on patients' age.[54] Unfortunately, the former study as well as one case report did not specify the histological types or tumour stages of pulmonary carcinoma.[125] However, case reports indicated involvement in patients diagnosed with large and small cell lung cancer and papillary-acinar adenocarcinoma.[206-210] Other reports showed ovarian involvement in pulmonary papillary serous carcinoma,[211,212] adenocarcinoma of the fetal lung type,[213] and pulmonary blastoma.[214]

Melanoma and malignant blue naevus

For melanoma, no studies reporting on autotransplantation of ovarian tissue, or the epidemiology of ovarian involvement were retrieved from the literature. However, numerous case reports were identified describing ovarian metastases from melanoma.[215-239] Apart from cases of ovarian metastases from cutaneous melanomas of different body locations, reports on choroidal melanomas metastasizing to the ovaries were published.[218,232,233] In one study a patient having ovarian involvement from a malignant blue naevus of the vulva was described.[240]

Bone and soft tissue tumours

For patients with bone and soft tissue tumours, autotransplantation has only been described for Ewing sarcoma. No oncological relapse was observed during follow-up after autotransplantation.[9,46,47,241] RT-PCR analysis of ovarian tissue obtained for autotransplantation purposes from 8 Ewing sarcoma patients showed involvement in one case.[16] Nevertheless, other studies reporting on histological analysis of ovarian tissue from Ewing sarcoma patients did not find any sign of ovarian metastases.[12,17] Three case reports were retrieved describing ovarian metastasis from Ewing sarcoma in one 13-year-old and two 15-year-old girls.[67,242,243]

Reports on histological analysis of ovarian tissue aiming to identify the presence of minimal residual disease have been published for patients with osteosarcoma as well as rhabdomyosarcoma. Fortunately, no sign of ovarian involvement was found in any of these diseases.[12,17] However, part of the patient groups were subjected to chemotherapy before harvesting the ovarian tissue. Case studies did report on ovarian involvement in patients with osteosarcoma[244] and rhabdomyosarcoma.[170,243,245,246] By means of xenotransplantation of ovarian tissue from 5 patients with sarcoma, no tumour components could be detected.[46]

For the remaining malignancies of the bone or soft tissue, only case studies were

obtained. Ovarian metastases were described in patients with (haem)angiosarcoma,[67,247] chondrosarcoma,[67,248] desmoplastic small round cell tumour,[249-254] clear cell sarcoma,[255] and other tumours.[256]

Other tumours

The only remaining indication for which ovarian tissue autotransplantation has been performed is neurectodermic tumour, but disease status after follow-up was not explicitly stated.[32,257] In another study where ovarian tissue from a patient with neurectodermal tumour was analysed histologically, no visible tumour components were observed.[12]

Data on histological analysis were available for patients who had ovarian tissue cryopreservation for medulloblastoma and neuroblastoma and suggested no ovarian involvement.[12,17] Nevertheless, case reports and autopsy data indicated that ovarian involvement in neuroblastoma does occur.[170,243,258-262]

Other case reports described ovarian involvement in pancreatic neuroendocrine tumours,[263-265] thyroid carcinoma,[266-269] malignant thymoma,[270-272] malignant adrenal rest tumour,[273] and goblet cell carcinoid.[243,274-288] Retinoblastoma,[170,289] mesothelioma,[103] tumours of the salivary glands,[290-292] and other tumours were also shown to have the capacity to metastasise to the ovaries.[293]

Discussion

Principal findings and implications for clinical practice

This review aimed to gain insight in the risk of recurrent oncological disease that is added to a cancer survivor's natural risk of cancer recurrence when ovarian tissue autotransplantation is performed.

Great concern

For some oncological diseases, a relatively high risk of reintroduction of malignant disease by means of autotransplantation could be derived from the literature available. In leukaemia, a clear risk of ovarian involvement and disease recurrence after transplantation has been shown by different methods. Therefore, autotransplantation of ovarian tissue should be considered unsafe in survivors of this blood borne malignancy.

Serious reasons for concern

For other tumour types, the drawing of conclusions based on the available literature proved to be more difficult. Almost exclusively based on clinical and autopsy studies

describing populations containing patients with different disease stages, we can conclude that it is justified to have serious concerns about oncological recurrence after ovarian tissue autotransplantation in survivors of gastric or colorectal cancer.

Most clinical studies on the prevalence of ovarian metastases in endometrial cancer were based on populations with different tumour stages and suffered from small sample sizes. Despite this, it is clear that ovarian metastases occur in different endometrial cancer stages, including FIGO I. As two studies concerning these low-stage patients reported contradictory results,[60,91] clinical decision-making will remain difficult in survivors from endometrial cancer.

Less concern

When it comes to cervical cancer, clinical studies suggest that ovarian involvement is not very common in, especially low-stage, cervical carcinoma patients. Nevertheless, data from histological examination of ovarian tissue are scarce. Although clinical data are reassuring when it comes to autotransplantation safety in patients with low-stage disease, the impact of different histological types of cervical cancer should be further evaluated.

Negative results from histological examination and xenotransplantation of ovarian tissue from breast cancer patients suggest a relatively low risk of disease recurrence in breast cancer survivors who have their ovarian tissue autotransplanted. However, information on the influence of different histological types (lobular versus ductal carcinoma) on the risk of ovarian involvement remains scarce. Another factor that might influence the risk of oncological relapse in breast cancer survivors is the restoration of a pre-menopausal hormonal status after autotransplantation. Although the exact impact of hormonal changes is unknown, they might theoretically play a role in patients with hormone dependent breast tumours.[294]

Least concern

Results from histological examination and xenotransplantation of ovarian tissue from patients in a fertility preservation programme suggest a low risk of disease recurrence following autotransplantation in lymphoma survivors. Moreover, follow-up data from ovarian tissue autotransplantation procedures are reassuring. Although safety can never be guaranteed, ovarian tissue autotransplantation can certainly be considered in lymphoma survivors.

Appropriateness of included studies

Follow-up after ovarian tissue autotransplantation

Reports on autotransplantation procedures are, at least theoretically, the 'Golden standard' when trying to estimate the risk of reintroducing malignancy. In clinical practice,

however, we observed that oncological follow-up of cancer survivors is still short and not always described comprehensively. Some patients who had their ovarian tissue transplanted had already received chemotherapy before ovarian tissue harvesting, whilst others had not.[32] As chemotherapy might influence the presence of viable cancer cells in the ovarian graft, this factor should be kept in mind when interpreting results.

Although difficulties in determining whether an oncological relapse is due to a reintroduction of tumour cells via the transplant will probably always remain, clinical data may give an indication. For instance, a solid tumour near the transplant is more likely to raise suspicion than a tumour at a distant location. Nevertheless, even with signs of oncological relapse in the area of the transplant, it will be difficult to establish whether or not this is a result of reintroduction of tumour cells via the graft.

Histology, PCR and xenotransplantation

For most types of cancer there is no substitute for microscopic examination of the ovarian cortex.[295] When a cancer cell is found, it is difficult to determine whether this cell is viable and has the capacity to recolonise the patient and cause oncological relapse.[15] PCR is a highly sensitive technique to detect DNA or RNA from metastatic cells. Unfortunately, only a limited number of tumour types have chromosomal aberrations that provide tumour-specific PCR targets.[13] Another limitation of PCR for the analysis of minimal residual disease is that the detection of tumour specific DNA or RNA does not necessarily mean that viable cancer cells are present in the ovarian cortex.

Different PCR results may be obtained from different parts of the same tissue fragment.[15] As the tissue fragment that is being analysed for residual malignant cells can no longer be autotransplanted, this examination does not guarantee safety regarding tumour reintroduction. This so-called 'sample bias' also applies to analysis by histology or xenotransplantation. Xenotransplantation provides better insight in the viability of the cancer cells available present in the graft. However, it is unknown to what extent xenotransplantation results are applicable to the human situation since the recipient animals have a compromised immune system and different strains may lead to different results.[296] Finally, as the minimal follow-up period of recipient animals needed for detection of tumour cells in the ovarian tissue has never been specified, some xenotransplantation studies may have missed ovarian involvement due to short follow-up.

Clinical studies and autopsy data

Clinical and autopsy studies provided the largest groups of patients from which an incidence of ovarian involvement in various types of malignancies could be determined. Notwithstanding this important strength, results should be interpreted with caution as they highly depend on the selection criteria of the particular study as well as on the study group characteristics.

It is almost certain that not all factors influencing ovarian metastasis in patients with a certain type of malignancy are known. For instance, in a multivariate analysis, histological tumour type and blood vessel invasion proved to be independent predictors of ovarian involvement in cervical carcinoma.[297] For some tumour types, such as gastric cancer, most data originated from a single continent. The impact on ovarian involvement in a certain type of malignancy by ethnical, environmental or cultural factors is, however, largely unknown.

Follow-up of a group of patients with a certain malignancy may lead to the diagnosis of ovarian metastases long after the detection of the primary tumour. It is difficult to determine whether the development of ovarian metastases during follow-up indicates that cancer cells would already have been present at the time of ovarian tissue cryopreservation shortly after diagnosis of the malignancy. This remark holds true for both autopsy and clinical studies.

Implications for future research

An important topic for further research is the development of alternative procedures to avoid transmission of cancer cells via autotransplantation, such as in vitro maturation of primordial follicles.

As these alternative procedures have not yet been introduced in clinical practice, future research should also focus on the safety aspects of ovarian tissue autotransplantation. Studies aimed at the detection of cancer cells in the ovarian tissue from patients in a fertility preservation programme should be performed, especially for those cancer types for which data are still scarce.

When it comes to clinical studies, many reports provided only very limited information on patient and disease characteristics of their study population. A more comprehensive registration of these data in future studies would provide better possibilities to compare the characteristics of patients from a particular clinical study with a patient seen in clinical practice.

Implications for fertility preservation choices

Several decades may lie between ovarian tissue harvesting and the actual autotransplantation of the ovarian tissue. During this period, new techniques aimed at avoiding the reseeding of the cancer through the transplant may become available. In vitro maturation of primordial follicles, xenografting of ovarian tissue, purging malignant cells from ovarian tissue, and transplantation of isolated follicles have all been proposed as future applications that could be combined with cryopreservation of ovarian tissue.[298-301] Although these techniques have not yet resulted in pregnancies in human beings, the approaches may provide salient options to girls and adolescents later in life.[302,303] For these reasons, one should not refrain from ovarian tissue cryopreservation

because of uncertainties regarding autotransplantation safety when it comes to young patients. Obviously, in these instances the patient should be counselled extensively about the possibility that also in the future her cryopreserved tissue may not be safe for autotransplantation.

Registration of autotransplantation procedures

The most reliable data regarding autotransplantation safety will be obtained from the follow-up of cancer survivors after ovarian tissue autotransplantation. This implies that data on the follow-up of all autotransplantations performed globally should be available to all experts in the field. In the current situation, data on adverse outcomes of ovarian tissue autotransplantation might be unavailable to other clinicians due to publication bias. In addition, published information reaches other specialists in the field after a certain delay. These factors could be overcome when an international database would be initiated, in which information on all procedures, as well as follow-up, would be registered and kept up-to-date.

Conclusion

Based on current literature, it is advisable to refrain from ovarian tissue autotransplantation in survivors of leukaemia. The safety of autotransplantation should be comprehensively discussed with survivors of all other malignant diseases. The most reassuring data regarding autotransplantation safety were found for lymphoma patients.

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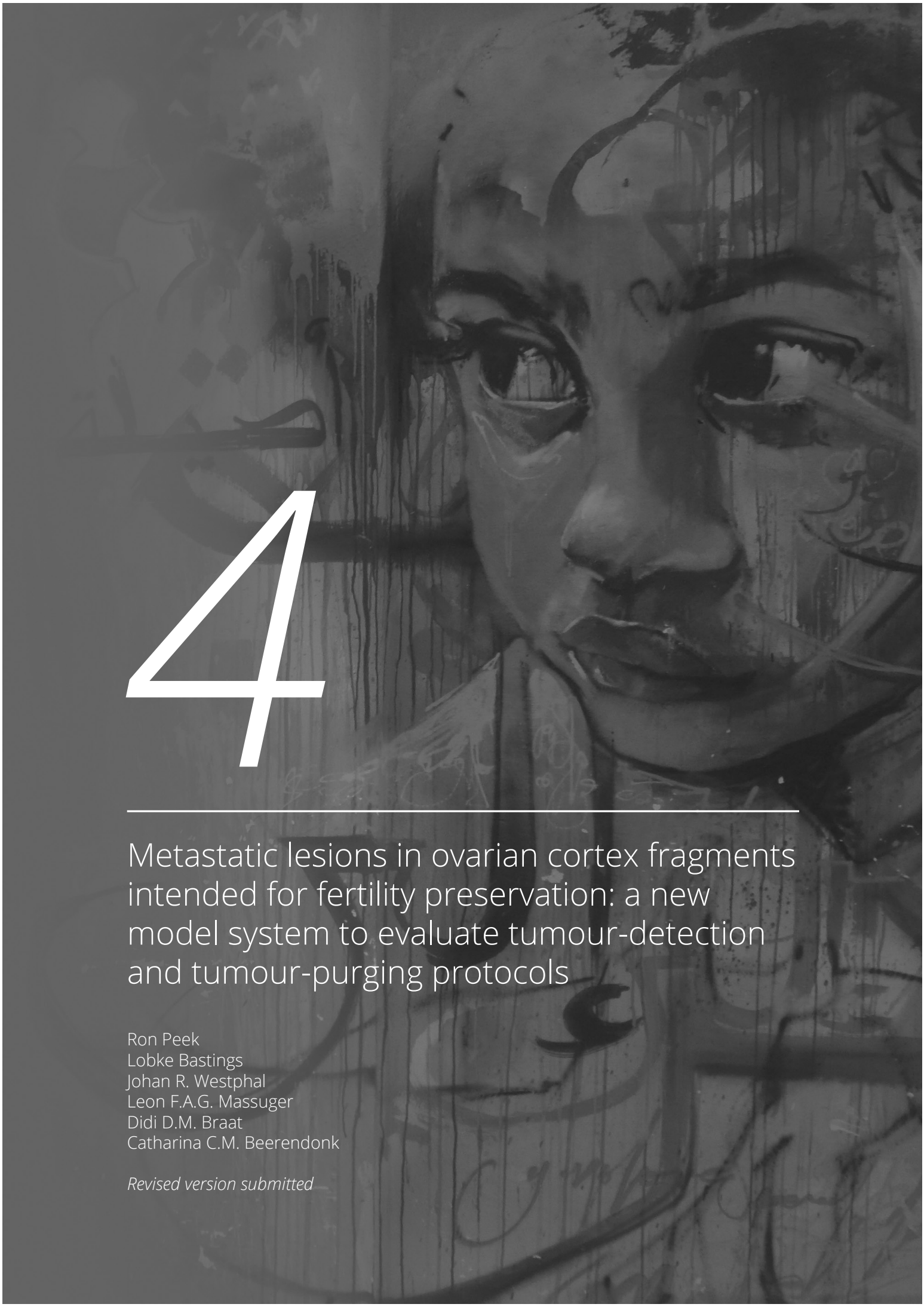

4

Metastatic lesions in ovarian cortex fragments intended for fertility preservation: a new model system to evaluate tumour-detection and tumour-purging protocols

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Abstract

Study question: Is it possible to mimic ovarian metastatic disease by inducing tumours in ovarian tissue in order to create a model-system for developing strategies to detect cancer cells and prevent cancer cell transmission via ovarian tissue autotransplantation in cancer survivors?

Summary answer: Injection of bovine or human ovarian cortex fragments with cells from different cancer types led to the formation of proliferating tumour masses and newly formed small metastatic lesions.

What is known already: Autotransplantation of ovarian tissue to restore the reproductive potential of cancer survivors comes with the major concern of cancer cells possibly being present in the cryopreserved ovarian tissue. Patient-derived ovarian cortex fragments containing metastasised cancer cells are not sufficiently available to develop effective strategies aiming to enhance or guarantee the safety of ovarian tissue autotransplantation. To date, no model system to generate these cortex fragments has been described.

Study design, size, duration: The ability of injected human leukaemia, lymphoma, Ewing's sarcoma or breast cancer cells to proliferate and form tumour-like structures in bovine and human ovarian cortex tissue was assessed. Bovine tissue was analysed at day 4 and 10, and human tissue was analysed at day 4 and day 7 after injection of cancer cells for the presence of proliferating tumour cell masses.

Participants/materials, setting, methods: Experiments were performed with ovarian tissue from women after prophylactic salpingo-oophorectomy. Bovine ovarian tissue was obtained at a local slaughterhouse. The maximum culture period of ovarian tissue was determined by a glucose-uptake assay. Human cancer cell lines were obtained from various sources. Tumour formation in ovarian tissue was assessed using histology and immunohistochemistry.

Main results and the role of chance: All cell lines tested were capable of forming tumour-like structures in bovine and human ovarian cortex tissue. Proliferation of cancer cells within the ovarian tissue was confirmed by immunohistochemical staining of the nuclear proliferation marker Ki-67. In human ovarian tissue, the lymphoma and breast cancer cells displayed metastatic capacity in vitro and produced small metastases near the original lesions.

Limitations, reasons for caution: The tumour model presented in this paper was based on the growth of human cancer cell lines in ovarian cortex tissue. It is unknown to what extent these cells behave differently from malignant cells of ovarian metastases disseminated from a primary tumour in patients opting for fertility preservation.

Wider implications of the findings: The model system may significantly contribute to efforts aimed at enhancing the safety of ovarian tissue grafting by preventing the transmission of cancer cells. Development of methods to more efficiently detect cancer cells in human ovarian tissue and protocols for purging malignant cells from intact or suspensions of ovarian cortex may benefit from this model system.

Introduction

Cryopreservation of ovarian cortex tissue has become an established technique to preserve fertility in patients awaiting a fertility-threatening treatment.[1] When the patient has overcome her disease and experiences difficulties to conceive due to the side effects related to her treatment, the cortex tissue can be autotransplanted in an attempt to restore her fertility. This option for fertility preservation is offered primarily to cancer patients scheduled for gonadotoxic chemo- and/or radiotherapy. A major concern of autotransplanting ovarian tissue in former cancer patients, however, is the reintroduction of malignant cells that may have metastasised to the graft.[2-4] Ovarian tissue derived from mice with lymphoma has been shown to transfer the disease to healthy recipients.[5] For cryopreserved and xenotransplanted human ovarian tissue from patients with acute lymphoblastic leukaemia (ALL), transmission of cancer cells via the graft to murine recipients has been convincingly demonstrated.[6] In patients, two relapses have been reported after autotransplantation of ovarian tissue; one in a patient with cervical cancer,[7] and one in a patient with breast cancer.[8] It should be noted, however, that it is unclear whether these relapses were due to the transplantation procedure. At present, autotransplantation of cortex tissue of patients with acute lymphoblastic leukaemia is considered an unsafe option for fertility preservation in view of the relative high risk of transmission of malignant cells through the graft.[2,6]

Inspection of ovarian cortex fragments for the presence of tumour cells prior to autotransplantation would obviously eliminate the risk of tumour transmission. This concept, however, is currently hampered by the lack of sensitive and specific detection methods as well as by the fact that detection procedures render the fragment under inspection unfit for autotransplantation.[2] An option to improve the safety of ovarian cryopreservation would be eliminating malignant cells from ovarian cortex tissue.[9,10] Ideally, the cortex fragments should, prior to autotransplantation, be purged from the malignant cells they potentially contain, while leaving the ovarian tissue (including follicles and oocytes) unaffected. This may be achieved by treating the cortex with anti-neoplastic agents in vitro. Assessing the effect of anti-neoplastic agents on different malignancies generally occurs with tumour cell lines that are cultured in vitro. For several types of cancer it has been demonstrated that tumour cells that are cultured conventionally react very differently to chemo- and radiotherapy than tumour cells that are attached to, and interact with, extracellular matrix components (ECM) in vivo.[11-14] Very recently, the physical effects of the microenvironment on tumour cell properties have been demonstrated for breast cancer,[15] a type of malignancy that frequently metastasises to the ovary.[16,17] Malignant cells growing in tissue in general, and in cortical tissue in particular, may therefore display very different properties with regard to cell-cell interactions, expression of cell (surface) antigens, cell motility etc. As a consequence, their sensitivity to tumour purging protocols may also differ from that of cultured cancer cells. It is therefore essential that ovarian cortex fragments with metastatic lesions,

in which the cancer cells are in close contact with the ECM of the ovarian cortex, are sufficiently available for the development of clinically relevant purging protocols. Ideally, contaminated patient-derived ovarian cortex fragments should be used. This kind of tissue is, however, hardly ever available for research purposes.

In the current study, we aimed to develop an in vitro culture system that mimics the in vivo microenvironment of ovarian metastases from various types of cancer as closely as possible. To this end, we induced lesions in ovarian cortex fragments from both bovine and human origin. During culture several of the cell lines that were tested displayed the capacity to migrate through the ovarian tissue, and form new small metastases next to the original lesion. The resulting tumour-containing fragments could be used as a model system to design protocols to reduce or even eliminate malignant cells from ovarian tissue or from partially purified follicles of patients before autotransplantation. In addition, these experimentally contaminated cortex fragments could be used to further optimise methods currently used for the detection of small numbers of malignant cells in ovarian cortex. Improved detection of contaminating cancer cells and efficient purging of ovarian tissue will lead to a broader application of fertility preservation by ovarian cryopreservation, including patients suffering from malignancies with high metastatic capacity to the ovary.

Methods

Study design

In view of the paucity of human ovarian tissue for experimental purposes, our initial experiments were conducted with cryopreserved/thawed cortex fragments from bovine ovaries. The viability of ovarian tissue during long-term culture was monitored by a glucose uptake assay. Using this assay, we determined the maximum length of the culture period for bovine and human ovarian cortex tissue before a significant loss of tissue viability occurred. Subsequently, bovine ovarian cortex was injected with tumour cells from various types of cancer to study tumour formation during in vitro culture. After successfully using bovine tissue, tumour induction was also performed in human tissue using one cell line for each of the four malignancies tested.

Ethical approval

The study was approved by the Radboud university medical center (Rumc) regional ethics committee.

Ovarian tissue preparation

Intact bovine ovaries were collected at a local abattoir essentially as described previously.[18,19] Human ovarian tissue was obtained after informed consent from premenopausal women (aged ≤ 45) who underwent a prophylactic laparoscopic salpingo-oophorectomy at the Rumc, Nijmegen, the Netherlands. Human ovarian tissue was derived from the second ovary that was dissected during surgery and immediately transferred to the laboratory in Custodiol at 4°C (Dr. Franz Köhler Chemie GmbH, Bensheim, Germany). Ovaries were placed on a precooled surface of 4°C. The cortex was scraped free from the medulla using forceps and scalpels. The remaining ovarian cortex was cut into fragments of approximately 5 x 8 mm and 1.5 - 2 mm thick.

Ovarian tissue cryopreservation and thawing

Bovine cortex fragments were cryopreserved in NuncCryoTubes with 1.5 ml of Dulbecco's modified Eagle medium (DMEM; PAA laboratories, Pasching, Austria) and 2% fetal calf serum (FCS; Gibco, Breda, The Netherlands) containing 10% dimethylsulphoxide (DMSO; Sigma-Aldrich, Zwijndrecht, Netherlands) for 15 min. Vials were kept at -80°C overnight and subsequently stored in liquid nitrogen. Human cortex fragments were transferred to Nunc CryoTubes filled with 1 ml of Leibovitz's medium (Lonza; Verviers, Belgium) containing 24 mg/ml human albumin (Albuman; Sanquin, Amsterdam, The Netherlands) and 10% DMSO (CryoSure-DMSO; Wak-Chemie Medical GmbH, Steinbach, Germany) and transferred to a CryoLogic programmable temperature controller (CL-3300, Cryosolutions, 's Hertogenbosch, the Netherlands) set at 0°C. Temperature was lowered at a rate of 2°C/min to -9°C at which the CryoTubes were seeded manually. After 10 min at -9°C seeding was confirmed and the temperature was lowered to -40°C at a rate of 0.3°C/min and subsequently to -120°C at 8.5°C/min before the tubes were stored in liquid nitrogen. For thawing, Cryotubes were placed at 37°C for several minutes until part of the ice had melted. Ovarian fragments were removed from the CryoTube and incubated for 10 min each at room temperature in DMEM/2% FCS with 0.25 M of sucrose, DMEM/2% FCS with 0.1 M of sucrose and DMEM/2% FCS without sucrose, respectively.

Determining the maximum length of the tissue-culture period

The ovarian tissue's viability during culture was monitored using a glucose uptake assay.[20] This assay quantitatively measures the glucose uptake by ovarian tissue during in vitro culture. Cortex fragments were cultured separately in 5 ml of DMEM high glucose (PAA laboratories, Pasching, Austria) with L-Glutamine supplemented with 10% FCS and 40 µg/ml gentamycin (Sigma-Aldrich Company Ltd, Gillingham, U.K.), in a 6-well plate (TPP, Trasadingen, Switzerland) at 37°C in humidified air with 5% CO_2. Conditioned culture

medium was collected and replaced by fresh medium at day 4 and 7, and collected at day 14. The conditioned medium was measured for glucose content using an Architect i2000 (Abbott Diagnostics, IL, USA). Glucose uptake of each tissue fragment was corrected for its weight and the length of the culture period. Variation in glucose uptake between two tissue fragments from the same ovary was usually within 20%. The mean glucose uptake/mg tissue/hour (nmol) was determined for 3 separate cortex fragments from each of the three bovine ovaries (bA-bC), and for 3 (pA and pB) and 2 (pC) separate cortex fragments derived from each of the three human ovaries tested.

Cell lines

The Chronic Myelogenous Leukaemia cell line K-562, the Burkitt's Lymphoma cell line Raji, the Large Cell Lymphoma cell line SU-DHL-6 and the Breast Cancer cell line MCF7 were obtained from the ATCC (Bethesda, MD, USA). The Ewing sarcoma cell lines EW8 (EWS-FLI1: exon 7/6) and ES2 (EWS-FLI1: exon 10/6) were a kind gift from the Pediatric Preclinical Testing Program (PPTP; Center for Childhood Cancer, Nationwide Children's Hospital, Columbus, OH, USA)[21,22] Cell lines MCF7, EW8 and ES2 were maintained as monolayers in medium comprising DMEM /10% FCS/40 µg/ml gentamycin (MCF7) or Roswell Park Memorial Institute medium (RPMI: PAA laboratories, Pasching, Austria) supplemented with 10% FCS/40 µg/ml gentamycin (EW8 and ES2). Cell lines K-562, Raji and SU-DHL-6 were cultured in suspension in RPMI/10% FCS/40 µg/ml gentamycin. All cell lines were incubated in 37°C, 5% CO_2 and saturated humidity.

Injection and culture of ovarian cortex fragments

Cell lines were maintained in T75 culture flasks (Corning, NY, USA) until approximately 80% confluence was reached. Cell were harvested and washed two times with 10 ml of medium (DMEM high glucose /10% FCS/40 µg/ml gentamycin). After counting, cells were diluted in cold (4°C) medium to a concentration of 2x103 cells/µl. Thawed ovarian cortex fragments were held with forceps and injected 5 times with 5 µl cell suspension per injection, using a needle with a 0.4 mm outer diameter. After injection, tissue fragments were briefly washed in 50 ml of medium and cultured in 5 ml of medium in a 6-well plate (TPP, Trasadingen, Switzerland) at 37°C in humidified air with 5% CO2. After 4 days of culture, some of the injected tissue fragments were harvested for histological and immunohistochemical analysis, while the remaining fragments were transferred to a well containing 5 ml of fresh medium and incubated for an additional 6 days (bovine tissue) or 3 days (human tissue) before harvesting and subsequent analysis.

Histology and immunohistochemistry

Tissue fragments were fixed overnight in Bouin's solution (Sigma-Aldrich) and embedded

in paraffin. Several series of 4 µm-thick sections were made, each separated by a layer of 100 µm tissue, to detect the presence of malignant cells. Sections were stained with standard hematoxylin/eosin (HE) and the Azan trichrome stain that highlights connective tissue of ovarian tissue in darkblue and cellular nuclei in red.[19] Cell proliferation was assessed by immunohistochemical staining of the Ki-67 protein.[23] Cell-type specific detection in histological sections was performed by staining with anti-HLA-DR (Thermo Fisher Scientific, Aalst, Belgium) for Burkitt's lymphoma (Raji); anti-CD99 (Thermo Fisher Scientific) for Ewing sarcoma (EW8 and ES2) and anti-cytokeratin 8/18 (BD Biosciences) for breast cancer (MCF7). To visualise tumour formation by leukaemia cells (K-562), anti-CD45 (DAKO, Heverlee, Belgium) was used for bovine tissue and anti-CD15 (Becton Dickinson Biosciences, Breda, The Netherlands) for human tissue. None of the antibodies gave significant background on non-injected bovine or human tissue. Stained tissue sections were analysed by conventional light microscopy and photographed.

Results

Determining the maximum length of the tissue-culture period

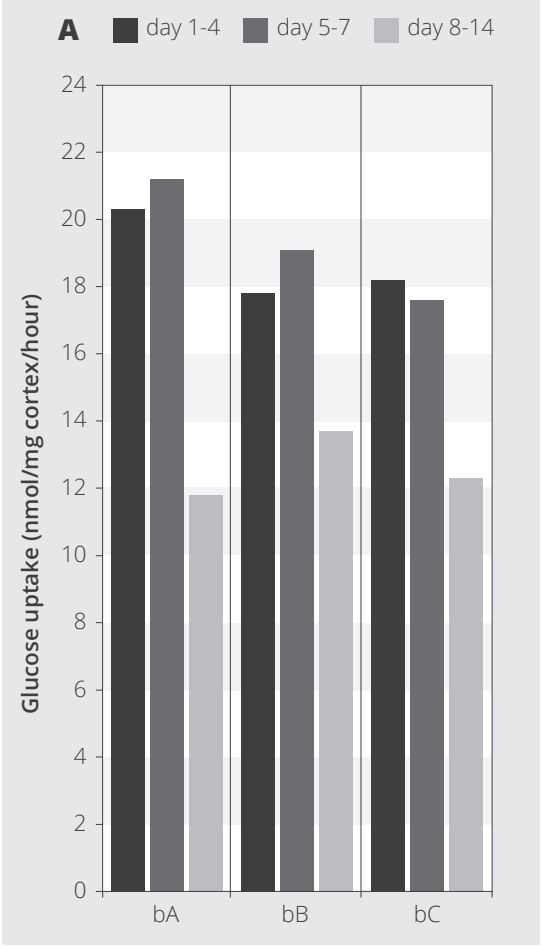
Glucose uptake by cortical tissue from three different cows (bA-bC) during in vitro culture was monitored to quantify the viability of cultured ovarian tissue (Figure I A). Using this assay, we determined the maximal duration of the culture period before a significant loss of tissue viability occurred in bovine ovarian cortex. At day 7 of culture no significant decrease in glucose-uptake was observed compared to day 4, which is in line with our previous experiments.[20] Extending the culture period up to 14 days led to a mean reduction in glucose-uptake of 34%. Subsequent tumour induction experiments with bovine tissue were therefore performed within the first 10 days of culture. Analogous to the bovine tissue, human cortex fragments from ovaries of three different patients (pA-pC) showed a comparable level of glucose uptake at day 4 and day 7. At day 14, a mean reduction in glucose uptake of 59% was measured (Figure I B). Culturing of human ovarian tissue was therefore not performed beyond day 7 in subsequent tumour induction experiments.

Induction of tumours in bovine ovarian cortex fragments

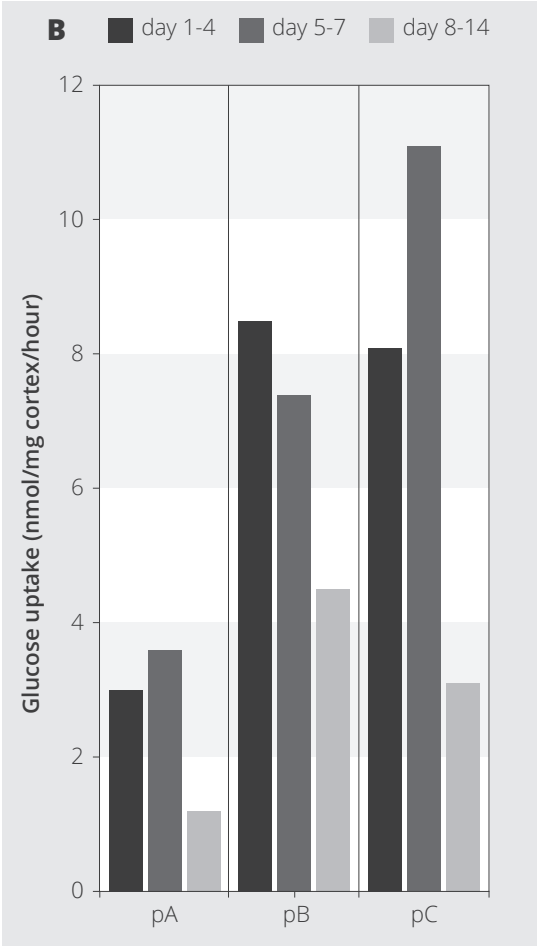
Tissue sections stained with hematoxylin-eosin (HE) derived from tissue injected with leukaemia cells that had been cultured for 4 days (Figure II A-D) showed that the lesions introduced by the injection were partially filled with viable leukaemia cells (Figure II A). High mitotic activity was demonstrated by staining of the proliferation marker Ki-67 (Figure II B). At day 10 of culture (Figure II E-H) the lesions were completely packed with leukaemia cells, and new small foci of cells appeared near the original lesions (Figure II

E). The extensive proliferation that was observed at day 4 was not apparent at day 10 of culture, with only few cells staining positive for Ki-67 (Figure II F). Leukaemic masses could be clearly distinguished from the ovarian cortex by staining with Azan, a stain that distinguishes between cells and ECM. Similar to the HE staining, foci of small masses of leukaemic cells were observed at day 10 of culture using this stain (Figure II C and E). Specific staining of tissue sections with the cell-surface marker CD43 (Figure II D and H) showed that the observed cell masses are indeed leukaemia cells and that these cells were in close contact with the ovarian tissue. Similar results were obtained with

Figure I. Glucose uptake by ovarian cortex fragments in vitro

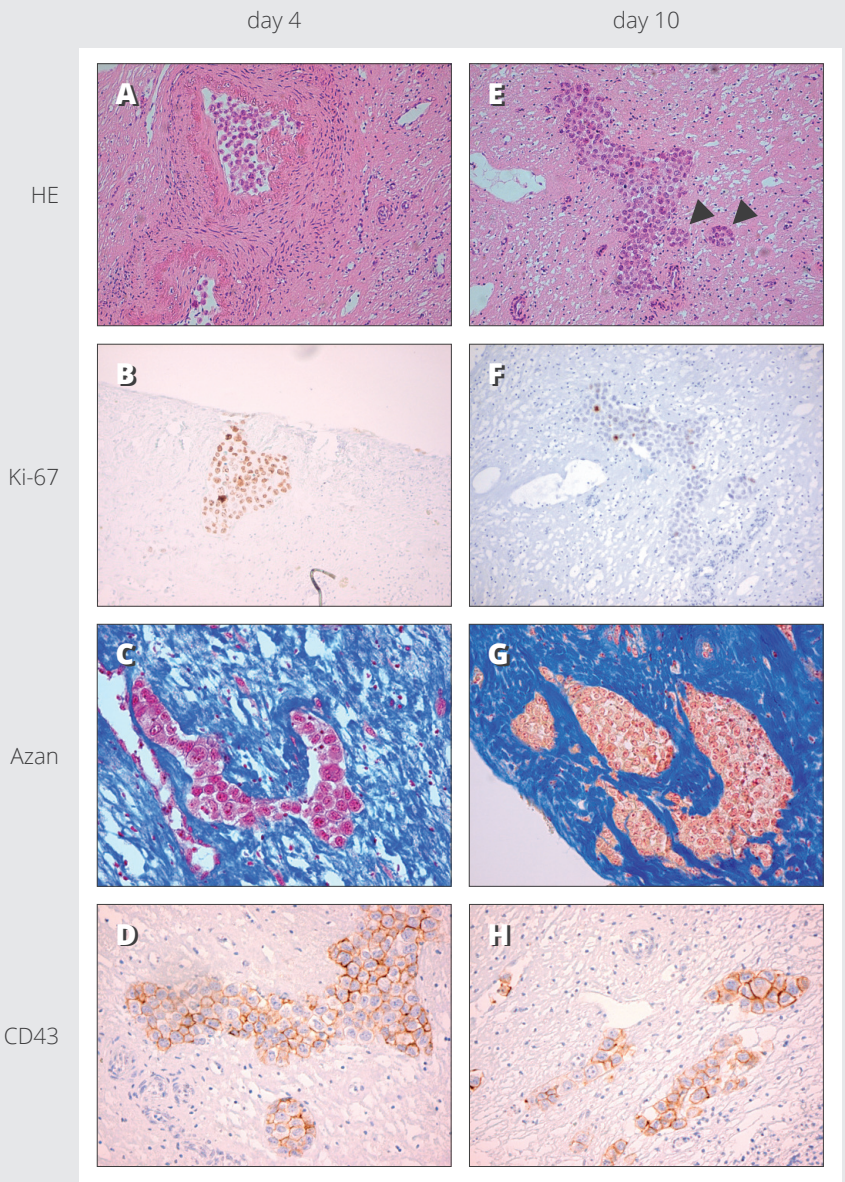


(A): Bovine ovarian cortex fragments from three animals (bA-bC) were cultured and glucose uptake (nmol/mg tissue/hour) was determined for three different time periods during culture; day 1-4, 5-7 and 8-14. For each time period, the mean glucose uptake for three individual tissue fragments is shown.



(B): Glucose uptake by human ovarian cortex fragments from three patients (pA-pC) during day 1-4, 5-7 and 8-14 of culture. For patients pA and pB, the mean glucose uptake was determined from 3 tissue fragments. For patient pC, mean glucose uptake is presented for 2 tissue fragments.

Figure II. Histological and immunohistochemical analysis of tumour formation by leukaemiacells in bovine ovarian cortex tissue.



Bovine ovarian cortex was injected with K-562 leukaemia cells and cultured for 4 days (A-D) or 10 days (E-H). Tumour formation was visualised by histochemical staining with HE (A and E) or with the Azan trichrome stain (C and G). Proliferation of leukaemia cells was assessed by immunohistological staining of the Ki-67 proliferation marker (B and F). Specific detection of leukaemia cells was performed by staining of the cell surface marker CD43 (D and H). Arrowheads point to newly formed foci of cancer cells. Original magnification was 100x for A-C and E-G, and 400x for D and H.

ovarian cortex fragments experimentally contaminated with lymphoma, Ewing sarcoma or breast cancer cells, although the formation of new foci of cancer cells during culture was less prominent than for leukaemia cells (data not shown).

Induction of tumours in human ovarian cortex fragments

Analogous to the bovine tissue, human ovarian cortex fragments were injected with cancer cell lines representing 4 different malignancies and cultured for 4 (not shown) or 7 days (Figure III A-D). HE and Azan staining of cortex fragments injected with leukaemia cells revealed efficient formation of densely packed tumour masses throughout the cortex fragment (Figure III A-C). At day 7 of culture, the leukaemia cells were still mitotically active, as judged by staining of the proliferation marker Ki-67 (Figure III C), but formation of small foci of leukaemia cells, as observed in the bovine fragments was not apparent in the human tissue. Staining of the cell-surface marker CD15 confirmed that the cell masses consisted of leukaemia cells and demonstrated close contacts between tumour cells and ovarian tissue (Figure III D). The lymphoma cells were also capable of establishing proliferating, tumour-like structures in human ovarian cortex (Figure III E-H), but in contrast to the leukaemia cells, large-scale migration of single cells through the ovarian tissue was observed after staining with anti-HLA-DR (Figure III H). Results of the tissue fragments injected with Ewing sarcoma cells were similar to those obtained with leukaemia cells (Figure IV A-D), with little evidence for the development of new metastases near the original lesions after staining with anti-CD99 (Figure IV D). In ovarian cortex fragments injected with breast cancer cells (Figure IV E-H), large-scale formation of different-sized, newly formed metastases was observed that were clearly visible in the sections stained with Azan or with an antibody against cytoskeleton 8/18 (Figure IV G and H, respectively).

Discussion

In the present study, we describe a model system consisting of experimentally induced tumours in ovarian tissue from bovine and human origin. This model can be used for the development and optimisation of protocols aiming at the detection and elimination of cancer cells in ovarian tissue intended for fertility preservation purposes.

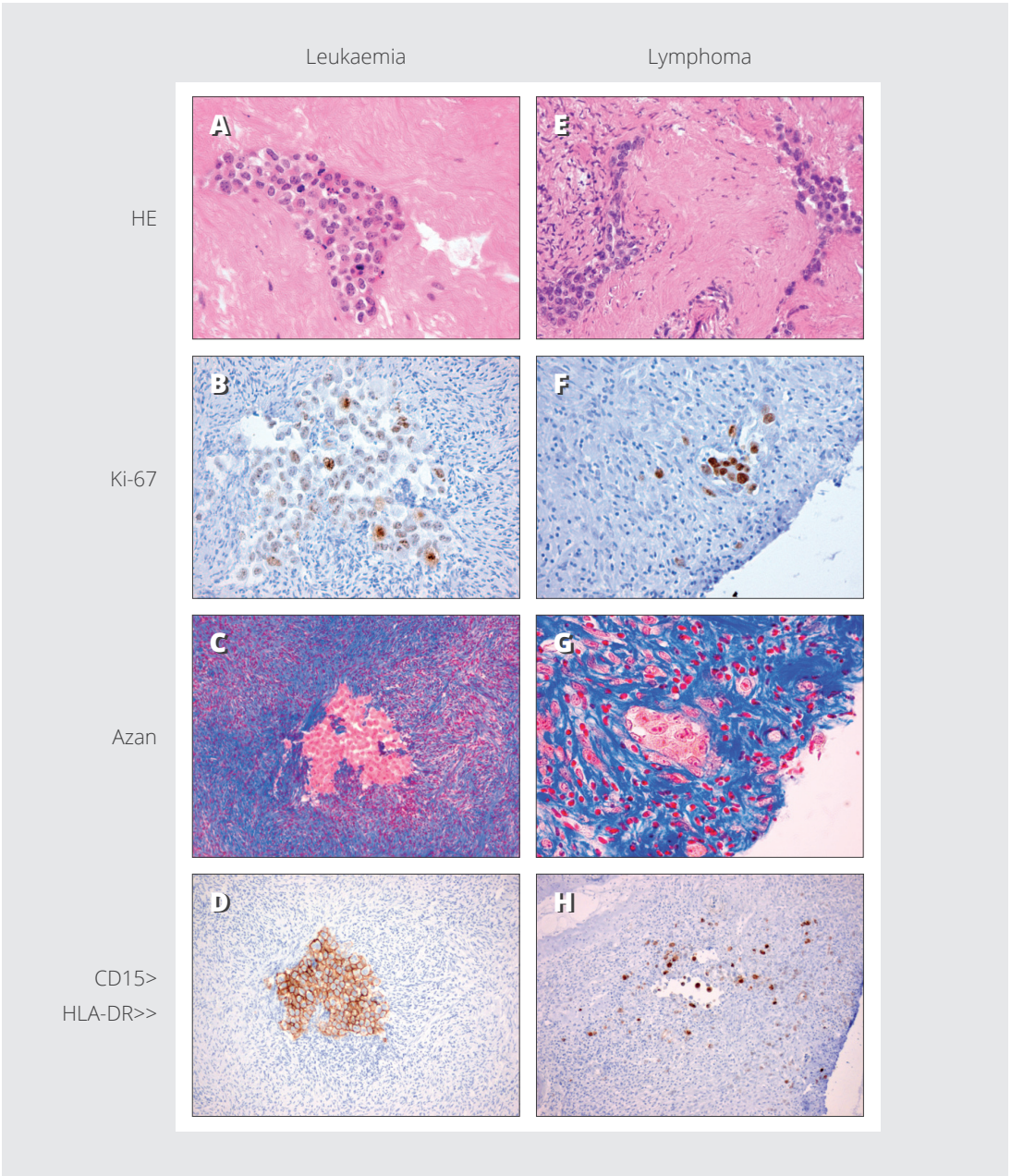
All medical treatments that involve the transplantation of tissue of cancer patients have the theoretical potential to transmit occult tumour cells that are present in the transplant to the recipient. In fact, transmission of donor tumour cells to the recipient leading to malignant disease has been described in kidney, liver, and heart transplants, providing proof of concept for this theory.[24-26] Obviously, tumour cells that are present in ovarian cortex fragments at the time of tissue collection also have the potential to lead to reintroduction of malignant disease after auto-transplantation. Although

cryopreservation of ovarian tissue is usually offered to girls and women with cancer at a stage of their disease where metastasis to their ovaries is unlikely, the presence of tumour cells in the ovarian tissue can never be ruled out completely. Therefore, it remains of crucial importance to develop and use highly sensitive detection techniques to determine whether ovarian tissue from cancer patients obtained for autotransplantation purposes is contaminated with malignant cells.

The analysis of ovarian cortex tissue for the presence of cancer cells is usually performed by standard histology, immunohistochemistry or by PCR, providing that molecular markers are available. In a few cases xenografting of immunodeficient mice was used.[6,27,28] Remarkably, PCR has been the technique of choice to positively identify the presence of malignant cells in ovarian tissue, while histology and immunohistochemistry showed negative results in all published cases.[6,27,29-33] Despite its sensitivity, the value of PCR is limited due to the absence of appropriate molecular markers in most patients. Furthermore, (RT)PCR does not necessarily detects living cells but might also detect only tumour cell DNA or RNA. It is therefore of great importance to enhance the sensitivity of histology and immunohistochemistry for the detection of malignant cells in ovarian tissue. The model system presented in the current paper will contribute to this process by providing ovarian cortex strips with proliferating malignant cells of various types of cancer in the relevant microenvironment.

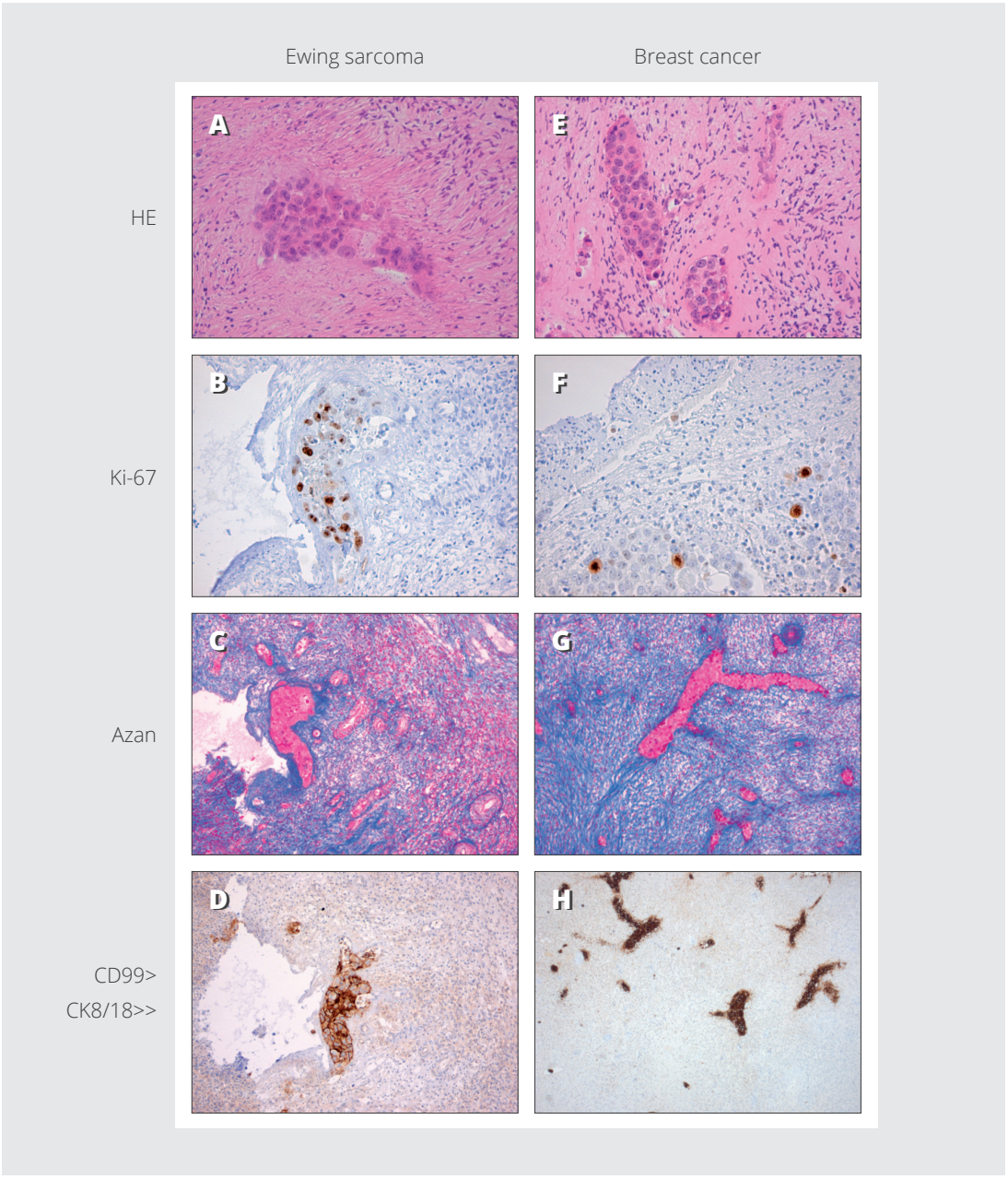
The most obvious approach to avoid the risk of malignant cell reintroduction after ovarian cortex retrieval and retransplantation is to isolate mature and fertilisable oocytes from the tissue, thereby omitting autotransplantation of the intact tissue.[34] Unfortunately, follicular culture is difficult and viable embryos from in vitro-cultured follicles have only been obtained in mice.[35] A more recently developed and promising approach to avoid the reintroduction of cancer cells by ovarian grafting is the construction of an artificial ovary in which purified follicles and somatic ovarian cells are embedded in a biodegradable matrix of fibrin and thrombin. By creating such a three-dimensional matrix, a more physiological environment was created to promote follicular development.[9] Since not only follicles but also stromal cells are required for creating an artificial ovary, it is of critical importance that the ovarian cell suspension required for such an artificial ovary is free of cancer cells. For research purposes, the potential co-purification of malignant cells with ovarian cells may be studied by spiking the partially dissolved ovarian tissue suspension with cancer cells. These spiked malignant cells will, however, not be in close cell-cell contact with ovarian components as is the case for cancer cells from actual metastases in patients' tissue. The lack of physical cell-cell interaction might well influence their behaviour during the purification process required to obtain cancer cell free ovarian components for the construction of an artificial ovary. Obviously, our model system of experimentally induced proliferating tumours in ovarian tissue, in which the malignant cells are in close contact with the ovarian tissue, might be very useful to develop reliable techniques to purify follicles and stromal cells from patients' tissue, without contaminating malignant cells. Purging of ovarian tissue by T-cell mediated tumour cell lysis,[10] chemotherapy, or

Figure III. Histological and immunohistochemical analysis of tumour formation by leukaemia and lymphoma cells in human ovarian cortex tissue



Human ovarian cortex tissue was injected with K-562 leukaemia cells (A-D) or Raji lymphoma cells (E-H). Tumour formation is shown after 7 days of culture by staining with HE (A and E) or the Azan trichome stain (C and G). Proliferation of cancer cells was determined by immunohistological staining of Ki-67 (B and F). Specific detection of leukaemia and lymphoma cells was performed by staining for the cell surface markers CD15 and HLA-DR (D and H, respectively). Original magnification was 100x.

Figure IV. Histological and immunohistochemical analysis of tumour formation by Ewing sarcoma and breast cancer cells in human ovarian cortex tissue



Human tissue was injected with ES-2 Ewing sarcoma cells (A-D) or MCF-7 breast cancer cells (E-H). Specific detection of Ewing sarcoma and breast cancer cells was performed by staining for CD99 and CK 8/18 (D and H, respectively). For further details see legend to figure III.

hyperthermia[36,37] might also be an approach to eliminate cancer cells. In contrast to the purging methods that apply tumour cell lysis or the construction of an artificial ovary, the latter two methods have the advantage that the ovarian tissue does not have to be in suspension, and hence the integrity of the ovarian tissue will not be compromised. Still, extreme caution must be taken to avoid damage to the follicles using any of these methods. Also for the development of reliable purging techniques it is important that the cancer cells that are to be eliminated are in close contact with the ovarian tissue. Tumour microenvironment and the ECM in particular will modify the biological response of malignant cells to purging.[13,38] Gastric cancer cells were found to be highly chemo-resistant, but regained chemo-sensitivity when re-attached to the appropriate ECM.[12] Chemo- and radiotherapeutic responses of leukaemia cells were also found to depend on adhesion to ECM.[11] More recently a significant relation between the physical structure of the microenvironment and breast tumour cell behaviour was described.[15] These data indicate the importance of the microenvironment on cancer cell behaviour regarding cell-cell (physical) interactions and responses to therapy. In conclusion, our model system in which cells from different types of cancer are present as small tumours within the ovarian tissue, might significantly contribute to the development of methods for efficient detection of contaminating cancer cells, the safe purification of ovarian components and the elimination of malignant cells from intact ovarian cortex tissue.

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PART B

Efficacy and efficiency

5

Efficacy of ovarian tissue cryopreservation in a major European centre

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Abstract

Purpose: To evaluate the effect of cryopreservation and thawing of ovarian tissue from oncological patients opting for fertility preservation on ovarian tissue viability.

Methods: In this prospective cohort study, the ovarian tissue viability before and after cryopreservation and thawing was measured for 25 newly diagnosed oncological patients who had their ovarian tissue cryopreserved. Outcome measures were follicle integrity (histology), follicle viability (Calcein viability assay), steroid hormone production (oestradiol and progesterone production *in vitro*) and overall tissue viability (glucose uptake *in vitro*). This study was conducted at a Cryobank for storage of ovarian tissue in a university hospital.

Results: Cryopreserved/thawed ovarian tissue showed a decreased glucose uptake when compared to tissue that had not been cryopreserved. In addition, a diminished oestradiol and progesterone production was observed after cryopreservation and thawing, despite the fact that numbers of viable follicles as determined by the Calcein viability assay were comparable. Histological examination revealed a higher percentage of degenerated follicles after cryopreservation and thawing.

Conclusions: Ovarian tissue cryopreservation and thawing impairs the viability of ovarian tissue in oncological patients opting for fertility preservation.

Introduction

Ovarian tissue can be harvested for the purpose of fertility preservation and cryopreserved before the onset of gonadotoxic therapy. When a patient is cured from her (oncological) disease but has developed ovarian insufficiency, the tissue can be thawed and autotransplanted in an attempt to restore fertility. Thus far, at least 30 live births have been reported following cryopreservation and autotransplantation of ovarian tissue.[1] Various methods for ovarian tissue cryopreservation and thawing[2-9] are being used worldwide. Although the efficacy of cryopreservation of ovarian tissue may thus differ from one centre to another, information regarding these differences is scarce.[10,11] This is remarkable given the fact that the use of cryopreservation of ovarian tissue has increased considerably in the past decade[5,12-22] with tissue from at least 2500 patients now being stored at a limited number of highly experienced centers.[23]

Obviously, pregnancies and live births[24,25] as well as living follicles identified after xenotransplantation[26] indicate that there must be (partial) follicle survival after cryopreservation/thawing according to the protocols of experienced centres. Nevertheless, it remains unknown from such data whether there is any room to improve these frequently used protocols in order to obtain higher pregnancy rates. Furthermore, patients cannot be counselled about the technique's success chances based on this information. Although there are studies that *quantify* the impact of slow-freezing on human ovarian tissue viability, these studies do not per definition focus on the protocols that are currently being used by the major centres.[8,27-38] Moreover, most of these studies did not take the viability of the stromal cell compartment into account.[27-38] This compartment, however, is essential for post-autotransplantation neovascularisation, follicle survival, and life span of the ovarian graft[39] and is considered to be more sensitive to ischemic and cryoinjury than primordial follicles.[8,40]

The aim of our current study was to assess the efficacy of the cryopreservation and thawing protocols from one of the largest centres for ovarian tissue cryopreservation in the world, the Cryobank Bonn (Academic Hospital Bonn, Germany), with respect to the survival of follicles as well as cortical stromal tissue. By overnight transport, this Cryobank – where ovarian tissue is stored from more than 1080 patients – receives tissue from hospitals throughout Germany. Orthotopic autotransplantation of ovarian tissue in 30 patients who had their tissue cryopreserved in Bonn has resulted in three live births and one additional pregnancy that has ended in a spontaneous abortion, providing proof of concept for the methods used at this facility.[2] In the current study we provide a frame of reference regarding the impact of cryopreservation-thawing on ovarian tissue viability and aim to facilitate future efforts to optimise cryopreservation and thawing methods that are currently being used worldwide.

Methods

Study design, patients and study approval

Following informed consent and approval of the institutional ethics committee, up to 10% of the ovarian tissue from each patient who had her tissue cryopreserved at the Cryobank Bonn before the start of gonadotoxic cancer treatment from January through November 2012 was available for this study. As we required twelve 3-mm cortex biopsies per patient for our experiments, only those patients for whom twelve biopsies required less than 10% of the tissue were included in this prospective cohort study. For each patient, we investigated ovarian tissue viability directly after the tissue's arrival at the Cryobank, as well as after cryopreservation and thawing using four measurements (Figure I). Subsequently, results obtained before and after cryopreservation were compared.

Study procedures

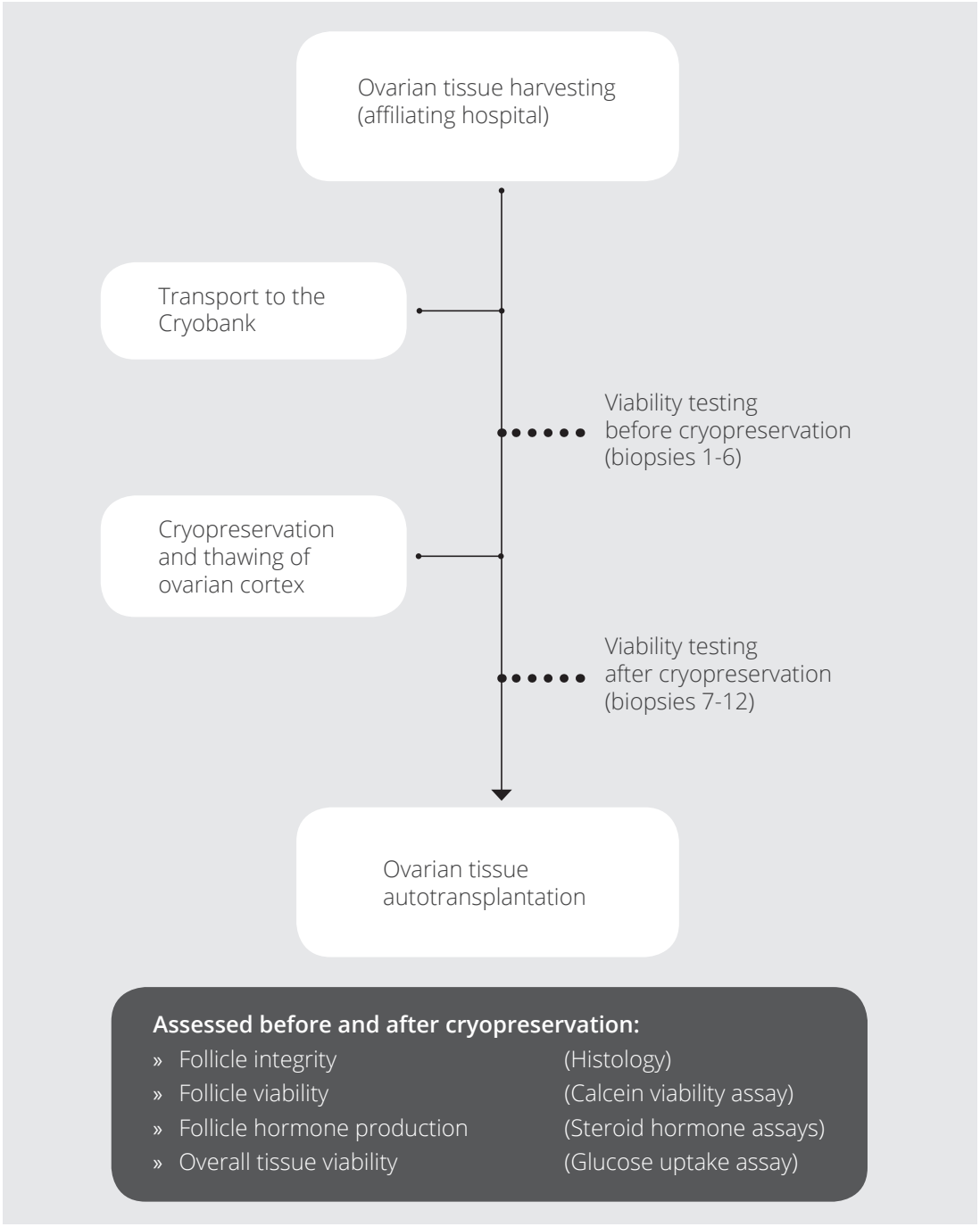
Ovarian tissue harvesting, transport and preparation

For each patient, (part of) one ovary was laparoscopically dissected at one of the Cryobank's affiliating hospitals and transferred into a tube with cold Custodiol (Dr. Franz Köhler Chemie GmbH, Bensheim, Germany). This tube was placed in a transport box (DeltaT, Giessen, Germany) containing six cool packs (4°C) and a temperature sensor for overnight road transport (± 22 hours) to the central facility at the Cryobank Bonn. Immediately after arrival, the medulla was removed and cortex strips (10x5x1 mm) were prepared for clinical purposes on a culture dish placed at a precooled surface (0-4°C) using precision forceps and scalpels. Of the remaining cortex tissue, twelve biopsies were obtained for each patient using a 3-mm diameter biopsy punch (pfm medical ag, Cologne, Germany). Of these 3-mm biopsies, six were cryopreserved and stored in MVE Vapor phase storage tanks (MTG, Technology for life, Bruckberg, Germany) for a period varying from 24 hours to 7 months and six were directly used as fresh control tissue.

Cryopreservation

The Cryobank's slow-freezing protocol was modified from a procedure published by Gosden and Isachenko.[41,42] Nunc CryoTubes (Sigma-Aldrich, St. Louis, MO, USA) were filled with 1.7 ml Leibovitz's L-15 GlutaMAX cryomedium (Gibco, Carisbad, CA, USA), 10% CryoSure-DMSO (Wak-Chemie Medical GmbH, Steinbach, Germany), and 10% serum substitute supplement (SSS; Irvine Scientific, Santa Ana, CA, USA) and precooled to 2°C. After placing six ovarian biopsies in the CryoTubes, the tubes were cryopreserved in a programmed freezer (IceCube 14S-A, SY-LAB, Neupurkersdorf, Austria). CryoTubes were pre-incubated at 2°C for 40 minutes, and then cooled at 2°C/min until automatic seeding at -6°C. After successful ice nucleation, CryoTubes were further cooled (0.3°C/min up to

Figure I. Study design



The chain of events preceding autotransplantation of ovarian tissue according to protocols of the Cryobank Bonn. In a prospective cohort study, the viability of follicular and stromal cell compartments of the ovarian cortex was assessed directly after the tissue's arrival at the Cryobank as well as following cryopreservation and thawing for the same patients.

-40°C; 10°C/min up to -140°C) and stored at -150°C in MVE Vapor phase storage tanks (MTG, Technology for life, Bruckberg, Germany).

Thawing

After removal from the liquid nitrogen, CryoTubes were kept at room temperature for 30 seconds and then placed in a 37°C water bath for 2 minutes. Using a continuous dilution protocol modified from Isachenko,[43] ovarian cortex strips were transferred to a sterile container with 10 ml Dulbecco's Phosphate Buffered Saline (DPBS; Gibco), 0.75 M sucrose (MP Biomedicals, Eschwege, Germany), 10% Fetal Bovine Serum (FBS; PAA Laboratories GmbH, Cölbe, Germany), and 0.1 mg/ml Pen/Strep (Lonza, Basel, Schwitzerland). After incubation under continuous agitation (15 min), a solution of 50 ml DPBS / 10% FBS / 0.1 mg/ml Pen/Strep was added with 100 ml/h using a perfusion device. Subsequently, the cortex strips were transferred to 5 ml pre-warmed (37°C) HEPES-buffered culture medium (Gamete, Cook Medical Europe LTD, Limerick, Ireland) and incubated under continuous agitation for 15 min. This step was repeated once using fresh Gamete medium, after which the tissue was washed three times (5 min) in the tissue culture medium.

Outcome measures

Before and after cryopreservation and thawing (Figure I), the viability of the ovarian cortex was assessed using four parameters focussed at follicle viability and integrity as well as the overall viability of the cortex, including the stromal tissue.

Follicle viability and integrity

Histology

To assess follicle integrity, one 3-mm cortex biopsy per patient was fixed in Bouin's solution (Sigma-Aldrich), dehydrated and embedded in paraffin wax for the fresh and cropreserved-thawed situation. Eight-µm sections were stained with haematoxylin and eosin and evaluated using light microscopy. Follicles were categorised into morphologically normal or degenerated follicles at primordial, primary, secondary, pre-antral or antral stages according to criteria predefined by Gougeon et al..[44] Follicles were considered to be degenerated when pyknotic nuclei, cytoplasm shrinkage, or disorganised granulosa cells were observed.[44] Two 8-µm sections, together covering 14 mm^2, were evaluated and all visible follicles were scored.

Calcein viability assay

The viable follicle count was determined using a Calcein AM (Promega, Mannheim, Germany) follicle viability staining. This staining was performed at day 0 (the day the fresh tissue arrived in Bonn or the day of tissue thawing) and at day 7 of tissue culture,

as described in the next paragraph ("Steroid hormone production"). Calcein AM is a non-fluorescent cell-permeant compound that can be hydrolysed by intracellular esterases, producing the strongly fluorescent Calcein. One 3-mm cortex biopsy (7 mm^3) was incubated in a 0.2% Calcein AM/DPBS solution supplemented with 1 mg/ml Collagenase type 1A (Sigma-Aldrich) at 37°C in humidified air with 5% CO_2 for two hours. During this incubation, the suspension was resuspended by pipetting every 30 minutes. The reaction was terminated by adding an equal volume of DPBS, after which all viable follicles that could be observed in the suspension using fluorescence microscopy at 495 nm were counted.

Steroid hormone production

To assess the follicles' developmental potential, we measured in vitro produced oestradiol (E2) and progesterone (P4) in a 7-day culture. Four cortex biopsies were cultured separately in a 24-well plate (TPP, Trasadingen, Switzerland) at 37°C in humidified air with 5% CO_2. Each well contained 2 ml of Dulbecco's Modified Eagle Medium (DMEM), High Glucose (4.5 g/L) supplemented with L-Glutamine (PAA Laboratories), 10% FBS and 0.1% Pen/Strep (GIBCO; 10.000 units penicillin/ml and 10.000 µg streptomycin/ml). No gonadotrophins were added to the culture medium. For day 0 measurements, unconditioned medium was collected approximately four hours after warming in the cell culture incubator. Conditioned supernatant was collected at day 4, and replaced by 2 ml of fresh medium, which was collected at day 7. E2 and P4 concentrations of the day 0 (control), and day 4 and 7 conditioned medium samples were determined by established chemiluminescent-microparticle-immunoassays using an Architect i2000 system (Abbott Diagnostics, IL, USA).[45] The limits of sensitivity and interassay coefficients of variation for these assays were 10 pg/ml and <7.4% for E2 and 0.1 ng/ml and <6.2% for P4. The total amount of E2 and P4 (picograms) produced during the day 0-4 and day 4-7 culture periods was calculated per hour of culture for each series of four biopsies. The E2 and P4 production of each biopsy were corrected for the weight of the biopsy that had produced the hormones.

Overall tissue viability

Glucose uptake assay

The tissue's overall viability was examined by measuring its glucose uptake from the culture medium (per mg tissue per hour) during the same 7-day culture as described above for the steroid hormone assays. As stromal cells cover large parts of the ovarian cortex, this assay, modified from Gerritse et al.,[46] is thought to give a good reflection of the viability of this cell type. Glucose concentrations in the culture medium (day 0, 4, 7) were measured using an Architect analyser. At the end of the culture, biopsies were weighed (mg) and the amount of glucose consumed (nanomoles) per mg tissue per hour was calculated for day 0-4 and day 4-7 for each biopsy.

Although glucose uptake has earlier been correlated to the extent of tissue damage sustained by *bovine* ovarian cortex,[46] we first confirmed this in a control experiment for the *human* situation. In this experiment, series of four cryopreserved-thawed biopsies that were cultured for seven days were exposed to either room temperature for one night, or 50ºC for 20 minutes, or snap-freezing without using a cryoprotectant. In addition, a control experiment using medium only as well as a control experiment using cryopreserved-thawed biopsies that were not exposed to damaging conditions were performed.

Data-analysis

Data-analysis was performed using IBM SPSS Statistics version 21 (IBM corporation, New York, USA). For all tests, differences with a *p*-value of 0.05 or less were considered to be statistically significant. E2 and P4 production (per mg tissue per hour), glucose uptake (nmol per mg tissue per hour), and the viable follicle counts were compared before versus after cryopreservation and thawing using related-samples Wilcoxon Signed Rank tests. Data were presented as median and interquartile range (IQR). To address the effect of a patient's age or preoperative AMH level on cryodamage, we performed ANCOVA analyses with the differences in glucose uptake, follicle viability count and steroid hormone production between the fresh versus the cryopreserved/thawed situation as dependent variable. ANCOVA analyses were also performed to assess the influence of the duration of cryopreservation, i.e. the length of the interval between the moment of cryopreservation and thawing.

Results

Patient characteristics

All 25 study participants (table I) had an indication for gonadotoxic cancer therapy and had their ovarian tissue cryopreserved for reasons of fertility preservation. None of the participants had received prior gonadotoxic treatment.

Follicle viability and integrity

Histology and Calcein viability assay

When looking at the entire study population, a larger percentage of degenerated (primordial) follicles was observed in the cryopreserved-thawed tissue when compared to the fresh controls (table I). Most follicles were in the primordial or primary stage, although a small proportion of follicles in more advanced stages were observed. A

relatively large number of histological sections suffered from a low number, or even a complete absence, of follicles, presumably due to the fact that we only used a single 3-mm biopsy because of a paucity of cortex tissue available for research purposes. After proteolytic digestion of biopsies for the Calcein viability assay, more follicles could be evaluated. With this assay, a similar number of viable follicles were observed before (median: 93; IQR 47-206) and after (median: 93; IQR 40-192) cryopreservation (p = 0.647). At culture day 7, similar follicle counts were obtained when compared with day 0. In the fresh tissue, a median number of 85 follicles (IQR 39-165) was observed, whereas a median number of 91 follicles (IQR 29-156) was observed after cryopreservation and thawing (p = 0.747). For some individual patients, however, remarkable differences in follicle counts before and after cryopreservation were found (table I). Furthermore, cortex pieces were heterogeneous with respect to follicle density as some patients had fewer and others had considerably more follicles in cryopreserved/thawed tissue when compared to fresh.

Steroid hormone production

In the unconditioned medium, before starting the cultures, E2 and P4 were below detection level. No significant difference was observed in the weight of biopsies that were cultured before versus after cryopreservation (data not shown). During the first four days of culture, the median E2 production was reduced with 45% after cryopreserved-thawed biopsies when compared to the fresh biopsies after correction for biopsy weight ($p < 0.001$; figure IIa). With prolonged culture, the E2 production in both cultures diminished. There was no statistically significant difference in the median E2 production at culture day 7 (p = 0.166; figure IIa). The production of P4 increased during the cultures, with a significantly higher P4 production at the end of the culture in the fresh biopsies when compared to the cultures of cryopreserved-thawed biopsies. After correction for biopsy weight (figure IIb), the level of P4 before cryopreservation was similar to the level after cryopreservation and thawing during day 0-4 (p = 0.339). For day 4-7 a median reduction of the tissue's P4 production of 55% (p = 0.004) was observed.

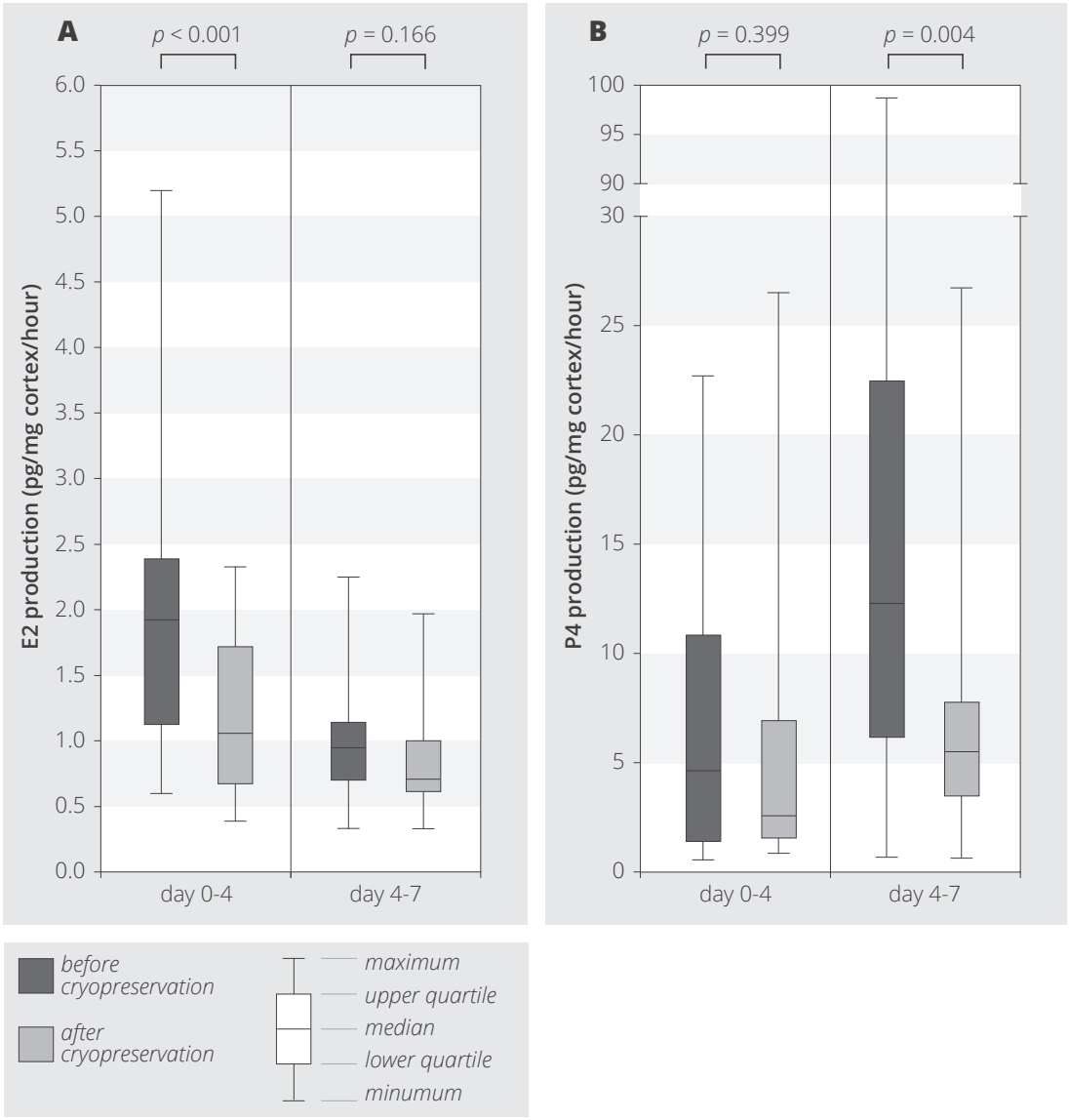
Table I. Patient characteristics, histology and follicle viability

N	Age	Cancer type	AMH level (µg/L)	Transport Temp (°C)	Viable follicle count (7 mm^3 cortex tissue)		Histology (14 mm^2 cortex tissue)	
					Before cryopreservation	After cryopreservation	Before cryopreservation	After cryopreservation
1	24	Rectal	1,6	5	65	62	2 primordial (1 D)	1 primordial
2	31	Breast	0,6	4,5	47	39	1 primordial	-
3	29	Cervix	no sample	4	47	28	-	4 primordial
4	21	HL	no sample	4	173	291	11 primordial, 4 primary, 6 secondary	1 primordial (D), 8 primary,
5	33	HL	1,9	6	102	113	7 primordial (1 D)	2 primordial (D), 1 primary (D)
6	39	Breast	1,6	4	32	24	5 primordial (2 D)	-
7	21	HL	2,9	6,5	248	310	31 primordial, 2 primary	-
8	20	HL	3,6	5,5	231	183	48 primordial, 4 primary	-
9	27	HL	2,1	5,5	128	133	-	-
10	23	Chondrosarcoma	1,8	5,5	128	46	-	-
11	33	Breast	1,8	4,5	93	135	6 primordial, 6 primary, 1 secondary	18 primordial, 1 primary
12	26	Cervix	no sample	6,5	74	24	-	-
13	29	Breast	2,1	4,5	83	220	3 primordial	3 primordial, 1 antral
14	21	NHL	1,1	5	291	91	19 primordial, 5 primary	-
15	35	Breast	0,6	7	38	40	-	1 secondary
16	31	Breast	no sample	6	286	350	-	-
17	21	HL	2,5	5,5	400	233	-	-

18	31	Breast	1,4	2	53	160	-	-
19	24	HL	1,8	6,5	129	150	-	5 primordial (2 D)
20	36	Breast	1,9	10	18	27	-	-
21	20	B-cell lymphoma	2,5	5,5	180	200	-	-
22	35	Breast	1,9	5,5	10	13	1 primordial (D)	-
23	31	Breast	0,8	5,0	34	46	-	-
24	34	Breast	2,9	5,5	56	76	-	2 primordial (1 D)
25	18	Breast	2,1	6,0	258	93	-	9 primordial (6 D)
Median (IQR)							**Total**	
	29 (21-33)			5.5 (4.5-6.0)	93 (47-206)	93 (40-192)	162 follicles of which 5 degenerated (97% intact) primordial follicles: 112 (5 degenerated; 96% intact)	57 follicles of which 13 degenerated (77% intact) primordial follicles: 45 (12 degenerated; 73% intact)

Characteristics of patients at the time of ovarian tissue cryopreservation and results of the Calcein follicle viability staining and histology ('-'no follicles were observed in the histologically evaluated biopsy). N = patient number. HL = Hodgkin's lymphoma. NHL = Non-Hodgkin's lymphoma. Transport temp = Temperature measured in the transport box at arrival in Bonn (°C). (D) = Degenerated follicle as observed during histological examination. IQR = Interquartile range.

Figure II. Steroid hormone production by ovarian cortex

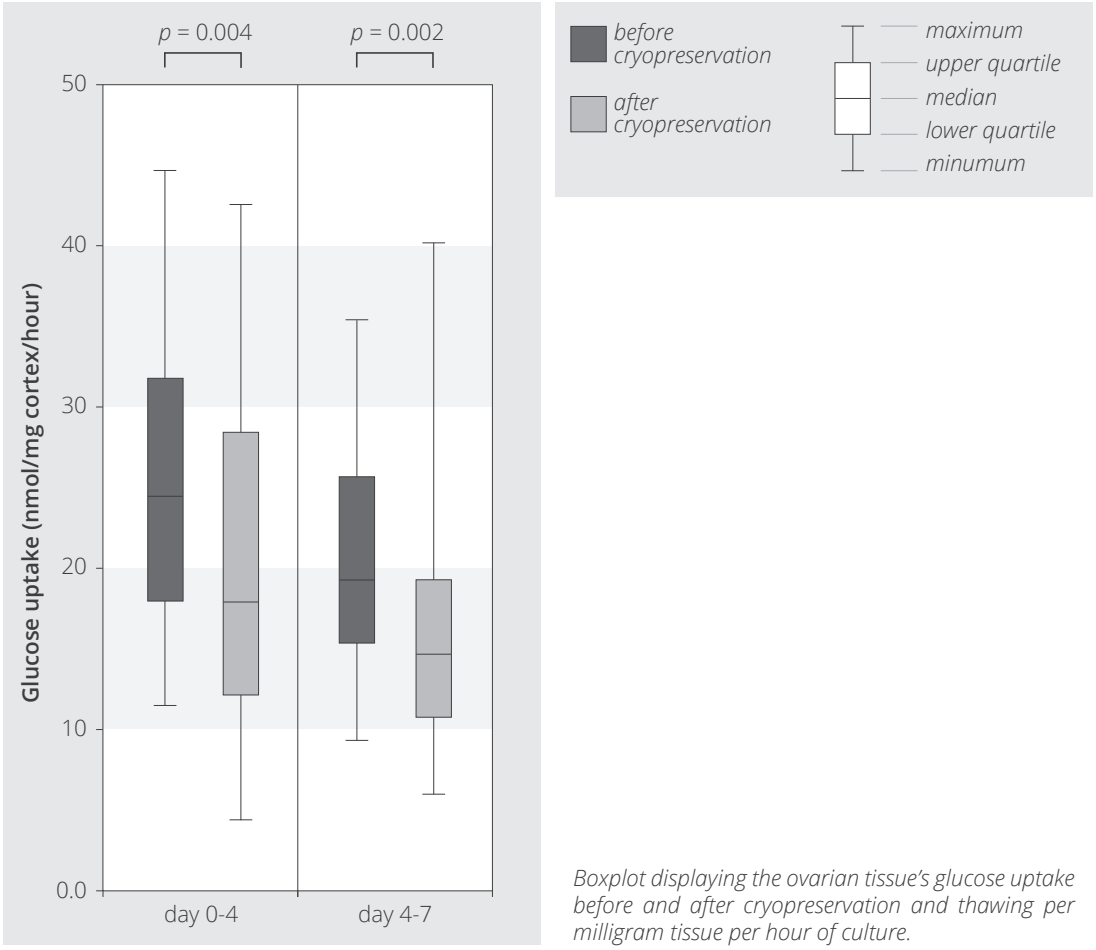


Boxplots displaying the ovarian tissue's E2 and P4 production before and after cryopreservation and thawing.

Overall tissue viability

Our control experiments revealed that the glucose uptake assay has the capacity to indicate various extents of tissue damage in human ovarian tissue. Four cryopreserved-thawed biopsies that were cultured for seven days had a mean glucose uptake of 16.9 nmol glucose per milligram tissue per hour (SD 5.2). Biopsies that were exposed either

Figure III. Glucose uptake by ovarian cortex



Boxplot displaying the ovarian tissue's glucose uptake before and after cryopreservation and thawing per milligram tissue per hour of culture.

to room temperature for one night, or 50ºC for 20 minutes, or snap-frozen without using a cryoprotectant had a glucose uptake of 2.9 nmol/mg/hour (SD 2.1), 15.8 nmol/mg/hour (SD 5.1) and a non-detectable glucose uptake respectively. Medium only showed stable glucose concentrations during the incubation period (day 0: mean: 23.2 mmol/L, SD 0.5; day 4: 24.5 mmol/L, SD 0.7; day 7: 24.3 mmol/L; SD 0.2).

At day 0, a median glucose concentration in the medium of 23.5 nmol/L was observed (range 21.9 – 25.0 nmol/L). Cryopreserved and thawed ovarian tissue from the patients in our study cohort showed a lower glucose uptake than tissue that had not been cryopreserved, indicating a reduced viability of the stromal cell compartment (Figure III). During the first culture period (day 0-4) a reduction in the median glucose uptake per mg per hour of 27% was observed after cryopreservation and thawing when compared to the tissue that had not been cryopreserved ($p = 0.004$). For the second culture period (day 4-7) this reduction in median glucose uptake was 24% ($p = 0.002$)

Effects of age, AMH, and duration of cryopreservation on cryodamage

A higher age and lower AMH-level were statistically significantly associated with a lower number of viable follicles observed with the Calcein AM viability assay for fresh as well as cryopreserved/thawed tissue. Despite this, the effect of cryopreservation and thawing on the tissue and follicle viability was comparable for younger and older patients. The differences in the glucose uptake, steroid hormone production, and number of viable follicles measured for the fresh versus cryopreserved/thawed tissue were not statistically significantly influenced by age, AMH, or the length of the interval between cryopreservation and thawing (data not shown).

Discussion

This prospective cohort study indicates that the viability of young oncological patients' ovarian tissue is impaired as a result of the cryopreservation and thawing process when using protocols from the Cryobank Bonn. Although various large centres for cryopreservation of ovarian tissue exist,[12,13,15,17,18,21,24] to the best of our knowledge, cohort studies in which the impact of currently used cryopreservation/thawing protocols on both follicle and stromal cell survival in young cancer patients is measured have not been published before. Earlier studies considering the impact of slow freezing techniques other than evaluated here, generally focussed on follicle viability only[27-38] and often described patient populations without an indication for fertility preservation (e.g. patients applying for a sterilization, cystectomy, or caesarean section).[8,27-29,32,34,35,38]

Although we observed a similar number of viable follicles with intracellular enzyme activity (Calcein viability assay) before and after cryopreservation/thawing in this cohort of 25 young cancer patients, ovarian tissue damage after cryopreservation was indicated by a decreased percentage of normal, intact follicles of 77% after versus 97% before cryopreservation (histology). The high percentage of normal follicles in the fresh tissue suggests that the transport of ovarian tissue did not have a deleterious effect on the follicles. The observed percentages of tissue viability impairment as a result of cryopreservation and thawing are roughly consistent with the 70-80% follicle survival reported earlier for various slow-freezing protocols.[8,27,29,30,35,38,39] In addition, we observed a 24-27% reduction in overall tissue viability using the glucose uptake assay. This decreased glucose uptake indicates a reduced metabolism of the tissue during culture as a result of cryodamage. As the ovarian cortex tissue mainly exists of stromal cells, the glucose uptake assay is thought to predominantly reflect the viability of this compartment. However, the decline in the median glucose uptake per milligram tissue per hour observed in our study (24-27%) was considerably higher than the increase in apoptosis in stromal cells (11%) observed in a single prior study analysing ovarian tissue

from women with benign cysts.[8] It is known from situations of trauma, hemorrhagic shock, organ transplantation, and autoimmune diseases that damage sustained by ischemic insult may lead to so-called ischemia/reperfusion-associated tissue damage as soon as this tissue is neovascularised.[47] After neovascularisation, the ischemic tissue activates the immune system that may lead to further tissue injury.[47-49] For this reason it is essential that during optimisation of cryopreservation/thawing protocols not only the viability of the follicles should be taken into account, but also the viability of the stromal cell compartment.

Various techniques to examine ovarian tissue viability have been reported in the literature,[8,28,34,50-53] but a golden standard does not exist. With respect to the results from our current study, one should bear in mind that only small biopsies were available for histology and the Calcein assay, meaning that some sample bias with respect to the density of follicles in the tissue did occur. Furthermore, the numbers of follicles that could be histologically evaluated was limited. Although steroid hormone production has earlier been associated with ovarian tissue damage,[30,37,54-56] the clinical relevance of measuring steroid hormone production is severely restricted. Namely, developing follicles produce larger amounts of steroid hormones than primordial and primary follicles,[57] whereas one specifically aims to preserve the more numerous small follicles that have the ability to mature after autotransplantation. From steroid hormone measurements *in vitro*, it remains unknown which part of the reduction in steroid hormone production after cryopreservation (varying from 11 to 55%) could be attributed to the expected loss of antral follicles during cryopreservation, and which part reflects the loss of small follicles. E2 levels are expected to rise as a result of granulosa cell proliferation, whereas P4 is produced after luteinisation of these cells in a more advanced stage of folliculogenesis.[57,58] Possibly, this was the reason why P4 levels rose in the second culture period, after the fourth day of culture. As alternative methods to assess the viability of ovarian tissue, measuring the tissue's uptake of bromodeoxyridine during culture has been proposed.[59] To assess follicle viability, follicle isolation and 3D culture with evaluation of the follicle structures and diameter have been suggested.[59]

In conclusion, the current study indicates that cryopreservation/thawing of ovarian tissue from cancer patients following protocols from one of the largest centres for ovarian tissue cryopreservation in the world (Cryobank Bonn) significantly impairs the viability of the tissue. Although these findings cannot be extrapolated to other protocols, various other clinics have comparable freezing protocols to the DMSO-based protocol that was evaluated in the current paper.[6,13,60,61] Nevertheless, ethylene glycol is also used as a cryoprotectant in a major centre reporting live births.[26] With respect to thawing, a variety of protocols has been reported.[26,60,61] Consistently with a small number of earlier studies, the current study showed a reduction in stromal cell viability as a result of cryopreservation and thawing. For this reason, it is advisable to – apart from assessing follicle viability – include measurements on stromal cell viability in future studies evaluating cryopreservation and thawing procedures. To assess follicle survival

after cryopreservation and thawing, a viability assay and histology are considered useful, whereas the relevance of steroid hormone assays is restricted. By optimising cryopreservation as well as thawing procedures, the viability of ovarian tissue after cryopreservation and thawing - and conceivably the clinical outcome - may be improved in the near future. To investigate whether cryodamage results from cryopreservation and/or thawing, the viability of ovarian tissue from the same patients should ideally be investigated after various combinations of cryopreservation and thawing protocols. This information is especially relevant as tissue has been cryopreserved for a large number of patients who may consider thawing and autotransplantation of their tissue in the future.[23] Furthermore, the effects of ovarian tissue transport on the tissue's condition, which could not be measured in the current study, should be further investigated. Since fertility preservation by means of cryopreservation of ovarian tissue is no longer considered experimental by some[24] and is applied on an increasing scale, efforts to optimise the laboratory phases have become imperative. The data presented in the current study can be used as a frame of reference for the evaluation and modification of currently used protocols.

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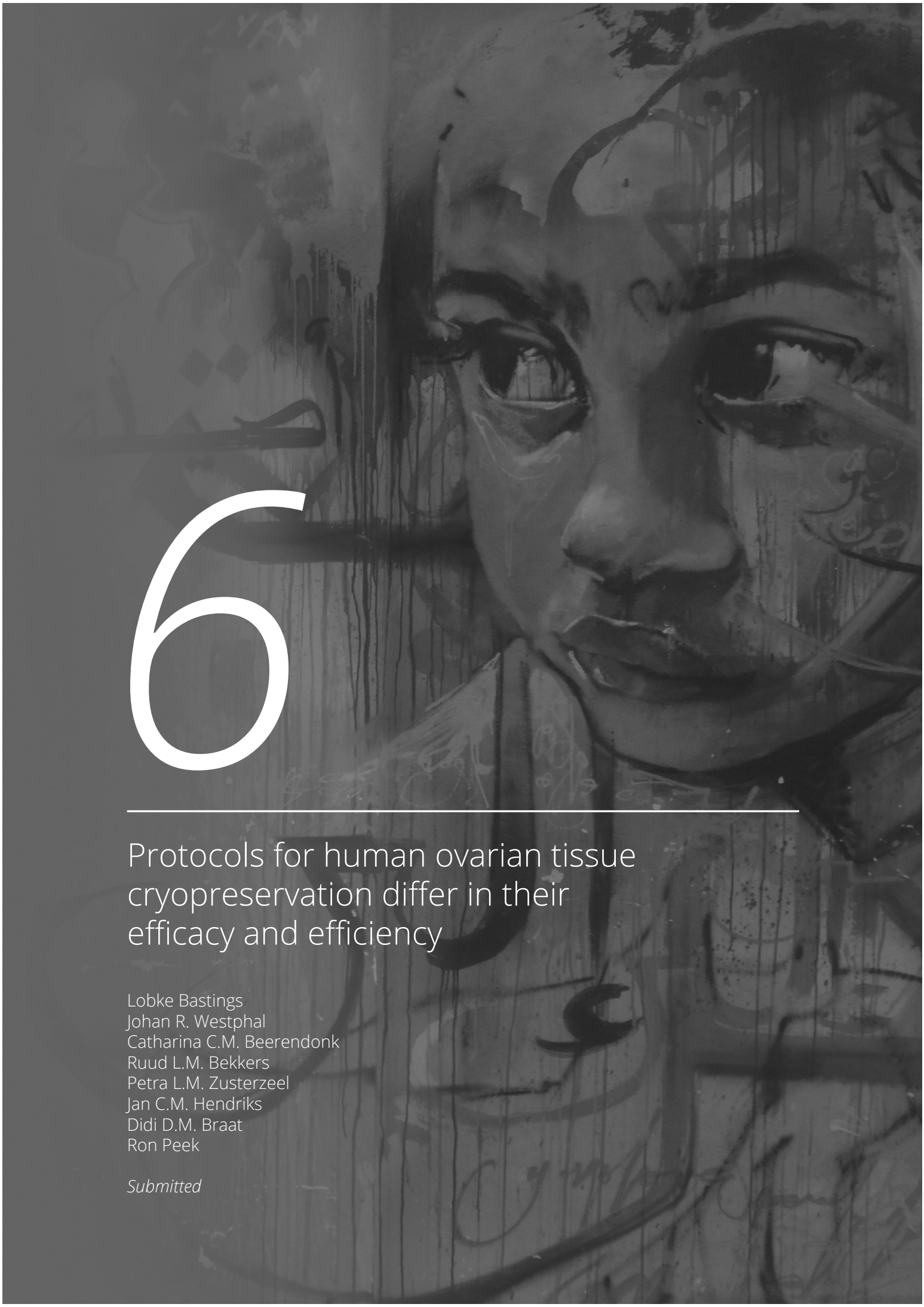

6

Protocols for human ovarian tissue cryopreservation differ in their efficacy and efficiency

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Abstract

Objective: To study the effect of ovarian tissue cryopreservation and thawing according to two very different protocols on the viability of human ovarian cortex tissue.

Design: In a prospective cohort (November 2012 – November 2013), the viability of human ovarian tissue was assessed before and after cryopreservation/thawing. To differentiate between cryopreservation ($_C$) versus thawing ($_T$) related effects, all four combinations of the protocols A and B (A_CA_T, A_CB_T, B_CA_T, B_CB_T) were studied.

Setting: This study was conducted at the Radboud university medical center, Nijmegen, the Netherlands.

Patients: After informed consent, ovarian tissue was obtained from women who received a prophylactic salpingo-oophorectomy (pre-menopausal, ≤ 45 years).

Interventions: Tissue viability was measured before versus after cryopreservation and thawing according to A_CA_T, A_CB_T, B_CA_T, and B_CB_T.

Main outcome measures: Before and after cryopreservation/thawing, a Neutral Red viability assay (percentage of living follicles), histology (percentage of morphologically normal follicles), and a glucose-uptake assay (overall tissue viability) were performed.

Results: A total of 21 women (median age: 40 years; range 32-45) participated. The choice of the cryopreservation method affected tissue viability, with a higher viability after protocol B_C using DMSO when compared to A_C using ethylene glycol as cryoprotectant. No statistical differences were found between the two thawing protocols. The tissue's morphology was best retained after cryopreservation/thawing combination B_CA_T.

Conclusion: Protocols for cryopreservation and thawing of ovarian tissue differ in their effects on ovarian tissue. Evaluation of clinically used protocols is warranted.

Introduction

The survival of paediatric, adolescent, and young adult cancer patients has significantly improved during the past decades.[1,2] For this reason, issues related to quality of life after cancer have become an essential part of oncological care. With many types of oncological therapy posing a threat to the ovarian function,[3] techniques aimed at preserving fertility in girls and young women have emerged and evolved.[4] One of the techniques for fertility preservation involves cryopreservation of ovarian tissue before the start of gonadotoxic anti-cancer treatment.

Although a considerable number of live births have been reported after auto-transplantation of cryopreserved ovarian tissue,[5] data on the pregnancy rates of ovarian tissue cryopreservation and autotransplantation are not yet available.[6, 7] Several studies have focussed on the efficacy of slow-freezing and thawing procedures, using human or animal ovarian tissue.[8-13] Worldwide, numerous protocols for cryopreservation and thawing of ovarian tissue are used,[14-20] meaning that the clinical outcome of fertility preservation by ovarian tissue cryopreservation may vary between centres. In the current study, we investigated the effects on the viability of human ovarian tissue of two very different protocols for human ovarian tissue cryopreservation and thawing.[14,21] We analysed the effects of cryopreservation and thawing as described by these two protocols separately, as cryopreserved tissue is currently stored for many patients who may request thawing and autotransplantation in the future[22] and may therefore benefit from an alternative thawing procedure.

Methods

Eligibility criteria and study design

The effects of the two selected cryopreservation and thawing protocols (protocol A_CA_T[23] and B_CB_T[24]) on overall ovarian tissue viability, follicle viability, and tissue morphology were investigated in a four-arm design:

» A_CA_T: cryopreservation and thawing protocol of centre A[23]
» B_CB_T: cryopreservation and thawing protocol of centre B[24]
» A_CB_T: cryopreservation protocol A followed by thawing protocol B
» B_CA_T: cryopreservation protocol B followed by thawing protocol A

As we aimed to investigate the effects of all four combinations of cryopreservation and thawing methods with each ovary, we could not use tissue from young cancer patients applying for ovarian tissue cryopreservation because of scarcity as well as ethical reasons. For this reason, premenopausal women aged ≤ 45 years were considered eligible for this prospective cohort study if they underwent a prophylactic laparoscopic salpingo-oophorectomy at the Radboud university medical center (Rumc), Nijmegen,

the Netherlands, between November 2012 and November 2013. Informed consent was obtained prior to surgery. Cortex fragments derived from the second dissected ovary were used for the study. When possible, tissue from each ovary was included in all four arms of the study. In addition, a fresh control was analysed for each ovary before cryopreservation/thawing. In case of insufficient material (due to size of the ovary, presence of large follicles, and/or extensive damage as a result of cauterisation during surgery), the material was only used in three or less of the study arm protocols.

Ethical approval

All study procedures were approved by the Rumc's regional ethics committee.

Ovarian tissue preparation

At the operating theatre, the second ovary dissected during surgery was collected in cold Custodiol (4°C; Dr. Franz Köhler Chemie GmbH, Bensheim, Germany). This ovary was immediately transferred to the laboratory (within 5-10 min), where it was placed on a pre-cooled surface (4°C). The medulla was removed from the cortex using precision forceps and scalpels, after which ovarian cortex fragments of approximately 5x5mm were prepared. For each of the four study arms as well as the fresh control, two to three cortex fragments were used.

Ovarian tissue cryopreservation and thawing

The ovarian tissue cryopreservation and thawing protocols selected for this study differ considerably. Briefly, protocol A consists of a cryopreservation protocol using ethylene glycol combined with a thawing protocol based on short washes to remove the cryoprotectant. Protocol B uses dimethylsulfoxide (DMSO) as a cryoprotectant and has a significantly longer thawing procedure based on continuous dilution.

Cryopreservation protocol A_C

The protocol described by Schmidt et al.[23] was followed as closely as possible. Tissue fragments were equilibrated in 30 ml of cryomedium, consisting of 0.1 mol/L sucrose (purity ≥99.5%; Sigma-Aldrich, Zwijndrecht, the Netherlands) and 1.5 mol/L ethylene glycol (Merck Millipore, Cat. No 100949) in phosphate-buffered saline (PBS; Braun, Melsungen, Germany) for 30 minutes on a tilting table at 4ºC. Instead of using a Planer Freezer (Planer K10; Planer Ltd, UK), ovarian cortex fragments were frozen in Nunc CryoTubes (Sigma-Aldrich, St. Louis, MO, USA) in 1 ml cryomedium in a CryoLogic programmable temperature controller (CL-3300, Cryosolutions, 's Hertogenbosch, the Netherlands) using CryogenesisTM V5 software (Cryosolutions). CryoTubes were transferred to the

CryoLogic set at 0ºC. Subsequently, the temperature was lowered at a rate of 2ºC/min until a temperature of -9ºC was reached. At this stage, CryoTubes were seeded manually with a cotton swab dipped in liquid nitrogen. The temperature remained at -9ºC for 10 minutes after which seeding was checked. Next, the temperature was decreased at a rate of 0.3ºC/min to -40ºC and subsequently with 8.5ºC/min to -120ºC before the CryoTubes were stored in liquid nitrogen. This freezing protocol was comparable to the original protocol, expect for the duration at which the temperature remained at -9ºC (5 versus 10 minutes) and the rapidity at which the temperature was lowered after reaching -40ºC (8.5ºC/min to -120ºC versus 10ºC/min to -140ºC in the original protocol).[23]

Thawing protocol A_T

Thawing of ovarian tissue was performed as described by Schmidt et al.[23] According to this protocol, the tissue was thawed rapidly at 37ºC and then rinsed in three consecutive solutions for 10 minutes each to wash out the cryoprotectant. Rinsing solutions consisted of PBS/ 0.25 mol/L sucrose/0.75 mol/L ethylene glycol; PBS/0.25 mol/L sucrose; and PBS. In total, this resulted in a thawing procedure of approximately 35 minutes.

Cryopreservation protocol B_C

According to cryopreservation protocol B_C,[24] a freezing solution was prepared consisting of Leibovitz's L-15 GlutaMAX cryomedium (Gibco, Carisbad, CA, USA), containing 10% dimethylsulfoxide (1.4 mol/L; CryoSure-DMSO; Wak-Chemie Medical GmbH, Steinbach, Germany), and 10% serum substitute supplement (SSS; Irvine Scientific, Santa Ana, CA, USA). Nunc CryoTubes were filled with 1.7 ml cold medium (2°C) and a cortex fragment. Instead of using the program as described for the IceCube (14S-A, SY-LAB, Neupurkersdorf, Austria) in the original protocol, (Dittrich et al., 2012) the CryoTubes were frozen in the CryoLogic programmable temperature controller using the program as stated under "Cryopreservation protocol A_C". This freezing protocol was comparable to the original protocol, except for an incubation step in the programmable freezer at 2°C for 40 minutes, the seeding temperature (-6°C instead of -9ºC) and the rapidity at which the temperature was lowered after reaching -40ºC (8.5ºC/min to -120ºC versus 10ºC/min to -140ºC in the original protocol).

Thawing protocol B_T

Thawing protocol B_T was based on continuous dilution of the cryoprotectant during approximately 1.5 hours.[24] The CryoTubes were removed from the liquid nitrogen and, after 30 seconds at room temperature, placed in a 37°C water bath for 2 min. Directly after thawing, the ovarian cortex fragments were transferred to a solution of 10 ml Dulbecco's Phosphate Buffered Saline (DPBS; Gibco), 0.75 M sucrose (MP Biomedicals, Eschwege, Germany), 10% serum substitute supplement (SSS; Irvine Scientific, Santa Ana, USA), and 0.1 mg/ml Pen/Strep (Lonza, Basel, Switzerland) and incubated under

continuous agitation for15 min at room temperature. After this first incubation step, 50 ml of a solution consisting of DPBS with 10% SSS and 0.1 mg/ml Pen/Strep was added using a perfusion device set at 100ml/h. At complete infusion (30 min), the cortex fragments were transferred into a 5 ml pre-warmed (37°C) Hepes buffered medium (Gamete, Cook Medical Europe LTD, Limerick, Ireland) and incubated for another 15 minutes. After repeating this step once, the tissue was washed three times for 5 min in tissue culture medium used for the glucose uptake assay (described below).

Outcome measures

For the fresh control as well as the four study arms, the overall ovarian tissue viability, follicle viability and tissue morphology were examined using the following methods.

Glucose uptake

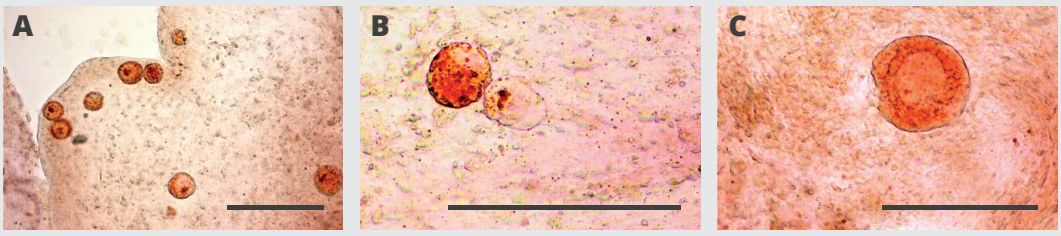
The ovarian tissue's overall viability was assessed using a glucose uptake assay modified from Gerritse et al.[25] This assay quantitatively measures the glucose uptake by ovarian tissue during in vitro culture. In addition to prior experiments with bovine tissue,[25] we first confirmed that the glucose uptake differed with the extent of ovarian tissue damage in the human situation. This pilot experiment showed that the glucose uptake of cryopreserved/thawed ovarian tissue varies with the extent of damage inflicted. No glucose uptake was found after snap-freezing ovarian tissue without a cryoprotectant, whereas a reduced uptake (nmol /mg tissue / hour of culture) of 2.9 nmol and 15.8 nmol glucose were found after exposure to room temperature for one night or to 50ºC during 20 minutes, respectively. When no damaging interventions were performed, the tissue took up 16.9 nmol glucose / mg / hour.

For each glucose uptake assay performed, four cortex biopsies were prepared using a 3-mm diameter biopsy punch (pfm medical ag, Cologne, Germany). Each ovarian biopsy was cultured separately in 2 ml Dulbecco's Modified Eagle Medium High Glucose (4.5 g/L) with L-Glutamine (DMEM, PAA Laboratories) supplemented with10% Fetal Bovine Serum (FBS; PAA Laboratories GmbH, Cölbe, Germany) and 0.1% Pen/Strep (GIBCO; 10.000 units penicillin/ml and 10.000 µg streptomycin/ml) in a 24-well plate (TPP, Trasadingen, Switzerland) at 37°C in humidified air with 5% CO_2. Conditioned culture medium was collected and replaced by fresh medium at day 4 and collected at day 7, when the culture was ended. After culture, biopsies were weighed and the glucose content in the medium of day 0 (control), and in the conditioned medium of day 4 and 7 were measured using an Architect i2000 system (Abbott Diagnostics, IL, USA). The glucose uptake of each biopsy during the culture period from day 0-4 and day 4-7 was corrected for its weight (mg) and the duration of the culture period.[25] Then, for each series of four biopsies cultured under the same conditions, the mean glucose uptake/mg/hour (nmol) was calculated for the culture period from day 0-4 and 4-7.

Percentage of living follicles

The viability of ovarian follicles was assessed using a Neutral Red (NR) assay modified from Kristensen et al.[26] With this metabolic assay, based on the ability of living cells to incorporate and bind neutral red in their lysosomes,[27] living follicles were stained red whereas dead follicles remained transparent (Figure I). Multiple fragments (<1 mm3) were cut from various parts of the cortex and incubated in 5 mL Ultraculture (Lonza) supplemented with 1 mg/mL Collagenase type IA (Sigma-Aldrich, Steinheim, Germany) at 37°C for 1h to soften and partially dissolve the tissue. Subsequently, the suspension was centrifuged at 400xg for 5 min after which the tissue pellet was resuspended in NR solution, consisting of 4.8 ml McCoy medium (Gibo, Life Technologies, New York, USA), 75 µL Neutral Red (Sigma-Aldrich), 2 µL Albuman (200g/l; Sanquin, Amsterdam, the Netherlands), 50 µL Insulin-Transferrin-Selenium (ITS-G, Gibco), 200 units penicillin (Gibco), and 200 µg streptomycin (Gibco). The NR suspension containing the ovarian tissue was incubated for 90 minutes at 37°C in humidified air with 5% CO_2. Next, the partly dissolved tissue fragments were removed from the bottom of the tube and placed on a glass slide. By gently pressing a cover slip on the softened tissue fragments a squash preparation was obtained. Living (stained red) as well as dead (non-stained) follicles were counted in this preparation using light microscopy (Figure I). The percentage of living follicles was documented for each condition for which a NR viability assay was performed, provided that at least 20 follicles could be analysed. Results obtained from samples with less than 20 follicles were considered unreliable and therefore excluded from further analysis.

Figure I: Follicles visualised by Neutral Red staining



(A): Overview of living follicles of various sizes as visualised with the Neutral Red Viability Stain. Due to the thickness of the squash preparation, not all follicles are in focus.

(B): Fresh tissue with a viable follicle (red) and dead follicle (transparent).

(C): Close-up of a living follicle.

Bars represent 200 µM.

Tissue morphology

For histology, a fragment of approximately 5x5 mm was fixed in Bouin's solution (Sigma-Aldrich), and subsequently embedded in paraffin wax. Histological examination of 8 µm haematoxylin and eosin stained sections was performed using light microscopy. For each fragment, three sections, separated by 100 µm tissue, were evaluated. Follicles were scored as morphologically normal or degenerated (in case of cytoplasm shrinkage, disorganised granulosa cells, pyknotic nuclei) and categorised as primordial, primary, secondary or antral according to predefined criteria.[28] We focussed at the percentage of degenerated/dead follicles rather than the total number of follicles observed per study arm, as the density of follicles in various parts of the cortex of the same ovary may vary considerably.[29]

Statistical methods

At first it needs to noted that negative values of glucose uptake were considered to be the result of measurement error inherent to measuring glucose uptake as such. Therefore these values were not treated differently from any other value of glucose uptake in the following analyses.

A linear mixed model for repeated measurements was used to study differences between protocols used for cryopreservation and for thawing on each of variables of the viability of human ovarian tissue, separately. Note that, this way, it is possible to find small within-subject differences relative to a large between subject (biological) variation. The dependent variables were the glucose uptake and the percentage of living follicles, respectively. The independent categorical variables were cryopreservation (two protocols: A_C, B_C), thawing (two protocols: A_T, B_T) and the time point of measurement (two levels: day 0-4, day 4-7).The intercept of each patient was treated as a random variable, in order to allow different levels for different patients. Initially all interaction terms between the categorical variables were included in the linear part of the model. However these terms were omitted from the final model presented, as these did not statistically significant improve the fit to the data (Likelihood-Ratio test). The estimated mean differences between the levels of each variable with the appropriate 95% confidence interval are presented. Statistical analyses were performed by using SAS® version 9.2 for Windows (SAS institute Inc. Cary, NC, USA). $P < 0.05$ was considered statistically significant.

Results

Patient characteristics

A total of 21 women (median age: 40 years; range 32-45) participated in this study. Of them, 20 were healthy BRCA 1 or 2 mutation carriers who did not receive prior gonadotoxic

therapy, whereas one woman had her ovaries removed because of breast cancer after chemotherapy (TAC). Ten women used contraceptives (oral: n=5; intrauterine device: n=4; NuvaRing: n=1), ten had regular menstrual cycles, and one had had a hysterectomy in the past. This last participant was considered premenopausal as she had a normal level of anti-müllerian hormone (AMH 3.2 µg/L) without menopausal symptoms. For 4 women, only part of the study arms could be evaluated, because of a small ovary (n=2), the presence of large follicles (n=1), or cauterization damage (n=1).

Glucose uptake

In table I, the observed median glucose uptake per milligram ovarian tissue per hour of culture is shown for all series of four biopsies cultured. In addition, data on biopsy weights and their culture durations (day 0-4) are presented. For day 4-7, similar results were obtained for the culture duration, albeit one day shorter (fresh: median: 71.8 hours, range: 66.5 - 94.3; thawing protocol A_T: 71.3; 66.3 - 72.0 hours; thawing protocol B_T: 72.0; 66.5 - 73.0 hours). As expected, the highest glucose uptake was found for fresh tissue (Table I). Furthermore, tissue cryopreserved according to protocol B_C had a higher glucose uptake when compared to A_C. In Table II, the estimated differences of the glucose uptake between the cryopreservation and thawing protocols – as obtained by a linear mixed model for repeated measurements – are presented. According to this model, tissue that is cryopreserved using protocol B_C is estimated to have a significantly higher glucose uptake (6.8 nmol/ mg / h higher) than tissue cryopreserved using protocol A_C (Table II; Figure IIa). A small difference in glucose uptake (1.7 nmol / mg / h) was found in favour of the shorter thawing protocol A_T when compared to B_T, but this difference did not reach statistical significance (p = 0.051; Table II).

Percentage of living follicles

A mean number of 80 follicles (SD 49) were evaluated in each ovarian tissue sample using the Neutral Red viability assay after having excluded samples with <20 follicles (n=21 out of n=98). Similar numbers of follicles could be counted before and after excluding the samples with <20 follicles for each of the study arms (data not shown). In Table I, the observed percentages of living follicles obtained with the Neutral Red viability assay are presented. In accordance with the results regarding the tissue's glucose uptake, tissue that was cryopreserved according to protocol B_C showed a statistically significant higher percentage of living follicles when compared to tissue cryopreserved according to protocol A_C (Table II; Figure IIb). In contrast to the superiority of protocol B_C with respect to cryopreservation, no significant difference between thawing protocols A_T and B_T with respect to the percentage of living follicles was observed. (Table II).

Table I. The observed median (range) of the glucose uptake and the percentage of living follicles for each combination of cryopreservation protocol and thawing protocol

Protocol			Glucose uptake (nmol / mg / h)									Living follicles (%)	
			Culture conditions				Glucose uptake						
Cryopre-servation	Thawing	N	Biopsy weight (mg) Median (range)		Duration, day 0-4 (hours) Median (range)		Culture days 0-4 Median (range)		Culture days 4-7 Median (range)		N	Median (range)	
Fresh	Fresh	21	5.5	(1.9 - 12.1)	96.0	(91.0 – 100.0)	23.5	(8.3 - 32.7)	20.3	(8.8 - 28.3)	11	94.5	(89.1 - 98.3)
A_C	A_T	18	5.8	(1.5 – 11.6)	98.0	(94.5 – 101.0)	8.7	(-0.5 - 29.5)	8.1	(1.0 - 26.8)	16	72.5	(26.3 - 95.4)
A_C	B_T	19	5.8	(1.3 – 12.9)	96.0	(91.0 – 101.8)	7.1	(-0.5 - 25.3)	6.5	(-5.5 - 29.0)	15	76.6	(20.0 - 95.7)
B_C	A_T	20	5.4	(1.5 - 11.5)	98.0	(94.5 – 101.0)	17.3	(0.8 - 31.3)	18.2	(0.5 - 30.9)	17	87.5	(65.5 - 96.1)
B_C	B_T	20	5.1	(1.8 - 14.8)	96.0	(91.0 – 101.8)	16.3	(1.9 - 24.1)	16.8	(3.2 - 23.9)	14	88.7	(18.2 - 100.0)

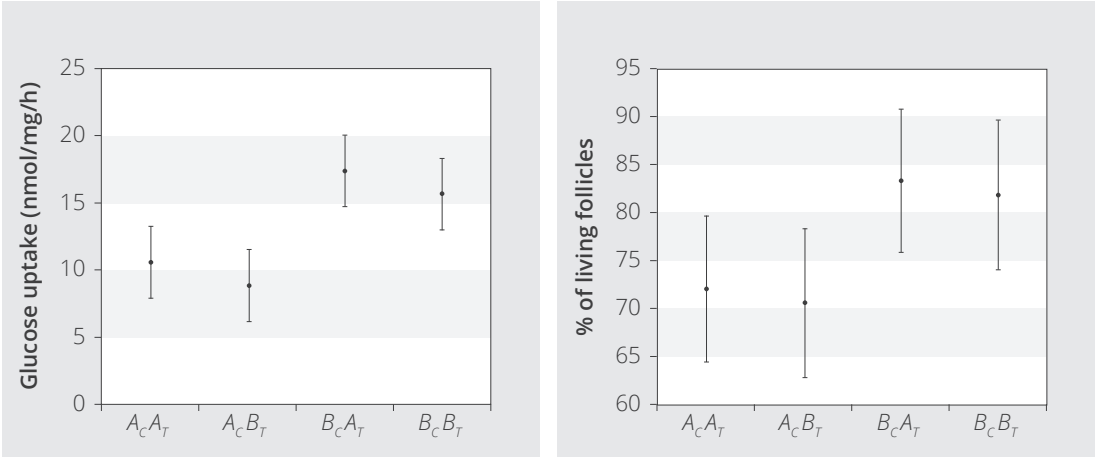
The glucose uptake is expressed per milligram of ovarian cortex per hour of culture. The percentage of living follicles results from the Neutral Red viability assays in preparations where at least 20 follicles could be counted. A_C = Cryopreservation protocol A; A_T = Thawing protocol A; B_C = Cryopreservation protocol B; B_T = Thawing protocol B. N = number of patients.

Table II. The estimated mean difference (95% confidence interval (CI)) of the glucose uptake and the percentage of living follicles between the cryopreservation protocols, thawing protocols, and culture periods, using a linear mixed model for repeated measurements

	Protocol / Day	Glucose uptake (nmol / mg / h)			Living follicles (%)		
		Estimated difference			Estimated difference		
		mean (95% CI)		*p*-value	mean (95% CI)		*p*-value
Cryopreservation	A_C	0.0	(reference)		0.0	(reference)	
	B_C	6.8	(5.0; 8.6)	<0.001	11.3	(4.6; 18.0)	0.002
Thawing	A_T	0.0	(reference)		0.0	(reference)	
	B_T	-1.7	(-3.5; 0.0)	0.051	-1.5	(-8.1; 5.1)	0.651
Culture period	Day 0-4	0.2	(-1.6; 1.9)	0.842	not measured		
	Day 4-7	0.0	(reference)				

A_C = Cryopreservation protocol A; A_T = Thawing protocol A; B_C = Cryopreservation protocol B; B_T = Thawing protocol B.

Figure II. The estimated mean (95% confidence interval) glucose uptake (nmol / mg / h; figure a) and percentage of living follicles (figure b) for the four study arms.



A_C = Cryopreservation protocol A; A_T = Thawing protocol A; B_C = Cryopreservation protocol B; B_T = Thawing protocol B.

Correlation of the percentage of living follicles and glucose uptake

Although the cryopreservation methods and thawing methods had similar results on both the glucose uptake by the ovarian tissue at day 0-4 and the percentage of living follicles, the correlation between these outcomes was poor in all four study arms (Spearman correlation <0.3, *p*-values 0.33–0.99). Similar results were found regarding the correlation between the glucose uptake during day 4-7 and the percentage of viable follicles.

Tissue morphology

Histological examination of the haematoxylin and eosin stained sections from fresh tissue revealed that 99% of the follicles were morphologically normal. After cryopreservation/thawing according to the four study arms, 89% (A_CA_T), 85% (A_CB_T), 93% (B_CA_T) and 84% (B_CB_T) of the follicles were morphologically normal, whereas the remaining follicles showed cytoplasm shrinkage, disorganised granulosa cells or pyknotic nuclei as a sign of follicle degeneration. The majority of the total of 2039 follicles observed were in the primordial or primary stages. For each of the four study arms as well as the fresh tissue, 2-3% of the follicles were in their secondary or antral stages.

Discussion

The current study revealed significant differences between the effects of two protocols for human ovarian tissue cryopreservation. The different effects of the two cryopreservation protocols (A_C and B_C) cannot be explained by the freezing procedure of the programmable temperature controller, as the same equipment and program was used for both A_C and B_C. The aspects at which protocol A_C differed from protocol B_C were the incubation time in the cryoprotective solution prior to transfer to the CryoLogic programmable temperature controller (30 minutes in protocol A_C versus a few minutes in protocol B_C) and the type of cryoprotectant used (1.5 mol/L ethylene glycol / 0.1 mol/L sucrose in protocol A_C versus 1.4 mol/L DMSO in protocol B_C). Previous studies compared DMSO and ethylene glycol – with or without adding sucrose – in slow freezing protocols for the cryopreservation of animal or human ovarian tissue or isolated follicles. In accordance with the results of the current study, a better follicle ultrastructure and morphology and a higher percentage of living follicles were found after cryopreservation using DMSO when compared to ethylene glycol for human, sheep or goat ovarian tissue.[8,10,30,31] However, DMSO did not improve the preservation of the follicle survival and ultrastructure when compared to ethylene glycol in bovine or agouti tissue.[9,32]

The two thawing procedures investigated in this study had major differences with respect to the solutions used as well as the length of the thawing process. This makes it difficult to separately consider the effects of the factor time and of various components used in the thawing solution on the tissue's viability. The exact reason for the effects on tissue viability notwithstanding, it is clear that protocol A_T was more time effective and required less human effort and material than protocol B_T. As a slightly higher tissue's glucose uptake was observed for the shortest and simplest thawing protocol (A_T), this protocol can be considered superior to protocol B_T for clinical practice.

In contrast to most studies concerning cryopreservation, we did not only evaluate follicle viability and structure, but also assessed the viability of the entire ovarian tissue by measuring the tissue's glucose uptake. As stromal cells contribute most to the cellular volume of the ovarian cortex, results from the glucose uptake assay will predominantly reflect the viability of this compartment. Stromal cells are essential for the neovascularisation of an ovarian graft after autotransplantation[33] and thus for follicle growth and maturation after transplantation. Prior studies – and presumably also the results of the current study regarding the effects of the two thawing protocols – have suggested that stromal cells may be more vulnerable to external damaging factors than follicles.[19,34] Given the importance of the stromal compartment in the function of the ovarian graft, thinking beyond follicle viability is vital when evaluating cryopreservation and thawing methods.

To the best of our knowledge, the current study is the first to assess the separate effects of two cryopreservation and thawing protocols on the viability of human ovarian tissue in a series of more than 20 patients. As BRCA mutation carriers donated their tissue for this study, the effects of various cryopreservation and thawing protocols should be further evaluated in a population of young patients applying for fertility preservation. Namely, the distribution of follicles, stromal cells, and collagen in the ovarian cortex alters as a result of ageing[35] and the BRCA-1 mutation has been associated with a diminished ovarian reserve.[36] Furthermore, cryopreservation and thawing protocols might be modified or optimised over time, as was described for the cryopreservation protocol using ethylene glycol investigated here by adding human serum albumin.[21] The impact of this change in protocol was not evaluated by the team using the protocol in clinical practice and it is therefore unknown to what extent this modification would have changed the results of our current study. Lastly, it remains unknown from our data to what extent the impairment of the ovarian tissue's viability observed in this study influences pregnancy rates after autotransplantation.

Several implications for clinical practice and future research can be derived from the findings of the current study. First of all, our findings suggest that it is important to evaluate clinically used cryopreservation and thawing methods in order to optimise the outcomes of the technique and presumably also pregnancy chances. The clinical use of cryopreservation and thawing protocols should be based on evidence resulting from laboratory research as long as pregnancy chances for the various protocols cannot

be given. As stromal cells may be more vulnerable to external damaging factors than follicles while being essential for the ovarian graft's function, future studies should also focus on the viability of this cell type.

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PART C

Patient-centeredness, timeliness, and equitability

7

Referral for fertility preservation counselling in female cancer patients

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Abstract

Study question: What changes can be detected in fertility preservation (FP) counselling (FPC) over time and what are the determinants associated with the referral of newly diagnosed female cancer patients, aged 0 - 39 years, to a specialist in reproductive medicine for FPC?

Summary answer: Although the absolute number of patients receiving FPC increased over time, only 9.8% of all potential patients (aged 0-39 years) were referred in 2011 and referral disparities were found with respect to patients' age, cancer diagnosis, and healthcare provider-related factors.

What is known already: Referral rates for FPC prior to the start of gonadotoxic cancer treatment are low. Determinants associated with low referral and referral disparities have been identified in previous studies, although there are only scarce data on referral practices and determinants for FPC referral in settings with reimbursement of FP(C).

Study design, size, duration: We conducted a retrospective observational and questionnaire study in a Dutch university hospital. Data on all female cancer patients counselled for FP in this centre (2001-2013), as well as all newly diagnosed female cancer patients aged 0-39 years in the region (2009-2011) were collected.

Participants/materials, setting, methods: Data were retrieved from medical records (FPC patients), cancer incidences reported by the Dutch Cancer Registry (to calculate referral percentages), and referring professionals (to identify reasons for the current referral behaviour).

Main results and the role of chance: In 2011, a total of 9.8% of the patients were referred for FPC. Patients aged 20 – 29 years or diagnosed with breast cancer or lymphoma were referred more frequently compared to patients under the age of 20 years or patients diagnosed with other malignancies. The absolute numbers of patients receiving FPC increased over time. Healthcare provider related determinants for low referral were: not starting a discussion about fertility related issues, not knowing where to refer a patient for FPC, and not collaborating with patients' associations.

Limitations, reasons for caution: Actual referral rates may slightly differ from our estimation as there may have been patients who did not wish to receive FPC. Sporadically, patients might have been directly referred to other regions or may have received ovarian transposition without FPC. By excluding skin cancer patients, we will have underestimated the group of women who are eligible for FPC as this group also includes melanoma patients who might have received gonadotoxic therapy.

Wider implications of the findings: The low referral rates and referral disparities reported in the current study indicate that there are opportunities to improve referral practices. Future research should focus on the implementation and evaluation of interventions to improve referral practices, such as information materials for patients at oncology departments, discussion prompts, or methods to increase the awareness of physicians and patients of FP techniques and guidelines.

Introduction

Current clinical guidelines recommend oncological healthcare providers to refer girls and young women who are newly diagnosed with cancer to a specialist in reproductive medicine.[1-3] Young cancer patients undergoing gonadotoxic treatment may wish to receive information about fertility preservation (FP) from such a specialist, regardless of diagnosis or prognosis.[4-6] However, despite patients' preferences, guideline recommendations and a rapid evolution of FP techniques in the past years,[7-9] the proportion of patients who are being referred for FP counselling (FPC) still remains low (1.0 – 20.6%).[10-13]

Although not consulting a FP specialist could very well be a conscious, well-informed choice for some young cancer patients, others may not have had the chance to consider FPC. Indeed, it is well-known that care organisational factors contribute to the current low referral rates, as well as the oncological healthcare provider's gender and his or her knowledge, attitude, and perceptions regarding FP.[14-18] Disparities in referral patterns and access to FP have also been observed with respect to patients' demographic, clinical, and socioeconomic characteristics.[11,19] Nevertheless, FPC seems to be of high importance, as concerns about infertility and unmet informational needs can have a severe psychological impact later in life.[20-22]

Financial aspects play a key role in patients' FP decision-making[23,24] as well as physicians' decisions to refer a patient for FPC or not.[11,14,16] Since most studies on FP referral are performed in the USA, where not all patients receive reimbursement from health insurance, referral practices presented in these studies are probably not generalisable for countries with full FP reimbursement. The few European studies that discussed FPC referral practices did not concern the entire female population eligible for FP or did not provide information about the ages or diagnoses of patients who did not receive FPC.[12,14,25]

In the current study, we aimed to describe changes in FPC referral patterns over time as well as current referral practices in a demarcated region in the Netherlands, where all legal citizens are obliged to have health insurance covering FP(C). As a second aim, we studied current referral practices, including referral rates and patient and healthcare provider related determinants associated with FPC referral. Indeed, being informed about the current referral practices, possible disparities, and reasons underlying the current referral practices, is a first step towards the design of interventions aiming at improving referral practices.

Methods

Study design

The current retrospective observational and questionnaire study was conducted in the

region of the Radboud university medical center (Rumc), Nijmegen, the Netherlands – and consisted of two parts:

» Part 1: 'FPC and the introduction of new techniques': the characteristics of all female cancer patients who received FPC, their referring professionals, FP counsellors, and FP choices in the Rumc between 2001 and 2013 were analysed and related to the time points at which new FP techniques were introduced.
» Part 2: 'Current state of referral': Actual referral rates were calculated for newly diagnosed cancer patients of various ages and with various oncological diseases. After a questionnaire study, characteristics of referring professionals, their reported knowledge, attitude and perceptions towards FP were related to their referral behaviour.

Ethical approval

In the Netherlands, approval for this study by an ethics committee was not required.[26]

Setting

FPC in the Netherlands

In the Netherlands, FPC for female cancer patients is performed by gynaecologists specialised in reproductive medicine who are working at Dutch university hospitals (eight in total) or (in a minority of cases) by a gynaecologist with a special interest in FP, working at a non-university hospital. Patients receive multidisciplinary oncological care and can be referred by any medical specialist involved for specialised FPC and FP care. Dutch breast cancer patients are surgically treated by surgeons and therefore they also need referral to a gynaecologist for information about FP. To which hospital a patient should be referred for FPC is not predefined, but depends on travel distance, FP options available, and the oncological healthcare provider's and patient's personal preference. All legal citizens of the Netherlands are obliged to have a health insurance policy covering FPC and FP, meaning that patients had no financial reasons to refrain from it.

Content of FPC at the Rumc

At the Rumc, during FPC, gynaecologists specialised in reproductive medicine informed each patient about her estimated risk of future infertility and the FP options applicable in her individual situation. For each FP option, the procedure, current experience, pregnancy chances, adverse effects, risks, and ethical questions were addressed. In those cases where the patient had an intermediate or high risk of premature ovarian insufficiency (>20%) and where FP options could be offered, patients could choose their preferred FP technique, or to refrain from FP. Cryopreservation of ovarian tissue was only offered to patients with a risk of premature ovarian insufficiency of at least 50%. Cryopreservation

of embryos after IVF or ICSI as well as laparoscopic ovarian transposition could be offered at the Rumc since before 2001. Cryopreservation of ovarian tissue and vitrification of oocytes became available in the Netherlands in 2005 and 2007 respectively, whereas these techniques could be offered in the Rumc from December 2009 and June 2011, respectively. In the intervening years, patients could be referred to other hospitals if they wished to receive ovarian tissue cryopreservation or vitrification of oocytes.

Communication and education about FP in the Rumc region

To outreach and educate the referring professionals, various courses and presentations on FP were organised in our region and country in the most recent years. Those courses and presentations considered all options for female FP and were not directed towards healthcare providers of patients with a certain oncological diagnosis or age. Educational sessions were accessible for all interested physicians and nurses in the community and not directed towards certain centres. Before 2010, FP less often was a topic of education and information on FP was generally provided individually to oncological healthcare providers after specific questions or based on specific patient cases. Throughout the years, there was continuous availability of gynaecologists specialised in reproductive medicine who could offer (telephonic) advise on FP to colleagues.

Data collection and analysis

Part 1: FPC and the introduction of new techniques

Demographic and medical data were retrospectively collected from the medical files of all female patients diagnosed with invasive cancer who ever consulted a FP specialist for FPC at the Rumc. This resulted in a study period ranging from January 2001 through December 2013. For each patient, information was obtained about her age, educational level, partner relationship status, parity, diagnosis, estimated risk of amenorrhea based on planned oncological treatment,[27] year of referral, and the profession and institute of her referring healthcare provider. In addition, the counsellor's experience with FPC, the FP options offered, and a patient's FP choice were documented. Experienced counsellors were defined as gynaecologists who had previously counselled more than 10 female patients.

To evaluate the changes in absolute numbers of FPC and the characteristics of patients referred for FPC over time, we described patients' characteristics for three relevant time periods based on the FP techniques available. These time periods were 2001 - November 2009 (cryopreservation of embryos and ovarian transposition available as FP options), December 2009 - May 2011 (addition of cryopreservation of ovarian tissue to the available FP options), and June 2011 - 2013 (addition of vitrification of oocytes to the available FP options). All data were analysed with IBM SPSS statistics version 20.0 for Windows, 2011, Armonk, NY, USA, using descriptive statistics.

Part 2: Current state of referral

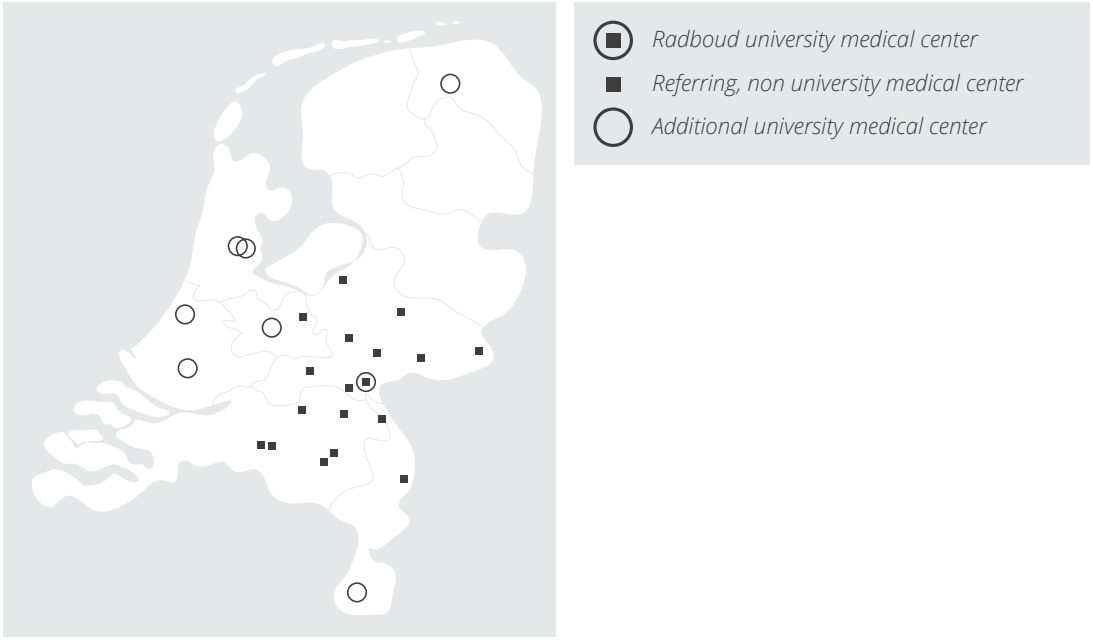
Referral rates and disparities with respect to patients' age and diagnosis
Based on epidemiological data, actual referral rates for FPC as well as patient-related determinants for FPC were identified. To obtain information about patients who were not referred for FPC, epidemiological data on the incidence of invasive cancer in hospitals in the Rumc region was obtained from the Dutch Cancer Registry for the three most recent years for which the Cancer Registry completed her data, i.e. 2009, 2010, and 2011. Data on skin cancer were excluded, as in this relatively large patient group, the majority of patients was thought not to have an indication for gonadotoxic therapy. Based on past referrals, the Rumc region was defined as a region with eighteen hospitals having a driving distance of up to 90 km from the Rumc (Figure I). Five gynaecologists with a known special interest in FP who worked in the Rumc region but not at the Rumc itself, were asked for the number of patients they counselled in 2009, 2010 and 2011 as well as their patients' diagnoses and ages. Girls and women aged 0-39 years who were newly diagnosed with an invasive tumour and who received FPC were compared with those who did not receive FPC with respect to diagnosis and age using descriptive statistics.

Characteristics of healthcare providers and referral practices
To obtain information about which characteristics of healthcare providers are related to referral behaviour, we conducted an electronic questionnaire study among a selected group of healthcare providers. Based on the top three most frequently occurring groups of oncological diagnoses among young Dutch women aged 18-39 years (breast cancer; cancer of blood/bone marrow/lymph nodes; and gynaecological cancer), we selected healthcare providers involved in the care for these patients at the time of diagnosis, namely surgeons, specialised "mammacare" nurses, oncologists, specialised oncology nurses, haematologists, oncological gynaecologists and radiotherapists. A total of eight hospitals (including the Rumc) and one radiotherapeutic centre were included based on their location within a range of 35 km from the Rumc. The electronic questionnaire was partly based on a literature search and partly contained factors associated with multidisciplinary working and collegial contact. The questionnaire contained 6 multiple choice, 5 likert-scale and 3 open questions, as shown in table III. Two categories of healthcare provider related determinants for FPC referral were identified:

- 'characteristics of referring specialist': gender,[15,18] age,[18] profession,[10] years working in this profession, number of young cancer patients treated per year, involvement in scientific research about female cancer patients, involvement in patients' associations. The term patients' association refers to organisations aiming to act on behalf of patients as well as to inform about specific diseases or health problems. In addition, patients' associations try to collaborate with medical specialists to develop guidelines and information materials for patients.
- 'knowledge, attitude and perceptions towards FP': FP knowledge,[18] attitude towards FP,[15,18,] expected patients' interest in FP.[15,16]

In each hospital, the departments of the selected healthcare providers were contacted by telephone to identify all eligible professionals by name and to obtain their (personal) e-mail address. Questionnaires were distributed to each person's e-mail address using SurveyMonkey (Survey Monkey Inc, Palo Alto, CA, USA) in December 2013, one week after having sent a personal paper letter to all invited persons to announce our study. Invited healthcare providers received reminders after 2, 5 and 9 weeks. Descriptive statistics, Fisher's exact tests and independent samples Student t-tests were used to compare responders to non-responders and to explore characteristics of the referring professionals that might be the reason for (not) referring for FPC. Differences with a two-sided *p*-value of ≤ 0.05 were considered to be statistically significant.

Figure I. University medical centres and referring hospitals in Rumc region



Map of the Netherlands showing eight university medical centres including the Radboud university medical center and the Rumc region with eighteen referring non-university hospitals within a driving distance of 90 km.

Results

Part 1. FPC and the introduction of new techniques

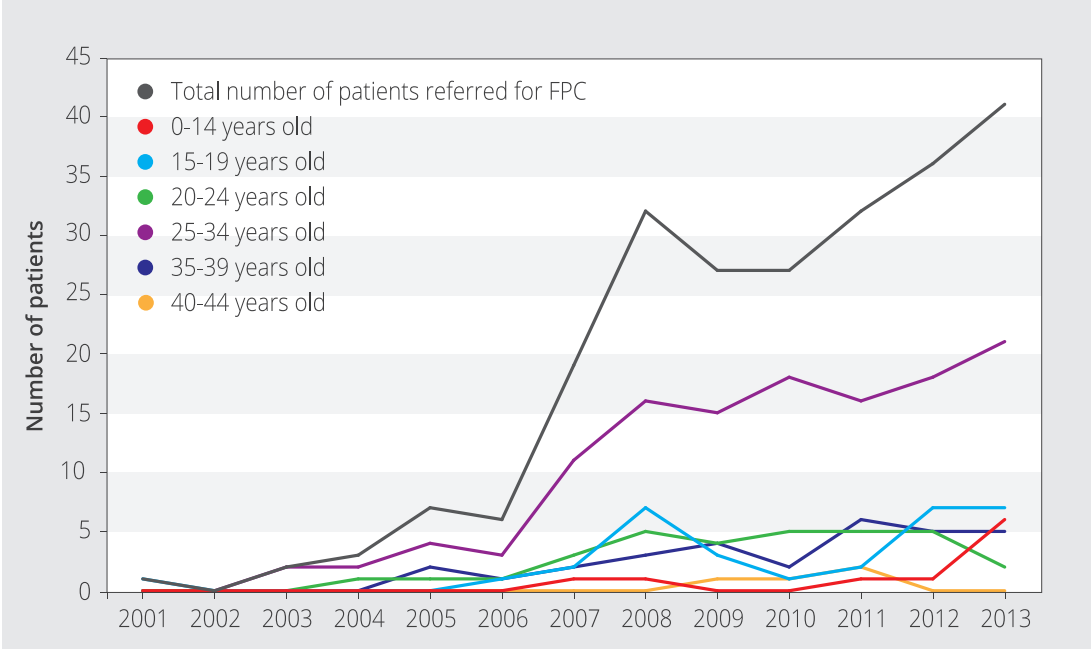
Referring specialists

A total of 233 patients received FPC at the Rumc between January 2001 and December 2013. Most patients were referred by an internist (46%), surgeon (27%), or oncological gynaecologist (14%). The remaining patients were referred by a paediatrician (6%), another medical specialist (5%), or contacted the reproductive gynaecologist on their own initiative (2%) Of all patients referred, 231 were from the Rumc's region. Large differences were found in the numbers of patients referred per hospital, with 175 of the 231 (75.8%) patients coming from four of the eighteen hospitals. These four hospitals included the Rumc and three referring hospitals with a central location in the region (number of hospital beds 380-850). [28-30] All 233 patients were referred by 131 unique healthcare providers, of whom a majority (n= 106; 80.9%) referred only one or two patients. Six medical specialist (4.6%) referred five patients or more with a maximum of seven patients.

Numbers of consultations and FP options offered

The annual number of consultations for FPC increased during our study period, with a sharp increase in 2006 and a renewed increase in the most recent years (2010-2013; Figure II). The increase observed since 2006 could mainly be attributed to a higher number of patients aged 24-34 years old being counselled, whereas since 2010 an increased annual number of FPCs was especially observed for patients aged 0-19 years and 24-34 years (Figure II). Patients were counselled by 12 gynaecologists of whom four were experienced counsellors (n>10 patients counselled previously), informing 88.0% of all patients. FP was offered to 181 patients (77.7%). In the last time period (since June 2011) in which all FP techniques were available, FP could be offered to an increasing proportion of the patients counselled.

Figure II. Patients referred for fertility preservation counselling (FPC) between the years 2001 and 2013



Characteristics of patients receiving FPC

In table I, the characteristics of all patients receiving FPC are provided for the three relevant time periods. At the time we conducted our study, 26 of these patients were deceased (median follow up from date of FPC to date of death 22.0 months; 95% Confidence Interval (CI) 5.8-38.2). It is remarkable that most patients were counselled because of breast cancer, lymphoma and gynaecological malignancies. Nevertheless, relatively more patients with other diagnoses received FPC from June 2011 through 2013 when compared to the years before. Moreover, patients counselled in the most recent period were younger and more frequently lacked a (stable) partner relationship than patients counselled before. For a significant number of patients (n=83) information on the exact cancer therapy was not yet available at the moment of FPC, in most cases because tumour staging was not yet completed. Despite this, our results illustrate that relatively more patients with a low risk of ovarian failure after cancer therapy were seen for FPC in recent years. Patients who were offered FP in the most recent time periods more frequently chose to proceed with a FP technique – especially the vitrification of oocytes – than patients counselled beforehand.

Table I. Female cancer patients who received fertility preservation counselling (FPC) by a specialist in reproductive medicine

	Total N=233	2001 – Nov 2009 N (%)	Dec 2009 – May 2011 N (%)	June 2011 - 2013 N (%)
Patients counselled	233	94	41	98
Mean age at counselling in years (SD)	27.1 (7.3)	27.5 (6.5)	28.7 (5.7)	26.1 (8.5)
Educational level (N=24 missing)				
Primary level/lower vocational education	35	9/86 (10.5)	9/37 (24.3)	17/86 (19.8)
Secondary school/higher vocational education	76	32/86 (37.2)	12/37 (32.4)	32/86 (37.2)
Higher education/university	98	45/86 (52.3)	16/37 (43.3)	37/86 (43.0)
Diagnosis				
Breast cancer	112	49/94 (52.1)	20/41 (48.8)	43/98 (43.9)
Leukaemia	16	8/94 (8.5)	4/41 (9.7)	4/98 (4.1)
Lymphoma	51	23/94 (24.4)	7/41 ((17.1)	21/98 (21.4)
Gynaecological cancer	24	6/94 (6.4)	7/41 (17.1)	11/98 (11.2)
Gastro-intestinal	8	1/94 (1.1)	2/41 (4.9)	5/98 (5.1)
Bone/soft tissue cancer	13	5/94 (5.3)	1/41 (2.4)	7/98 (7.1)
Neurological cancer	5	1/94 (1.1)	0/41 -	4/98 (4.1)
Others	4	1/94 (1.1)	0/41 -	3/98 (3.1)
Stability relationship (N=21 missing)				
Partner, living together/married	121	60/89 (67.4)	20/36 (55.6)	41/87 (47.1)
Partner, living apart and unmarried	38	15/89 (16.9)	7/36 (19.4)	16/87 (18.4)
No relationship	53	14/89 (15.7)	9/36 (25.0)	30/87 (34.5)
Parity (N=1 missing)				
Nulliparous	184	75/94 (79.8)	30/40 (75.0)	79/98 (80.6)
Parous	48	19/94 (20.2)	10/40 (25.0)	19/98 (19.4)
Risk of amenorrhea based on planned therapy at time of counselling (N=83 missing)				
High risk (>80% risk of amenorrhea)	57	21/66 (31.8)	12/27 (44.5)	24/57 (42.1)
Intermediate risk (20-80% risk of amenorrhea)	36	19/66 (28.8)	7/27 (25.9)	10/57 (17.5)
Low risk (<20% risk of amenorrhea)	51	22/66 (33.3)	7/27 (25.9)	22/57 (38.6)
Very low/no risk of amenorrhea	1	1/66 (1.5)	0/27 -	0/57 -
Unknown risk of amenorrhea	5	3/66 (4.6)	1/27 (3.7)	1/57 (1.8)
Desire to have children in the future (N=61 missing)				
Yes	141	62/68 (91.2)	22/27 (81.5)	57/77 (74.0)
No/not yet	31	6/68 (8.8)	5/27 (18.5)	20/77 (26.0)
FP offered				
Yes	181	73/94 (77.7)	30/41 (73.2)	78/98 (79.6)
No	52	21/94 (22.3)	11/41 (26.8)	20/98 (20.4)
Number of FP options offered				
1	105	40/73 (54.8)	13/30 (43.3)	52/78 (66.7)
2	51	22/73 (30.1)	12/30 (40.0)	17/78 (21.8)
3	25	11/73 (15.1)	5/30 (16.7)	9/78 (11.5)

Which FP options offered							
Ovarian transposition	18	5/117	(4.3)	4/52	(7.7)	9/113	(8.0)
Cryopreservation of embryo's	113	54/117	(46.1)	20/52	(38.5)	39/113	(34.5)
Cryopreservation of ovarian tissue	91	42/117	(35.9)	17/52	(32.7)	32/113	(28.3)
Vitrification of oocytes	60	16/117	(13.7)	11/52	(21.1)	33/113	(29.2)
FP used if offered							
Yes	107	33/73	(45.2)	17/30	(56.7)	57/78	(73.1)
No	75	40/73	(54.8)	13/30	(43.3)	21/78	(26.9)
FP option chosen							
Ovarian transposition	16	4[a]/34	(11.8)	4[b]/20	(20.0)	8[c]/60	(13.3)
Cryopreservation of embryo's	61	24[a]/34	(70.6)	11/20	(55.0)	26/60	(43.3)
Cryopreservation of ovarian tissue	17	5/34	(14.7)	5[b]/20	(25.0)	7[c]/60	(11.7)
Vitrification of oocytes	20	1/34	(2.9)	0/20	-	19/60	(31.7)

FP = Fertility preservation N=number.
From 2001 to November 2009, the FP options available were cryopreservation of embryo's after IVF/ICSI and laparoscopic ovarian transposition. In December 2009 cryopreservation of ovarian tissue was added to the available options followed by the vitrification of oocytes in June 2011.
a In this time period one patient chose for a combination of IVF and ovarian transposition
b In this time period three patients chose for a combination of cryopreservation of ovarian tissue and ovarian transposition.
c In this time period three patients chose for a combination of cryopreservation of ovarian tissue and ovarian transposition.

Part 2. Current state of referrals

Referral percentage

In the eighteen hospitals belonging to the Rumc's region, a total of 1169 women aged 0 – 39 years were diagnosed with invasive cancer in 2009, 2010 or 2011. In 2011, the total percentage of cancer patients referred for FPC was 9.8%. In table II, the percentages of patients referred with various diagnoses or age categories are presented. The five gynaecologists with a known special interest in FP who worked outside the Rumc indicated to have counselled a total of 18 patients in the years 2009 - 2011, although three out of five gynaecologists could only provide a number of patients counselled for FP and not their ages and additional characteristics.

Patient determinants associated with referral

Patients aged 20 – 29 years were referred most frequently (23.0% in 2011), while patients up to the age of 20 years were only scarcely referred (4.5% in 2011; table II). Referral disparities were also found with respect to diagnosis, with breast cancer or lymphoma patients being referred most frequently (Table II). It is remarkable that none of the patients with neurological cancer, cancer of the head or neck, lung, urinary tract, thyroid or adrenal gland, or eye were referred from 2009 through 2011.

Healthcare provider related determinants associated with referral

A total of 172 healthcare providers were found eligible for our electronic questionnaire study of whom 103 (59.9%) responded to our questionnaire. After comparing responders with non-responders, more women responded (66.0% versus 49.3% amongst the non-responders; $p = 0.039$). Differences were also found in profession with more than 75% of nurses responding, 50%-75% of haematologists, surgeons, oncologists and oncological gynaecologists responding and 36% of radiotherapists responding ($p = 0.001$).

Of the 103 respondents, 84 answered the question about how often they refer patients for FPC. As presented in table III, only those 84 responding healthcare providers were divided into two groups for comparison: the group who stated to often or always refer young female cancer patients for FPC and the group who stated to rarely or never refer. Healthcare providers less frequently reported referring for FPC if they were a radiotherapist or did not collaborate with patients' association(s). Other determinants predicting non-referral included restricted knowledge of the healthcare provider about where to refer patients for FPC, having patients who rarely or never ask about fertility related issues, and rarely or never initiating a discussion about fertility related issues on one's own initiative. Although just not reaching statistical significance, professionals who were working in their profession for a longer period tended to refer for FPC more often.

Table II. Proportion of newly diagnosed female cancer patients referred for fertility preservation counselling (FPC)

	2009-2011 FPC patients / total (%)		2009 FPC patients / total (%)		2010 FPC patients / total (%)		2011 FPC patients / total (%)	
Patients, N (%)	100 / 1169	(8.6)	29/91	(7.4)	32/380	(8.4)	39/398	(9.8)
Age in categories, N (%)*								
0-4 years	0/60	-	0/16	-	0/23	-	0/21	-
5-9 years	0/25	-	0/9	-	0/10	-	0/6	-
10-14 years	1/37	(2.7)	0/6	-	0/13	-	1/18	(5.6)
15-19 years	6/56	(10.7)	3/18	(16.7)	1/16	(6.3)	2/22	(9.1)
20-24 years	16/75	(21.3)	4/21	(19.0)	5/25	(20.0)	7/29	(24.1)
25-29 years	27/132	(20.5)	9/42	(21.4)	8/45	(17.8)	10/45	(22.2)
30-34 years	27/266	(10.2)	6/94	(6.4)	11/78	(14.1)	10/94	(10.6)
35-39 years	14/518	(2.7)	4/185	(2.2)	4/170	(2.4)	6/163	(3.7)
Diagnosis, N (%)*								
Breast cancer	47/469	(10.0)	13/171	(7.6)	15/142	(10.6)	19/156	(12.2)
Leukaemia	8/83	(9.6)	4/28	(14.3)	2/22	(9.1)	2/33	(6.1)
Lymphoma	19/128	(14.8)	6/37	(16.2)	5/38	(13.2)	8/53	(15.1)
Gynaecological cancer	10/147	(6.8)	2/51	(3.9)	5/47	(10.6)	3/49	(6.1)
Gastro-intestinal cancer	4/75	(5.3)	1/23	(4.3)	2/29	(6.9)	1/23	(4.3)
Bone/soft tissue cancer	3/42	(7.1)	0/9	-	0/19	-	3/14	(21.4)
Neurological cancer	0/66	-	0/23	-	0/28	-	0/15	-
Others**	0/159	-	0/49	-	0/55	-	0/55	-

Numbers of female patients who received FPC in the region of the Rumc, Nijmegen, the Netherlands, in the years 2009, 2010, or 2011 as a percentage of the total number of female patients (age 0-39 years) newly diagnosed with an invasive tumour in the same region and timeframe according to the Dutch Cancer Registry. FPC = Fertility preservation consultation with a specialist in reproductive medicine. N= number.

** Three of the five gynaecologist who performed FPC in other hospitals in the Rumc region could only provide the absolute number of patients counselled for FP in 2009, 2010 and 2011, but no information about age of diagnosis. Per year, data about age and diagnoses of three patients is missing.*

*** The group of patients with 'other' malignant diseases consisted of patients with cancer of the head or neck (n=23), lung (n=28), urinary tract (n=30), thyroid or adrenal gland (n=67), eye (n=4), and patients with a primary tumour of unknown origin (n=7).*

Table III. Determinants of FPC referral based on self-reported behaviour of healthcare providers

	Often/ Always refers patients for FPC (N=52)	Rarely/ Never refers patients for FPC (N=32)	*p*-value*
Gender			*p* = 1.000
Man	19	11	
Woman	33	21	
Age in years (mean, SD)			*p* = 0.552
Missing (N=1)	45.9 (9.5)	44.7 (7.4)	
Current profession			*p* = 0.009
Surgeon	10	3	
Nurse	14	9	
Radiotherapist	2	6	
Oncologist	15	5	
Haematologist	8	1	
Oncological Gynaecologist	3	8	
Years working in this profession (mean, SD)	10.7 (8.3)	7.6 (5.8)	*p* = 0.050
Involved in clinical scientific research about female cancer patients			*p* = 0.498
Yes	22	17	
No	29	15	
Missing (N=1)			
Collaboration with patient association(s)			*p* = 0.007
Yes	33	10	
No	19	22	
Number of patients aged 18-41 years treated in 2013 (mean, SD)			*p* = 0.889
Missing (N=8)	16.7 (14.7)	16.1 (21.0)	
Do patients ask about fertility issues?			*p* = 0.001
Yes, often/always	42	15	
No, rarely/never	10	17	
Do you bring up fertility related issues yourself?			*p* = 0.004
Yes, often/always	51	25	
No, rarely/never	1	7	
Do you know where to refer patients for FPC?			*p* = 0.019
Yes	52	28	
No	0	3	
I don't refer patients myself	0	1	
I think FPC and FP is an important part of the patients' treatment for cancer			*p* = 0.551
Yes, agree/totally agree	51	28	
No, disagree/totally disagree	1	2	
Missing (N=2)			

I think my knowledge about the FP options is good			$p = 0.640$
Yes, agree/totally agree	34	18	
No, disagree/totally disagree	18	13	
Missing (N=1)			
I think the currently available FP options are good			$p = 0.215$**
Yes, agree/totally agree	40	18	
No, disagree/totally disagree	3	4	
I don't know what FP options are currently being offered	9	9	
Missing (N=1)			
I think the current success rates of the different FP methods are good			$p = 0.727$**
Yes, agree/totally agree	9	4	
No, disagree/totally disagree	14	9	
I don't know what the current success rates are	29	18	
Missing (N=1)			

**p-value resulting from comparison of the two groups with Fisher's exact texts and independent samples Student t-tests.*
***Only the responders who had an opinion about this subject ("Yes, agree/Yes, totally agree/No, disagree/ No, totally disagree") were selected for the analysis.*

Discussion

The current study revealed that – despite the fact that patients in our study had no financial reasons to refrain from FP – only 9.8% of the female cancer patients aged 0-39 years were referred to a specialist in reproductive medicine in 2011. In accordance with earlier research,[11] referral disparities were identified with respect to diagnosis and patients' age. In the same country as we conducted our study, the Netherlands, poor referral rates (2%) were suggested previously for adult female cancer patients diagnosed in the years 2002 to 2007.[25] Nevertheless, this percentage was not adjusted for the fact that FPC was also performed in various other hospitals in the country and may therefore very well be an underestimation of the actual referral rate. With regard to the referral of children and teenagers, referral rates in our population (4.5% for patients aged 0-19 years) were higher than the percentage of girls referred in a study from the UK (1%), although this study was conducted over 8 years ago.[12] Possibly as a result of the reimbursement of FP in our country, we found a high uptake of FP services in the most recent years (58% of all patients who received FPC) when compared to an American study in which 36.5% of the patients proceeded with FP after FPC in 2009 and 2010.[31] Apart from the differences in the reimbursement of FP, these high uptake rates of FP techniques combined with the very low referral rates for FPC in our country with reimbursement might also illustrate that the patients who actually consult an FP specialist are already part of a very selected and interested population.

Several hypotheses can be put forward when thinking about the causes of the poor

referral rates observed. Referral rates may be low because some of the patients who were newly diagnosed with cancer might not have had an indication or wish for FPC.[10] Although we excluded skin cancer patients, a minority of the other patients may not have needed gonadotoxic therapy or may have had a poor prognosis. With a mean age of 29.4 years of women giving birth to their first child in the Netherlands,[32] some of the patients may already have had a completed family. Indeed, patients older than 30 years were less frequently referred than adult patients below this age. It is remarkable that the majority of the healthcare providers questioned in this study reported that they 'often' or 'always' refer their patients for FPC, whereas the referral percentages obtained from the current study were below 10%. Presumably, deliberate decisions to refrain from referral for FPC as described above play a role in the discrepancy between these two observations.

Apart from these deliberate decisions to refrain from FPC, low referral may be caused by barriers for discussing FP with a patient and referring her. The results of the current study identified that initiating a discussion about fertility related issues, knowing where to refer a patient for FPC, and collaborating with patients' associations were the main healthcare provider related facilitators for adhering to guidelines recommending referral for FPC.[1-3] Although initiating the discussion about fertility was found to be a determinant associated with referral in our current study, professionals who frequently referred did not differ from those who did not frequently refer for FPC with respect to their self-reported knowledge, attitude, and perceptions towards FP. A lack of knowledge or other reasons for discomfort of oncological healthcare providers with bringing up the topic of FP[33] could hamper a comprehensive discussion about FP. The tendency towards the more frequent referral by experienced healthcare providers as observed in this study may reflect an effect from 'training on the job' with respect to discussing the topic of FP with patients. Furthermore, the fact that the number of FPC's at the Rumc increased after the publication of an important guideline on FP in 2006[34] and the establishment of new FP techniques (approximately 2010-2011) suggests that technical evolution of the field and awareness as a result of more information and guidelines becoming available contribute to oncologists' tendency to refer their patients for FPC. The key role of an increased awareness of the importance of FP is also illustrated by our finding that healthcare providers who are involved in patients' associations were found to refer for FPC more frequently.

One of the most important strengths of our study was the fact that we based our conclusions on epidemiological data combined with current practices, knowledge, and attitudes as self-reported by referring healthcare providers. Furthermore, we were the first to investigate the referral percentages for both paediatric and adult female cancer patients in a country where bias from financial reasons to refrain from FP(C) was excluded, in contrast to prior studies where financial factors influenced FPC referral and decision-making.[24,31,35] Although we have estimated the current referral rates as good as possible, it is theoretically possible that we missed a small number of patients who were

directly referred to another region of the country, or who received ovarian transposition without prior FPC by a specialist in reproductive medicine. As a second limitation, we were uninformed about other characteristics than age or diagnosis of those patients who did not have FPC, such as the level of education, the partner relationship, parity, and risk of infertility. For this reason, we could not obtain information about the extent to which there were referral disparities regarding these factors. Determinants related to the referring healthcare providers were self-reported and not actually tested (e.g. subjective knowledge about FP). Furthermore, responders and non-responders to our questionnaire may differ with respect to their attitude and knowledge about FP, which might have biased the results.

The low referral rates, referral disparities, and determinants associated with FPC referral practices presented in the current study indicate opportunities to improve referral practices and adherence to guidelines, even in a setting with reimbursement of FP(C).[1-3] To overcome physician-related determinants for low referral, interventions that would increase physicians' knowledge, skills and training with respect to FPC; increase awareness and positive attitudes regarding FP; and increase time available for discussing FP, have been considered helpful by other studies.[14,17,18,36] In accordance with this need for knowledge, skills and training, our study findings also suggest that training on how to discuss fertility issues would indeed contribute to higher referral rates. Training of oncological healthcare providers has already been suggested in various studies, with seminars about FP[10], psycho-education to facilitate the discussion[17] or joint training events with gynaecologists specialised in reproductive medicine to improve information exchange.[14] Various decision-aids on the topic of FP could be offered to patients to help them to make a well-informed decision.[37,38] For healthcare providers, the ability to call an expert for advise on FP at any time, such as initiated by the Oncofertility Consortium,[39] may facilitate the discussion of FP with a patient. Future research should focus on the implementation and evaluation of these and other interventions aiming at the improvement of referral practices, such as information materials regarding FP for patients at oncology departments, discussion prompts, or methods to increase the awareness of physicians about FP techniques and guidelines. Furthermore, special attention should be paid to referral for FPC in paediatric populations, and specific determinants associated with the referral of children for FP, as we identified this group of patients to be referred less frequently than adult patients.

In conclusion, this study revealed the need for improving the referral of young female cancer patients for FPC and the need to overcome referral disparities and determinants associated with low referral.

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8

Deciding about fertility preservation after specialist counselling

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Abstract

Study question: How do female patients experience fertility preservation (FP) consultation (FPC) with a specialist in reproductive medicine and subsequent decision-making on FP?

Summary answer: Most patients had positive experiences with FPC, but negative experiences were found to be associated with decisional conflict and decision regret.

What is known already: When confronted with a need for gonadotoxic treatment, girls and young women will have to make an irreversible decision with regard to FP. Patients may experience decisional conflict and develop regret about their decision during follow-up. Patients' opportunities to ask questions during FPC and their knowledge about FP have been inversely related to decisional conflict.

Study design, size, duration: A questionnaire on experiences with FPC, designed after qualitative research, was retrospectively distributed to 108 patients to whom FP was offered after FPC between July 2008 and July 2013. Aiming to minimise recall bias, we defined a subgroup of patients counselled since 2011 who had not yet tried to conceive after FPC.

Participants/materials, setting, methods: Patients were aged ≥16 years and had either cancer or a benign disease that required gonadotoxic therapy. They received FPC in a single university hospital in the Netherlands. Apart from patients' experiences, patients' characteristics, decisional conflict, and decision regret were assessed.

Main results and role of chance: A total of 64 patients (59.3%) responded to the questionnaire. Patients generally had positive experiences with FPC, but indicated room for improvement. Negative experiences were associated with decisional conflict regarding the FP decision (not enough time for counselling: $p < 0.0001$; not having the opportunity to ask all questions during FPC: $p < 0.0001$; not feeling supported by the counsellor during decision-making: $p = 0.0003$; not all applicable options were discussed: $p = 0.0001$; benefits and disadvantages of FP options were not clearly explained: $p = 0.0005$). Decisional conflict was related to decision regret ($p < 0.0001$). In the subgroup of patients counselled after 2011 who had not tried to conceive (n = 33), similar results as for the total study population were found for the association of patient experiences with decisional conflict.

Limitations, reasons for caution: Given our retrospective design, we were not informed about the causality of the associations observed. We studied Dutch patients counselled in a single centre who were at least 16 years old when filling in the questionnaire. This may limit the generalisability of our data to other settings and populations.

Wider implications of the findings: More attention should be paid to improving FPC care. Interventions aiming at improving patients' comprehension of the topic of FP and their feelings of being supported in decision-making are advisable.

Introduction

Saving the potential to have biological children after cancer seems to be of high importance to many girls and young women with cancer.[1] Cancer therapy may have gonadotoxic side effects and thereby threaten fertility.[2,3] Various fertility preservation (FP) techniques have evolved with the aim of safeguarding the childbearing capacity of patients with cancer or benign diseases requiring gonadotoxic treatment.[4-6] As FP techniques should ideally be performed before the start of gonadotoxic treatment, decision-making on FP needs to take place within a very short time frame.

To enable patients to make a well-informed decision on FP in the burdensome time period after cancer diagnosis, clinical guidelines advise FP consultation (FPC) with a counsellor specialised in reproductive medicine.[7-9] With respect to FPC care, it has been found that patients who have had opportunities to ask questions and who had extensive knowledge about FP had less decisional conflict (difficulties in decision-making) than those who had less knowledge or who felt less opportunities to ask questions.[10-12] Decisional conflict is defined as a state of uncertainty about a course of action[13] and may very well be a forerunner of regretting the FP decision made.[12]

In American studies where not all citizens have the costs of FP techniques reimbursed, 28.8% of the patients indicated FP treatment to be cost prohibitive,[14] whereas 48% reported that costs influenced their FP decision.[15] Patients who reported that the costs of FP influenced decision-making had a median decisional conflict score of 37.5 on a scale of 0-100, whereas others scored 21.9.[10] In a second study, patients for whom FP services were cost prohibitive had a mean decisional conflict score of 56.3, compared with 32.8 for patients who indicated that FP services were *not* cost prohibitive.[14] Due to the health insurance system in the Netherlands where we conducted our study, patients do *not* have to pay for FPC and FP techniques. For this reason we could exclude decisional conflict based on financial concerns and investigate the actual association between FPC experiences and decisional conflict. In the current study, we aimed to investigate how female patients experienced FPC and FP decision-making. As a second aim, we investigated the interplay between patients' FPC experiences, decisional conflict, and decision regret. With the results of the current study conducted in a setting with reimbursement, we aim to gain insight in the importance of FPC care and to contribute to developing interventions that might help patients to make high-quality FP decisions.

Methods

Study design

We performed a single centre, cross-sectional study querying women after FPC, via a questionnaire which was partly developed specifically for this study and partly consisted

of validated scales. The questionnaire was distributed by mail to patients who received FPC between July 2008 and July 2013. Data from the questionnaire were complemented with data from the medical files.

Eligibility criteria and setting

Our study was conducted at the department of reproductive medicine of a single academic hospital in the Netherlands, the Radboud university medical center (Rumc). Patients were counselled about FP by one of the counsellors (seven gynaecologist and one nurse practitioner), of whom two gynaecologists actively participated in the Netherlands Network for Fertility preservation (NNF). Available FP techniques were ovarian transposition, the cryopreservation of embryos, cryopreservation of ovarian tissue (since 2009), and vitrification of oocytes (since 2011). Patients had the option to be referred to other centres for techniques that were not yet available at the Rumc. Information leaflets from the NNF about FP techniques were developed during the study period and provided if relevant. For the current study, women to whom FP was offered after FPC between July 1, 2008 and July 1, 2013 were considered eligible. For reasons of ethical approval, participants had to be at least 16 years of age at the time we conducted our study (November 2013). Women who were deceased (n=9), severely diseased as a result of their diagnosis (n=5), or who had severe psychological problems (n=1), were excluded (Figure I).

Ethical approval

The Rumc's institutional ethics committee approved all study methods for patients who were at least 16 years of age at the time we conducted our study.

Questionnaire development

As we wished to interview a subset of patients that would best reflect our study population, women were recruited for the interviews via either the Rumc's 'Adolescent and Young Adult Cancer Taskforce' or via their gynaecologist (C.C.M.B.). All women who visited their gynaecologist during follow-up were consecutively invited to participate in semi-structured, individual interviews (45-75 minutes) with a single interviewer (L.B.). Of six patients contacted in total, one refused to participate. The patients interviewed had various oncological diagnoses, ages, and partner-relationship situations at the time of FPC. Patients made a variety of FP decisions and differed from each other with respect to their medical and reproductive outcome. Patients were asked how they experienced FPC and FP decision-making and how they would reflect on their decision. As soon as saturation was achieved and no new themes came up after five interviews, no new interviews were performed. All interviews were recorded digitally, transcribed verbatim,

and coded by two medical researchers (L.B. and S.A.E.P.) independently following the concepts of grounded theory methodology.[16] Disagreement was solved by consensus and a crosscheck by a qualitative research expert (W.L.D.M.N.). Based on the interviews, we developed the questionnaire items regarding baseline characteristics and patients' experiences with FPC.

Questionnaire outcome measures

Baseline and clinical characteristics

Based on the interviews, a total of 19 open and multiple-choice items relating to the patient's current situation and situation at FPC were included in the questionnaire. Items covered partner relationship, parental status, strength of the wish to have a child, educational level, current health status, patients' reproductive outcome, and the use of assisted reproduction with or without cryopreserved material.

Experiences with FPC

Ten Likert scale items (five points) covered the relevant aspects of patients' experiences with FPC (strongly disagree – strongly agree), as indicated by our interviewees. The exact formulations of the individual Likert scale items in the questionnaire are provided in Figure II and Table II. High scores on the items reflected positive experiences. In addition to the Likert scales, the questionnaire contained a free-response section in which patients could provide additional comments about their counselling experiences if they wished.

Decisional conflict

Patients ease or difficulty with FP decision-making was measured using a Dutch translation of the validated Decisional Conflict Scale (DCS).[17] This scale includes 16 items measuring 'a state of uncertainty about a course of action' during medical decision-making (strongly agree - strongly disagree).[13] We asked patients to reflect on the time period in which they decided on FP when filling in the questions. Given the retrospective design of our study, item 15 ('I expect to stick with my decision') was removed. Decisional conflict was thought to be greater when a person felt uninformed; was unclear about personal values; or felt unsupported at the time of decision-making. For this reason, the DCS contains the following subscales: 'Informed', 'Values clarity' 'Support', 'Uncertainty' and 'Effective decision'.[13] As an example, one of the items was: "I had enough advice to make a choice". Items for decisional conflict were converted to a final score of 0 to 100 with higher scores representing higher decisional conflict. Scores below 25 are associated with an absence of decisional conflict, whereas scores exceeding 37.5 are associated with decision delay and feeling unsure.[13]

Decision regret

A patient's current regret regarding her past FP decision was assessed using the validated Decision Regret Scale (DRS; five items), a five-point Likert scale measuring distress or remorse after a healthcare decision.[18] As an example, one of the items was: "The choice did me a lot of harm". As no validated Dutch version was available, the scale was translated to Dutch by one of the authors (L.B.) and back to English by an English native speaker. Only subtle translation flaws were identified and after three back-translation cycles, the Dutch and English versions were considered accordant. Sum-scores ranged from 5 to 25, with higher scores representing greater regret.[18]

Data collection

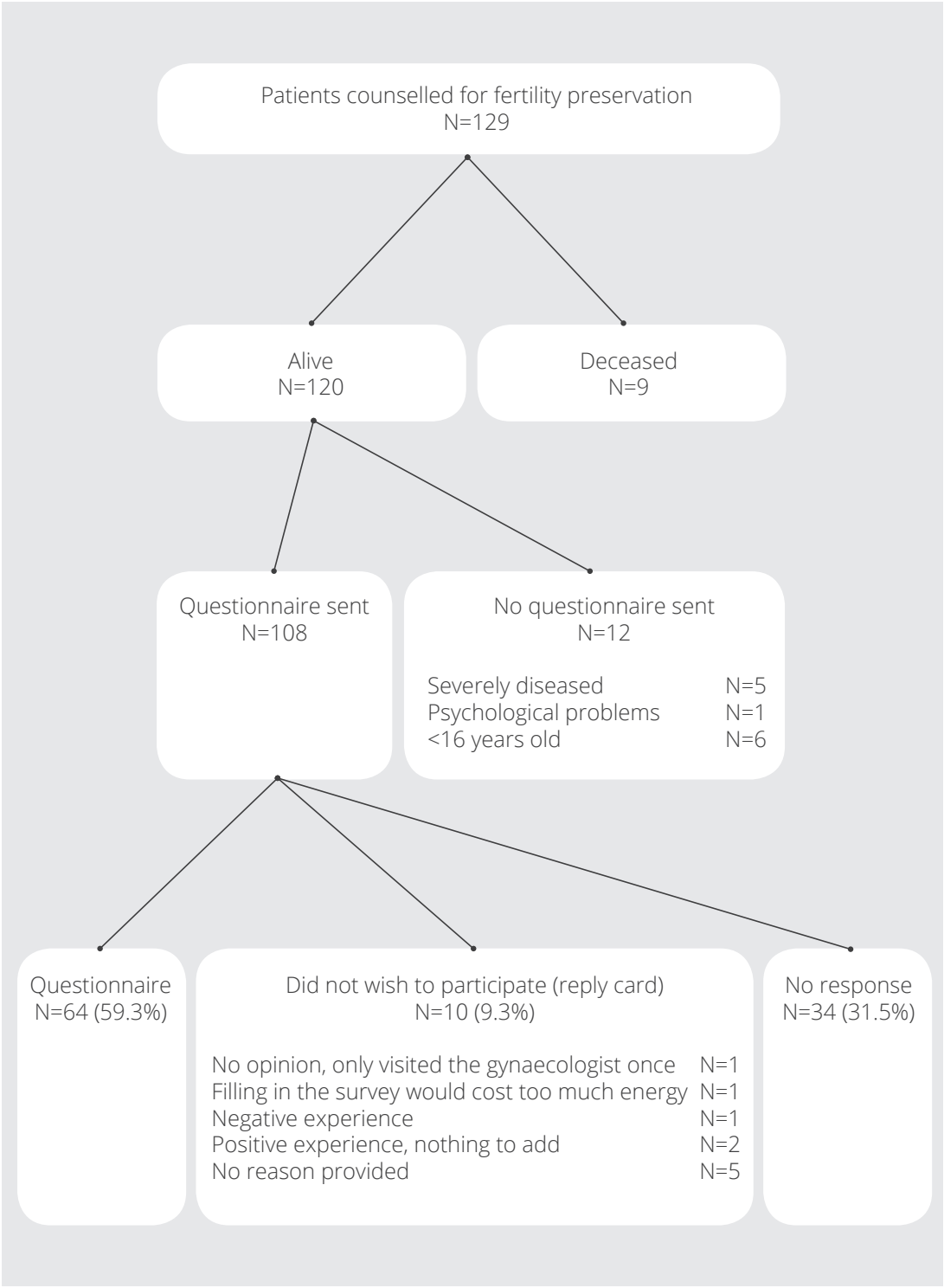
To ensure that we would not distribute our questionnaire to deceased or severely diseased patients, broad information about patients' current health status was obtained from their FP counsellors or general practitioners. Subsequently, eligible patients (Figure I) received a paper version of the questionnaire by mail, together with a reply card on which they could indicate that they did not wish to participate. Patients received this questionnaire in either March 2013 (FPC before October 2012) or November 2013 (FPC between October 2012 and July 2013 and patients counselled before October 2012 who reached the age of 16 years between March and November 2013). If we did not receive a completed questionnaire or reply card after three weeks, one reminder was sent. Data retrieved from the questionnaire were supplemented with baseline and clinical information from medical files as well as information on the counsellor's experience with FPC and participation in the NNF.

Data analysis

Data-analysis was performed using IBM SPSS Statistics version 20 for Windows (IBM corporation, Armonk, NY, USA). Differences with a two-sided *p*-value of ≤ 0.05 were considered to be statistically significant. Characteristics of responders and non-responders were compared using independent samples Student *t*-tests and Chi square tests. The internal consistency of the questionnaire's items on FPC experiences was assessed by measuring Crohnbach's alpha ($\alpha > 0.70$: reliable). Using ANCOVA, the influence of FPC experiences and other determinants on the overall decisional conflict score (1) and the relation of this DCS score with decision regret (2) were assessed. As we performed multiple tests when assessing the influence of various variables on decisional conflict, a Bonferroni correction was performed. In addition to the ANCOVA, we obtained a Spearman correlation coefficient for the association between the DCS and DRS.

Apart from our total study population, we defined a subgroup of patients for whom we repeated the abovementioned ANCOVA analyses and Spearman correlations for the following reasons. We could not exclude patients' recall bias with respect to their FPC

Figure I. Flowchart: Eligibility and response



experiences based on their knowledge about their (favourable or unfavourable) medical and reproductive outcome and current feelings of regret.[19,20] Patient who have decision regret may have cognitive dissonance (discomfort resulting from a bad decision) and try to cope with this dissonance by changing their opinion or reflection about the decision.[19] To minimise this bias, we defined a subgroup of women who were counselled since 2011 and who did not try to conceive after FPC. This subgroup also represents patients counselled in the time period in which all FP techniques were available at the Rumc.

Results

Response

Out of the 108 patients who received our questionnaire, 64 patients (59.3%) participated (Figure I). Patients who did not fill in the questionnaire (n=44) had similar baseline and clinical characteristics, follow-up characteristics and counsellor's characteristics as responders.

Participants

Out of the 64 patients who returned the questionnaire, 60 patients completely filled in the DCS. The characteristics of these 60 responders are provided in Table I. A total of 33 responders to whom FP was offered had received FPC in the time since 2011 and had not tried to conceive.

Patients' experiences with FPC

The ten items of our questionnaire measuring patients' experiences with FPC were reliable for our sample (Crohnbach's $\alpha = 0.79$). In general, patients had positive experiences with FPC (Figure II). Despite this, a significant proportion of the respondents saw opportunities to improve patient involvement and support, the counsellor's awareness of patients' personal importance of specific issues for decision-making, and the extent to which FP options offered were appropriate for the patient's individual situation (Figure II). Some patients indicated that they missed essential information during FPC in the free-response section. This included information about gestational carriers, the influence of hormonal stimulation on a hormone-dependent tumour, or the influence of hormonal stimulation and ovarian enlargement on the risk that chemotherapy would result in ovarian failure.

Decision-making and decisional conflict

At the free-response section of the questionnaire, patients revealed that their FP decision

was mainly dependent on a difficult trade off between their risk of ovarian failure and their wish to start oncological therapy as soon as possible. A significant number of patients indicated that their young age, the recent start of their partner relationship, and/or the short period of time to make a decision complicated their decision-making processes. Patients had a median overall score of 25.0, with 11 patients (18%) having scores exceeding the score of 37.5 associated with decision delay and feeling unsure.[13] The highest conflict was indicated for the 'Uncertainty' and 'Values clarity' subscale (Table II). For the subgroup of 33 patients who had not tried to conceive and who were counselled since 2011, a comparable pattern was seen (Table II). In this group, only two patients (6%) had overall scores above 37.5.

Patients' FPC experiences in relation to decisional conflict

The influence of patient experiences and other determinants on the overall DCS score are provided in Table III. Although trends were observed for various baseline characteristics, the only items that remained statistically significantly associated with patients' decisional conflict after a Bonferroni correction concerned patient experiences with FPC. Especially negative experiences with the most basic ingredients of FPC, namely items related to the patient's ability to obtain enough information about FP options, were correlated with decisional conflict (Table III). In addition, patients who did not feel supported by their counsellor reported higher conflict. In the subgroup of participants (n=33) who had not tried to conceive after FPC and who had been counselled at some time since 2011, similar results as for the total study population were found for the association of patient experiences with decisional conflict (data not shown).

Decision regret

Patients had a median score on the decision regret scale (DRS) of 8 (Table II). An ANCOVA assessing the influence of decisional conflict (DCS overall score) on decision regret (DRS) revealed that the decisional conflict and regret were closely related to each other (B = 0.21; 95% confidence interval (CI) = 0.15; 0.27; p <0.0001; Spearman's rho 0.74). The DCS overall score remained related to decision regret in our subgroup (B = 0.18; 95% confidence interval (CI) = 0.10; 0.26; p < 0.0001; Spearman's rho 0.57).

Table I. Baseline characteristics and determinants of experiences with counselling and decision-making

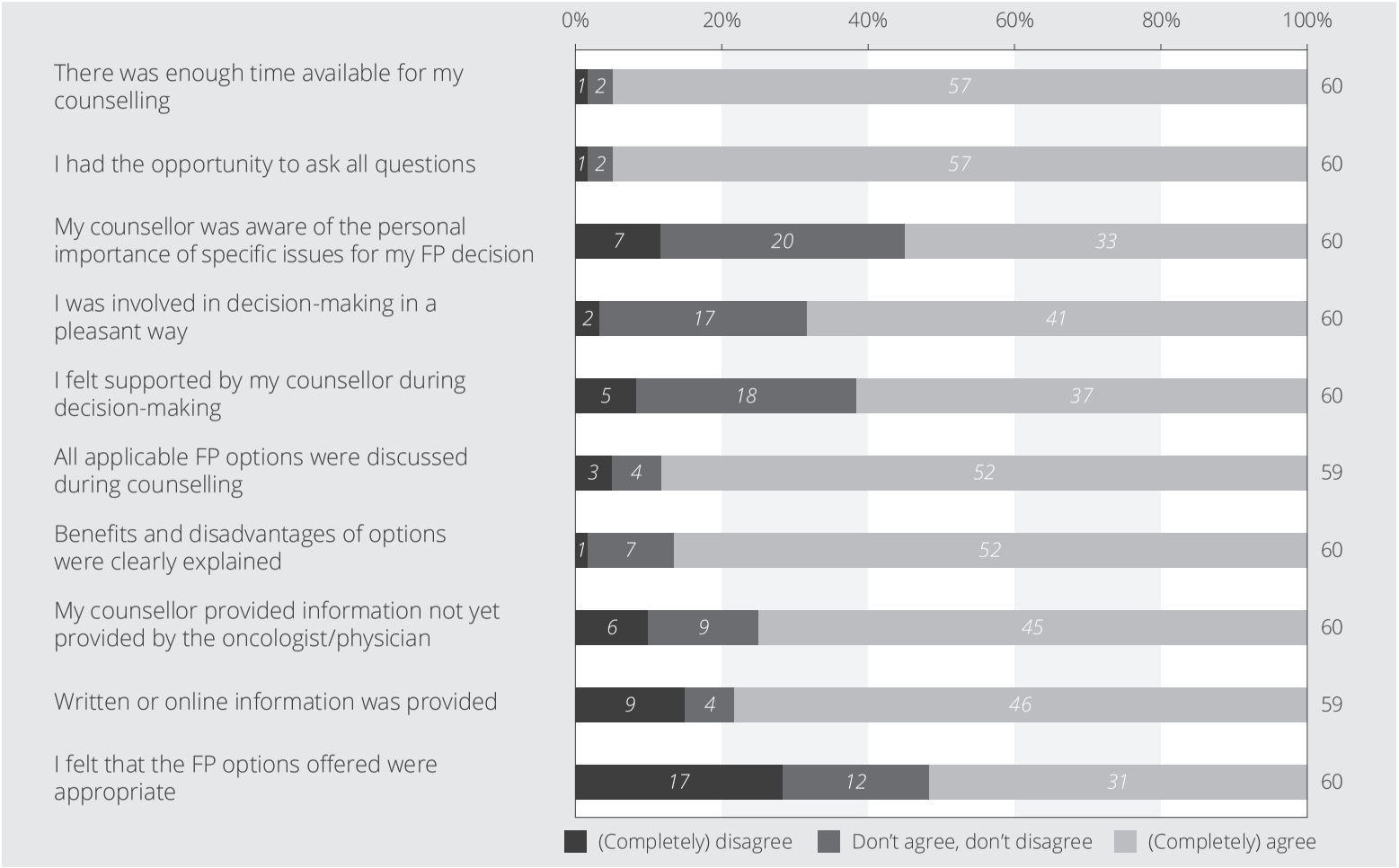
	Participants who completed the DCS (N=60)	
Baseline and clinical characteristics at counselling		
Age, years (mean, SD)	28.9	(5.7)
Diagnosis (number, percentage)		
Benign disease (nephrotic syndrome)	1	1.7%
Breast cancer	36	60.0%
Lymphoma	11	18.3%
Leukaemia	-	-
Gynaecological malignancy	5	8.3%
Bone or soft tissue tumour	4	6.7%
Tumour of the gastro-intestinal tract	3	5.0%
Central nervous system tumour	-	-
Partner relationship (number, percentage)		
No partner relationship	6	10.0%
Partner, living apart	14	23.3%
Partner, living together	40	66.7%
Parity (number, percentage)		
Nulliparous	51	85.0%
Parous	9	15.0%
Wish to conceive (1-10; mean, SD)	6.8	(2.8)
Level of education (number, percentage)		
Primary school or lower vocational education	3	5.0%
Secondary school or higher vocational education	17	28.3%
Higher education or university	40	66.7%
FP counsellor		
Experience		
≤ 10 consultations with female patients for FP counselling	13	21.7%
11 - 25 consultations with female patients for FP counselling	12	20.0%
> 25 consultations with female patients for FP counselling	35	58.3%
Counsellor actively participated in the NNF (number, percentage)		
Yes	39	65.0%
No	21	35.0%
FP options		
Patients to whom at least one FP option was offered (number, percentage)	60	100%
IVF or ICSI offered	38	63.3%
Cryopreservation of ovarian tissue offered	26	43.3%
Vitrification of oocytes offered	26	43.3%
Ovarian transposition offered	5	8.3%
FP options performed (number, percentage)		
No FP	26	43.3%
IVF or ICSI	21	35.0%
Cryopreservation of ovarian tissue*	2	3.3%
Vitrification of oocytes	8	13.3%
Ovarian transposition*	4	6.7%

Follow-up		
Follow-up, years (mean, SD)	2.0	(1.3)
Period of counselling: July, 2008 – January, 2011 (number, percentage)	26	43.3%
Period of counselling: January 2011 – July 2013 (number, percentage)	34	56.7%
Current health status		
(Cancer) treatment successfully completed, follow-up	36	60.0%
Current treatment	22	36.7%
Diseased	2	3.3%
Current partner relationship (number, percentage)		
No	12	20.0%
Same partner as during FPC	46	76.7%
Other partner as during FPC	2	3.3%
Current wish to have a child (1-10; mean, SD)	6.5	(3.0)
Tried to conceive after FPC (number, percentage); (1 missing)		
No	51	86.4%
Yes	8	13.6%
Conceived after FPC (number, percentage); (1 missing)		
Yes (all spontaneous conceptions)	6	10.2%
No	53	89.8%
Takes care for children who did not live in the family at the moment of FPC		
No	56	93.3%
Yes, biological children from my current partner and myself	4	6.7%
Adopted children	0	0%
Children from my current partner	0	0%

NNF = Netherlands Network for Fertility Preservation; FP = Fertility preservation; FPC = Fertility preservation consultation

** One patient received both cryopreservation of ovarian tissue and ovarian transposition.*

Figure II. Patients' experiences with Fertility Preservation Consultation (FPC)



Percentages of patients (completely) disagreeing; not agreeing and not disagreeing; or (completely) agreeing with 10 Likert scale items assessing experiences with the communication and patient involvement during FPC as well as the content of FPC.

Table II. Decisional conflict and regret scores

	All participants (N=60)		Participants in subgroup (N=33)	
Decisional conflict				
Overall score (median, IQR)	25.0	(18.8; 35.0)	23.3	(17.5; 28.3)
Informed	25.0	(16.7; 33.3)	25.0	(16.7; 25.0)
Values clarity	33.3	(25.0; 47.9)	33.3	(25.0; 50.0)
Support	25.0	(8.3; 33.3)	25.0	(8.3; 25.0)
Uncertainty	29.2	(16.7; 41.7)	25.0	(16.7; 37.5)
Effective decision	25.0	(8.3; 33.3)	8.3	(0.0; 25.0)
Decision regret				
Decision regret scale (median, IQR)	8	(5; 12)	7	(5; 12)

Discussion

This is the first study conducted in a European country with reimbursement of FP that quantitatively assessed patients' experiences with FPC in the context of decisional conflict and regret. In accordance to prior findings,[10,15] the majority of patients in this study were satisfied with FPC. Although some patients wished for more information about specific subjects (e.g. the influence of hormones and ovarian enlargement on the risk that chemotherapy would cause ovarian failure), information could sometimes not be provided as our knowledge in the field is still limited.

The results of this study were consistent with earlier studies establishing a link between the extensiveness of counselling and decisional conflict or even regret. Indeed, lower regret and conflict have been observed among cancer survivors who received FPC when compared with patients who did not benefit from this care.[14,21] Furthermore, negative associations with decisional conflict have been reported for patients' fertility-related knowledge and opportunities to ask questions.[10,11] It was remarkable that participants in the current study had lower decisional conflict scores (median 25.0) than patients from the USA (29.7 and 31.3).[10,14] Presumably, decisional conflict is higher in the American setting with not all citizens having the costs of FP reimbursed.[10,14] Compared with 36.5% and 40.9% of the patients proceeding with an FP technique after FPC in the USA,[10,14] 56.7% of the patients in our sample underwent FP. Despite these differences in decisions, the levels of decision regret found in the current study (median score: 8) were comparable to those previously reported for American FPC patients (mean score: 8.4). Significantly higher regret scores were obtained in an Australian population of breast cancer patients counselled more recently (mean score after conversion to a scale of 5-25: 14.8).[12]

Table III. Determinants of decisional conflict

	B	95% CI	*p*-value*
Baseline and clinical characteristics at counselling			
Age, years	-0.01	(-0.63; 0.61)	1.0
Diagnosis (reference: tumour of the gastro-intestinal tract)			
Benign disease (nephrotic syndrome)	5.00	(-26.82; 36.82)	0.8
Breast cancer	9.61	(-6.95; 26.17)	0.3
Lymphoma	3.49	(-14.47; 21.44)	0.7
Gynaecological malignancy	7.67	(-12.46; 27.79)	0.4
Bone or soft tissue tumour	4.58	(-16.47; 25.63)	0.7
Partner relationship (reference: partner, living together)			
No partner relationship	2.81	(-8.56; 14.19)	0.6
Partner, living apart	10.55	(2.48; 18.62)	0.011
Parity (reference: parous)			
Nulliparous	-1.93	(-11.77; 7.91)	0.7
Wish to conceive (1-10)	-0.58	(-1.85; 0.68)	0.4
Level of education (reference: higher education or university)			
Primary school or lower vocational education	12.55	(-3.56; 28.66)	0.12
Secondary school or higher vocational education	-0.33	(-8.12; 7.46)	0.9
FPC experiences			
Enough time available for counselling	**-10.59**	**(-15.28; -5.89)**	**<0.0001**
Had the opportunity to ask all questions	**-12.86**	**(-17.14; -8.57)**	**<0.0001**
Counsellor was aware of personal importance of specific issues for FP decision	-2.82	(-6.82; 1.19)	0.17
Involved in decision-making in a pleasant way	-4.88	(-8.77; -0.98)	0.015
Supported by counsellor during decision-making	**-6.51**	**(-9.89; -3.13)**	**0.0003**
All applicable FP options were discussed	**-7.49**	**(-11.14; -3.83)**	**0.0001**
Benefits and disadvantages of options were clearly explained	**-8.28**	**(-12.79; -3.77)**	**0.0005**
Counsellor provided information not yet provided by the oncologist/physician	-3.30	(-7.02; 0.42)	0.081
Written or online information was provided	-3.93	(-6.97; -0.89)	0.012
FP options offered were appropriate	-2.04	(-4.48; 0.40)	0.099
FP counsellor			
Experience (reference: > 25 consultations)			
≤10 consultations	0.16	(-8.77; 9.09)	1.0
11-25 consultations	-0.04	(-9.24; 9.16)	1.0
Counsellor actively participated in the NNF (reference: yes)			
No	-0.78	(-8.15; 6.60)	0.8
FP options			
IVF or ICSI offered (reference: no)	-5.02	(-13.38; 3.35)	0.2
Cryopreservation of ovarian tissue offered (reference: no)	0.39	(-7.86; 8.63)	0.4
Vitrification of oocytes offered (reference: no)	2.01	(-6.30; 10.33)	0.6
Ovarian transposition offered (reference: no)	-5.32	(-19.03; 8.39)	0.4
FP performed (reference: no)	-7.59	(0.78; 14.40)	0.030

Follow-up			
Follow-up, years	3.19	(0.69; 5.70)	0.013
Current health status (reference: diseased)			
(Cancer) treatment successfully completed, follow-up	2.56	(-4.77; 9.90)	0.5
Current treatment	12.01	(-8.02; 32.03)	0.2
Current partner relationship (reference: other partner as during FPC)			
No	-8.61	(-29.27; 12.04)	0.4
Same partner as during FPC	-11.98	(-31.5; 7.56)	0.2
Current wish to have a child (1-10)	-0.11	(-1.32; 1.10)	0.9
Tried to conceive after FPC (reference: no)			
Yes	-7.84	(-17.98; 2.31)	0.13
Conceived after FPC (reference: no)			
Yes	-7.39	(-18.95; 4.18)	0.2

After a Bonferroni correction (p≤0.05 / 38 statistical tests), variables for which a p-value of p≤ 0.0013 was found were considered statistically significantly related to decisional conflict. Unstandardised coefficients (B) with 95% confidence intervals (CIs) in parentheses are demonstrated here. Items with a statistically significant association with the Decisional Conflict Scale (DCS) score are provided in bold. Example 1: For each year that patients were older, patients' mean overall DCS score was 0.01 points lower. Example 2: The mean DCS score of nulliparous women was 1.93 points lower than that of parous women (reference).

NNF = Netherlands Network for Fertility Preservation; FP = Fertility preservation; FPC = Fertility preservation consultation
** P-values and CIs resulting from ANCOVA with the overall score on the DCS as a dependent outcome variable.*

The associations found between patients' experiences with FPC, current regret, and recalled decisional conflict about the FP decision may have several explanations. First of all, there may be a causal relationship between the quality of FPC and patients' ease or difficulty in decision-making. Various studies have indicated an association between patients' limited FP knowledge and decisional conflict.[11,12,22] Secondly, patients' current feelings of decision regret may have altered their reflections on their FPC and FP decision-making that took place in the past.[19] It has been shown that recalled decisional conflict can fluctuate during time, as the same patients reported various levels of recalled decisional conflict at three time-points across the first year of follow-up after FPC.[12] As a third explanation for the associations found, patients' experiences, decisional conflict, and regret can have (a) common cause(s).

The fact that we could not clarify whether the associations between patients' experiences, decisional conflict, and decision regret were causal was one of the limitations of our study. Apart from this important limitation, we recruited female patients who were at least 16 years of age when filling in our questionnaire, limiting the generalisability of our results to paediatric populations. Our sample size was not sufficient to create a

multivariable model or to study subgroups of patients with a specific diagnosis, partner relationship status, educational level, or age. Nevertheless, important strengths of this study were the fact that the results were obtained via a systematic mixed methods approach in a setting where financial reasons to refrain from FP did not play a role. Moreover, our study evaluated FPC provided by various counsellors.

Several conclusions and implications for the clinical practice can be drawn from this study. As long as a causal relationship between the quality of FPC, decisional conflict, and regret is not refuted, attempts should be made to optimise care in order to attain a higher quality of FP decisions. To optimise care, interventions aiming to improve patients' comprehension of the topic of FP and their feelings of being supported should be considered. Patients suggested written material before and after FPC,[15,23] the opportunity to meet a psychosocial counsellor,[15] and the use of a decision aid.[24] Moreover, additional contact with the FP specialist following FPC[22] and decision aids[12,25] have been shown to improve patients' knowledge. Experiences with FPC and FP decision-making should be further investigated in prospective studies to obtain information about the causality of the association between the quality of FPC, decisional conflict, and regret. With sufficiently large samples, researchers could investigate whether there are subgroups of patients at risk for developing regret who may be helped with personalised interventions.

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9

General Discussion

General Discussion

In this thesis, we aimed at improving the quality of female fertility preservation care by addressing some important questions concerning its safety (Part A), efficacy and efficiency (Part B), and patient-centeredness, timeliness and equitability (Part C). In this final chapter, our main findings are discussed in the light of quality improvement. We conclude with a number of implications for clinical practice, as well as recommendations for future research.

Part A - Safety of ovarian tissue autotransplantation in cancer survivors

Main findings

Why is there concern about the safety of ovarian tissue autotransplantation in cancer survivors? (Chapter2)
Cancer cells may be present in ovarian tissue available for autotransplantation. There is concern about the risk of reintroducing malignant disease via the autotransplantation of ovarian tissue during cancer survivorship.

How should we assess the risk of oncological relapse due to the reintroduction of tumour cells via an ovarian tissue transplant? (Chapter 2)
To date, there is no diagnostic test with which the risk of recurrent cancer due to ovarian tissue autotransplantation can be determined with a sufficient sensitivity and specificity. Data on the follow-up after ovarian tissue autotransplantation, the detection of cancer cells in ovarian tissue, and the incidence of ovarian metastases in patients with various tumour types provide guidance.

What is the risk of reintroducing malignant disease via ovarian tissue autotransplantation in survivors of various malignant diseases? (Chapter 3)
The magnitude of this risk differs with cancer diagnosis, with survivors of leukaemia having the highest risk.

Is it possible to mimic the growth of (metastatic) cancer cells in ovarian tissue to develop techniques for tumour cell purging protocols that would be useful to apply in clinical practice? (Chapter 4)
Yes, the injection of bovine or human ovarian cortex fragments with cells from different cancer types led to the formation of proliferating tumour masses and newly formed small metastatic lesions.

Interpretation and discussion of the main findings

Various strategies aimed at improving the safety of ovarian tissue cryopreservation and autotransplantation have been proposed in the literature. Most strategies use alternative approaches to restore fertility that would eliminate the need for autotransplantation and the inherent risks of this procedure.[1-5] Others have focussed on strategies to reduce the risk that living cancer cells are present in the ovarian graft at the time of autotransplantation.[6-9]

As illustrated before, it is difficult to determine how many living cancer cells are actually present in the tissue fragments available for autotransplantation (Chapter

2). Alternative techniques for the detection of cancer cells with a possible higher sensitivity and specificity, including flow cytometry are being studied.[10] However flow cytometry[10,11] does not overcome the problem of sample bias between various cortex pieces of the same ovary as it remains impossible to examine the piece of ovarian tissue that will be actually transplanted. Apart from the difficulties in detecting cancer cells in ovarian tissue, it is currently unknown how many viable cancer cells are needed to cause cancer recurrence.[12] During the transplantation of testicular tissue from rats with T-cell leukaemia to healthy rats, a total of 20 cancer cells proved to be enough to cause relapse in recipient animals.[13] Although such data are obviously not available for the human situation, lessons can be learned from blood transfusions and organ transplantations from donors who turned out to have cancer, or developed cancer shortly after the transplantation. In healthy individuals receiving blood products from donors who developed cancer within 5 years after the blood donation, no excess risk of cancer was found.[14] In contrast to these reassuring data for *blood* transfusions, donor transmitted cancer has been described in recipients of donor *tissue* contaminated with cancer cells.[15,16] In more than 140.000 organ transplantation procedures, 15 recipients of a kidney, liver, or heart transplant suffered from cancer as a result of cancer cell transmission.[16] To what extent these data can be translated to the situation of ovarian tissue autotransplantation is difficult to say, as in this situation, small tissue fragments are being transplanted rather than large organs. In addition, there is no need for immunosuppression after ovarian tissue autotransplantation as the patient receives her own tissue.

Presumably as a result of the difficulties with determining the actual risk of cancer recurrence in humans, the categorisation of oncological diseases as high or low-risk differs between various recent studies, including our own systematic review in Chapter 3.[17-19] Risk classifications may not only need to be based on cancer diagnosis, but also on the moment of ovarian tissue cryopreservation. Although it is considered important to harvest ovarian tissue before the start of gonadotoxic therapy to prevent tissue damage, it has been suggested that in leukaemia patients, ovarian tissue may best be cryopreserved during a remission phase that is induced with chemotherapy with a low risk of ovarian damage, but before bone marrow transplantation.[12] Namely, no oncological disease was observed in mice receiving ovarian transplants from patients in a remission phase.[12] The exact differences in the risk of recurrent disease after autotransplantation of tissue that was cryopreserved during a remission phase versus an active disease phase still needs to be determined. This is also true for the type and dose of chemotherapy administered prior to ovarian tissue cryopreservation would yield the optimal result with respect to both safety and ovarian tissue survival.

The concept of purging metastatic cells from ovarian tissue has not been extensively studied. In a single study using human ovarian tissue brought into suspension, breast cancer cells could be partially purged using activated lymphocytes and antibodies.[20] Unfortunately, effective cancer cell purging from intact and viable ovarian tissue

fragments containing tumour metastases has not yet been reported. For the purging of cancer cells from bone marrow transplants, purging techniques using viruses, monoclonal antibodies, hyperthermia, or *ex vivo* chemotherapy have been described.[6-9] Although the biophysical effects of hyperthermia are not yet fully understood,[21,22] tumour apoptosis and a disruption in the expression of key mitotic regulators have been observed after hyperthermia.[22,23] Hyperthermia in combination with intraperitoneal chemoperfusion has been clinically applied for intra-abdominal cancer (HIPEC: heated intra-peritoneal chemotherapy).[21,24] To date, hyperthermia and other approaches for tumour cell purging to enhance the safety of ovarian tissue autotransplantation have not yet been investigated for clinical application. Purging techniques could very well be investigated using the model-system developed in Chapter 4.

As the safety of autotransplantation of ovarian tissue in cancer survivors cannot yet be guaranteed, attempts have been made to find alternative approaches for fertility preservation. Embedding isolated follicles in an "artificial ovary" is one option that may become clinically available in the future to prevent the need for ovarian tissue autotransplantation.[1,2] With this technique, follicles are isolated from the ovarian tissue and mixed with fibrinogen to form a biodegradable scaffold in which follicles and ovarian cells are transplanted. Autografting in mice followed by the retransplantation of such an artificial ovary has recently led to survival and growth of murine follicles.[1] Another strategy eliminating the need for autotransplantation of ovarian tissue includes xenotransplantation. Cryopreserved and thawed human ovarian tissue fragments have been successfully xenotransplanted to mice, resulting in follicle development and successful oocyte retrieval.[25] However, major ethical issues on the safety of this technique for offspring, as well as the risk of transmitting murine infectious diseases should still be resolved.[26]

Two last alternatives to the autotransplantation of ovarian tissue include *in vitro* maturation and the retrieval of oogonial stem cells. It has been attempted to isolate and mature primordial ovarian follicles from cryopreserved/thawed ovarian tissue using *in vitro* follicle growth and maturation. Although mice have been born after *in vitro* maturation of primordial follicles since 1996,[27,28] it remains challenging to develop a culture system that would allow *in vitro* growth and maturation of *human* oocytes from the most immature stages to fully developed oocytes.[3,4] Furthermore, it is currently unknown whether the establishment of genome imprinting – essential for pre- and postnatal growth and metabolism – is correct for *in vitro* matured human oocytes.[5,29] Another possible future alternative to the autotransplantation of ovarian fragments includes the retrieval of oogonial stem cells from ovarian tissue.[5] Although it is a central belief that females are born with a finite number of oocytes, mitotically active germ cells known as oogonial stem cells have recently been identified in human ovarian tissue.[30] For fertility preservation, oogonial stem cells retrieved from (cryopreserved) ovarian tissue could be injected into the patient's remaining ovary where they could theoretically result in neo-oogenesis and generate a new population of follicles. [5] In addition, oocytes

might be derived from oogonial stem cells that could be used for *in vitro* fertilisation.[5,31] However, the existence of oogonial stem cells in humans has been debated and this field of research is promising, but still very much in its infancy.[5,31]

To summarise, a wide range of strategies to enhance or even guarantee the safety of restoring fertility after ovarian tissue cryopreservation are currently being investigated. These approaches include the development of diagnostic tools for detecting cancer cells in ovarian tissue, purging techniques to eliminate cancer cells from ovarian tissue, and strategies to restore reproductive potential without the need to transplant the ovarian graft.

Part B - Efficacy and efficiency of ovarian tissue cryopreservation and thawing

Main findings

What is the efficacy of ovarian tissue cryopreservation and thawing using the protocols from a major European centre? (Chapter 5)
In our hands, the viability of human ovarian tissue was not fully preserved during cryopreservation and thawing according to a clinically successful protocol.

What is the difference in the efficacy and efficiency of two very different protocols for cryopreservation and thawing on the viability of ovarian cortex tissue? (Chapter 6)
We found a cryopreservation protocol using dimethylsulfoxide (DMSO) to be associated with a statistically significantly better follicle and overall tissue viability after thawing when compared to a protocol using ethylene glycol. With respect to thawing, a short and efficient protocol led to a similar percentage of living follicles and overall tissue viability as a lengthy thawing protocol based on continuous dilution.

Interpretation and discussion of the main findings

As we found that protocols for cryopreservation and thawing of ovarian tissue differ in their effects on tissue viability, it is very likely that the efficacy of ovarian tissue cryopreservation could be improved (Chapter 5 and 6). Information about efficacy might also facilitate efficiency, as the protocol costing the least time, human effort, material, and money can be chosen if it preserves tissue viability at least as good as other, less efficient protocols. Contradictory results have been reported for the efficacy of various slow-freezing and thawing protocols. After cryopreservation using DMSO, a better follicle ultrastructure and morphology and a higher percentage of living follicles were found when compared to ethylene glycol for human, sheep or goat ovarian tissue.[32-35] These results are concordant with the results obtained from our study (Chapter 6) using human material. However, in studies using bovine or agouti tissue, DMSO did not improve the preservation of the follicle survival and ultrastructure when compared to ethylene glycol.[36,37] Apart from the type of cryoprotectants used, the concentration of the

cryoprotectant has been related to the ovarian tissue's viability post cryopreservation and thawing.[36,38] In contrast, limited attention is being paid to the effects of various thawing techniques, with most studies evaluating tissue viability after a single combined cryopreservation/thawing protocol or various cryopreservation methods combined with a single thawing protocol.[35,36,38,39] As significant ischaemic injury is thought to occur after the transplantation of an avascular ovarian graft, it seems vital to strive for the best graft survival during the cryopreserevation and thawing process. As the integrity of the stroma is essential for the neovascularisation process after grafting,[40] it is essential that during optimisation of cryopreservation/thawing protocols not only the viability of the follicles is taken into account, but also the viability of the stromal cell compartment. An improved preservation of the stromal tissue ultrastructure has been suggested with the use of vitrification instead of slow-freezing.[41,42] Nevertheless, contradictory findings have been reported with regard to the effects of vitrification of human ovarian tissue, with some recommending slow-freezing techniques and others recommending vitrification.[43]

To solve the problem of ischaemia after the autotransplantation of avascular cortex fragments, the cryopreservation and autotransplantation of an intact ovary – including its vascular pedicle – has been proposed. This technique allows immediate revascularisation of the transplant via vascular anastomoses.[44] The procedure, however, is complex and requires advanced laboratory and surgical skills.[40,45] In addition, the autotransplantation of an entire organ greatly enhances the risk of reintroduction of the neoplastic disease via tumour cells that have metastasised to the ovary. Finally, the elevated risk of vascular thrombosis in the small anastomosed vessels[40] could lead to the loss of the entire ovary. Given these difficulties with the transplantation of an entire ovary, others researchers have focussed on how to minimise ischaemic damage after the autotransplantation of ovarian cortex fragments. Strategies proposed to minimise damage after ovarian fragment transplantation include the administration of antioxidants to neutralise oxygen free radicals,[40,46] growth factors,[40,46,47] platelet rich plasma containing a high concentration of growth factors,[48] or gonadotrophins[40,46,49] to upregulate vascular endothelial growth factor (VEGF).[40] However, the efficacy of these promising strategies needs to be further investigated. Attention has also been paid to spingiosine-1-phosphate as an angiogenic factor. The transplantation of human ovarian tissue to mice treated with sphingiosine-1-phosphate resulted in an increased density of blood vessels and stromal cells when compared to the transplantation of fragments to untreated mice.[50] As an alternative to medication, angiogenesis has been mechanically stimulated in patients receiving autotransplantation by creating a wound bed one week before transplanting the ovarian cortex fragments to the granulation tissue (two steps laparoscopy).[51,52]

To summarise, knowledge about the effects of various cryopreservation and thawing protocols on pregnancy chances after autotransplantation is limited. However, based on studies measuring the effect of various protocols on ovarian tissue viability, it can

be concluded that protocols should be evaluated and improved. Various strategies have been proposed aiming at reducing ischaemic damage after ovarian tissue autotransplantation, although the clinical efficacy of these strategies still needs to be determined.

Part C - Patient-centeredness, timeliness, and equitability of female fertility preservation care

Main findings

Are there any changes in the numbers and characteristics of Dutch female cancer patients receiving fertility preservation consultation during time? (Chapter 7)
The number of patients counselled annually increased during our study period (2001 – 2013). Patients who were counselled since June 2011 tended to be younger and more frequently lacked a (stable) partner relationship than patients counselled earlier. Furthermore, patients who were counselled since June 2011 more frequently proceeded with fertility preservation.

What are the current referral rates of girls and young women with cancer for fertility preservation consultation in a setting with reimbursement of fertility preservation services? (Chapter 7)
In 2011, 9.8% of the newly diagnosed girls and young women with cancer were referred for fertility preservation consultation with a specialist in reproductive medicine.

Which determinants influence the referral of women for fertility preservation? (Chapter 7)
Referral disparities were identified with respect to age and diagnosis. Furthermore, the referral of female cancer patients for fertility preservation depended on a physician's profession, collaboration with patients' associations, initiation of discussion about fertility preservation with the patient, and the physician's knowledge about where to refer a patient for further counselling.

How do Dutch female patients experience consultation and decision-making with respect to fertility preservation? (Chapter 8)
In general, patients had positive experiences with fertility preservation consultation.

Are patients' experiences with fertility preservation consultation associated with decisional conflict during decision-making? (Chapter 8)
Patients' negative experiences with fertility preservation consultation are related to decisional conflict.

Does decisional conflict during decision-making with respect to fertility preservation relate to decision regret? (Chapter 8)
High decisional conflict with respect to the fertility preservation decision is associated with decision regret.

Interpretation and discussion of the main findings

"Hearing that my fertility was in jeopardy was almost more devastating than the cancer diagnosis." (a female patient reflecting on the time of diagnosis)[53]

This statement illustrates the need for high quality fertility preservation counselling and care for female patients who are confronted with the double jeopardy of a cancer diagnosis and infertility risks. Despite this need, only a minority of the newly diagnosed cancer patients receives sufficient information and support to make a high quality

fertility preservation decision. In accordance with our findings (Chapter 7), low referral percentages for fertility preservation consultation of 1% to 20.6% were found in other studies,[54-57] even though guidelines recommend referral.[58,59] When patients are being referred to a specialist in reproductive medicine, this referral is not always timely, as chemotherapy may have started already.[60] Patients commented that the need for a quick decision did not allow them to obtain enough knowledge, make a decision, and feel confident about this decision.[60,61] Despite this, the median time from diagnosis to fertility preservation consultation has been shown to be relatively long, with a median interval between diagnosis and referral of 18 days and a median time from referral to consultation of 5 days.[62]

In accordance with our own study (Chapter 7), disparities in the referral for fertility preservation consultation have been observed with respect to patients' demographic, socioeconomic status and clinical characteristics,[56,63] illustrating inequity in the access to care. In order to be able to design strategies to improve access to female fertility preservation care for all patients, the barriers and facilitators for referral need to be elucidated first. One of the barriers for referral appears to be the doctors' interpretation of patients' wishes, needs and capabilities. In accordance with our finding that referral depends on a patient's age and diagnosis (Chapter 7), patients in a study from the United States of America had a three times higher chance if they were below the age of 35 years and a 10 times higher chance of referral if they had breast cancer instead of another oncological disease.[56] Moreover, patients in this study had a two times higher chance to be referred if they were Caucasian and a four times higher chance if they were nulliparous.[56] In another study, trends towards a better referral of parous women, women below the age of 35, and highly educated women were observed.[63] Patients were less often referred if they were uninsured[56] or when their physician perceived that they could not afford the treatment.[64] According to British oncologists, the most common barriers to initiate a discussion regarding fertility preservation (one of the determinants of non-referral in our study in Chapter 7) were illness of the patient that would not allow delay in treatment to pursue fertility preservation (93%), a patient's poor prognosis (88%), and a hormonally sensitive tumour (72%).[64] Apart from these three most important barriers for the discussion of the topic with a patient, oncologists reported that their discussion was influenced by the parity, sexual orientation, partner relationship status, and wealth of the patient.[64]

Organisational factors that were reported to contribute to the current low referral rates, include time available to discuss fertility preservation,[64-66] the availability of informational resources,[67] and the lack of referral sites or collaborating reproductive specialists.[66,67] Indeed, oncological specialists in our own study (Chapter 7) also referred patients more often if they knew where they could refer patients. In contrast to our own findings, fertility preservation has been found to be more frequently discussed by female physicians, physicians younger than 50 years of age, physicians who had knowledge about fertility preservation, a positive attitude towards fertility preservation,

and physicians who routinely asked patients about fertility preservation.[66,68] Oncologists themselves indicated that a lack of knowledge and perceived poor success rates of fertility preservation were major barriers for initiating discussions about fertility preservation.[64]

After having identified the various determinants related to the suboptimal referral practices and adherence to guidelines, strategies to overcome barriers and improve clinical practice should be developed, implemented, and evaluated. Proposed strategies include seminars on the topic in the community[55], psycho-education to facilitate the discussion[67] or joint training events with gynaecologists specialised in reproductive medicine.[64] As not knowing where to refer a patient was found to negatively influence referral in our study (Chapter 7), information materials or the ability to call an expert at any time may facilitate referral. Nevertheless, an evaluation of the effects of these interventions has not yet been performed and there might be additional useful strategies as well.

After referral for fertility preservation consultation, patients need to make an irreversible choice for their future in a very troublesome time period of their lives. Given the nature of the fertility preservation decision, it seems vital for fertility preservation counsellors to provide care that is respectful of and responsive to individual patient preferences, needs, and values, and to ensure that patient values guide the decision (i.e. to provide patient-centered care).[69] Although patients confronted with the decision for fertility preservation have reported difficulties in decision-making, patients who received counselling from a specialist in reproductive medicine reported lower difficulties in decision-making[70] and lower levels of decision regret than those who did not receive this opportunity.[71] In our study (Chapter 8), patient experiences, decisional conflict, and decision regret were associated with each other. This is in accordance with previous studies pointing out an association between the amount of information provided to patients, patients' knowledge on the topic, and their opportunities to ask questions on the one hand, and reduced decisional conflict or even regret on the other hand.[72-75] Except for studies focussing on patients' knowledge, previous studies have not addressed a possible association between care that is responsive to patients' preferences, values, and needs and the development of decisional conflict or decision regret. Physicians are searching for the exact informational needs of cancer patients with various diagnoses, the preferred communication strategies, and the ideal timing of information provision regarding a broad spectrum of topics.[76,77] Namely, apart from concerns about becoming infertile, (parents of) patients expressed concerns regarding their (child's) own health, pubertal development, menopause, and survivorship.[76,78-81] Some patients were concerned that oncological treatment would affect their genetic material, possibly leading to birth defects or an increased risk of cancer in their offspring.[78,79,81] Cancer survivorship has been associated with avoidance and delay in approaches to sexual relationships[82] as well as with sexual dysfunction.[83] Moreover, young cancer survivors are less likely than their peers to enter long-term partner-relationships and some cancer patients reported to fear that their partner-relationship would crumble as a result at the prospect of

possibly being infertile.[82] This is remarkable as female survivors reported to feel under pressure to establish permanent relationships at a young age to avoid jeopardising their chances of motherhood.[82]

To summarise, the timeliness and equitability of female fertility preservation care can be improved, whereas little is known about the patient-centeredness of this care. Numerous barriers have been identified for starting the discussion about fertility preservation with patients and referring them to a specialist in reproductive medicine. Various strategies to overcome these barriers have been proposed, but still need to be implemented and evaluated.

Safeguarding the ovaries from gonadotoxic effects

As fertility preservation techniques come with concerns and difficulties, medical approaches have been proposed to safeguard the ovarian function during cancer treatment. Examples of these approaches are the use of GnRH agonists or oral contraceptives during cancer treatment.[84] However, despite GnRH agonists being frequently used in clinical practice to protect the ovaries from the effects of chemotherapy,[85] evidence for the effectiveness of (menopausal symptoms inducing) GnRH agonists[58,86-88] and oral contraceptives to preserve ovarian function is insufficient.[89,90] More recently, attention has shifted to AS101 and sphingiosine-1-phosphate as potential drugs providing protection against the adverse effect of follicle loss during chemotherapy.[52,91,92] As chemotherapy induces damage to growing follicles with actively dividing cells, chemotherapy might lead to an enhanced recruitment of primordial follicles into the growing follicle pool. AS101 is thought to reduce this enhanced activation of primordial follicles during chemotherapy and thereby protect the ovarian function.[91,93-95] The safety and effectiveness of AS101 is now being investigated in a phase II clinical trial.[91] Apart from AS101, the anti-apoptotic agent sphingiosine-1-phosphate has also been shown to reduce chemotherapy-induced apoptotic follicle death. In mice receiving a transplant of human ovarian tissue, co-treatment with sphingiosine-1-phosphate during doxorubicin or cyclophosphamide chemotherapy resulted in lower follicle apoptosis.[92] As an alternative to the medical protection of the ovaries from the gonadotoxic side effects of treatment, other forms of cancer treatment without gonadotoxic side effects hold promise. Examples of such types of non-gonadotoxic cancer therapies that might eliminate the need for fertility preservation in the future include cancer immunotherapy,[96,99], the genetic modification of T-cells,[96,100] and the administration of ascorbate (vitamin C)[101] or hyperthermia.[23]

Implications for clinical practice and future research

Although several implications for clinical practice and research were already mentioned, we will outline the main implications of this thesis here.

Implications for clinicians and laboratory healthcare professionals

» Oncological healthcare providers should discuss fertility preservation with (the parents of) all newly diagnosed female cancer patients of paediatric or reproductive age and refer to a specialist in reproductive medicine.
» To ascertain that patients have enough knowledge to make a high quality decision, fertility preservation counsellors should invest time and effort in counselling, as well as the design of written/online information.
» Laboratories should evaluate the effect of their own cryopreservation and thawing protocols on ovarian tissue viability and modify protocols where needed.
» When planning ovarian tissue autotransplantation, it is recommended to consider the actual scientific knowledge regarding the efficacy of factors that might enhance the process of neovascularisation of the ovarian graft after transplantation.

Implications for (parents of) patients and patients' associations

» Patients should be assertive and ask their physicians for all information they need.
» Patients' organisations can support patients and increase the chance that information on fertility preservation will come across. This could be done via online information, newspapers or magazines, information leaflets, or other communication strategies.
» By participating in research, patients facilitate quality improvement in female fertility preservation care.

Implications for policy makers and healthcare organisations

» To gain knowledge about the safety and efficacy of ovarian tissue autotransplantation, an international register should be initiated by the ASRM and ESHRE, containing clinical information of all cases of autotransplantation including details on the oncological and reproductive follow-up.
» Training as well as an (online) overview with basic information as well as contact persons should be available to oncological healthcare providers to facilitate their discussion about fertility preservation with a patient.
» Hospital boards of hospitals in which fertility preservation counselling and techniques are offered should acknowledge the importance of high quality fertility preservation care and facilitate fertility preservation services.

Implications for future research

» Future research should focus on interventions to enhance or even guarantee the safety of restoring the reproductive potential after ovarian tissue cryopreservation.
» It is important to investigate the effects of clinically used ovarian tissue cryopreservation and thawing protocols on tissue viability in order to identify the protocol that best preserves tissue viability.
» There is an urgent need to design, implement, and evaluate strategies to improve timely referral and equal access to female fertility preservation in clinical practices.
» Study interventions that might improve the information-provision and patient-centeredness of female fertility preservation care are highly needed.
» Research on non-gonadotoxic cancer therapy should continue.

Final conclusions

This thesis demonstrates that various aspects of the quality of female fertility preservation care could be improved. We identified that the risk of cancer recurrence as a result of tumour cell transmission during ovarian tissue autotransplantation depends on diagnosis. As safety with autotransplantation cannot be guaranteed, it is advisable to develop interventions to enhance the safety of restoring the fertility potential after ovarian tissue cryopreservation. These interventions could very well be investigated using the model-system mimicking ovarian tumour cell involvement presented in this thesis. With respect to the efficacy and efficiency of ovarian tissue cryopreservation and thawing, we illustrated the importance of evaluating and improving cryopreservation and thawing protocols in order to optimise ovarian tissue survival. Finally, we identified opportunities to improve the patient-centeredness, equity and timeliness of female fertility preservation care, even in a setting where fertility preservation is reimbursed. Strategies to improve every patient's access to female fertility preservation care should be designed and implemented as soon as possible. In conclusion, healthcare professionals as well as researchers should invest in quality improvement in female fertility preservation care.

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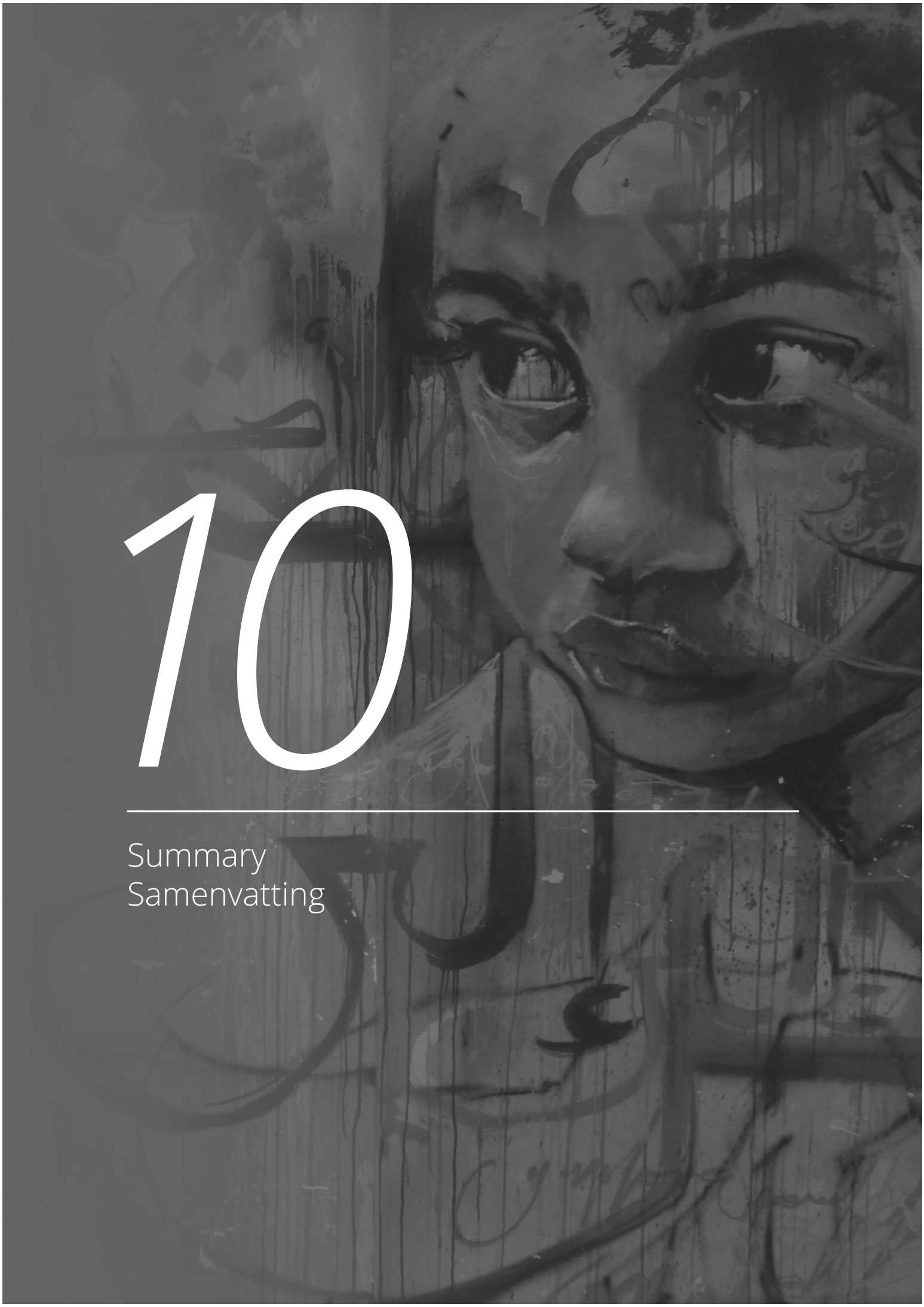

10

Summary
Samenvatting

Summary

The rationale for this thesis on quality of care in female fertility preservation is outlined in the *General Introduction,* **Chapter 1**. In the Netherlands, 2300 to 2400 girls and young women (age 0 – 40 years) are diagnosed with cancer each year. As the survival of young oncological patients has improved considerably during the past decades, issues relating to their quality of life after cancer have become an integral part of oncological care. One of these issues concerns the fertility of cancer survivors. Various types of oncological therapy negatively affect the ovarian and uterine function, which may lead to premature ovarian insufficiency and fertility problems. The field of fertility preservation offers techniques to help patients retain their ability to procreate. These techniques include embryo cryopreservation, oocyte vitrification, cryopreservation and autotransplantation of ovarian tissue, or transposition of the ovaries outside the radiation field. Several questions related to the quality of female fertility preservation care remain unanswered. The aim of this thesis is to address some of these important questions and thereby contribute to improving the quality of female fertility preservation care. In the three parts of this thesis, we focussed on the safety of ovarian tissue autotransplantation in patients who survived cancer **(Part A)**, the efficacy and efficiency of cryopreservation and thawing of ovarian tissue **(Part B)**, and the timeliness, patient-centeredness and equitability of fertility preservation care in general in the Dutch setting **(Part C).**

Safety

In **Part A**, we investigated the safety of ovarian tissue autotransplantation. As shown in **Chapter 2**, cancer cells may be present in the ovaries of oncological patients at the time of cryopreservation. Cancer cells present in the ovarian graft will be reintroduced at the time this tissue is autotransplantated, possibly leading to cancer recurrence. Investigating the ovarian tissue using histology, polymerase chain reaction (PCR), or xenotransplantation prior to autotransplantation, cannot guarantee the absence of cancer cells nor the safety of transplantation. This implicates that, to facilitate clinical decision-making and patient counselling, information should be obtained on the risk of cancer recurrence resulting from the autotransplantation of ovarian tissue in survivors of various types of cancer. In **Chapter 3,** the medical literature on this risk was reviewed. Apart from studies on patients' follow-up after ovarian tissue autotransplantation, we included studies presenting information regarding the detection of cancer cells in ovarian tissue from oncological patients by histology, PCR or xenotransplantation. Moreover, studies on ovarian metastases in patients with various oncological diseases who had *not* cryopreserved their ovarian tissue were included. The 289 included studies revealed that survivors of leukaemia should be most concerned about oncological recurrence after autotransplantation. For this reason, it is advisable to refrain from

autotransplantation in leukaemia survivors. In addition, serious reasons for concern were found for patients with gastric, colorectal, or endometrial cancer. Patients with breast or cervical cancer have less reason for concern, although the risk of recurrent disease after autotransplantation should be further specified for various histological tumour subtypes. The most reassuring data on the safety of ovarian tissue autotransplantation were found for patients with lymphoma. As the safety of ovarian tissue autotransplantation cannot be guaranteed for patients with any oncological diagnosis, the risk of cancer recurrence due to the transmission of cancer cells in the graft should be comprehensively discussed with survivors of all types of cancer.

If cancer cells could be eliminated from the ovarian graft without damaging the ovarian tissue, autotransplantation of cortex fragments would be more safe and available to all patients eligible for fertility preservation, regardless of the type of primary tumour. For the development of efficient tumour-purging methods, cortex fragments containing metastases are essential. As such cortex fragments are not sufficiently available for research purposes, we developed a model system for generating metastases-like structures in cortex fragments, presented in **Chapter 4**. Bovine and human ovarian cortex fragments were injected with human Ewing's sarcoma, leukaemia, breast cancer, or lymphoma cells and subsequently cultured *in vitro* for up to 10 days. Formation of metastases-like structures was monitored at different time points. All investigated types of cancer cells were able to form tumour and metastases-like structures in this model system. Proliferation of cancer cells in the ovarian cortex was confirmed by staining with Ki-67, an antigen that is specifically expressed by proliferating cells. Immunohistochemical staining using cancer cell-specific antibodies revealed single neoplastic cells migrating through the ovarian tissue. With this model system, approaches for tumour-purging can be tested preclinically.

Efficacy and efficiency

Part B focusses on the efficacy and efficiency of ovarian tissue cryopreservation and thawing. The effect of cryopreservation and thawing on the viability of human ovarian tissue was explored in **Chapter 5**, as an optimal function of the autotransplanted graft depends on the vitality of the graft after cryopreservation and thawing. In collaboration with one of the most experienced centres in Europe, the Cryobank Bonn (Germany), we investigated the viability of ovarian tissue before versus after cryopreservation/thawing for 25 newly diagnosed cancer patients. When compared to fresh tissue, cryopreserved/thawed tissue had a reduced overall viability, as was indicated by a decreased tissue glucose uptake during *in vitro* culture. In addition, ovarian follicles in cryopreserved/thawed tissue produced lower hormone levels and had higher percentages of morphologically abnormal follicles when compared to fresh tissue. In contrast, the numbers of viable follicles as determined by the Calcein viability assay were comparable before versus

after cryopreservation and thawing. As our study revealed that the overall tissue and follicle viability were not optimally preserved during cryopreservation and thawing, we set out to gain more knowledge about the separate effects of various cryopreservation procedures as well as thawing procedures on ovarian tissue viability. In **Chapter 6**, we investigated the effects of two very different protocols for cryopreservation and thawing. We found that a cryopreservation protocol using DMSO as a cryoprotectant resulted in a better tissue survival when compared to a cryopreservation protocol using ethylene glycol. With respect to thawing, a relatively short protocol led to a similar tissue survival as a lengthy continuous dilution protocol.

Patient-centeredness, timeliness, and equitability

In **Part C**, the current female fertility preservation care was evaluated in the light of patient-centeredness, timeliness and equitability. Fertility preservation starts with comprehensive counselling on the options available. For this reason, the referral of female cancer patients for fertility preservation consultation with a specialist in reproductive medicine was outlined in **Chapter 7**. A total of 233 female cancer patients received fertility preservation consultation at the Radboud university medical centre (Rumc) in the years 2001 - 2013. The annual number of consultations increased during the study period, with a sharp increase after 2006, when a new international guideline on fertility preservation was published. Most patients were counselled because of breast cancer, lymphoma, or a gynaecological malignancy. Patients who were counselled in the most recent years were younger and more frequently without a stable partner relationship than patients counselled earlier. In 2011, 9.8% of the female patients aged 0-39 years who were newly diagnosed with cancer in the Rumc's region according to the Dutch Cancer Registry were referred for fertility preservation consultation. Referral disparities were identified with respect to diagnosis and age, with children being referred less often than older patients and women with breast cancer, lymphoma, and leukaemia being referred more often than patients with other malignancies. The referral of female cancer patients for fertility preservation depended on a physician's profession, collaboration with patient associations, discussion about fertility preservation with the patient, and knowledge about where to refer a patient for further counselling. These results illustrate that strategies to improve referral practices and to reduce disparities are urgently needed.

In **Chapter 8**, we investigated the experiences of female cancer patients with respect to fertility preservation counselling by a specialist in reproductive medicine and decision-making on fertility preservation. A questionnaire on experiences with fertility preservation consultation – designed after qualitative research – was retrospectively distributed to 108 patients to whom fertility preservation was offered between July 2008 and July 2013. Using the questionnaire, patients' characteristics, patients' experiences, decisional

conflict, and decision regret were assessed. A total of 64 patients (59.3%) responded. In general, these patients had positive experiences with fertility preservation consultation, but also indicated room for improvement with respect to patient involvement and support, and the counsellor's awareness of patients' personal importance of specific issues for decision-making. Moreover, patients did not always consider the FP option(s) offered to be appropriate in their situation. Patients' negative experiences with the consultation were associated with higher decisional conflict regarding the fertility preservation decision. Furthermore, high decisional conflict during decision-making was related to high decision regret. Given our retrospective design, we were not informed about the causality of the associations observed. However, as long as a causal relationship between the quality of the fertility preservation consultation, patients' decisional conflict, and regret is not refuted, attempts should be made to optimise care in order to attain a higher quality of FP decisions.

In **Chapter 9**, the *General Discussion*, the research questions formulated in the *General Introduction* are addressed. The outcomes of this thesis are discussed in the light of quality improvement in female fertility preservation care. We elaborate on various approaches that can be used to improve the safety, efficacy and efficiency of the cryopreservation, thawing and autotransplantation of ovarian tissue. Furthermore, we discuss how the referral and counselling of female patients for fertility preservation could be improved. The discussion is concluded with some implications for clinical practice as well as recommendations for future research.

Samenvatting

De reden voor het schrijven van dit proefschrift over behoud van vruchtbaarheid voor meisjes en vrouwen van wie de vruchtbaarheid bedreigd wordt, is beschreven in de *Algemene introductie*, **Hoofdstuk 1**. In Nederland worden jaarlijks 2300 tot 2400 meisjes en vrouwen in de leeftijd van 0 tot 40 jaar gediagnosticeerd met kanker. Aangezien de overleving van jonge kankerpatiënten gedurende de afgelopen decennia duidelijk is verbeterd, komt in de zorg de kwaliteit van leven na genezing van kanker steeds meer centraal te staan. Eén van de aspecten van kwaliteit van leven na overleving van kanker betreft de vruchtbaarheid. Verschillende vormen van behandeling van kanker kunnen namelijk de functie van de eierstokken en baarmoeder negatief beïnvloeden met als gevolg een verminderde vruchtbaarheid. Er zijn verschillende technieken beschikbaar die als doel hebben de vruchtbaarheid zo goed als mogelijk te behouden. Hieronder vallen het invriezen van embryo's of eicellen, het invriezen van eierstokweefsel met als doel dit weefsel na genezing terug te transplanteren, of het verplaatsen van de eierstokken buiten het bestralingsgebied. Echter, verschillende vragen ten aanzien van de kwaliteit van zorg rondom het behoud van vruchtbaarheid voor meisjes en vrouwen zijn nog onbeantwoord. Het doel van dit proefschrift was om enkele van deze belangrijke vragen te beantwoorden en hiermee bij te dragen aan een verbetering van de kwaliteit van zorg op dit gebied. In de drie delen van dit proefschrift hebben we ons gericht op de veiligheid van het terug transplanteren van eierstokweefsel bij vrouwen die kanker overleefden **(Deel A)**, de effectiviteit en efficiëntie van het invriezen en ontdooien van eierstokweefsel **(Deel B)** en de tijdigheid, patiëntgerichtheid en gelijke toegang tot de zorg voor behoud van vruchtbaarheid in Nederland **(Deel C)**.

Veiligheid

In **Deel A** hebben we de veiligheid van het terugtransplanteren van eierstokweefsel bij overlevenden van kanker onderzocht. Zoals te lezen is in **Hoofdstuk 2** kunnen er kankercellen aanwezig zijn in de eierstokken op het moment dat eierstokweefsel wordt ingevroren na de diagnose kanker. Deze kankercellen worden terug in het lichaam gebracht op het moment dat het eierstokweefsel waarin ze zich bevinden wordt terug getransplanteerd. Dit kan mogelijk leiden tot een terugkeer van de kanker. Verschillende huidige methoden voor het aantonen van kankercellen in het eierstokweefsel voorafgaand aan de transplantatie geven geen absolute zekerheid en kunnen daarom niet garanderen dat de transplantatie veilig is. Dit betekent dat er, ten behoeve van de klinische besluitvorming en de voorlichting aan patiënten, meer informatie nodig is over het risico van terugkeer van de kanker als gevolg van het terugtransplanteren van eierstokweefsel bij overlevenden van verschillende vormen van kanker. In **Hoofdstuk 3** hebben we daarom de beschikbare medische literatuur ten aanzien van

dit risico samengevat. Naast de studies waarin er werd gezocht naar kankercellen in eierstokweefsel van kankerpatiënten, bekeken we ook studies die beschreven hoe de gezondheid was van patiënten na een transplantatie. Daarnaast zochten we studies die informatie gaven over de aanwezigheid van uitzaaiingen in de eierstok bij verschillende soorten kanker. Deze studies lieten zien dat de kans op terugkeer van de ziekte het grootst was bij overlevenden van leukemie. Het is daarom af te raden eierstokweefsel bij overlevenden van leukemie terug te plaatsen. Ook bij patiënten met maag-, darm- of en baarmoederkanker is het risico op terugkeer van de ziekte aanzienlijk. Bij patiënten met borst- of baarmoederhalskanker was er minder reden tot bezorgdheid, hoewel de risico's verder zouden moeten worden gespecificeerd voor verschillende tumor subtypen. De meest geruststellende gegevens ten aanzien van de veiligheid werden gevonden voor patiënten met een lymfoom. Echter, omdat de veiligheid van het terugtransplanteren van eierstokweefsel bij overlevenden van kanker in geen enkel geval gegarandeerd kan worden, dient het risico op terugkeer van de kanker uitgebreid met hen besproken te worden alvorens eventueel tot een transplantatie wordt overgegaan.

Wanneer kankercellen uit het eierstoktransplantaat zouden kunnen worden verwijderd zonder het weefsel te beschadigen, zou het terugtransplanteren veiliger worden en beschikbaar komen voor alle patiënten, ongeacht hun diagnose. Voor de ontwikkeling van methoden waarmee tumorcellen uit weefsel kunnen worden verwijderd is eierstokweefsel nodig waarin zich uitzaaiingen bevinden. Omdat dergelijk weefsel onvoldoende beschikbaar is voor wetenschappelijk onderzoek, hebben we een modelsysteem ontwikkeld voor het genereren van structuren lijkend op uitzaaiingen in eierstokfragmenten, zoals beschreven in **Hoofdstuk 4**. Eierstokfragmenten van runderen en van mensen werden geïnjecteerd met cellen van verschillende vormen van kanker, namelijk een Ewing sarcoom, leukemie, borstkanker en lymfoom. De geïnjecteerde fragmenten werden gekweekt gedurende 4, 7, of 10 dagen. Alle soorten kankercellen die we onderzochten bleken in staat om structuren te vormen gelijkend op tumoren en uitzaaiingen in het eierstokweefsel. Deling van de kankercellen in het weefsel werd bevestigd met het aantonen van een eiwit (Ki-67) dat alleen aanwezig is in delende cellen. Met behulp van kankercel-specifieke antilichamen lieten we zien dat de kankercellen zich verplaatsten door het eierstokweefsel. Met dit modelsysteem kunnen mogelijke methoden voor het verwijderen van tumorcellen uit eierstokweefsel worden onderzocht.

Effectiviteit en efficiëntie

Deel B betreft de effectiviteit en efficiëntie van het invriezen en ontdooien van eierstokweefsel. Voor het ontstaan van zwangerschappen na de autotransplantatie van ovariumweefsel is een goede functie van het eierstoktransplantaat essentieel. Daarom hebben we in **Hoofdstuk 5** onderzocht wat het effect is van het invriezen en ontdooien

van eierstokweefsel op de vitaliteit van het eierstokweefsel. In samenwerking met één van de meest ervaren centra in Europa, de Cryobank Bonn (Duitsland), hebben we de vitaliteit van het eierstokweefsel voor en na invriezen en ontdooien bij 25 kankerpatiënten onderzocht. In vergelijking met vers eierstokweefsel had het ingevroren en ontdooide weefsel een verminderde vitaliteit, zoals bleek uit een verminderde opname van glucose door het weefsel tijdens een kweekprocedure. Hiernaast produceerden de follikels in het eierstokweefsel minder hormonen en was een groter percentage van de follikels abnormaal van vorm na invriezen en ontdooien. Desondanks was er voor en na invriezen een even groot aantal levende follikels. Aangezien er in deze studie aanwijzingen waren dat de vitaliteit van het eierstokweefsel bij het invriezen en ontdooien niet optimaal behouden bleef, wilden we meer kennis vergaren over de geïsoleerde effecten van verschillende invries- en ontdooiprotocollen. In **Hoofdstuk 6** hebben we daarom de effecten van twee zeer verschillende protocollen voor het invriezen en ontdooien van eierstokweefsel onderzocht. Een invriesprotocol dat gebruik maakte van het antivriesmiddel dimethylsulfoxide (DMSO) bleek te resulteren in een betere overleving van het eierstokweefsel dan een invriesprotocol gebruik makend van ethyleen glycol. Voor wat betreft het ontdooien van het weefsel bleek een kort protocol te leiden tot een vergelijkbare overleving van het weefsel na ontdooien als een langer ontdooiprotocol.

Patiëntgerichtheid, tijdigheid en gelijke toegang

In **Deel C** wordt de huidige zorg rondom behoud van vruchtbaarheid voor meisjes en vrouwen geëvalueerd in het licht van patiëntgerichtheid, tijdigheid en toegankelijkheid tot zorg. Het gebruik van methoden om de vruchtbaarheid te behouden begint met een goede uitleg over alle mogelijke opties aan de patiënt. Daarom hebben we de verwijzing van vrouwelijke kankerpatiënten voor voorlichting over behoud van vruchtbaarheid door een specialist in de voortplantingsgeneeskunde in kaart gebracht in **Hoofdstuk 7**. Van 2001 tot en met 2013 werden er in het Radboudumc 233 vrouwelijke kankerpatiënten voorgelicht. Het aantal vrouwen dat jaarlijks werd voorgelicht steeg gedurende de studieperiode, met een scherpe toename sinds 2006, tevens het moment waarop een nieuwe internationale richtlijn werd gepubliceerd. De meeste patiënten die voorgelicht werden hadden borstkanker, een lymfoom of een gynaecologische vorm van kanker. Patiënten die voorgelicht werden in de meest recente jaren gaven vaker aan geen stabiele relatie te hebben dan patiënten die langer geleden werden voorgelicht. In 2011 werd 9.8% van alle vrouwelijke kankerpatiënten van 0-39 jaar die in de regio van het Radboudumc werden gediagnosticeerd met kanker volgens de Nederlandse Kanker Registratie verwezen voor voorlichting over behoud van vruchtbaarheid. Verschillen in verwijspercentages werden gevonden op basis van diagnose en leeftijd. Kinderen werden minder vaak verwezen werden dan oudere patiënten en vrouwen met borstkanker, lymfoom of leukemie werden vaker verwezen dan patiënten met andere vormen van kanker. Of vrouwelijke kankerpatiënten werden verwezen voor behoud van

vruchtbaarheid hing af van het specialisme van de verwijzer, zijn/haar samenwerking met patiëntorganisaties, in hoeverre het onderwerp met de patiënt besproken werd en of de verwijzende arts kennis had over waar hij/zij een patiënt naartoe kon verwijzen. Deze resultaten laten zien dat er verbetering mogelijk is voor wat betreft het verwijsgedrag voor behoud van vruchtbaarheid en dat het terugdringen van ongelijkheden in de toegang tot deze zorg noodzakelijk is.

In **Hoofdstuk 8** onderzochten we de ervaringen van vrouwelijke patiënten met de voorlichting over behoud van vruchtbaarheid zoals zij die door een specialist binnen de voortplantingsgeneeskunde kregen. Hiervoor hebben we een vragenlijst ontwikkeld die we hebben verspreid onder 108 patiënten die tussen juli 2008 en juli 2013 voorlichting hadden ontvangen. Met behulp van deze vragenlijst werden de ervaringen van patiënten, hun algemene karakteristieken, hun moeilijkheden tijdens het maken van een keuze en spijt ten aanzien van hun keuze in kaart gebracht. In totaal vulden 64 vrouwen (59.3%) de vragenlijst in. Zij rapporteerden positieve ervaringen met de voorlichting, maar gaven ook aan dat er ruimte voor verbetering bestond ten aanzien van het betrekken van de patiënt bij het keuzeproces, het geven van steun en de mate waarin de arts die de voorlichting gaf zich bewust was van de zaken die de patiënt het meest van belang vond in het keuzeproces. In zijn algemeenheid bleken patiënten met negatieve ervaringen met de voorlichting meer moeilijkheden te hebben met het maken van een keuze ten aanzien van behoud van vruchtbaarheid. Daarnaast bleken mensen die meer moeilijkheden hadden met het maken van deze keuze vaker spijt te hebben over de door hen genomen beslissing. Gezien het feit dat wij vrouwen achteraf vroegen de vragenlijst in te vullen, weten we niet of er hier sprake is van oorzakelijke verbanden. Echter, zolang een oorzakelijk verband tussen de kwaliteit van voorlichting over behoud van vruchtbaarheid, moeilijkheden bij de besluitvorming en spijt over de beslissing niet ontkracht is, dient er gestreefd te worden naar het verbeteren van de kwaliteit van zorg om hierdoor betere beslissingen te verkrijgen.

In **Hoofdstuk 9**, de *Algemene discussie* worden de onderzoeksvragen uit Hoofdstuk 1 beantwoord. De resultaten van dit proefschrift worden bediscussieerd in het licht van verbetering van de kwaliteit van zorg rondom behoud van vruchtbaarheid voor meisjes en vrouwen. We bespreken verschillende strategieën voor de verbetering van de veiligheid, effectiviteit en efficiëntie van het invriezen, ontdooien en terugplaatsen van eierstokweefsel. Hiernaast bediscussiëren we hoe de verwijzing en voorlichting ten aanzien van behoud van het vruchtbaarheid kan worden verbeterd. Aan het einde van de discussie worden aanbevelingen gedaan voor de klinische praktijk en voor toekomstig wetenschappelijk onderzoek.

11

Dankwoord
Bibliografie
Curriculum Vitae

Dankwoord

Ik ben aan het einde gekomen van mijn promotietraject! Maar niet zonder de hulp van velen....
Een aantal personen wil ik hier graag in het bijzonder noemen.

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Curriculum Vitae

Lobke Bastings werd op 27 juni 1985 als oudste van twee kinderen geboren te Blerick en groeide op in het Noord-Brabantse Mierlo. Met een duidelijke wens geneeskunde te gaan studeren, doorliep zij het gymnasium aan het Strabrecht College te Geldrop. In 2003 kon zij aansluitend aan het VWO starten met de studie geneeskunde aan de Universiteit van Maastricht. Zij verhuisde naar een kamer aan de Lage Barakken en werd actief binnen het bestuur van MUSTANGH Foundation (Maastricht University Students Twinning A North Ghanaian Hospital). Al in het tweede studiejaar werd haar interesse in het specialisme Verloskunde en Gynaecologie gewekt. Een keuzecoschap in Paramaribo, Suriname, was hierna de eerste *echte* kennismaking met het vak. Teruggekeerd in Nederland werd Lobke lid van de stageplanningsgroep voor het co-schap Verloskunde en Gynaecologie. In 2007 behaalde zij haar doctoraal-diploma *(cum laude),* waarna de nog resterende reguliere co-schappen van het vijfde studiejaar volgden. Voor de invulling van het zesde studiejaar koos Lobke voor een gecombineerde klinische en wetenschappelijke 'semi-arts stage' Voortplantingsgeneeskunde in het Maastricht Universitair Medisch Centrum. In mei 2009 slaagde zij vervolgens voor haar arts-examen.

Vanuit Maastricht verhuisde Lobke naar Enschede waar zij haar eerste werkervaring opdeed bij de afdeling Verloskunde en Gynaecologie in het Medisch Spectrum Twente. Om naast klinisch werk ook wetenschappelijk onderzoek te kunnen doen trok zij in 2010 naar 'de academie', waar zij als fertiliteitsarts aan de slag kon. In deze periode ontstonden de eerste ideeën voor het onderzoek dat later tot dit proefschrift zou leiden. Onderbroken door een leerzame en gezellige periode als ANIOS Verloskunde en Gynaecologie in het Jeroen Bosch Ziekenhuis in 's-Hertogenbosch, keerde zij in 2011 als promovenda en fertiliteitsarts terug naar het Radboudumc. Vanaf 2012 kwam het wetenschappelijk onderzoek in een stroomversnelling terecht door de toekenning van een Junioronderzoekersubsidie door het Radboud Institute for Health Sciences (RIHS). Het proefschrift dat nu voor u ligt is hiervan het resultaat.

Inmiddels werkt Lobke als ANIOS Verloskunde en Gynaecologie in het Catharina Ziekenhuis in Eindhoven. Aansluitend zal zij per 1 januari 2015 starten met haar specialisatie tot gynaecoloog in het Canisius-Wilhelmina Ziekenhuis in Nijmegen (opleider: Dr. M.P.M.L. Snijders).

Lobke is getrouwd met Jim Offerman en woont in Nijmegen.