Safeguarding ovarian tissue autotransplantation in cancer patients

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‘È bello sognare di raggiungere qualcosa che si vuole,
ma è fantastico tentare di raggiungerla sul serio’

Francesco Amadori, 15 januari 1932, Cesena, Italië

Voor mijn ouders en zusjes

Table of contents

Chapter 1

General introduction and thesis outline

Based on:
Non-invasive methods for detecting tumor cells in cortical ovarian strips prior to autotransplantation

Submitted

According to the Dutch Cancer Registry, there were 752,133 cancer survivors in the Netherlands on January 1, 2016,[1] comprising 4.4% of the Dutch population.[2] Of these survivors, 417,326 were female and 3.8% of them were aged < 40 years, resulting in 16,034 female cancer survivors of reproductive age.[1] These survival rates have been continuously rising due to better screening methods for early cancer detection and enhanced treatment modalities, such as chemotherapy and radiotherapy.[3] Unfortunately, chemotherapy and pelvic radiotherapy might be highly gonadotoxic and result in premature ovarian failure/insufficiency, which is defined as amenorrhea due to premature depletion of functional ovarian follicles in women < 40 years.[4] Consequently, these treatments may cause severe morbidities related to estrogen deficiency. These include for instance osteoporosis, increased risk of cardiovascular disease and reduced emotional well-being.[5] In addition, these treatments may have a detrimental impact on future fertility, all of which will profoundly influence the cancer survivor's quality of life. The risk of fertility impairment increases with patient age and is associated with the type, dose and duration of chemotherapy and radiotherapy administered.[6,7]

In order to spare young female cancer patients the deleterious effects of gonadotoxic therapies, much effort has been devoted to preserving fertility. The currently available fertility preservation options for women who receive chemotherapy, whether or not in combination with pelvic radiotherapy, are cryopreservation of either embryos, oocytes, or ovarian tissue.[8] Although cryopreservation of embryos and oocytes are at present the only established options, both methods have several disadvantages. Firstly, they require hormonal stimulation for optimal oocyte harvesting. As a result, these methods cannot be applied to prepubescent girls nor women who lack sufficient time to undergo oocyte retrievals. Secondly, normal ovarian function cannot be restored. Cryopreservation and subsequent autotransplantation of ovarian tissue may overcome these limitations.

Cryopreservation and autotransplantation of ovarian tissue

Cryopreservation of ovarian tissue is the only option available for prepubescent girls and patients who require immediate gonadotoxic treatment because of aggressive malignancies.[8] Furthermore, it is the only fertility preservation method by which ovarian function can be reinstated. To become eligible for ovarian tissue cryopreservation, several selection criteria should be met. These selection criteria include among others: age < 35 years, a good ovarian reserve, a realistic chance of survival, and a high risk of premature ovarian insufficiency (> 50%).[9,10] The selection criteria are indicative. For instance, cryopreservation of ovarian tissue has also been performed in patients who were slightly older.[11-13]

Preferentially, ovarian tissue is cryopreserved before the start of gonadotoxic treatment. After laparoscopic unilateral oophorectomy, the ovary is transferred to the laboratory. At the laboratory, the ovaries are cut into halves and the medulla is removed. The remaining cortex is then trimmed to a thickness of 1-2 mm and subsequently cut into fragments measuring

approximately 5-10 mm in diameter. Following this, the cortical ovarian fragments are frozen, usually according to a slow-freezing procedure, and stored in liquid nitrogen at -196 °C.[12] When the patient experiences premature ovarian failure due to the anticancer treatment and wishes to conceive, the cortical ovarian strips can be thawed and transplanted back to the remaining ovary or a peritoneal window (orthotopic transplantation), or to the abdominal wall or the forearm (heterotopic transplantation).[14] The first case of successful orthotopic autotransplantation in terms of resumption of menses was reported in 2001,[15] followed by the first live birth in 2004.[16] Until now, more than 86 live births have been reported following autotransplantation of ovarian tissue, either by spontaneous conception or by in vitro fertilization.[17] Restoration of ovarian activity has been established in 93% of cases.[14]

Experimental nature

Because of these promising results, some scientists now advocate that autotransplantation of frozen-thawed ovarian tissue should no longer be considered experimental.[18,19] Nevertheless, the American Society for Reproductive Medicine (ASRM) has not yet acknowledged this procedure.[20] The Dutch National Health Care Institute shares the position of the ASRM. This institute recently sent a letter to the Minister of Health, Welfare and Sport to point out that ovarian tissue autotransplantation is not in accordance with the current state of medical science.[21] The underlying reason for this is twofold: firstly, the efficiency of ovarian tissue autotransplantation remains unclear, as pregnancies may occur in the setting of premature ovarian insufficiency and ovarian function may resume after chemotherapy, and secondly, its safety has not been determined for certain types of cancer at risk of ovarian involvement.

Safety concerns with ovarian tissue autotransplantation

The safety of ovarian tissue autotransplantation cannot be ensured, as it has not yet been possible to rule out the presence of malignant cells in the cortical ovarian tissues that are transplanted. This shortcoming is attributable to the fact that after examination by the currently available tumor detection methods (i.e. histology/immunohistochemistry, PCR and xenotransplantation) the cortical ovarian tissues can no longer be used for transplantation to the patient. As a result, the current tumor detection approach includes assessment of only one or two cortical ovarian fragments that are not transplanted, whereas cortical ovarian tissue fragments that are placed back remain unchecked.[22] Autotransplantation of ovarian tissue thus entails a risk of reimplanting malignant cells in the recipient.

Several studies have found that the risk of reintroducing cancer varies widely among various tumor types, with the highest and lowest risk for leukemia and lymphoma, respectively.[7,23,24] The results presented in these studies were mainly based on histology, PCR and xenotransplantation tests that were performed on single ovarian tissue fragments. These reports are valuable with respect to providing insight regarding the extent to which cancer cells disseminate following

transplantation of ovarian tissue. For instance, they showed that there is a genuine risk of reimplanting leukemic cells following ovarian tissue autotransplantation, since PCR tests frequently showed positive molecular markers in ovarian tissues.[25-27] On the other hand, in patients with lymphoma the risk of reimplanting malignant cells may be underestimated, as evaluating a randomly selected ovarian tissue fragment does not necessarily eliminate the possibility that micrometastases are present in the strips that are ultimately transplanted.[22,28,29]

Evaluation of the current tumor detection approach

In order to determine whether examining cortical ovarian strips that will not be transplanted can accurately predict the absence of tumor cells in the ovarian tissue that will be transplanted, we must understand the localization and morphology of metastatic disease in the ovary. Figure 1 illustrates the different morphological features of disseminated tumor cells in ovarian tissues. For example, if tumor cells are homogenously scattered in the ovarian parenchyma (Figure 1a), examining one or two cortical ovarian strips will suffice to determine the presence of tumor cells in the actual ovarian autografts. On the other hand, if tumor cells are restricted to a relatively small region in the ovarian cortex (Figure 1b and Figure 1c), this approach could lead to an incorrect diagnosis. Then, cortical ovarian strips that are examined might be devoid of tumor cells, whereas cortical ovarian tissues that are transplanted could harbor a metastasis, potentially causing recurrent disease.

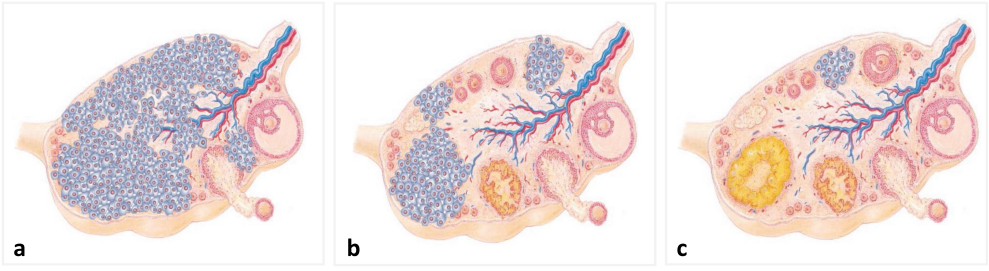

Figure 1. Morphological features of disseminated tumor cells within ovarian tissues

Three examples are shown: **(a)** diffuse seeding without any discernable pattern, **(b)** multiple distinct nodules separated by uninvolved ovarian tissue and **(c)** a solitary metastasis. The current tumor detection approach can be considered inadequate if disseminated tumor cells manifest as a solitary metastasis or multiple distinct nodules within ovarian tissues. In those cases, the absence of metastatic disease in the remaining ovarian autografts cannot be guaranteed when the results of testing one fragment are negative for the presence of tumor cells. The images are adapted from Cummings.[30] Tumor cells are depicted as blue cells with a red nucleus.

Morphology and localization of ovarian metastases

A detailed review of the literature on the morphology and localization of ovarian metastases derived from primary neoplasms in which cryopreservation of ovarian tissue is performed, is summarized below.

Breast cancer

Breast cancer is the most commonly diagnosed malignancy in women. Approximately 7% of cases diagnosed in Western Europe and the United States are younger than 40 years of age.[31-33] The reported prevalence of ovarian metastases in breast cancer patients ranges from 13-47% (Table 1).[23,34] The majority of these women are diagnosed with advanced breast cancer.[35-38] With respect to histological subtype, lobular breast carcinomas are more likely to invade the ovary than ductal carcinomas.[39,40] Kasilag et al. examined 23 premenopausal patients with ovarian metastases.[38] Four of these cases had diffuse seeding with no discernable pattern, 11 cases had hilar involvement, and one case had a mixed pattern. The remaining seven cases had involvement at the ovarian surface. Gagnon et al. examined 59 ovarian metastases derived primarily from advanced breast tumors.[41] Up to one-third of the ovarian tumors were <1 mm in diameter, and typical Indian-file and/or ductal patterns were identified in 75% of cases. In 10% single tumor cells were seen.

Cervical cancer

Cervical cancer is the second most commonly diagnosed cancer in women in developing countries and the seventh most common cancer in developed countries.[42] More than one-fourth of women with cervical cancer are of reproductive age.[43] Metastases from the cervix to the ovary are present in up to 4% of premenopausal women with FIGO stage Ia-IIb cancer,[23,44] and are more prevalent in adenocarcinoma than in squamous cell carcinoma.[45-47] Strikingly, Natsume et al. reported that 14% of patients with stage Ib and stage II cervical adenocarcinoma have ovarian metastases (Table 1).[47] The two cases described showed lymphovascular invasion and in one of these two cases the pelvic lymph nodes appeared to be involved. Lymphatic spread may be a plausible route for ovarian metastases, as metastases are frequently found in the presence of lymphatic permeation.[44,48] The finding of metastatic lesions in the hilus of two patients with stage IIb adenocarcinoma and nodal involvement supports this hypothesis.[49] In addition to localization in the ovarian hilus, microscopic metastases can also manifest in the ovarian cortex with histological features similar to the endocervical tumor.[45,50]

Endometrial cancer

Endometrial cancer is the most common malignancy in the female genital tract in Western countries. It is usually diagnosed in elderly women, although approximately 5% of cases appear under the age of 40.[51,52] Women with an unfulfilled child wish may be considered for conservative

endocrine therapy if an absolutely certain diagnosis of an early-stage well-differentiated carcinoma is made.[53,54] Ovarian involvement occurs in 2-42% of young women with FIGO stage I-IV endometrial cancer (Table 1).[23,55-58] Bilateral ovarian involvement, a multi-nodular growth pattern, surface implants, and prominent lymphovascular permeation within and/or adjacent to the ovary should raise suspicion of a metastasis rather than a primary ovarian malignancy.[39,58]

Colorectal cancer

With respect to colorectal cancer, autotransplantation of frozen-thawed ovarian tissue was performed in a 28-year-old patient with invasive anal carcinoma.[59] In this patient, serial sections of six ovarian specimens measuring 2 mm in diameter revealed no malignant cells. Overall, the percentage of ovaries containing bowel tumor cells is approximately 3%, but can reach 33% among premenopausal women (Table 1).[60] The most common microscopic findings are tall columnar cells, dirty or segmental necrosis, and a 'garland-like' growth pattern.[39,61]

Leukemia

Leukemia is the most common hematological cancer among young girls and adolescent females[40] and can be classified into four subgroups: acute lymphoblastic leukemia (ALL), chronic lymphoblastic leukemia (CLL), acute myeloid leukemia (AML), and chronic myeloid leukemia (CML). Because PCR tests have consistently yielded positive results for the presence of a disease-specific molecular marker in cortical ovarian strips and xenotransplantation into nude mice has induced tumors, autotransplanting ovarian tissue obtained from leukemia patients is currently considered too dangerous.[26,27] The likelihood of ovarian metastases occurring during the disease course of leukemia is generally up to 62% (Table 1),[23] and differs between the various subtypes. For example, an analysis of 4728 autopsy reports found ovarian metastases in 41-50% of patients with ALL, 21-30% of patients with CLL or AML, and 11-20% of patients with CML.[62] However, few studies have examined the precise location at which the leukemic cells are trapped within the ovary, although Reid and co-workers reported a large mass of leukemic cells in the ovarian medullas of a one-year-old girl with AML and a nine-year-old girl with ALL.[63]

Lymphoma

Bittinger et al. emphasized the need for a technique that can examine every cortical ovarian strip used for transplantation purposes.[29] They examined two 10-mm ovarian fragments of one ovary obtained from a patient with stage IIIb Hodgkin's lymphoma. Surprisingly, conspicuous malignant cells were found in one fragment, whereas the other fragment was completely devoid of tumor cells. In an autopsy study, Kyono et al. found ovarian metastases in 13% of lymphoma patients, although the stage of their disease was not specified (Table 1).[64] Lastly, Monterroso et al. described eighteen patients under the age of 40 with advanced-stage malignant lymphoma and ovarian involvement.[65] These secondary tumors were composed primarily of small, non-cleaved

cells consistent with aggressive Burkitt's type lymphoma and were distributed throughout the ovarian parenchyma. In addition, some cells were arranged in an Indian-file pattern and were more prominent in the periphery of the ovarian cortex.

Bone and soft tissue tumors

Osteosarcoma, Ewing's sarcoma, and chondrosarcoma are the most prevalent bone cancers in children and young adults.[66-68] Among soft tissue tumors, rhabdomyosarcoma is the most common form and is often diagnosed in early childhood.[69] Although bone tumors rarely metastasize to the ovaries, several cases have been reported. For example, a 23-year-old woman with a history of osteosarcoma presented with an ovarian mass seven years later.[70] Microscopically, the tumor consisted of rare foci containing osteoid material and sheets of malignant cells. Three cases of Ewing's sarcoma have also been described with a predominantly diffuse growth of small cells.[71-73] Sporadically, discrete nodules of tumor separated by uninvolved ovary were present. Regarding chondrosarcoma, the ovarian tumor of an 18-year old woman showed multiple confluent nodules containing small groups and cords of cells as well as single cells in a background that focally showed cartilaginous differentiation.[72] With respect to rhabdomyosarcoma, Young reported the presence of large nodules of neoplastic cells situated primarily in the superficial ovarian cortex as well as in several vascular spaces.[74]

Table 1. The prevalence of ovarian metastasis according to cancer type in women < 50 years of age

Primary tumor	Prevalence of ovarian metastasis	References
Breast cancer	13.2 - 46.7%	23, 24
Cervical cancer	0.0 - 14.3%	23, 45
Endometrial cancer	1.9 - 41.7%	23, 56-58
Colorectal cancer	2.5 - 33.3%	23
Leukemia	<0.7 - 62.3%	23
Lymphoma	10.5 - 13.3%	23
Bone and soft tissue tumors	Not reported*	Not applicable

* A prevalence rate cannot be provided, since only single cases are reported.

Based on these findings, we conclude that metastases can be confined to a specific region in the ovarian cortex. Since the volume of a single cortical ovarian strip is extremely small compared to the volume of the entire ovarian cortex,[75] examining only one or two cortical ovarian fragments that are ultimately not transplanted carries a risk of reintroducing cancer in the patient. It should be noted, however, that the vast majority of published data regarding the histological features of ovarian metastases are derived from patients with advanced stage disease, whereas most patients undergo cryopreservation of ovarian tissue in an early stage of disease. Nevertheless, the paucity of available information does not necessarily mean that tumor cells do not reach the ovary in early stage disease.

Non-invasive tumor detection methods

One approach to safeguard the transfer of cortical ovarian tissue to the patient would be to develop methods that can be used to exclude the presence of metastases in ovarian autografts without affecting the tissue's viability. Two novel techniques might potentially be suitable: near-infrared fluorescence imaging and full-field optical coherence tomography.

Near-infrared fluorescence imaging

Near-infrared fluorescence (NIRF) imaging can be used to differentiate malignant tissue from healthy tissue in real time without affecting the tissue being examined. The advantages of using NIRF imaging rather than visible light include substantial deeper tissue penetration and less autofluorescence, thereby rendering sufficient contrast. Because the human eye is insensitive to near-infrared light, an imaging system is required to perceive the fluorescent light.[76] The imaging system is equipped with a spectrally resolved light source that excites a fluorophore and a charge-coupled device (CCD) camera to image the light emitted from the fluorophore.[77,78]

In the field of surgical oncology, NIRF imaging has been used successfully using the non-targeting agents indocyanine green and methylene blue to identify tissues that need to be resected, for instance tumors and affected lymph nodes, as well as structures that need to be spared, like ureters and bile ducts.[79-84] Due to extensive angiogenesis and decreased lymphatic drainage, known as the 'enhanced permeability and retention (EPR) effect' commonly seen in malignancies, these non-specific targeting NIRF agents freely pass through the capillary walls and accumulate in the surrounding tumor tissue, thereby revealing the location of cancer.[85] NIRF-labeled tumor-targeting moieties can be used to detect cancer with far more accuracy. These tumor-targeting probes consist primarily of two components: firstly, antibodies or peptides binding with high affinity to proteins at the cell surface of specific tumor cells, and secondly, a fluorophore that emits light in the near-infrared range (λ = 700-900 nm).[86,87] The moieties used to visualize these tumors exploit the hallmarks of cancer, including increased levels of growth factor receptors, limitless replicative potential, sustained angiogenesis, and increased proteolytic activity leading to tissue invasion and metastasis.[86]

Van Dam et al. were the first to show in-human use of a fluorescent agent beyond the NIR spectrum targeted to folate receptor alpha (folate-FITC) to increase the accuracy of cytoreductive surgery in patients with primary ovarian cancer.[88] The authors showed that ovarian metastases could be clearly identified using fluorescence imaging. Our group recently confirmed these results in a larger cohort of ovarian cancer patients,[89] and demonstrated that the intraoperative use of OTL38, a folate analogue conjugated to a NIRF dye, led to an additional 29% resection of disseminated ovarian cancer lesions that were otherwise not detected by standard visual and tactile inspection.[90] Figure 2 shows a peritoneal metastasis from a serous ovarian adenocarcinoma that was intraoperatively detected by OTL38. Burggraaf et al. demonstrated that a higher number of colorectal polyps could be endoscopically resected after intravenous administration of a fluorescently labeled peptide against c-Met than by conventional colonoscopy.[91] This probe was

also well tolerated by the patients. Two recently published phase I trials reported the feasibility of bevacuzimab-IRDye800CW targeting vascular endothelial growth factor (VEGF)-A in breast cancer and the therapeutic monoclonal antibody cetuximab targeting epidermal growth factor receptor (EGFR) in patients with head and neck cancer, respectively.[92,93] Lastly, the activatable fluorescent probe LUM015 could be safely administered to breast cancer patients and provided excellent tumor-to-normal tissue contrast after cleaving by proteases, which were overexpressed by the primary breast tumor.[94] The above-mentioned in-human studies emphasize the great potential of NIRF imaging in the field of oncology, including the detection of occult metastastic disease.

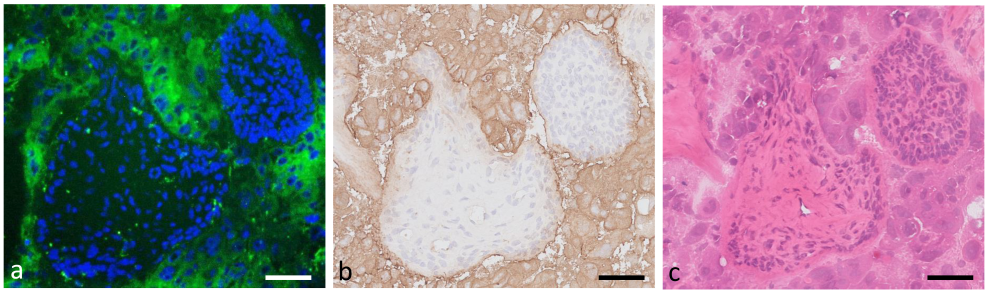

Figure 2. Histopathologic evaluation and fluorescence signal in ovarian cancer
Representative image of membranous and cytoplasmic accumulation of OTL38 in tumor cells visualized by fluorescence microscopy **(a)**. The fluorescence signal is indicated in green, and the nuclei were counterstained with DAPI (blue). The fluorescence pattern is consistent with FRα expression analyzed with immunohistochemistry **(b)**, and corresponds to a peritoneal metastasis derived from a serous ovarian adenocarcinoma, as observed on hematoxylin and eosin (H&E) staining **(c)**.

In order to use NIRF imaging for the detection of metastatic tumor cells in cortical ovarian tissue prior to cryopreservation, a tumor-specific NIRF probe should be intravenously administered to the patient before oophorectomy. After oophorectomy, the ovary can be dissected into cortical ovarian strips. Because these strips usually measure 5-10 mm in diameter and 1-2 mm in thickness,[11] an imaging system is required that allows detailed images to be obtained at a depth of at least 1 mm. A multiphoton microscope seems to be an appropriate device to achieve this.[95-97]

Full-field optical coherence tomography

Full-field optical coherence tomography (FF-OCT) generates real-time histology-like images from tissue samples at a depth of up to 500 μm.[98] The system consists of an upright microscope and a reference arm in the Linnik interferometric configuration. The tissue sample is placed under the objective and the light reflected or backscattered by the tissue sample interferes with the light reflected by the reference mirror. The returning light is then combined and collected by a detector, after which an *en face* tomographic image is created.[99,100] FF-OCT imaging can be

performed without the need of tissue manipulation or staining. Studies in breast and skin tissues revealed that several distinct tissue types can be distinguished based on their light-scattering properties.[101,102] For example, adipocytes scatter light poorly and therefore appear as dark rounded structures. In contrast, connective tissue scatters light more broadly and appears gray. Loose connective tissue has a 'wavy' appearance, whereas dense connective tissue appears more compact and organized. Figure 3 shows a representative FF-OCT image of primary invasive ductal breast cancer in which the collagen bundles can be clearly recognized. Until now, FF-OCT imaging has proven its efficacy in detecting lung,[100] kidney,[103] brain,[104] and prostate[105] cancer.

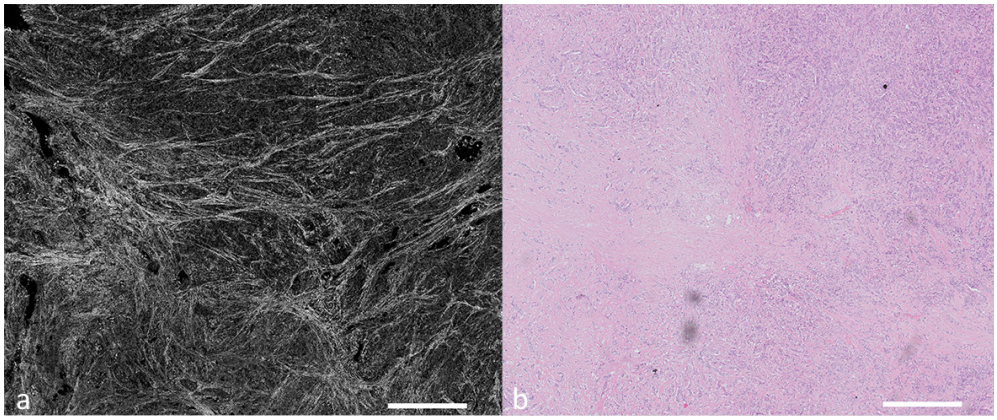

Figure 3. Representative FF-OCT image and corresponding histology image of primary invasive ductal breast cancer
Collagen bundles are clearly shown in the FF-OCT image **(a)**, corresponding to the stromal reaction in the haematoxylin-and-eosin staining **(b)**. Scale bars represent 500 µm.

This thesis

With the studies presented in this thesis, we aimed to make a step forward in determining the safety of ovarian tissue autotransplantation. Besides, we aimed to develop novel detection methods by which the actual ovarian autografts can be examined. This thesis therefore focuses on further unravelling the accuracy of the current tumor detection approach to establish the need for an alternative detection method by which every ovarian autograft can be examined, and the feasibility of detecting ovarian metastases by NIRF imaging and FF-OCT. Since breast cancer is one of the primary indications for cryopreservation of ovarian tissue and relatively much ovarian tissue is available from breast cancer patients due to prophylactic or therapeutic oophorectomies, this thesis is mainly dedicated to studies on ovarian metastases from primary invasive breast cancer.

Objectives of the work described in this thesis

- To establish the prevalence of ovarian metastases among young patients diagnosed with primary invasive breast cancer in the Netherlands **(chapter 2)**
- To investigate the clinicopathological characteristics of young women diagnosed with primary invasive breast cancer and ovarian metastases, and to identify risk factors for the development of ovarian metastases **(chapter 2)**
- To assess the distribution of breast tumor cells in ovarian tissues from young patients who were diagnosed with ovarian metastases derived from primary invasive breast cancer in order to evaluate the current approach for tumor detection in ovarian tissues considered for autotransplantation **(chapter 4)**
- To determine which cell-surface proteins are suitable as a target for tumor-specific imaging of ovarian metastases derived from primary invasive breast cancer **(chapter 3 and chapter 4)**
- To analyze whether invasive breast cancer tissues can be used to predict the most suitable target for the detection of ovarian metastases in a particular patient by tumor-specific imaging **(chapter 4)**
- To examine whether FF-OCT is an appropriate approach for the non-invasive detection of ovarian metastases **(chapter 5)**

In **chapter 6**, the general discussion, the findings of the work presented in this thesis are summarized and placed in a broader perspective. The general discussion is followed by a summary in Dutch.

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Chapter 2

Prevalence and risk factors of ovarian metastases in breast cancer patients < 41 years of age in the Netherlands: a nationwide retrospective cohort study

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Abstract

Background

Breast cancer is one of the primary indications for cryopreservation and subsequent autotransplantation of ovarian tissue. The safety of this fertility preservation method remains questionable, as the presence of disseminated breast tumor cells cannot yet be excluded in the ovarian autografts. We explored the prevalence of ovarian metastases among young breast cancer patients and determined risk factors for the development of ovarian metastases.

Methods

Using the nationwide database of the Dutch Pathology Registry (PALGA), we identified a cohort of 2648 women with primary invasive breast cancer at age < 41 years in the period 2000-2010 in the Netherlands who subsequently underwent an oophorectomy. From this source population, all cases who had histologically confirmed ovarian metastases were included. For each case of whom clinical data were available, one control without ovarian metastases who matched the time interval between breast cancer diagnosis and oophorectomy was selected. Data were collected on patient characteristics, diagnosis, treatment and follow-up.

Results

Ovarian metastases were found in 63 out of 2648 patients who met the inclusion criteria. The risk of developing ovarian metastases increased with time passed since breast cancer diagnosis. Multivariate logistic regression analyses showed significant association between tumor stage and the development of ovarian metastases ($p = 0.024$).

Conclusion

The prevalence of ovarian metastases was 2.4% among young breast cancer patients. Early ovary removal may reduce the risk of developing ovarian metastases. In breast cancer patients with tumors > 5 cm and/or inflammatory carcinoma, we recommend a cautious approach to ovarian tissue autotransplantation.

Introduction

Breast cancer is the most frequently diagnosed malignancy among women with worldwide around 230.000 new cases in 2015.[1] Approximately 5% of these women were aged younger than 40 years at the time of diagnosis.[2] In these young women, chemotherapy may result in premature ovarian failure[3] and could pose a threat to ovarian function and future childbearing potential. Fertility preservation is therefore of crucial importance. In addition to cryopreservation of embryos and oocytes, which are currently the most established options to preserve fertility, cryopreservation followed by autotransplantation of ovarian tissue is progressively emerging. This approach does not only offer young women the chance to conceive and have their own genetic offspring, but also provides the opportunity to restore their endocrine function.[4,5] In recent series, restoration of ovarian activity has been observed in 93% of cases[6] and 60 live births have now been reported.[7]

Despite these favorable outcomes, the safety of this method remains of great concern, since ovarian tissue may contain malignant cells derived from the primary invasive breast tumor. Previous studies, mainly comprising autopsies, prophylactic and therapeutic oophorectomies, showed that ovarian metastases occur in 13-47% of breast cancer patients.[8-10] By contrast, in early-stage breast cancer patients who were eligible for cryopreservation of ovarian tissue, immunohistochemical examination of cortical ovarian biopsies did not disclose any malignant cells.[11-13] Quantitative PCR analysis of frozen-thawed cortical ovarian fragments from patients with advanced-stage breast cancer revealed cells that expressed the mammaglobin B (*MGB2*) gene, which is associated with breast cancer.[14] However, whether these cells bear any malignant potential remains unclear.

Although the results with respect to cryopreservation of ovarian tissue are relatively reassuring, it should be stressed that only a few cortical ovarian fragments were included for analysis, since the current tumor detection methods (i.e. immunohistochemistry, PCR analysis) render the ovarian tissues unsuitable for autotransplantation. It therefore remains difficult to estimate the prevalence of ovarian metastases in breast cancer patients who are considered for ovarian tissue cryopreservation. Furthermore, as a consequence of this approach, malignant cells that have disseminated to the ovarian autografts cannot be excluded and might be reimplanted upon autotransplantation of ovarian tissue.

In this study, we aimed to explore the prevalence of ovarian metastases among young patients diagnosed with primary invasive breast cancer in order to assess the risk of reimplanting malignant cells following autotransplantation of ovarian tissue. In addition, we identified risk factors associated with the presence of ovarian metastases in young patients diagnosed with primary invasive breast cancer in order to more thoroughly define selection criteria for cryopreservation of ovarian tissue in breast cancer patients.

Methods

Patient selection and data collection of the study population

Via a nationwide search performed by PALGA, the Dutch histopathology and cytopathology network and archive that encompasses all pathology laboratories within the Netherlands,[15] a source population was compiled. This source population consisted of all patients who were diagnosed with primary invasive breast cancer at age < 41 years in the period 2000-2010 who subsequently underwent a unilateral or bilateral oophorectomy for any reason (n = 2648; Figure 1). From this source population, all patients who had histologically confirmed ovarian metastases derived from primary invasive breast cancer were selected (n = 69; cases). Patients who were diagnosed with primary ovarian cancer or a borderline ovarian malignancy were excluded (n = 44). From the remaining group of patients who had normal ovaries or benign ovarian abnormalities (n = 2535; controls), all patients who were treated in the same hospitals as the cases were taken (n = 2036). For each case of whom clinical data were available (n = 57), one control without ovarian metastases was included who matched the time interval between the diagnosis of breast cancer and oophorectomy (n = 57; matched controls).

Clinical data were extracted from the patient's files after approval by the medical ethical committee of the Leiden University Medical Center (protocol number P14.106) and the local ethical committee of the participating hospitals. Data were collected on patient characteristics, diagnosis of breast cancer, treatment and follow-up. Furthermore, data were sought on date of oophorectomy, age at oophorectomy, reasons to perform ovarian surgery and diagnosis.

From the primary invasive breast tumors in which the *HER2/neu* gene amplification status was not yet determined, formalin-fixed paraffin-embedded (FFPE) tissue samples were requested from the pathology laboratories. Following this, immunohistochemistry was performed on 3-µm thick FFPE tissue sections using primary antibodies against Her2/neu (ERBB2, rabbit polyclonal, Dako, Denmark), as described previously.[16] Primary invasive breast tumors that showed immunohistochemical reactions of 0 and 1+ were considered negative. In primary invasive breast tumors that showed 2+ or 3+ immunohistochemical reaction,[17] chromogenic silver in situ hybridization (SISH) was carried out using the Ventana SISH kit on Benchmark XT to establish the final *HER2/neu* status (amplification or no amplification).[18]

All patient samples and clinical data were handled in accordance with the medical ethics guidelines described in the Code of Conduct for Proper Secondary Use of Human Tissue of the Dutch Federation of Biomedical Scientific Societies (FMWV).[19]

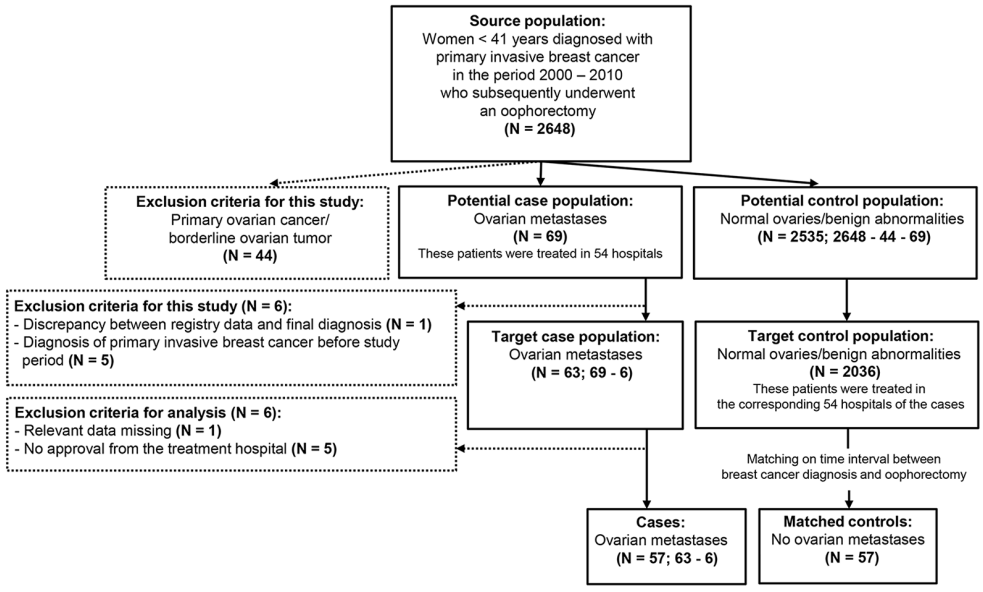

Figure 1. Flow chart of selection of cases and matched controls
The source population was compiled by the Dutch histopathology and cytopathology network. The exclusion criteria are indicated in the dotted boxes.

Validation of the control population

In order to estimate whether the matched controls to some extent also reflected women diagnosed with primary invasive breast cancer at age < 41 years whose ovaries remained in situ, the matched controls were compared to a cohort of patients who did not undergo an oophorectomy. To this end, all patients diagnosed with primary invasive breast cancer at age < 41 years who were treated in the corresponding 54 hospitals, were selected from the Dutch Cancer Registry (n = 7299; Figure 2). After notification by PALGA, patients who had undergone an oophorectomy or who were either not or double registered in the PALGA registry, were excluded (n = 2355). The remaining group of patients exclusively consisted of breast cancer patients who were younger than 41 years of age at the time of diagnosis and did not undergo ovarian surgery (n = 4944). From these patients, data on the diagnosis of breast cancer, staging and treatment were collected from the medical records by trained registry personnel using the registration and coding manual of the Comprehensive Cancer Center the Netherlands (CCCN). This group of patients was further indicated as CCCN controls in this study.

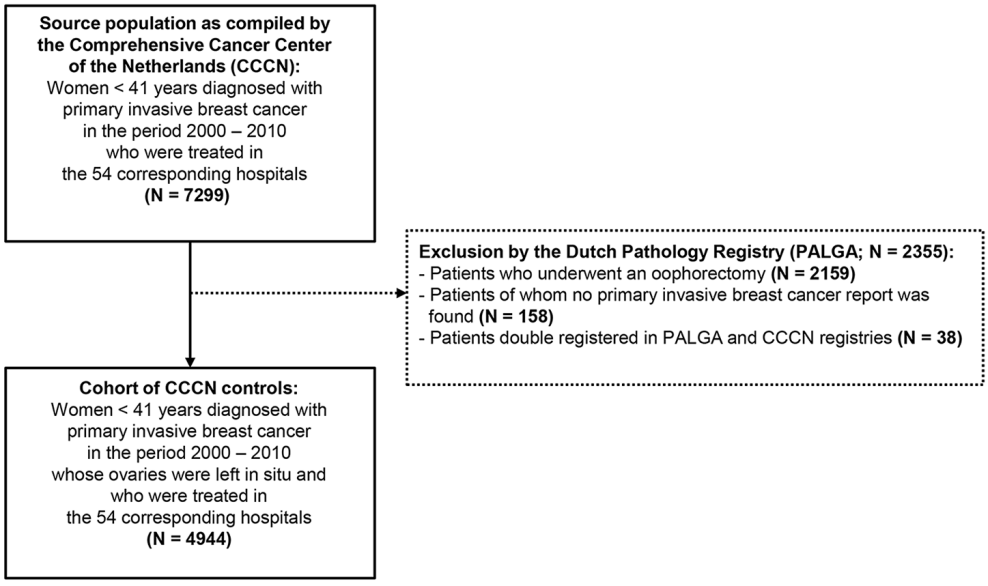

Figure 2. Flow chart of selection of patients diagnosed with primary invasive breast cancer at age < 41 years who did not undergo an oophorectomy

This cohort of patients was compiled by the Comprehensive Cancer Center the Netherlands (CCCN) and indicated as CCCN controls in the study. The exclusion criteria are indicated in the dotted box.

Statistical analysis

Statistical analysis was performed using SPSS version 23.0 (IBM, Armonk, NY). Logistic regression analyses were used to identify predictors for the development of ovarian metastases in the study population and for comparing the current control group with the cohort of CCCN controls. Missing values were accounted for by 10-fold multiple imputation, in which all risk factors and the case-control status in the imputation models were included. In some cases, logistic regression analyses could not be performed because of empty categories. In those cases, the Pearson Chi-square test was used. Factors that were associated with the development of ovarian metastases ($p < 0.100$) in univariate logistic regression models were included in multivariate logistic regression analyses. Survival rates were calculated according to the Kaplan Meier method. Statistical significance was assigned at the level of $p < 0.05$.

Results

Prevalence of ovarian metastases

According to the PALGA registry, 2648 patients were diagnosed with primary invasive breast cancer at age < 41 years in the period 2000-2010 who subsequently underwent a unilateral or bilateral oophorectomy (Figure 1). Among these women, 69 patients (2.6%) had histologically

confirmed ovarian metastases. Yet, in one patient the registry data did not correspond to the final pathological diagnosis. Moreover, in five patients the diagnosis of primary invasive breast cancer was made before the study period. Thus, strictly, ovarian metastases were found with a prevalence of 2.4% (63 out of 2642) in patients with primary invasive breast cancer at age < 41 years in the period 2000-2010 in the Netherlands.

Clinicopathological characteristics of the cases

Clinical data were available for 57 patients diagnosed with primary invasive breast cancer and ovarian metastases (Figure 1). The median age at the time of breast cancer diagnosis was 37.0 years (range 28-40 years). Ten patients (17.5%) were tested for the presence of a *BRCA* gene mutation and one of them resulted positive; the *BRCA* gene mutation status in the remaining patients was unknown. Forty-four patients (77.2%) were diagnosed with invasive ductal breast cancer and eight patients (14.0%) were diagnosed with invasive lobular breast cancer. The remaining five patients (8.8%) had invasive ductolobular breast cancer. Fifty-one patients (89.5%) had hormone-sensitive breast cancer. *HER-2/neu* gene amplification was observed in eight of 56 tumor samples tested (14.3%); of the remaining tumor, no tissue was available. The majority of patients had positive axillary lymph nodes and 41 patients (71.9%) had tumors larger than 2 cm in diameter of whom five patients presented with inflammatory breast cancer. Nine patients (15.8%) had distant metastases outside the ovary at the time of diagnosis of primary invasive breast cancer; eight patients had bone metastases of whom two had synchronous liver metastases, and one patient was diagnosed with both pulmonary and retinal metastases. Surgical resection of the primary breast tumor was performed by either breast conserving surgery (18 patients; 31.6%) or mastectomy (33 patients; 57.9%). Six patients (10.5%) did not undergo any surgical treatment, because of diffuse metastatic disease. Adjuvant chemotherapy was administered to 35 patients (61.4%), 36 patients (63.2%) underwent locoregional radiotherapy and 45 patients (78.9%) received hormonal treatment.

The median time between the diagnosis of breast cancer and oophorectomy was 48.7 months (range 0.3-141.8 months). Apart from the nine patients who already had distant metastases at the onset of breast cancer, 33 patients (57.9%) developed a locoregional or distant recurrence prior to oophorectomy. The presence of ovarian metastases was the first manifestation of recurrent disease in fifteen patients (26.3%). Thirty-four patients (59.6%) had ovarian metastases in both ovaries. In seven patients (12.3%) one or both fallopian tubes were involved, whereas in 33 patients (57.9%) the fallopian tubes were free of metastatic disease. Of the remaining 17 patients (29.8%), no data on the fallopian tubes were available. Seven patients (12.3%) had peritonitis carcinomatosa at the time of oophorectomy. The median duration of follow-up was 152.8 months (range 9.9-166.6 months). During follow-up, 43 patients (75.4%) died, all because of metastatic breast cancer. The median time from the diagnosis of ovarian metastases to death was 24.0 months (range 2.3-118.7 months). The 5-year disease-specific survival was 69.5%.

Risk factor analysis for the development of ovarian metastases

The time interval between the diagnosis of breast cancer and oophorectomy significantly differed between the 63 cases who were diagnosed with ovarian metastases and who met the inclusion criteria, and the 2535 controls without ovarian metastases in the source population, 47.0 and 32.0 months, respectively ($p = 0.002$). In order to identify baseline risk factors that are associated with the development of ovarian metastases, the time interval between the diagnosis of breast cancer and oophorectomy should be comparable between the cases and controls. Therefore, the 57 cases of whom clinical data were available, were matched on this time interval to an equally large cohort of controls (Figure 1).

Table 1 shows the indications for oophorectomy in the cases and the matched controls. The cases had significantly more often abnormal ovaries on preoperative transvaginal ultrasonography or MRI than the matched controls, 26.3% versus 3.5%, respectively ($p = 0.000$). The two matched controls who presented with abnormal ovaries were diagnosed with a serous cystadenoma and an epithelioid cell granuloma, respectively. The 42 cases who presented with normal ovaries on transvaginal ultrasound underwent oophorectomy because of prophylactic or therapeutic reasons. In those cases, the ovarian metastases were clinically indolent. This emphasizes the need to determine which young breast cancer patient is at risk of developing ovarian metastases.

Table 1. Indications for oophorectomy in patients diagnosed with primary invasive breast cancer at age < 41 years with and without ovarian metastases

	Cases		Matched controls		*p*-value
	N = 57	%	N = 57	%	
Indication for oophorectomy					0.000
Prophylactic because of breast cancer	11	19.3	39	68.4	
Therapeutic because of breast cancer	31	54.4	15	26.3	
Abnormal ovaries on ultrasound	15	26.3	2	3.5	
Unknown	0	0.0	1	1.8	

The cases with ovarian metastases were matched on the time interval between the diagnosis of breast cancer and oophorectomy to an equally large cohort of controls without ovarian metastases, as shown in the flow chart of Figure 1. The Pearson Chi-square test was used to compare the indications for oophorectomy between the cases and matched controls. *P*-values < 0.05 were considered statistically significant.

Table 2 shows the results of the univariate and multivariate logistic regression analyses that were performed in the matched case-control population. Univariate logistic regression analyses revealed that the risk of developing ovarian metastases significantly increased with tumor size and the presence of inflammatory breast cancer, the number of positive lymph nodes and the presence of distant metastases. In the multivariate logistic regression analyses, only a larger tumor size (i.e. > 5 cm) and the presence of inflammatory breast cancer was significantly associated with the development of ovarian metastases ($p = 0.024$). The presence of distant metastases could not be included in the multivariate logistic regression analyses, as none of the matched controls had clinical evidence of distant metastases at the time of breast cancer diagnosis.

Table 2. Clinicopathological characteristics of patients diagnosed with primary invasive breast cancer at age < 41 years with and without ovarian metastases

Characteristics	Cases		Matched controls		Univariate analysis	Multivariate analysis
	N = 57	%	N = 57	%	*p*-value	*p*-value
Age at diagnosis of breast cancer, years - median (range)	37	(28 - 40)	36	(27 - 40)	0.730	n.a.
Breast tumor localization					0.271	n.a.
Left	30	52.6	27	47.4		
Right	25	43.9	30	52.6		
Both	2	3.5	0	0.0		
Histological subtype					0.333	n.a.
Ductal	44	77.2	52	91.2		
Lobular	8	14.0	4	7.0		
Ductolobular	5	8.8	1	1.8		
Scarff-Bloom-Richardson grade					0.174	n.a.
I	5	8.8	5	8.8		
II	30	52.6	20	35.1		
III	22	38.6	31	54.4		
Missing	0	0.0	1	1.8		
Estrogen receptor					0.055	0.084
Negative	6	10.5	14	24.6		
Positive	51	89.5	43	75.4		
Progesterone receptor					0.167	n.a.
Negative	10	17.5	17	29.8		
Positive	45	78.9	39	68.4		
Missing	2	3.5	1	1.8		
Her2/neu receptor					0.101	n.a.
Negative	48	84.2	39	68.4		
Positive	8	14.0	15	26.3		
Missing	1	1.8	3	5.3		
Tumor stage[20]					0.001*	0.024*
T1	16	28.1	28	49.1		
T2	26	45.6	28	49.1		
T3	10	17.5	1	1.8		
T4	5	8.8	0	0.0		
Nodal status[20]					0.036*	0.510
N0	13	22.8	26	45.6		
N1	21	36.8	21	36.8		
N2	13	22.8	8	14.0		
N3	10	17.5	2	3.5		
Distant metastasis[20]					0.002*	n.a.
cM0	48	84.2	57	100.0		
cM1	9	15.8	0	0.0		

* Values are statistically significant; n.a. = not applicable.

The cases with ovarian metastases were matched on the time interval between the diagnosis of breast cancer and oophorectomy to an equally large cohort of controls without ovarian metastases, as shown in the flow chart of Fig 1. Univariate and multivariate logistic regression analyses were used to compare the cases and matched controls for the clinicopathological characteristics as indicated in the table. *P*-values < 0.05 were considered statistically significant.

Table 3. Clinicopathological characteristics of patients diagnosed with primary invasive breast cancer at age < 41 years with and without oophorectomy

Characteristics	Matched controls		CCCN controls		*p*-value
	N = 57	%	N = 4944	%	
Age at diagnosis of breast cancer, years - median (range)	36	(27 - 40)	37	(18 - 40)	0.347
Breast tumor localization					0.735
Left	27	47.4	2552	51.6	
Right	30	52.6	2376	48.1	
Both	0	0.0	15	0.3	
Missing	0	0.0	1	0.0	
Histological subtype					0.303
Ductal	52	91.2	4391	88.8	
Lobular	4	7.0	209	4.2	
Ductolobular	1	1.8	143	2.9	
Other	0	0.0	201	4.1	
Scarff-Bloom-Richardson grade					0.741
I	5	8.8	417	8.4	
II	20	35.1	1241	25.1	
III	31	54.4	2436	49.3	
Missing	1	1.8	850	17.2	
Estrogen receptor					0.047*
Negative	14	24.6	1117	22.6	
Positive	43	75.4	1543	31.2	
Missing	0	0.0	2284	46.2	
Progesterone receptor					0.070
Negative	17	29.8	1303	26.4	
Positive	39	68.4	1301	26.3	
Missing	1	1.8	2340	47.3	
Her2/neu receptor					0.809
Negative	39	68.4	1913	38.7	
Positive	15	26.3	733	14.8	
Missing	3	5.3	2298	46.5	
Tumor stage[20]					0.124
T1	28	49.1	2319	46.9	
T2	28	49.1	1989	40.2	
T3	1	1.8	360	7.3	
T4	0	0.0	197	4.0	
Missing	0	0.0	79	1.6	
Nodal stage[20]					1.000
pN0	26	45.6	2416	48.9	
pN1	21	36.8	1665	33.7	
pN2	8	14.0	560	11.3	
pN3	2	3.5	248	5.0	
Missing	0	0.0	55	1.1	
Distant metastasis[20]					0.100
cM0	57	100.0	4720	95.5	
cM1	0	0.0	224	4.5	

* Values are statistically significant.

Patients without ovarian metastases were indicated as matched controls, of whom selection is shown in the flow chart of Figure 1. Patients without oophorectomy were indicated as CCCN controls, of whom selection is illustrated in the flow chart of Figure 2. Univariate logistic regression analyses were used to compare the matched controls and CCCN controls for the clinicopathological characteristics as indicated in the table. *P*-values < 0.05 were considered statistically significant.

Validation of the control population

Table 3 shows that, apart from the fact that more hormone-sensitive breast tumors were diagnosed in the matched controls than in the CCCN controls, 75.4% compared to 31.2%, respectively (p = 0.047), no statistically significant differences were found. These data indicate that the clinicopathological characteristics of the matched controls broadly corresponded to those of women whose ovaries remained in situ.

Discussion

In the current Dutch nationwide retrospective cohort study, we found that ovarian metastases occurred in 2.4% of young women diagnosed with primary invasive breast cancer who subsequently underwent an oophorectomy. This percentage is much lower than the previously reported prevalence rates of 13-47%.[8-10] The discrepancy between our findings and those reported in the literature can be explained by the fact that the prevalence rates were explored in different patient populations. In previous studies, the prevalence rates were primarily derived from clinical studies in patients with disseminated breast cancer who underwent therapeutic oophorectomy, and autopsy reports of patients who died of metastatic breast cancer.[8-10] Our findings were based on a nationwide cohort mainly consisting of young breast cancer patients in whom the ovaries were either removed prophylactically because of a positive family history and/or the presence of a *BRCA* gene mutation, or therapeutically because of hormone-sensitive breast cancer. Hence, our findings provide more insight into the prevalence of ovarian metastases in the general population of young breast cancer patients. Nonetheless, some remarks on the establishment of this prevalence rate should be made. Firstly, the prevalence of ovarian metastases was solely substantiated among young breast cancer patients who underwent an oophorectomy. The reason for this was that ovarian metastases can only be diagnosed with certainty by microscopic examination.[21] The prevalence of ovarian metastases among young breast cancer patients whose ovaries remained in situ thus remains elusive. This point might also be considered as a strength of the current study, as our findings are exclusively based on a large cohort of young breast cancer patients in whom the presence of ovarian metastases could be determined. Secondly, it should be noted that the majority of the ovarian tissues were not completely examined, since sequentially cut tissue sections were often not obtained using standard pathology procedures. As a result, malignant cells might have been overlooked, thereby potentially resulting in an underestimation of the prevalence of ovarian metastases among young breast cancer patients. Thirdly, the time between breast cancer and the onset of ovarian metastases was on average 42 months, whereas in patients who undergo ovarian tissue cryopreservation an oophorectomy is usually performed soon after initial diagnosis. Fourthly, the majority of the patients included in this study were treated with chemotherapy, which may have treated distant metastases if present. Lastly, 26% of the cases underwent an oophorectomy because their ovary appeared abnormal on ultrasound.

Each of these factors might have affected the prevalence rate to some extent. Nonetheless, the prevalence rate based on the current study represents the closest possibility to come to a prevalence of ovarian metastases among young breast cancer patients who may undergo ovarian tissue autotransplantation, since frozen-thawed cortical ovarian fragments from patients who are willing to undergo ovarian tissue autotransplantation cannot be used to estimate the prevalence rate. Examination of cortical ovarian tissue fragments from deceased patients will certainly yield too small study populations to draw reliable conclusions from.

The most striking difference between the cases and controls in the source population was the difference in time interval between the diagnosis of breast cancer and oophorectomy. Due to the retrospective study design, it was impossible to find out why the ovaries were much earlier removed in the controls than in the cases. Nevertheless, these findings suggest that the risk of developing ovarian metastases increases with the passage of time. Hence, in young breast cancer patients who wish to preserve their fertility, it seems important to perform an oophorectomy soon after the diagnosis of breast cancer in order to reduce the risk for the development of ovarian metastases. Besides, some recommendations can be proposed with respect to the site of ovarian tissue autotransplantation. As long as there is no accurate alternative to the current tumor detection approach available by which the actual ovarian autografts can be examined, it would be advisable to transplant the cortical ovarian fragments back to the remaining ovary rather than, for instance, a peritoneal window. After all, transplantation of the cortical ovarian fragments to the remaining ovary enables the complete removal of the grafted ovarian tissues at a later stage by simply extirpating the entire ovary, for instance when the patient's family has been completed or when the ovarian grafts have ceased functioning. By contrast, in case the cortical ovarian fragments are transplanted to a peritoneal window, complete extraction of these fragments cannot be guaranteed, as it will be difficult to retrace the ovarian autografts within the peritoneum. Thus, transplantation to the remaining ovary should be preferred over transplantation to the peritoneum as it may further minimize the risk that tumor cells in the ovarian grafts ultimately develop into ovarian metastases. Lastly, in the patients who were diagnosed with ovarian metastases, it is plausible that tumor cells have disseminated very early after the onset of cancer and have long remained dormant before they formed overt metastases in the ovaries.[22,23] Our findings therefore do not alter the fact that minimal residual disease should be excluded in the actual ovarian autografts in order to avoid a cancer relapse following ovarian tissue autotransplantation.

Because the presence of ovarian metastases is inextricably linked to the time of oophorectomy, baseline risk factors could only be determined if the time interval between the cases and controls was comparable. The most suitable approach to achieve this would be to subject every young patient who is diagnosed with primary invasive breast cancer to a bilateral oophorectomy after a certain predefined time interval and subsequently evaluate whether ovarian metastases have developed. However, such an approach would obviously never be ethically acceptable. We

therefore circumvented this by matching the 57 cases, of whom clinical data were available, to an equally large cohort of controls on this time interval, making accurate risk factor analyses possible. These risk factor analyses showed that a larger tumor size (i.e. > 5 cm) and the presence of inflammatory breast cancer resulted in an increased risk of developing ovarian metastases. Yet, because the matched controls did not fully reflect the general population of young breast cancer patients without ovarian metastases, the magnitude of association between the tumor stage and the risk of developing ovarian metastases has limited value for clinical practice.

Although other reports stated that lobular breast cancers are more likely to metastasize to the ovary than ductal breast cancers,[21] we did not observe any significant differences in histological subtype between the cases and matched controls. This might be different in elderly women with breast cancer, as lobular breast cancers are more frequently diagnosed in older patients.[24]

Information on *BRCA* gene mutation status was available from 10 cases (17.5%) and 21 matched controls (36.8%). Compiled data from 18 studies reporting a total of 1187 women with *BRCA* mutations who underwent risk-reducing salpingo-oophorectomy revealed only two patients (0.17%) with metastatic breast cancer in the ovaries.[25] Hence, the presence of a *BRCA* gene mutation does not seem to be associated with the risk of developing ovarian metastases in patients with breast cancer and was therefore not taken into account in our risk factor analyses. Nevertheless, risk-reducing salpingo-oophorectomy is often recommended to *BRCA* gene mutation carriers to reduce their risk of developing primary ovarian cancer.[26]

As described above, patients were only enrolled in the current study if they had undergone ovarian surgery. Nevertheless, a comparison of our matched controls to young breast cancer patients whose ovaries remained in situ (CCCN controls) showed that the clinicopathological characteristics were broadly similar between the two groups. The reason that our matched controls were more often diagnosed with hormone-sensitive breast tumors relies on the fact that the indication for oophorectomy in these patients was primarily therapeutic. Hence, apart from the difference in hormone receptor expression, the intrinsic tumor characteristics of our matched controls were passably in line with those of young breast cancer patients whose ovaries remained in situ.

In conclusion, our research shows that secondary ovarian involvement is encountered in 2.4% of young breast cancer patients. In order to minimize the risk of developing ovarian metastases in young breast cancer patients who wish to preserve their fertility, we recommend early ovary removal followed by transplantation of cortical ovarian tissue fragments to the remaining ovary. Ultimately, when the patient's family has been completed or when the ovarian grafts have ceased functioning, the remaining ovary to which the cortical ovarian tissue fragments were transplanted should preferably be removed in order to keep the risk of developing ovarian metastases as low as possible. In addition, we suggest a cautious approach to ovarian tissue autotransplantation in patients diagnosed with tumors > 5 cm and/or inflammatory breast cancer.

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Supplementary figure S1. Data of patients diagnosed with primary invasive breast cancer at age < 41 years with and without ovarian metastases. The cases with ovarian metastases were matched on the time interval between the diagnosis of breast cancer and oophorectomy to an equally large cohort of controls without ovarian metastases, as shown in the flow chart of Figure 1. N.A. = Not applicable; NED = No evidence of disease; AWD = Alive with disease; DOC = Dead of other cause; DOD = Dead of disease; DSS = Disease-specific survival.
See online: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0168277

Supplementary figure S2. Data of patients diagnosed with primary invasive breast cancer at age < 41 years who did not undergo an oophorectomy. This cohort of patients was compiled by the Comprehensive Cancer Center the Netherlands (CCCN) and indicated as CCCN controls in the study.
See online: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0168277

Chapter 3

Identification of cell-surface markers for detecting breast cancer cells in ovarian tissue

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Abstract

Background

The safety of ovarian tissue autotransplantation in oncology patients cannot be ensured, as current tumor-detection methods compromise the ovarian tissue viability. Although non-destructive methods (for instance near-infrared fluorescence imaging) can discriminate malignant from healthy tissues while leaving the examined tissues unaffected, they require specific cell-surface tumor markers. We determined which tumor markers are suitable targets for tumor-specific imaging to exclude the presence of breast cancer cells in ovarian tissue.

Methods

Immunohistochemistry was performed on formalin-fixed paraffin-embedded specimens of ten ovaries from premenopausal patients. Additionally, we screened a tissue microarray containing tumor tissue cores from 24 breast cancer patients being eligible for ovarian tissue cryopreservation. The following cell-surface tumor markers were tested: E-cadherin, EMA (epithelial membrane antigen), Her2/neu (human epidermal growth factor receptor type 2), $\alpha v\beta 6$ integrin, EpCAM (epithelial cell adhesion molecule), CEA (carcinoembryonic antigen), FR-α (folate receptor-alpha), and uPAR (urokinase-type plasminogen activator receptor). For each tumor, the percentage of positive breast tumor cells was measured.

Results

None of the ten ovaries were positive for any of the markers tested. However, all markers (except CEA and uPAR) were present on epithelial cells of inclusion cysts. E-cadherin was present in the majority of breast tumors: ≥90% of tumor cells were positive for E-cadherin in 17 out of 24 tumors, and 100% of tumor cells were positive in 5 out of 24 tumors.

Conclusion

Of the markers tested, E-cadherin is the most suitable marker for a tumor-specific probe in ovarian tissue. Methods are required to distinguish inclusion cysts from breast tumor cells.

Introduction

Premature ovarian failure is the most common long-term major adverse effect in premenopausal women following chemotherapy.[1] Because loss of fertility can significantly decrease quality of life,[2] considerable effort has been devoted to offering these patients options for preserving their fertility. These options currently include cryopreservation of embryos and/or oocytes. Besides, autotransplantation of pretreatment cryopreserved ovarian tissue is becoming more prevalent and is considered predominantly feasible for both prepubescent girls and women who cannot postpone adjuvant therapy.[3-5]

Although autotransplantation of frozen-thawed ovarian tissue has improved greatly in recent years,[6-7] its safety is questionable for certain types of cancer at risk of ovarian involvement, as it remains uncertain whether the transplanted cortical ovarian strips contain metastatic cells. This uncertainty arises from the highly damaging effects of currently available tumor-detection methods (e.g., PCR, immunohistochemistry) on tissue viability.[8-9] Therefore, traditional screening is performed using a limited number of ovarian strips that are ultimately not transplanted. As a consequence of this approach, autotransplanting ovarian tissue involves the risk of reimplanting diseased cells that can lead to cancer relapse in some patients.

To safeguard the transfer of cortical ovarian tissue to the patient, methods must be developed in which tumor cells can be detected in ovarian autografts while preserving the tissue's reproductive function. Near-infrared fluorescence (NIRF) imaging might be a suitable approach, as this technique can safely distinguish malignant tissues from non-malignant tissues in real time while leaving the tissues viable.[10] A NIRF probe consists of a fluorophore that emits light in the near-infrared spectrum (λ = 700-900 nm) conjugated to an antibody or peptide with high affinity for a protein marker expressed selectively at the cell surface of tumor cells.[11-12]

The first step towards developing tumor-specific imaging is the identification of protein markers that are present selectively at the cell surface of tumor cells, but absent on cells within the normal ovarian cortex. Because breast cancer is one of the primary indications for cryopreservation of ovarian tissue[13-16] and breast cancer metastases in the ovaries have been reported with a prevalence ranging from 13 to 47%,[8,17] we examined a panel of cell-surface markers known to be expressed by breast cancer cells. This panel included human epidermal growth factor receptor type 2 (Her2/neu),[18-19] E-cadherin,[20] and carcinoembryonic antigen (CEA).[21] In addition, we tested several markers involved in tumor invasion and migration, including epithelial cell adhesion molecule (EpCAM),[22-23] $\alpha v\beta 6$ integrin,[24] urokinase-type plasminogen activator receptor (uPAR),[25-26] and epithelial membrane antigen (EMA, also known as MUC1).[27-28] Lastly, we included folate receptor alpha (FR-α), which is expressed in several tumor types but not in normal ovarian tissue.[29] We excluded cytokeratin CAM 5.2, gross cystic disease fluid protein-15 (GCDFP15), Wilms' tumor antigen-1 (WT1), mammaglobin 1, and cytokeratin 7 (CK-7), which were used previously by Sánchez-Serrano et al.[30] and Rosendahl et al.,[31] as these proteins are not expressed at the cell surface and therefore not suitable as a target for tumor-specific imaging.

In this study, we measured the expression levels of the above-mentioned markers in breast cancer cells obtained from patients who were potentially eligible for cryopreservation of ovarian tissue. In addition, we compared these expression levels to expression in normal ovarian tissues.

Material and methods

Control ovaries

Formalin-fixed, paraffin-embedded (FFPE) specimens of control ovaries obtained from premenopausal patients who underwent a unilateral or bilateral oophorectomy in 2001-2012 were selected from the archives of the Department of Pathology at the Leiden University Medical Center (LUMC). The clinical data were extracted from the patients' medical records. Indications for surgery included suspected malignancy in the contralateral ovary, early-stage uterine sarcoma, endometrial carcinoma, squamous cell carcinoma of the cervix, or enlarged ovary during pregnancy. *BRCA* mutation carriers and women with unknown *BRCA* mutation status were excluded. Patients who used a gonadotropin-releasing hormone (GnRH) agonist or oral contraceptives prior to oophorectomy were excluded to ensure that only functionally active ovaries were studied. A pathologist specialized in gynecology confirmed the absence of overt abnormalities in the ovaries by reviewing hematoxylin-and-eosin-stained sections. A total of ten control ovaries from ten different patients were included.

Breast cancer tissue

Breast tumor samples were collected from 24 patients who were potentially eligible for cryopreservation of their ovarian tissue based on the inclusion criteria established by the Dutch Network of Fertility Preservation.[32] All women were ≤35 years of age and were diagnosed with invasive breast carcinoma for which they were treated surgically at the LUMC in 1997-2009. The following data were obtained from the medical records: age at the time the tissue was obtained, TNM (tumor/node/metastasis) stage, histological subtype, Scarff-Bloom-Richardson (SBR) grade, and expression of the estrogen and progesterone receptors. All patients were eligible for adjuvant chemotherapy based on the current protocols, and none was diagnosed with distant metastases.

Immunohistochemistry

Immunohistochemistry was performed on 4-µm thick FFPE sections of control ovaries and 4-µm thick slices of a tissue microarray (TMA) containing invasive breast tumor cores. To generate the TMA, tissue biopsies measuring 1.0 mm in diameter were taken in triplicate from representative regions of the FFPE tumor samples and arrayed into a new recipient paraffin block using TMA Master (3DHistech, Hungary). The tissue sections were deparaffinized in xylene, rehydrated in a stepwise series of graded alcohol solutions, and rinsed in distilled water. After

blocking endogenous peroxidase activity with 0.3% hydrogen peroxide for 20 minutes, heat-induced antigen retrieval was performed by placing the slides in EnVision Flex Target Retrieval Solution high pH/low pH in PT Link (Dako, Denmark). EpCAM and αvβ6 integrin epitopes were unmasked by 30-minute incubation with 0.125% trypsin and 0.4% pepsin, respectively, at 37° C. The sections were incubated overnight in a humidified chamber at room temperature with primary antibodies against Her2/neu (ERBB2, rabbit polyclonal, Dako), E-cadherin (NCH38, mouse monoclonal, Dako), EpCAM (323/A3, mouse monoclonal, provided by the Department of Pathology, LUMC, the Netherlands), CEA (A0115, rabbit polyclonal, Dako), αvβ6 integrin (6.2A1, mouse monoclonal, Cell Essentials), uPAR (ATN615, mouse monoclonal, kindly provided by Prof. A.P. Mazar, Northwestern University, Evanston, IL), or EMA (E29, mouse monoclonal, Dako); all primary antibodies were used at their predetermined optimal dilution. Some sections were incubated in an antibody against FR-α (26B3.F2, mouse monoclonal, Biocare Medical) for 60 minutes in accordance with the manufacturer's instructions. After incubation with the primary antibody, the sections were rinsed with PBS, incubated with secondary antibody (anti-mouse or anti-rabbit EnVision; Dako) for 30 minutes, and visualized using liquid DAB+ substrate buffer (Dako). The sections were counterstained with Mayer's hematoxylin solution, dehydrated, and permanently mounted with Pertex (Leica Microsystems, Germany). For each immunostain, a positive control expressing the antigen of interest was included. The primary antibody was omitted as a negative control.

Image capture and quantification of immunoreactivity

The immunostained slides were scanned using a Pannoramic MIDI digital slide scanner (3DHistech, Hungary). Immunohistochemical staining of the ovary sections was evaluated by the primary researcher (I.P.) and an experienced pathologist specialized in gynecology (V.S.). In each breast tumor tissue core sample, the percentage of breast tumor cells and the percentage of positively stained membranes among the malignant cells were scored by two independent observers (I.P. and R.V.). In the event of a major discrepancy, the observers reached consensus regarding a final score. The tumor cell membranes were considered positive if they showed immunoreactivity of any intensity. A weighted scoring method based on the size of the tumor area in each tumor core was used to calculate the percentage of positive membrane-stained tumor cells in each sample.

Statistical analysis

Statistical analysis was performed using SPSS version 20.0 (IBM, Armonk, NY). Inter-observer agreement was calculated using the Pearson correlation coefficient. The suitability threshold for the putative NIRF probe targets was set at 80, 90, or 100% of tumor cells expressing the antigens.

Results

Control ovaries

A histological analysis showed that all ovaries contained follicles. The cortex of each ovary was negative for immunohistochemical staining by all markers tested. In contrast, all markers (except CEA and uPAR) were detected at the plasma membrane of epithelial cells in inclusion cysts (Figures 1a and 1b). These inclusion cysts were present in five of the ten ovaries. In addition, E-cadherin was expressed at moderate levels in the granulosa cells of primary follicles (Figure 2).

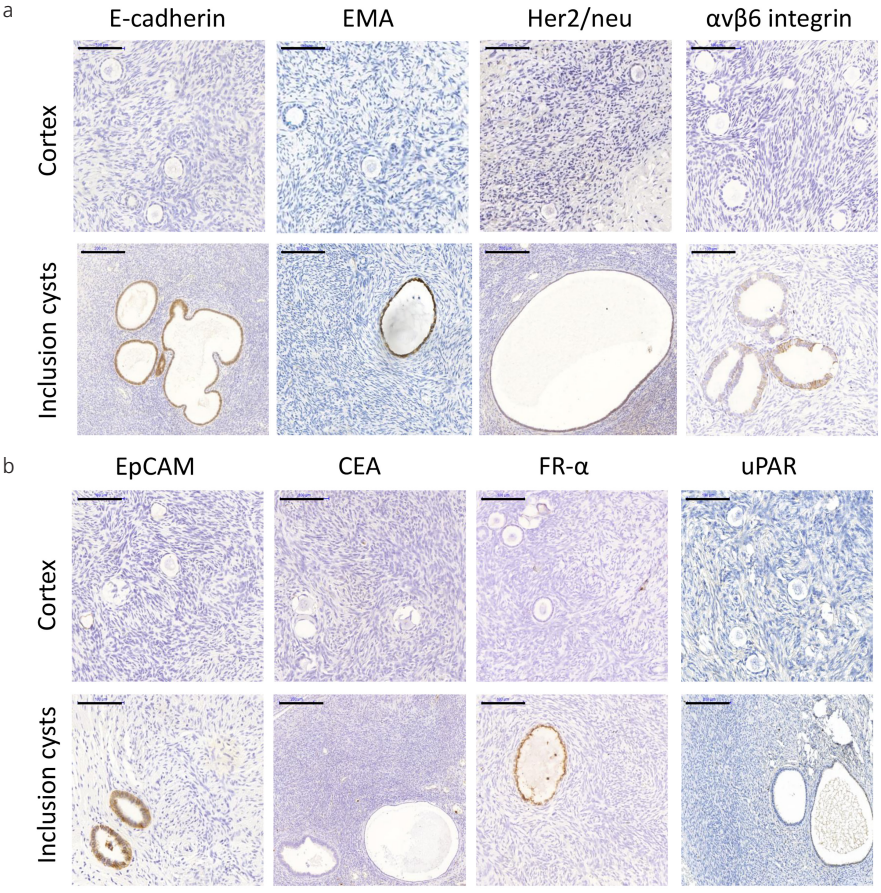

Figure 1. Immunohistochemical expression of the investigated markers in ovarian cortices and inclusion cysts

a. Immunohistochemical expression of E-cadherin, EMA, Her2/neu and αvβ6 integrin. Stromal cells stained negative, but E-cadherin, EMA, Her2/neu and αvβ6 integrin showed expression at the epithelial cells of inclusion cysts.

b. Immunohistochemical expression of EpCAM, CEA, FR-α and uPAR. Stromal cells stained negative, but EpCAM and FR-α showed expression at the epithelial cells of inclusion cysts. Scale bar in the upper panel represents 100 μm and scale bar in the lower panel represents 200 μm.

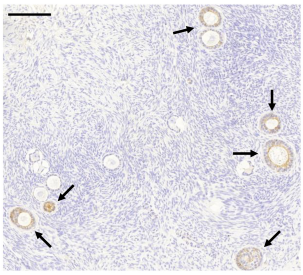

Figure 2. Immunohistochemical expression of E-cadherin in the granulosa cells of primary follicles in the ovarian cortex

Moderate expression of E-cadherin in the granulosa cells of primary follicles in the ovarian cortex is indicated by arrows. Scale bar represents 200 µm.

Breast cancer tissue

The median age at the time of diagnosis was 32 years (range 21-35 years) for the 24 patients included in the TMA analysis. Twenty-three patients were diagnosed with ductal breast cancer, and the remaining patient was diagnosed with lobular breast carcinoma. The characteristics of these 24 patients and their tumors are summarized in Table 1.

Table 1. Clinicopathologic characteristics of premenopausal patients with primary invasive breast cancer

Characteristic	N = 24
Age at diagnosis, years - median (range)	32.0 (21 - 35)
Tumor size, mm - median (range)	20.5 (10 - 45)
Tumor stage - no. (%)	
pT1	11 (45.8)
pT2	12 (50.0)
pT3	1 (4.2)
pT4	0 (0.0)
Lymph node involvement - no. (%)	
pN0	13 (54.2)
pN1	11 (45.8)
Scarff-Bloom-Richardson grade - no. (%)	
I	2 (8.3)
II	9 (37.5)
III	13 (54.2)
Histological subtype - no. (%)	
Ductal	23 (95.8)
Lobular	1 (4.2)
Estrogen receptor - no. (%)	
Negative	12 (50.0)
Positive	9 (37.5)
Unknown	3 (12.5)
Progesterone receptor - no. (%)	
Negative	15 (62.5)
Positive	6 (25.0)
Unknown	3 (12.5)

Table 2. The percentage of tumor cells in each tumor showing positive expression for the investi-gated tumor markers

Tumor nr.	E-cadherin	EMA	Her2neu	αvβ6 integrin	EpCAM	CEA	FR-alpha	uPAR tumor [a]	uPAR stroma [a]
1	100	90	34	11	95	0	0	3	0
2	93	91	63	54	40	5	6	0	0
3	90	84	100	98	92	100	3	0	0
4	58	35	76	31	40	24	0	0	0
5	94	38	60	85	71	81	98	0	0
6	80	69	35	50	22	18	7	0	0
7	97	82	68	9	100	4	0	0	0
8	5	52	50	90	35	74	52	0	3
9	94	100	78	95	0	15	25	0	0
10	100	100	88	71	2	13	0	0	0
11	88	78	47	93	67	0	33	6	14
12	95	77	77	24	83	20	0	0	0
13	50	74	40	2	81	31	0	0	0
14	94	100	96	90	10	2	3	0	0
15	100	37	13	100	100	95	80	11	8
16	91	89	100	40	98	76	0	0	0
17	100	38	100	41	100	28	0	0	0
18	54	50	48	78	0	27	52	10	0
19	63	100	96	93	8	67	0	0	0
20	95	13	34	43	94	77	3	10	7
21	100	95	100	62	13	21	0	0	2
22	94	77	45	58	21	0	1	3	0
23	92	94	55	35	42	74	100	7	0
24	97	65	11	31	82	4	5	0	0
Median	94	78	61	56	54	23	3	0	0

100% of tumor cells
90-99% of tumor cells
80-89% tumor cells

[a] uPAR expression was subdivided into tumor and stromal expression.

Expression of investigated markers

Microscopic quantification of marker levels was possible in all breast tumor samples. Strong correlation was obtained between the scoring results obtained by the two observers; the median R^2 was 0.746 (range 0.626-0.818). E-cadherin, EMA, Her2/neu, CEA, and uPAR staining was positive in both the plasma membrane and cytoplasm of the breast cancer cells, whereas αvβ6 integrin, EpCAM, and FR-α staining was confined to the membrane. In addition, uPAR staining was observed in stromal cells surrounding the tumor cells (Figure 3).

The median (range) percentage of positive tumor cells was 94% (5-100) for E-cadherin, 78% (13-100) for EMA, 61% (11-100) for Her2/neu, 56% (2-100) for αvβ6 integrin, 54% (0-100) for EpCAM, 23% (0-100) for CEA, and 3% (0-100) for FR-α. uPAR was expressed in extremely few tumor and stromal cells, 0% (0-11) and 0% (0-14), respectively (Table 2).

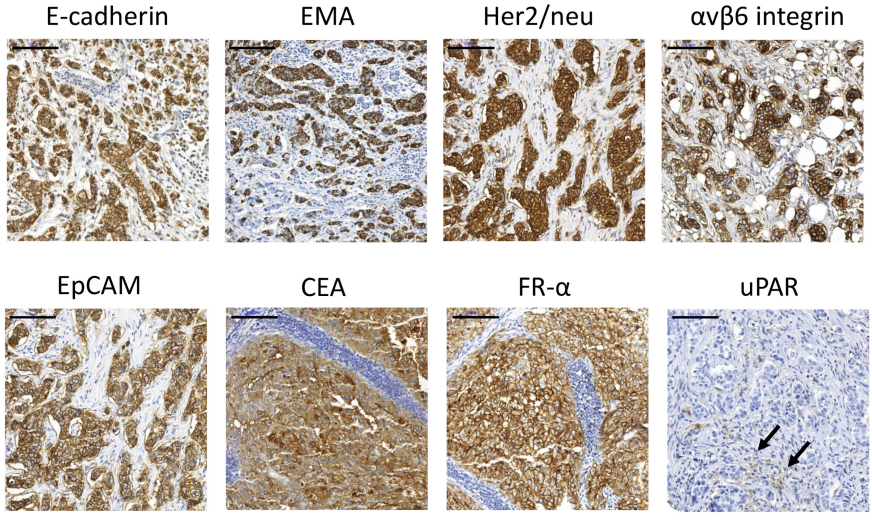

Figure 3. Immunohistochemical expression of E-cadherin, EMA, Her2/neu, αvβ6 integrin, EpCAM, CEA, FR-α and uPAR in invasive breast cancer

uPAR was barely expressed in stromal cells surrounding the tumor (arrows). Scale bars represent 100 μm.

Potential targets for imaging

Given that breast cancer is relatively heterogeneous and that the expression of antigens varied among the tumors examined (Table 2), targeting one membrane protein would likely be insufficient for detecting all possible tumor cells in each patient. Therefore, to facilitate the selection of possible targets, we used suitability thresholds set at 80, 90, and 100%, corresponding to the percentage of tumor cells that expressed the various antigens.

Figure 4 summarizes the suitability of each tumor marker for detecting invasive tumor cells in the 24 patients who were diagnosed with breast cancer. Based on this analysis, E-cadherin was

identified as the most suitable marker for detecting breast cancer cells; specifically, E-cadherin was present in 100% of cells in five tumors, and this marker was present in ≥90% of cells in 17 tumors. The seven tumors with <90% positivity for E-cadherin were positive for the markers EMA (1 tumor; 100% of cells detected), αvβ6 integrin (3 tumors; 78-93% of cells detected), EpCAM (1 tumor; 81% of cells detected), E-cadherin (1 tumor; 80% of cells detected), and Her2neu (1 tumor; 76% of cells detected). Two tumors had <80% positivity for all of the markers tested (Table 2). In these two tumors, 76% and 78% of the tumor cells were detected by the markers, corresponding to a maximum of 24% and 22% of undetected malignant cells, respectively.

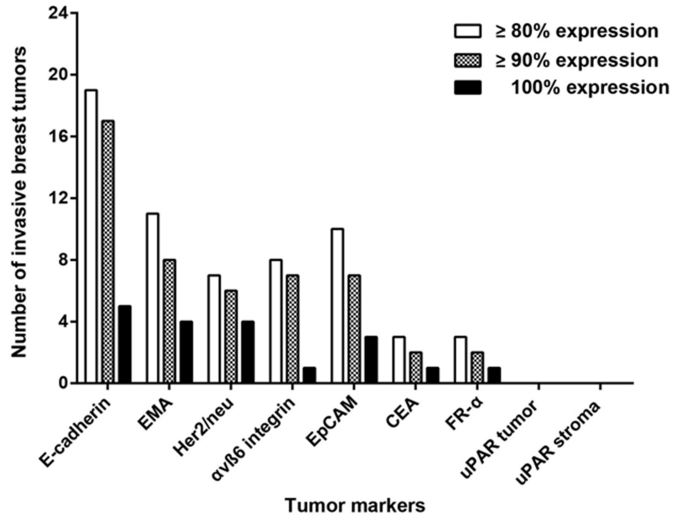

Figure 4. Suitability of tumor markers to use as a target for the detection of tumor cells in 24 premenopausal women with invasive breast cancer

Columns represent the number of tumors in which at least 80%, at least 90% or 100% of the tumor cells showed expression of the tumor markers. For uPAR, stromal cell expression is also shown.

Discussion

Here, we identified several proteins that could potentially serve as a suitable target for detecting breast cancer cells within ovarian autografts. One clear application for these markers is the use of NIRF imaging, a technique that can differentiate malignant tissues from non-malignant tissues without reducing the tissue's viability.[10,33-35] Designing a NIRF probe directed against E-cadherin shows particular promise, as E-cadherin was expressed by the majority (94%) of invasive breast tumor cells and was absent on the surface of normal ovarian cells. However, a combination of tumor-selective probes will likely be needed to detect all tumor cells. Based on our results, a combination of probes against E-cadherin, EMA and Her2/neu seems suitable.

Metastatic spread requires the local invasion of the surrounding host tissue by cells that originated from the primary tumor, followed by intravasation in blood and lymphatic vessels, ultimately leading to the dissemination of tumor cells.[36] E-cadherin and EpCAM mediate cell-cell adhesion, and the downregulation or loss-of-function of these proteins enables cells to escape from solid tumors.[19] E-cadherin and/or EpCAM are not necessarily expressed in all tumor cells; therefore, metastatic tumor cells might not be detected in some tissues. Furthermore, the majority of metastatic lobular breast cancer cells, which lack E-cadherin expression, will not be detected using a specific anti-E-cadherin probe, even though lobular breast cancer cells are more likely to invade ovarian tissue compared to cells derived from ductal carcinomas.[37-38]

As mentioned above, we considered tumor cell membranes positive if they showed immunoreactivity of any intensity. As a result, some tumor cells might be more positive than others for the investigated markers. Yet, for NIRF imaging, the staining intensity is less important as long as a significant tumor-to-background-ratio can be achieved.

None of the premenopausal ovaries in our cohort had positive staining in either the stromal cells or the ovarian surface epithelium. However, all markers (except CEA and uPAR) were expressed on epithelial cells in inclusion cysts. Consequently, conjugating antibodies against these markers to a NIR fluorophore will illuminate invasive breast cancer cells in the ovary, as well as inclusion cysts. Because inclusion cysts might differ from metastatic breast cancer cells with respect to their fluorescent configuration, it might be possible to distinguish between these structures. The same strategy might be used to distinguish granulosa cells from primary follicles that express E-cadherin. In addition, full-field optical coherence tomography (FF-OCT), a non-invasive imaging technique that mimics conventional histopathology, might be very useful. In the field of dermatological oncology, FF-OCT has already been proven capable of visualizing sebaceous glands and adipose tissue surrounding hair follicles as well as small malignant skin tumors.[39] On high magnification, fine architectural details on the subcellular level can be recognized. Therefore, it is expected that FF-OCT will also be able to distinguish inclusion cysts from metastatic tumor cells in ovarian tissue.

One strength of our study is that we examined the expression of tumor markers in tumor tissues obtained from young breast cancer patients who met the criteria for ovarian tissue cryopreservation. Moreover, only biologically active ovaries were analyzed, giving the results clinical relevance, as ovarian tissue is generally cryopreserved before ovarian failure has occurred.

On the other hand, this study has some limitations that merit discussion. First, a relatively small sample size was examined. However, normal ovarian tissue from premenopausal patients is not readily available. We excluded ovaries that were removed due to the presence of a *BRCA* gene mutation, as such samples could contain primary ovarian tumor cells,[40] that express the markers investigated in this study. To be certain, we also excluded ovaries from breast cancer patients with unknown mutation status. At the LUMC, normal ovarian tissue from premenopausal breast cancer patients was exclusively available from *BRCA* mutation carriers or women with unknown mutation status. As a consequence, ovaries from breast cancer patients were not included in

this study. Malignant cells may also be present in ovaries that were removed due to endometrial carcinoma, uterine sarcoma, cervical squamous cell carcinoma, or contralateral ovarian carcinoma; however, the risk of false-positive results was relatively low in our cohort, as all primary tumors were diagnosed at an early stage and the lymph nodes in these patients were clear. Second, the expression of the markers was evaluated on primary invasive breast tumors, since a substantial cohort consisting of ovarian tissues containing breast cancer metastases is scarce. Finally, we examined the expression of tumor markers in relatively small tumor cores using a TMA approach. However, the TMA technique is considered an accurate method for examining protein expression in breast cancer tissues.[41]

Recently, the intraoperative use of tumor-targeted fluorescent imaging yielded a high tumor identification rate and enabled the surgeon to detect metastases that could not be detected by visual observation.[42] For our purpose, tumor-specific NIRF probes could be administered intravenously prior to oophorectomy, after which the removed ovary is dissected into cortical ovarian strips. Detailed fluorescent images could then be obtained using multiphoton microscopy, which provides an inherent submicron spatial resolution that allows revelation of subcellular details with reduced phototoxicity and photobleaching.[43-44] Because the NIRF signal lies beyond the red end of the visible spectrum, the signal has enhanced tissue penetration, enabling the identification of fluorescently labeled tumor cells that are located deep within the tissue. Moreover, the low autofluorescence of the tissue at the emission wavelength of the probe provides a high tumor-to-background ratio.[45] Because of these features and the fact that cortical ovarian fragments can be imaged from both the upper and lower side, thereby increasing the imaging depth even further, NIRF imaging is a promising technique for detecting tumor cells in cortical ovarian strips up to 2 mm in thickness.

In conclusion, we report the identification of tumor markers that may serve as a target for detecting breast cancer cells in ovarian tissue using robust imaging techniques such as NIRF imaging. Based on our analysis, E-cadherin is likely the most suitable target for designing a tumor-specific probe. Further research will focus on examining the expression of these markers on breast cancer metastases in ovaries, refining methods to distinguish breast cancer cells from ovarian inclusion cysts, and examining the clinical feasibility of applying NIRF imaging to the field of fertility preservation.

Ethics approval

All patient samples and clinical data were handled in accordance with the medical ethics guidelines described in the Code of Conduct for Proper Secondary Use of Human Tissue of the Dutch Federation of Biomedical Scientific Societies (FMWV).[46]

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Chapter 4

Morphological and phenotypical features of ovarian metastases in breast cancer patients

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Abstract

Background

Autotransplantation of frozen-thawed ovarian tissue is a method to preserve ovarian function and fertility in patients undergoing gonadotoxic therapy. In oncology patients, the safety cannot yet be guaranteed, since current tumor detection methods can only exclude the presence of malignant cells in ovarian fragments that are not transplanted. We determined the need for a novel detection method by studying the distribution of tumor cells in ovaries from patients with breast cancer. Furthermore, we examined which cell-surface proteins are suitable as a target for non-invasive tumor-specific imaging of ovarian metastases from invasive breast cancer.

Methods

Using the nationwide database of the Dutch Pathology Registry (PALGA), we identified a cohort of 46 women with primary invasive breast cancer and ovarian metastases. The localization and morphology of ovarian metastases were determined on hematoxylin-and-eosin-stained sections. The following cell-surface markers were immunohistochemically analyzed: E-cadherin, epithelial membrane antigen (EMA), human epidermal growth receptor type 2 (Her2/neu), carcinoembryonic antigen (CEA), $\alpha v\beta 6$ integrin and epithelial cell adhesion molecule (EpCAM).

Results

The majority of ovarian metastases (71%) consisted of a solitary metastasis or multiple distinct nodules separated by uninvolved ovarian tissue, suggesting that ovarian metastases might be overlooked by the current detection approach. Combining the targets E-cadherin, EMA and Her2/neu resulted in nearly 100% detection of ductal ovarian metastases, whereas the combination of EMA, Her2/neu and EpCAM was most suitable to detect lobular ovarian metastases.

Conclusion

Examination of the actual ovarian transplants is recommended. A combination of targets is most appropriate to detect ovarian metastases by tumor-specific imaging.

Introduction

Cryopreservation of ovarian tissue is the only option to preserve fertility and restore ovarian activity in prepubescent girls and women who cannot postpone the start of adjuvant chemotherapy.[1] Although autotransplantation of frozen-thawed cortical ovarian tissue has resulted in more than 86 live births worldwide,[2] this method has not yet been endorsed by the American Society for Reproductive Medicine (ASRM).[3] One of the reasons that the ASRM committee has put forward is that the safety of the procedure has not been substantiated in patients with cancer. Cortical ovarian tissue may contain malignant cells that could lead to reseeding of cancer upon autotransplantation. This risk of reintroducing malignant cells cannot be eliminated, since the current tumor detection methods (e.g. PCR, immunohistochemistry) jeopardize the ovarian tissue's viability.[4] These methods can therefore only be used to examine cortical ovarian strips that are not transplanted. Hence, the presence of tumor cells in the actual ovarian autografts remains questionable.

Whether the current approach for tumor detection is accurate depends on the distribution of metastatic tumor cells in the ovarian tissue.[5,6] If tumor cells are diffusely dispersed throughout the ovary, examination of one or two cortical ovarian strips might be sufficient. By contrast, if tumor cells are confined to a specific area in the ovarian cortex, this approach is inadequate. Then, cortical ovarian strips that are examined may turn out to be devoid of tumor cells whereas ovarian fragments that harbor metastases may be transplanted, possibly resulting in cancer relapse.

The implementation of a detection method that allows examination of the cortical ovarian strips that will be transplanted, will significantly reduce the risk of transferring malignant cells. Near-infrared fluorescence (NIRF) imaging might be an appropriate approach, as this technique discriminates malignant cells from non-malignant tissue in real time while leaving the tissues viable.[7] A NIRF probe consists of a fluorophore that emits light in the near-infrared spectrum (λ = 700-900 nm) and an antibody or peptide with high affinity for a protein expressed specifically at the cell surface of tumor cells.[8,9]

In order to use tumor-specific imaging to exclude malignant cells in cortical ovarian autografts, tumor markers should be identified that are present at the cell surface of ovarian metastases. Since a substantial proportion of patients who undergo ovarian tissue cryopreservation is diagnosed with breast cancer,[10-12] we tested a panel of cell-surface markers known to be expressed by breast cancer cells, including E-cadherin,[13] epithelial membrane antigen (EMA, also known as MUC1),[14,15] human epidermal growth factor receptor type 2 (Her2/neu),[16,17] carcinoembryonic antigen (CEA),[18] $\alpha v\beta 6$ integrin,[19] and epithelial cell adhesion molecule (EpCAM).[20-22] The markers cytokeratin CAM 5.2, gross cystic disease fluid protein-15 (GCDFP15), Wilms' tumor antigen-1 (WT1), mammaglobin 1, and cytokeratin 7 (CK-7), which were used by Sánchez-Serrano et al.[23] and Rosendahl et al.,[6] were excluded, as they are not expressed at the cell surface and therefore not suitable as a target for tumor-specific imaging.

In this study, we assessed the distribution of breast tumor cells in ovarian tissues from patients with ovarian metastases and determined which cell-surface proteins are suitable as a target for tumor-specific imaging of ovarian metastases derived from invasive breast cancer. Because it is crucial to select a target prior to the administration of the NIRF probe, we also examined whether invasive breast cancer tissue can be used to predict the most suitable target for the detection of ovarian metastases in a particular patient.

Methods

Patient selection and tissue collection

Via a nationwide search performed by PALGA, the Dutch histopathology and cytopathology network that encompasses all pathology laboratories within the Netherlands,[24] a source population was compiled. This source population consisted of all patients who were diagnosed with primary invasive breast cancer at age < 41 years in the period 2000-2010 and who subsequently underwent an oophorectomy for any reason. From this source population, all patients who had histologically confirmed ovarian metastases from primary invasive breast cancer, were selected. Following this, hematoxylin-and-eosin (H&E) stained tissue sections and formalin-fixed paraffin-embedded (FFPE) tissue samples from the primary invasive breast tumors and their corresponding ovarian metastases were requested from pathology laboratories. If patients had locally recurrent breast cancer or a second primary invasive breast tumor prior to oophorectomy, FFPE tissue samples from these tumors were also requested. Clinical data were extracted from the patient's files after approval by the medical ethical committee of the Leiden University Medical Center (protocol number P14.106) and the local medical ethical committees of the participating hospitals.

Distribution of breast cancer cells in the ovary

The distribution of breast cancer cells in ovarian tissues was evaluated using the original H&E-stained sections by assessment of their localization and morphological features. The localization of breast cancer cells was determined as confined to the ovarian cortex and/or medulla. With respect to morphology, breast cancer cells were classified as a solitary metastasis, multiple distinct nodules separated by uninvolved ovarian tissue, or diffuse seeding without any discernable pattern.

Immunohistochemistry

Immunohistochemistry was performed on 4-µm thick FFPE sections of primary invasive breast cancers, locally recurrent breast cancers (if applicable) and their corresponding ovarian metastases. The tissue sections were deparaffinized in xylene, dehydrated in a stepwise series of graded alcohol solutions, and rinsed in distilled water. After blocking endogenous peroxidase activity with 0.3%

hydrogen peroxide for 20 minutes, heat-induced antigen retrieval was performed by placing the slides in EnVision Flex Target Retrieval Solution high pH (pH 9.0; E-cadherin, EMA) or in the same solution but low pH (pH 6.0; Her2/neu) in PT Link (Dako, Denmark). EpCAM and αvβ6 integrin epitopes were unmasked by 30-minute incubation with 0.125% trypsin and 0.4% pepsin, respectively, at 37° C. For CEA, no antigen retrieval was required. The sections were incubated overnight in a humidified chamber at room temperature with primary antibodies against Her2/neu (ERBB2, rabbit polyclonal, Dako), E-cadherin (NCH38, mouse monoclonal, Dako), EpCAM (323/A3, mouse monoclonal, provided by the Department of Pathology, LUMC, the Netherlands), CEA (A0115, rabbit polyclonal, Dako), αvβ6 integrin (6.2A1, mouse monoclonal, Cell Essentials), or EMA (E29, mouse monoclonal, Dako); all primary antibodies were used at their predetermined optimal dilution. After incubation with primary antibodies, the sections were rinsed with PBS, incubated with secondary antibodies (anti-mouse or anti-rabbit EnVision; Dako) for 30 minutes, and visualized using liquid DAB+ substrate buffer (Dako). The sections were counterstained with Mayer's hematoxylin solution, dehydrated, and mounted with Pertex (Leica Microsystems, Germany). For each immunostain, tissues expressing the antigen of interest were included as a positive control. Tissue sections stained without application of the primary antibody were used as a negative control.

Immunofluorescent triple staining

For immunofluorescent triple staining, the three most highly expressed markers for ductal and lobular ovarian metastases were chosen. In brief, FFPE sections of these ovarian metastases were deparaffinized as described above. Antigen retrieval was performed by placing the slides in EnVision Flex Target Retrieval Solution high pH (pH 9.0; Dako). Primary antibodies for ductal ovarian metastases: E-cadherin, EMA and Her2/neu. Primary antibodies for lobular ovarian metastases: EMA, Her2/neu and EpCAM. Secondary antibodies were all isotype-specific antibodies with Alexa Fluorochromes (LifeTechnologies, USA): anti-mouse IgG1-AlexaFluor488 (E-cadherin and EpCAM; green), anti-mouse IgG2a-AlexaFluor647 (EMA; red) and anti-rabbit-AlexaFluor546 (Her2/neu; orange). Sections were mounted with Vectashield containing DAPI (Vector Laboratories, USA). Primary invasive breast tumor samples that showed positive expression for all markers in previous experiments were used as a positive control. Tissue sections stained without application of primary antibodies were used as a negative control.

Image capture and quantification of immunoreactivity

The immunohistochemically stained slides were digitized using an IntelliSite Pathology Ultra-Fast Scanner 1.6 RA (Philips, The Netherlands). The percentage of malignant cells with immunohistochemically positive stained membranes were scored by two independent observers (I.P. and M.S.). In case of discrepancy, the observers reached consensus regarding a final score. The tumor cell membranes were considered positive if they showed immunoreactivity of any

intensity. The immunofluorescent stained slides were digitized using a Pannoramic MIDI digital slide scanner (3DHistech, Hungary). The percentage of malignant cells with immunofluorescent positive stained membranes were also scored by two independent observers (I.P. and B.H.).

Statistical analysis

Statistical analysis was performed using SPSS version 23.0 (IBM, Armonk, NY). Inter-observer agreement was calculated using the Pearson correlation coefficient. Scatter plots based on generalized estimating equations analysis were made to determine whether invasive breast cancer tissue can be used to predict the most suitable target for the detection of ovarian metastases in a particular patient.

Results

Patient selection and clinicopathological characteristics

According to the PALGA registry, 2648 patients were diagnosed with primary invasive breast cancer at age < 41 years in the period 2000-2010 in the Netherlands who subsequently underwent an oophorectomy (Figure 1). Among these patients, 63 patients had ovarian metastases. Of these 63 patients, tumor tissue samples were available from 46 patients. These 46 patients were included in this study.

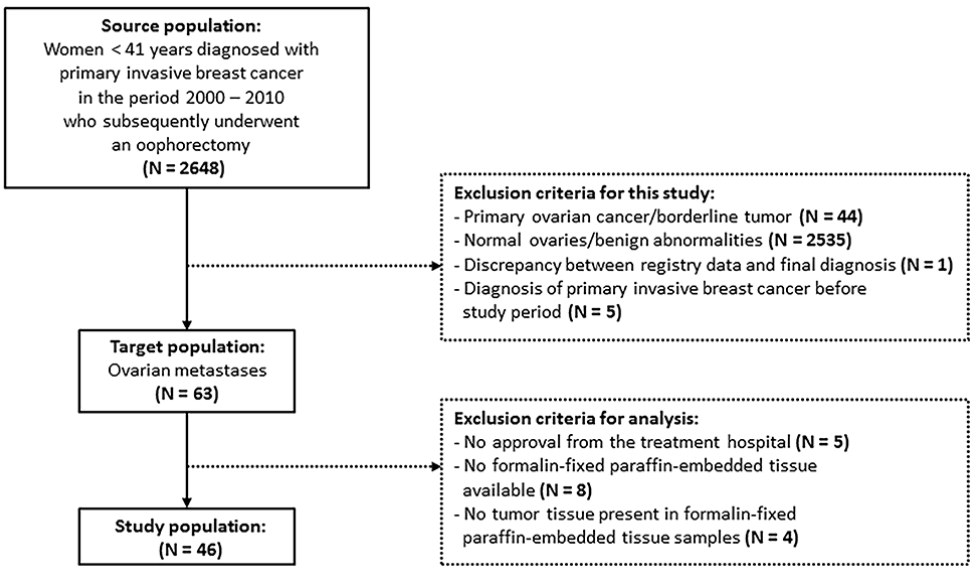

Figure 1. Patient selection and composition of the study population
The source population was compiled by the Dutch histopathology and cytopathology network. The exclusion criteria are indicated in the dotted boxes.

The clinicopathological characteristics of the 46 patients are shown in Table 1. The median age at the time of diagnosis was 36.5 years (range 28-40 years). Thirty-six patients were diagnosed with invasive ductal breast cancer and five patients were diagnosed with invasive lobular breast cancer. The remaining five patients had invasive ductolobular breast cancer. Almost 15% of patients had distant metastases outside the ovary at the time of breast cancer diagnosis. The median time between this diagnosis and oophorectomy was 41.9 months (range 0.3-141.8 months). In the majority of cases, the oophorectomy was done prophylactically or therapeutically because of breast cancer. In only one fourth of cases, the ovaries were removed because they appeared abnormal on ultrasound. Further patient and tumor characteristics are presented in Table 1.

Table 1. Clinicopathological characteristics of patients with primary invasive breast cancer and ovarian metastases

Clinicopathological characteristics	N = 46	%
Age at diagnosis of breast cancer, years - median (range)	36.5 (28 - 40)	-
BRCA gene mutation		
No	8	17.4
Yes, *BRCA* 1	1	2.2
Yes, *BRCA* 2	0	0.0
Unknown	37	80.4
Breast tumor localization		
Left	23	50.0
Right	21	45.7
Both	2	4.3
Most extensively performed breast surgery		
Needle biopsy	4	8.7
Breast conserving surgery	15	32.6
Mastectomy	27	58.7
Breast tumor histological subtype		
Ductal	36	78.2
Lobular	5	10.9
Ductolobular	5	10.9
Scarff-Bloom-Richardson grade		
I	4	8.7
II	19	41.3
III	15	32.6
Unknown	8	17.4
Estrogen receptor		
Negative	5	10.9
Positive	41	89.1

Table 1. Continued

Progesterone receptor		
Negative	8	17.4
Positive	38	82.6
Her2/neu receptor		
Negative	38	82.6
Positive	8	17.4
Tumor stage		
T1	11	23.9
T2	24	52.2
T3	7	15.2
T4	4	8.7
Nodal stage		
N0	14	30.4
N1	12	26.1
N2	10	21.7
N3	10	21.7
Distant metastasis		
cM0	39	84.8
cM1	7	15.2
Age at diagnosis of ovarian metastases, years - median (range)	40.0 (31 - 51)	-
Time between breast cancer and ovarian metastases, months - median (range)	41.9 (0.3 - 141.8)	-
Recurrent disease prior to oophorectomy		
No	15	32.6
Yes, locoregional recurrence	12	26.1
Yes, distant recurrence	19	41.3
Type of ovarian surgery		
Unilateral oophorectomy	0	0.0
Bilateral oophorectomy	46	100.0
Indication for oophorectomy		
Prophylactic because of breast cancer	9	19.6
Therapeutic because of breast cancer	25	54.3
Abnormal ovaries on ultrasound	12	26.1
Localization of ovarian metastases		
Left	4	8.7
Right	6	13.0
Both	29	63.0
Unknown	7	15.2

Localization and morphology of ovarian metastases

Of the 46 patients, 29 patients had metastases in both ovaries (Table 1). Therefore, the total number of ovaries that contained metastases was 75. The localization and morphology of these 75 ovarian metastases are shown in Table 2. In 14 ovaries (19%) the metastases seemed confined to the cortex, whereas in 53 ovaries (70%) both the cortex and medulla were involved (Table 2). In half of the ovaries multiple distinct nodules were seen, while in twenty percent a solitary metastasis was found. Diffuse seeding without any discernable pattern was observed in 29% of ovaries. Figure 2 shows examples of these morphological features.

Table 2. Localization and morphology of ovarian metastases derived from patients diagnosed with invasive breast cancer

Histological features	Ovarian metastases	
	N = 75	%
Localization of ovarian metastases		
Cortex	14	18.7
Medulla	8	10.7
Both	53	70.1
Morphology of ovarian metastases		
Solitary metastasis	15	20.0
Multiple distinct nodules separated by uninvolved ovarian tissue	38	50.7
Diffuse seeding without any discernable pattern	22	29.3
Fallopian tube involved		
No	55	73.3
Yes	5	6.7
Unknown	15	20.0

Of the 46 patients who were diagnosed with invasive breast cancer and ovarian metastases, 29 patients had metastases in both ovaries. The total number of ovaries that contained metastases was 75.

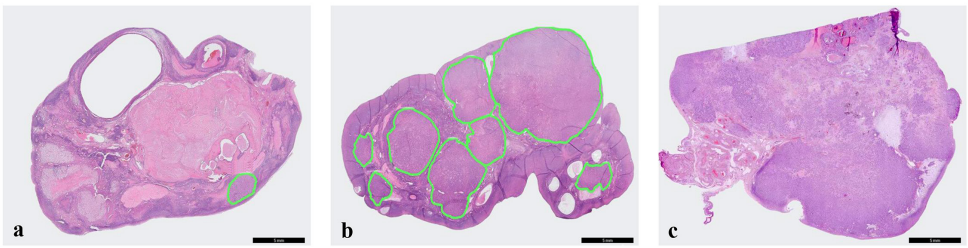

Figure 2. Localization of ovarian metastases derived from patients diagnosed with invasive breast cancer

Three examples are shown: **(a)** a solitary metastasis, **(b)** multiple distinct nodules separated by uninvolved ovarian tissue and **(c)** diffuse seeding without any discernable pattern. In order to clearly display the solitary metastasis in **(a)** and the multiple distinct nodules in **(b)**, a green line is drawn that delineates the metastases in the ovary. Scale bars represent 5 mm.

Expression of cell-surface proteins

Immunohistochemistry was performed to determine which cell-surface proteins are suitable as a target for tumor-specific imaging of ovarian metastases from invasive breast cancer. A strong correlation was observed between the scoring results obtained by the two observers; the median R^2 was 0.846 (range 0.640-0.960). Representative examples of the immunohistochemical stainings of the invasive breast tumor samples and their corresponding ovarian metastases are shown in Supplementary Figure S1.

Table 3 shows the mean percentage of positive tumor cells for the investigated markers in primary and recurrent invasive breast tumors and their ovarian metastases. Since loss of expression of the cell-adhesion molecule E-cadherin frequently occurs in invasive lobular carcinomas,[25] the expression of markers was examined by histological subtype. With respect to invasive ductal carcinomas, E-cadherin, EMA and Her2/neu were most suitable; these markers were present in 91, 84 and 81% of metastatic breast tumor cells in the ovaries, respectively. In invasive lobular carcinomas, the mean percentage of positively stained breast tumor cells in the ovaries was highest for EMA, Her2/neu and EpCAM; specifically, 64, 74 and 68%, respectively. In patients diagnosed with ductolobular breast cancer, targeting EMA would result in the detection of 99% of disseminated breast cancer cells in the ovaries.

Table 3. Immunohistochemical expression of the investigated markers in invasive breast tumors and their corresponding ovarian metastases

Marker	% of positive tumor cells in invasive ductal carcinoma				% of positive tumor cells in invasive lobular carcinoma				% of positive tumor cells in invasive ductolobular carcinoma			
	Breast tumors (n = 44)		Ovarian metastases (n = 58)		Breast tumors (n = 7)		Ovarian metastases (n = 10)		Breast tumors (n = 7)		Ovarian metastases (n = 7)	
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
E-cadherin	91	18	91	20	9	23	0	0	73	35	51	41
EMA	86	23	84	24	86	32	64	32	97	6	99	2
Her2/neu	76	35	81	31	88	26	74	26	80	34	67	38
CEA	56	40	57	39	73	32	59	26	62	32	56	33
αvβ6 integrin	51	40	45	39	54	35	38	28	45	30	29	35
EpCAM	36	42	38	39	38	46	68	26	19	29	35	29

SD = standard deviation

The mean percentages of immunohistochemically positive stained tumor cells are subdivided by histological subtype. Tumor cell membranes were considered positive if they showed immunoreactivity of any intensity. EMA, epithelial membrane antigen; Her2/neu, human epidermal growth receptor type 2; CEA, carcinoembryonic antigen; EpCAM, epithelial cell adhesion molecule.

Correlation between the expression of cell-surface proteins in invasive breast tumors and their corresponding ovarian metastases

In patients diagnosed with ductolobular breast cancer, the expression of EMA in the invasive breast tumors was in accordance with the expression in their corresponding ovarian metastases, showing small standard deviations (Table 3). Therefore, EMA would be the most suitable target to detect ductolobular ovarian metastases. By contrast, in patients diagnosed with ductal or lobular breast cancer large variations in expression among tumors were found. To understand whether in these patients invasive breast tumor tissues can be used to predict the most suitable target for the detection of ovarian metastases in an individual patient, scatter plots were made (Figure 3). For each patient, the percentage of positive tumor cells in primary and locally recurrent breast tumors (if applicable) was set against the percentage of positive tumor cells in their corresponding ovarian metastases. No correlation between these expressions could be substantiated, showing that ductal and lobular breast tumor tissues cannot be used to predict the most pertinent marker for the detection of their corresponding ovarian metastases.

Detection of ovarian metastases by a combination of markers

Figure 3 also shows that the use of one marker would not always be sufficient to detect all metastatic ductal or lobular breast cancer cells in the ovaries. The use of one marker (E-cadherin, EMA or Her2/neu) would result in the detection of 100% of tumor cells in 44 out of 58 ductal ovarian metastases (data not shown). With respect to the lobular subtype, EMA, Her2/neu or EpCAM was present in 100% of tumor cells in 4 out of 10 ovarian metastases.

To investigate whether a combination of markers would enable the detection of 100% of tumor cells in all ductal and lobular ovarian metastases, an immunofluorescent triple staining was performed. By combining the three most suitable markers for the ductal (E-cadherin, EMA and Her2/neu) and lobular (EMA, Her2/neu and EpCAM) subtypes, 100% tumor cell detection was accomplished in 53 out of 58 ductal ovarian metastases and in 7 out of 10 lobular ovarian metastases. Hence, cells within ovarian tissues that show membranous positivity for any of the three markers mentioned will be deemed malignant. In the remaining five ductal and three lobular ovarian metastases, the mean percentage of undetected metastatic cells was 5% (no range) and 25% (range 10-40), respectively. Figure 4 shows a representative image of the immunofluorescent triple staining in a lobular ovarian metastasis, in which the combination of EpCAM, EMA and Her2/neu led to the detection of all metastatic breast cancer cells.

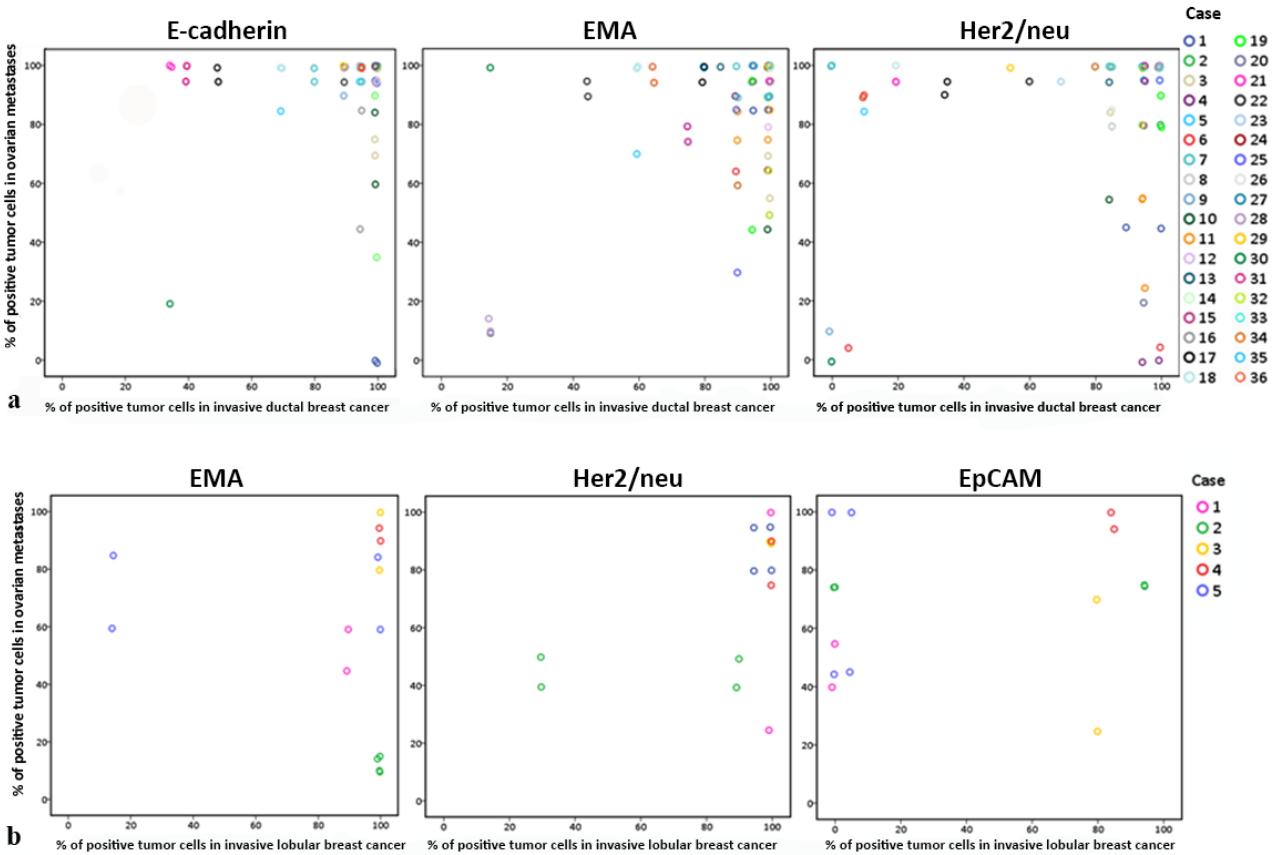

Figure 3. The correlation between tumor marker expression in breast tumors and ovarian metastases for individual patients

Upper panel **(a)** shows invasive ductal breast cancer and lower panel **(b)** represents invasive lobular breast cancer. For each patient, the percentage of positive tumor cells in primary and locally recurrent breast tumors (if applicable) was set against the percentage of positive tumor cells in their corresponding ovarian metastases. EMA, epithelial membrane antigen; Her2/neu, human epidermal growth receptor type 2; EpCAM, epithelial cell adhesion molecule.

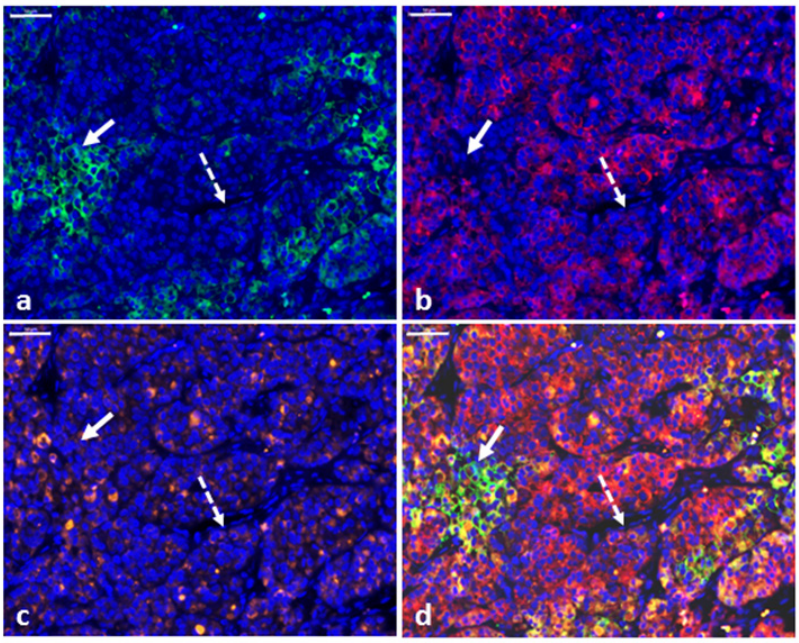

Figure 4. Detection of ovarian metastases by a combination of markers
Representative image of a lobular ovarian metastasis stained with DAPI counterstain and triple immunofluorescence for EpCAM **(a)**, EMA **(b)**, Her2/neu **(c)**, and the three stainings combined **(d)**. The solid arrow indicates tumor cells that are positive for EpCAM, but negative for EMA and Her2/neu. The dashed arrow indicates tumor cells that are positive for Her2/neu, but negative for EpCAM and EMA. Scale bars represent 100 µm. EpCAM, epithelial cell adhesion molecule; EMA, epithelial membrane antigen; Her2/neu, human epidermal growth receptor type 2.

Discussion

One of the purposes of the present study was to examine the histological features of ovarian metastases in breast cancer patients to evaluate the current tumor detection approach[4] in ovarian tissues considered for autotransplantation. We found that 71% of ovarian metastases consisted of a solitary metastasis or multiple distinct nodules separated by uninvolved ovarian tissue. These findings suggest that tumor cells might have been missed if the current tumor detection approach would have been used. The patients included in this study however, underwent oophorectomy after a median time interval of 42 months. In patients undergoing ovarian tissue cryopreservation an oophorectomy is performed soon after cancer diagnosis. In these patients, disseminated tumor cells may not yet have outgrown into overt metastases and may appear as micrometastases in the ovarian tissues.[23,26,27] The chance that tumor cells will then be overlooked is presumably

greater. We therefore recommend examination of the actual ovarian autografts on the presence of malignant cells prior to autotransplantation.

In our study, merely 63 out of 2648 patients (2.4%) who were diagnosed with primary invasive breast cancer at age < 41 years had ovarian metastases. For the determination of suitable targets for NIRF imaging, it might have been relevant to focus on malignancies with a higher risk of ovarian contamination in the actual patient population, for instance leukemia.[28-30] Nonetheless, breast cancer can be perfectly used as a starting point to investigate whether NIRF imaging is feasible for the detection of ovarian metastases.

Considering the expression of Her2/neu in primary invasive breast cancers and ovarian metastases a high percentage of Her2/neu positive tumor cells (67-88%) was found, as we considered tumor cell membranes positive if they showed immunoreactivity of any intensity. This is in contrast to the diagnostic setting, where Her2/neu overexpression is determined because of its potential prognostic value.[17,31] We applied a lower cut-off point, because for NIRF imaging the staining intensity is less important as long as a significant tumor-to-background-ratio can be achieved. In the NIR spectrum, non-specific fluorescence background signal is substantially decreased compared to wavelengths lower than NIR.[8] Hence, since ovarian stromal cells do not immunohistochemically express Her2/neu,[32] Her2/neu-targeting NIRF probes will detect metastatic breast cancer cells within ovarian tissues if these cells show immunohistochemical reactivity.

In individual patients, no correlation was found between the expression of the investigated markers in breast tumors and their corresponding metastases in the ovary. This might be due to the fact that breast cancer is known as a heterogeneous disease[17] or be in line with the hypothesis that disseminated tumor cells autonomously evolve from the primary tumor.[33] For the clinical application of these markers there should not be an obstacle, since a combination of three markers enhances the ability to detect breast tumor cells in ductal and lobular ovarian metastases. Furthermore, only the histological subtype of the invasive breast tumor needs to be known to determine which combination of markers is pertinent for the detection of the corresponding ovarian metastases, making the selection of suitable NIRF probes simple and straightforward.

For the non-invasive detection of metastases in the actual ovarian autografts by tumor-specific imaging, NIRF probes could be administered intravenously, after which the removed ovary is dissected into cortical ovarian strips. Subcellular detailed fluorescent images of tumor cells within ovarian autografts could then be obtained by multiphoton microscopy.[34] Beside breast cancer cells, inclusion cysts will likely also be illuminated by NIRF imaging, as we previously showed that in normal ovaries, all markers (except CEA) were expressed on epithelial cells in inclusion cysts.[32] Nevertheless, we additionally demonstrated that full-field optical coherence tomography (FF-OCT), which creates histology-like images without the need for tissue manipulation, can be perfectly used to differentiate between inclusion cysts and metastases in the ovary.[35] On a tomographic FF-OCT image, an inclusion cyst is characterized by a thin dark outer layer and lack of interior structure, whereas micrometastatic lesions from primary invasive ductal carcinomas

present as 'web-like' structures in which tumor cells appear light gray. Metastatic lesions derived from primary invasive lobular carcinomas often show an Indian file pattern, defined as infiltrating single rows of cells.[36] Since ovarian inclusion cysts are separately identifiable within the ovarian parenchyma,[32] a distinction between these structures can also be made. In addition, FF-OCT and NIRF imaging might be combined to enhance their sensitivity and specificity rates, as both methods are noninvasive.

The a priori probability that other benign epithelial ovarian abnormalities will be detected by our panel of cell-surface markers is low, since ovaries that present as an adnexal mass on preoperative ultrasonography are generally not used for ovarian tissue cryopreservation. In case primary ovarian cancer cells are present, these cells will be detected as E-cadherin,[37] EMA,[38] and EpCAM[39] are virtually always expressed in ovarian cancer, and approximately 33% of primary ovarian carcinomas show Her2/neu amplification.[40]

Conclusion

In conclusion, we showed that in young breast cancer patients with ovarian metastases, metastatic breast tumor cells may be confined to a specific area in the ovarian cortex. A non-invasive tumor detection technique by which cortical ovarian fragments that are transplanted can be examined, is recommended to minimize the risk of reintroducing metastatic tumor cells by ovarian tissue autotransplantation in breast cancer patients. NIRF imaging is a promising technique to discriminate malignant from benign tissues while leaving the examined tissues vital. Our research opens a new avenue for the development of tumor-specific NIRF probes that can be used for non-invasive detection of breast cancer metastases in ovarian tissues prior to autotransplantation.

Ethics approval and consent to participate

This study was approved by the medical ethical committee of the Leiden University Medical Center (protocol number P14.106) and the local medical ethical committees of the participating hospitals (Supplementary Table S2). Written human subject consent was not necessary, as the processing of personal data was performed according with the Wbp (Personal Data Protection Act). We received permission from the Dutch Pathology Registry (PALGA) to access the PALGA dataset. All patient samples and clinical data were handled in accordance with the medical ethics guidelines described in the Code of Conduct for Proper Secondary Use of Human Tissue of the Dutch Federation of Biomedical Scientific Societies (FMWV).[41]

Acknowledgements

The authors gratefully acknowledge the Dutch Pathology Registry (PALGA), the pathology laboratories, and the treatment hospitals for their collaboration. The authors also thank Rob Keyzer, BSc, Ronald L.P. van Vlierberghe, BSc for practical help, and Erik W. van Zwet, PhD for assistance with statistical analysis. These contributors have no conflict of interest.

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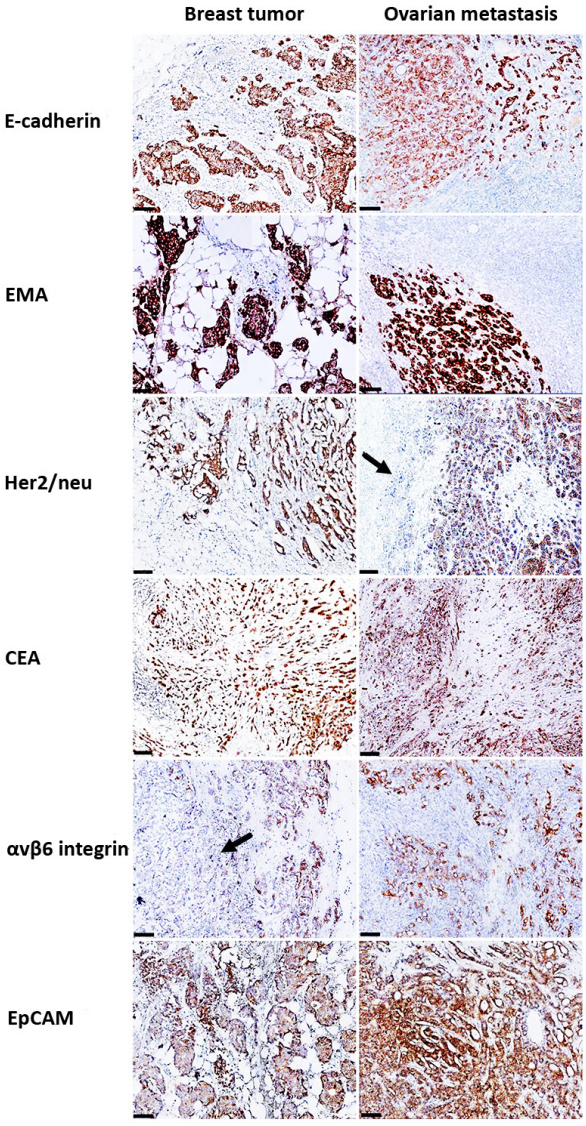

Supplementary Figure S1. Immunohistochemical expression of tumor markers in invasive breast tumors and their corresponding ovarian metastases

Arrows indicate tumor cells that show heterogeneous expression of markers. Scale bars represent 100 µm. EMA, epithelial membrane antigen; Her2/neu, human epidermal growth receptor type 2; CEA, carcinoembryonic antigen; EpCAM, epithelial cell adhesion molecule.

Supplementary Table S2. List of participating hospitals
An overview is given of the treatment hospitals that participated in the study.

Amphia hospital
Antoni van Leeuwenhoek hospital - Netherlands Cancer Institute
Diakonessenhuis Utrecht
Gelre hospital
Haga hospital
IJsselland hospital
Isala klinieken
Jeroen Bosch hospital
Leiden University Medical Center
Lievensberg hospital
Maastricht University Medical Center
Martini hospital
Meander Medical Center
Medisch Spectrum Twente
Onze Lieve Vrouwen Gasthuis
Rijnstate hospital
St. Anna hospital
St. Antonius hospital
St. Lucas Andreas hospital
St. Franciscus gasthuis
University Medical Center Groningen
University Medical Center Utrecht
Vlietland hospital
VU Medical Center
Zaans Medical Center
Ziekenhuisgroep Twente
Zuiderzee Medical Center

Chapter 5

Noninvasive detection of metastases and follicle density in ovarian tissue using full-field optical coherence tomography

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* These authors contributed equally to this article

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Abstract

Purpose

Autotransplantation of ovarian tissue can be used to restore fertility in cancer patients following gonadotoxic treatment. Whether this procedure is safe remains unclear, as current tumor detection methods render the ovarian tissue unsuitable for transplantation. Full-field optical coherence tomography (FF-OCT) is an imaging modality that rapidly produces high-resolution histology-like images without the need to fix, freeze, or stain the tissue. In this proof-of-concept study, we investigated whether FF-OCT can be used to detect metastases in ovarian tissue, thereby increasing the safety of ovarian tissue autotransplantation. We also evaluated whether cortical ovarian tissue and follicles remain viable following FF-OCT imaging.

Experimental design

Formalin-fixed, paraffin-embedded tissue samples were obtained from seven normal ovaries and fourteen ovaries containing metastases and/or micrometastases. These samples were deparaffinized and imaged using FF-OCT. The FF-OCT images were then compared to corresponding hematoxylin-and-eosin-stained tissue sections. Finally, we examined the effect of FF-OCT imaging on the viability of ovarian tissues and follicles in fresh bovine ovarian tissue using a glucose uptake and neutral red staining, respectively.

Results

FF-OCT illustrated both normal structures and metastases in ovarian tissue within minutes. Primordial follicles were readily identifiable. Finally, tissues and follicles remained viable following FF-OCT imaging for up to 180 and 60 minutes, respectively.

Conclusion

FF-OCT imaging is a promising method for the non-invasive detection of metastases, including micrometastases, in ovarian tissue. Moreover, this method facilitates the selection of cortical ovarian tissue with the highest density of primordial follicles, potentially increasing the likelihood of restoring ovarian function following ovarian tissue autotransplantation.

Introduction

Although advanced screening methods and novel treatment modalities represent a great leap forward in the treatment of cancer leading to substantially increased survival rates, new concerns have arisen as a consequence of these remedies, particularly among young women. Specifically, chemotherapy and/or pelvic radiotherapy can lead to premature ovarian failure due to accelerated follicle loss.[1] Therefore, preserving female reproductive capability is extremely important and has high priority. Currently, the most established fertility preservation procedures include freezing of embryos and/or oocytes. In addition, cryopreservation and subsequent autotransplantation of cortical ovarian tissue has become more prevalent. Because this method obviates the need for hormonal stimulation, it is deemed particularly suitable for prepubescent girls and young women who cannot delay the start of adjuvant therapy.[2] Autotransplantation of frozen-thawed ovarian tissue has been shown to restore ovarian activity in 93% of cases, and to date 60 resulting live births have been reported worldwide.[3-4] Despite these encouraging statistics, the safety of this procedure cannot yet be ascertained for certain types of cancer, as there is a potential risk of occult ovarian involvement at the time of tissue harvesting.[5-6] Due to the fact that the current tumor detection methods (e.g. histology, PCR analysis) render the tissue fragment unsuitable for transplantation, the presence of disseminated tumor cells can only be determined in the ovarian tissues that are ultimately not transplanted. Consequently, tumor cells that have metastasized to the actual ovarian autografts can be reimplanted, thereby potentially re-establishing cancer in the recipient. Previous reports have documented recurrences following autotransplantation in a patient diagnosed with breast cancer[7] and a patient diagnosed with cervical cancer.[8] However, whether these recurrences can be directly attributed to autotransplantation remains unclear. On the other hand, it is entirely plausible that malignant cells were reintroduced by autotransplantation in a patient with a granulosa cell tumor.[9]

To minimize the likelihood of reintroducing tumor cells, a technique that is capable of discriminating malignant from healthy tissues while leaving the examined tissues unaffected is needed. Full-field optical coherence tomography (FF-OCT) is a promising new imaging modality that satisfies these key criteria. Importantly, FF-OCT rapidly generates high-resolution histology-like images without the need to fix, freeze, or stain the tissue.[10] With FF-OCT, the image contrast is based on the light-scattering properties of different structures in the tissue. FF-OCT has already been used successfully to detect tumors in a wide variety of human tissues, including prostate,[11] skin,[12] brain,[13] lung,[14] and kidney[15] samples.

In this proof-of-concept study, we investigated whether FF-OCT can be used to visualize normal structures as well as metastases—including micrometastases—in human ovarian tissue. In addition, we evaluated whether cortical ovarian tissue and preantral follicles remain viable following FF-OCT imaging.

Materials and methods

Human ovarian tissue specimens

Formalin-fixed, paraffin-embedded (FFPE) specimens of normal ovaries obtained from premenopausal women who underwent prophylactic bilateral oophorectomy because of the presence of a *BRCA* gene mutation in the period 2001-2012 were selected from the archives of the Department of Pathology at the Leiden University Medical Center (LUMC). Patients who were previously treated with chemotherapy or used oral contraceptives prior to oophorectomy were excluded in order to ensure that only functionally active ovaries were included in the study. In addition, via a nationwide search performed by PALGA, the Dutch histopathology and cytopathology network and archive that covers all pathology laboratories within the Netherlands,[16] FFPE specimens of ovarian metastases were collected from women with primary invasive breast cancer at age < 41 years in the period 2000-2010. Ovarian metastases derived from other primary malignancies in which ovarian tissue cryopreservation is performed were collected from the archives of the Department of Pathology at the LUMC. Hematoxylin-and-eosin (H&E) stained tissue sections were obtained and digitized using an IntelliSite Pathology Ultra-Fast scanner 1.6 RA (Philips, Eindhoven, the Netherlands). Following this, the entire FFPE tissue blocks were deparaffinized and imaged using FF-OCT.

All patient samples and clinical data were handled in accordance with the medical ethics guidelines described in the Code of Conduct for the Proper Secondary Use of Human Tissue of the Dutch Federation of Biomedical Scientific Societies (FMWV).[17]

Bovine ovarian tissue specimens

Intact bovine ovaries were collected at an abattoir within 15 minutes after slaughtering and immediately transported on ice to the laboratory at the LUMC. The average transport time was 20 minutes. At the laboratory, each ovary was cut into two halves, and the medulla was removed. The remaining cortex was trimmed to a thickness of 1-2 mm and subsequently cut into fragments measuring 2-3 mm or 5-10 mm in diameter for a glucose uptake assay or a neutral red staining, respectively. Bovine cortical ovarian fragments were stored until imaging (ranging from 0 to 180 minutes) in 24-wells plates at 4°C. Each well contained 1.5 ml DMEM culture medium (Lonza BioPharma; Visp, Switzerland) supplemented with 10% FCS (Gibco, Thermo-Fisher; Waltham, MA) and 1% penicillin/streptomycin (MP Biomedicals; Santa Ana, CA).

Full-field optical coherence tomography

A commercially available LightCT FF-OCT system (LLTech; Paris, France) was initially used in this study.[18] During the course of the study, the LightCT scanner was upgraded to a newly developed FF-OCT system (LLTech), which is faster and can accommodate larger tissue samples. Both systems consisted of an upright microscope with a 10x objective with a central wavelength of 700 nm

with spectral width of 125 nm, and reference arm in the Linnik interferometric configuration.[19-20] The light reflected by the tissue interferes with the light reflected by the reference mirror, and the signal is then isolated from the scattered background light using a combination of four phase-shifted interferometric images. The LightCT scanner, which was used for the viability experiments, uses a 150-Watt white halogen lamp as the light source and generates a 0.8 mm x 0.8 mm *en face* image (with a maximum field of view 25 mm in diameter using image mosaicking) at an image rate of 35 Hz. The newly developed FF-OCT system, which was used to image the human ovarian tissues, uses an LED lamp as the light source and generates a 1.3 mm x 1.3 mm *en face* image (with a maximum field of view 45 mm in diameter) at an image rate of 75 Hz.

Image acquisition was similar for the two systems. In brief, the tissue was placed in the sample holder. Deparaffinized ovarian tissue was covered with saline, whereas fresh ovarian tissue was covered with DMEM culture medium (Lonza BioPharma) supplemented with 10% FCS (Gibco, Thermo-Fisher) and 1% penicillin/streptomycin (MP Biomedicals). An optical window was positioned above the tissue, and the tissue was gently flattened against this window. A layer of silicone oil was then applied between the optical window and the microscope objective. A macroscopic image was obtained using a wide-field camera, followed by FF-OCT images, which were made *en face*. The field of view encompassed the entire cortical ovarian fragment. With respect to human ovarian tissues, images were taken at a depth of 0-20 µm to ensure the best possible correspondence with the histology images. Considering the bovine cortical ovarian fragments, images were taken up to a depth of 100 µm, as it was the maximum depth at which high resolution could be retained. The number of images taken in depth depended on the time that was needed to achieve the predefined imaging time for the viability tests (ranging from 0 to 180 minutes). Both systems had 1.5 µm transverse resolution and 1 µm axial resolution.

Determining the viability of ovarian tissue using the glucose uptake assay

Bovine cortical ovarian tissue fragments measuring 2-3 mm in diameter were imaged using the LightCT scanner for 3, 10, 60, or 180 minutes. Fresh cortical ovarian samples that were not imaged served as a control, whereas cortical ovarian tissue samples in which cell death was induced by snap-freezing in liquid nitrogen served as a negative control. After imaging, the ovarian samples were transferred to 24-well plates for culturing. Each well was prepared in triplicate and contained three cortical ovarian samples in 1.5 ml DMEM culture medium (Lonza) supplemented with 10% FCS (Gibco, Thermo-Fisher) and 1% penicillin/streptomycin (MP Biomedicals). During culture, the medium was checked periodically for the presence of bacteria under a light microscope. The glucose content of the culture medium was measured using a blood-gas analyzer (Modular P Chemistry Analyzer; Roche Diagnostics, Indianapolis, IN). After four days in culture, the samples were weighed, and glucose consumption was measured and expressed per milligram of ovarian tissue per day, as previously described.[21-22]

Determining the viability of ovarian follicles using neutral red staining

The viability of preantral ovarian follicles was determined using a neutral red staining assay adapted from the protocol published by Kristensen et al.[23] Fresh bovine cortical ovarian fragments were imaged in triplicate using the LightCT scanner for 3, 10, 60, or 180 minutes. These ovarian fragments were 5-10 mm in diameter and 1-2 mm thick. Fresh cortical ovarian fragments that were not imaged served as a control, and cortical ovarian tissue fragments in which cell death was induced by snap-freezing in liquid nitrogen served as a negative control. After imaging, the ovarian fragments were cut into small pieces. These pieces were placed in 15-ml conical tubes containing 5 ml preheated Serum-free Ultraculture medium (Lonza) supplemented with 1 mg/ml collagenase type IA (Sigma-Aldrich, St. Louis, MO). During incubation, the cell suspensions were triturated through a 5-ml pipette to further disrupt the tissue. After incubation for 25 minutes at 37°C, the samples were centrifuged at 2500 rpm for 5 minutes. The supernatants were poured off, and each pellet of cells was incubated for 1.5 hours at 37°C in 4.8 ml McCoy's medium (Life Technologies, Carlsbad, CA) supplemented with 2 µl Albuman (200 g/L; Sanquin, Amsterdam, the Netherlands), 50 µl Insulin-Transferrin-Selenium (ITS-G; Life Technologies), 50 µl penicillin/streptomycin (MP Biomedicals), and 75 µl neutral red solution (3.3 g/L; Sigma-Aldrich). The number of red-colored (viable) and uncolored (non-viable) follicles in the partially dissolved ovarian tissue fragments were counted in ten high-power fields per specimen under a light microscope by two independent observers (I.P. and P.S.) who were blinded with respect to the samples.

Statistical analysis

Statistical analysis was performed using SPSS version 23.0 (IBM, Armonk, NY). Inter-observer agreement was calculated using the Pearson correlation coefficient. A multivariate linear regression model was used to compute the mean difference in the glucose uptake of cortical ovarian biopsies between the various FF-OCT exposure times. The values were adjusted for the number of ovaries included. To investigate the effect of FF-OCT imaging on the viability of preantral follicles, we compared the proportions of viable preantral follicles between the various FF-OCT exposure times.

Results

Human ovarian tissue specimens

FFPE tissue samples from seven normal ovaries (obtained from six premenopausal patients) and fourteen ovaries containing metastases and/or micrometastases (obtained from twelve patients) were deparaffinized and imaged using FF-OCT. In most cases, the FF-OCT images could be easily interpreted using the inverse setting. In this inverse setting, structures that reflect light the strongest appear black, whereas structures that reflect light poorly appear white.

Figure 1 shows representative FF-OCT images and corresponding histology images of the most clinically relevant structures in a normal ovary. The primordial follicles, which are small primary oocytes surrounded by a single layer of flattened granulosa cells,[24] appear as either round or crescent-shaped white structures, depending on the level at which they were imaged (Figure 1A-B). In primary follicles, the flat granulosa cells transform into a cuboidal structure and the zona pellucida forms between this layer and the oocyte. On the FF-OCT image, the oocyte appears somewhat darker than the zona pellucida, and the granulosa cells can be distinguished quite well (Figure 1C-D). Figure 1E-F shows a corpus rubrum, which is a small hemorrhage that forms immediately after ovulation. The corpus luteum is composed of an outer layer of smaller thecal-lutein cells and an inner layer of larger granulosal-lutein cells. On the FF-OCT images, the densely packed thecal-lutein cells reflected more light than the granulosal-lutein cells, which reflected light to the same extent as the corpus rubrum (Figure 1G-H). The corpus albicans (Figure 1I-J) and corpus fibrosum (Figure 1K-L), which are masses of fibrous scar tissues that form when the oocyte is not fertilized, showed high levels of reflection due to their collagen content. Lastly, an inclusion cyst (Figure 1M-N) was identified by its thin dark outer layer and lack of interior structure.

Figure 2A shows representative FF-OCT images of a sagittal section of an ovary containing micrometastases; this sample was derived from a primary invasive ductal breast carcinoma. Figure 2B-C and Figure 2D-E show magnified views of micrometastases measuring 0.9 mm and 1.7 mm, respectively. These metastatic lesions could be distinguished clearly from the surrounding stromal cells, as the stromal cells reflected considerably more light than the lesions. On the FF-OCT images, these lesions have a 'web-like' architecture in which the tumor cells appear light gray. Furthermore, a distorted ovarian cortex architecture (Figure 2D) was often seen in the presence of disseminated tumor cells. Figure 3 shows metastatic lesions within ovarian tissues obtained from three individual patients. Figure 3A-B depicts a solitary metastasis that originated from an invasive ductal breast carcinoma; the FF-OCT image shows the microglandular proliferation. In Figure 3C-D, the ovarian stroma is completely occupied by the metastatic lesion, which has a characteristic stromal component. The FF-OCT image of a metastasis originating from a primary endometrial carcinoma shows a typical cribriform morphology (Figure 3E), with good correspondence with the previously obtained H&E tissue section (Figure 3F). The morphological features of the remaining ten ovaries containing metastatic disease corresponded to those depicted in Figures 2 and 3. All types of normal ovarian features and metastatic lesions that were identified in the histology (H&E) sections were also detected by FF-OCT.

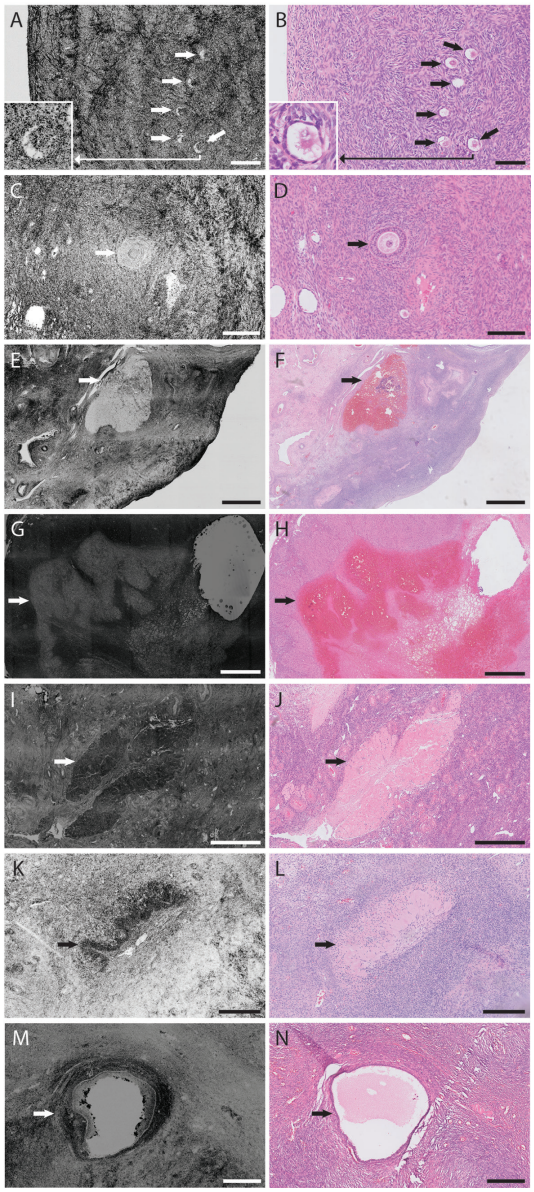

Figure 1. Example inverse FF-OCT images (left column) and corresponding histology images (right column) of normal ovarian tissues

The following normal ovarian structures are shown in the images: primordial follicles (A-B), a primary follicle (C-D), a corpus rubrum (E-F), part of a corpus luteum (G-H), a corpus albicans (I-J), a corpus fibrosum (K-L), and an inclusion cyst (M-N). In each image, the normal ovarian structures are indicated by an arrow. All but one primordial follicle were detected in the FF-OCT image (A) as compared to the corresponding histology image (B). This is due to a slightly different imaging depth. Scale bars represent 100 µm (A-B), 150 µm (C-D), 250 µm (K-L, and M-N), and 1 mm (E-F, G-H, and I-J). Insets show a characteristic primordial follicle (A-B) at three times higher magnification.

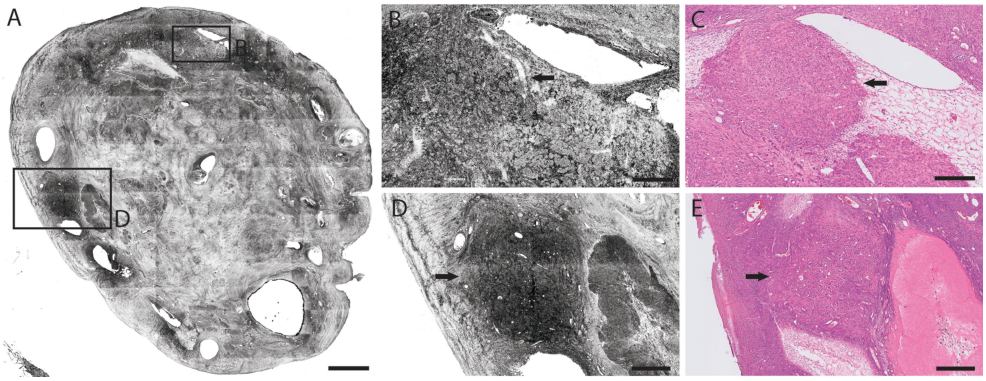

Figure 2. Example inverse FF-OCT images and corresponding histology images of ovarian metastases originating from a primary invasive ductal breast carcinoma

A sagittal view is shown in panel A, and panels B-E show magnified views of the micrometastases (indicated by arrows). Scale bars represent 2 mm (A), 250 µm (B-C), and 500 µm (D-E).

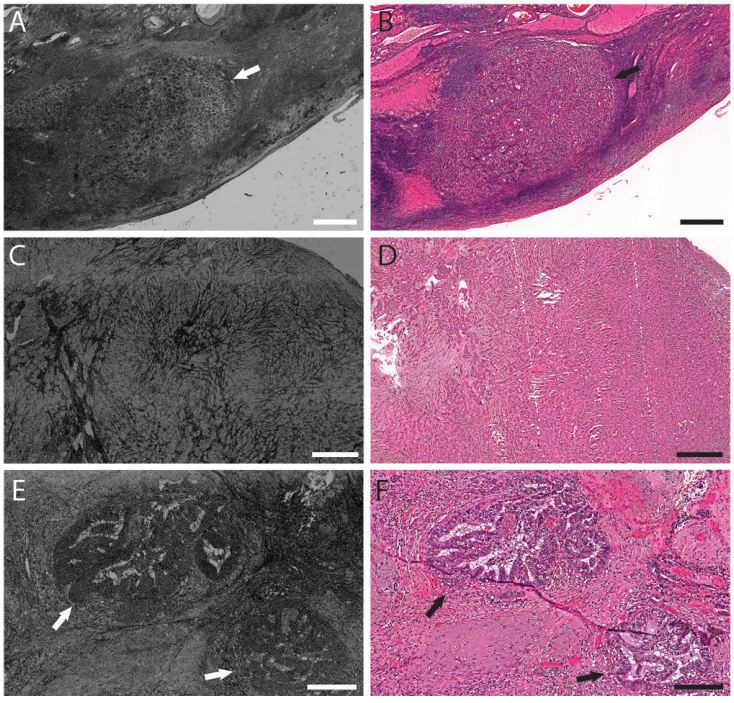

Figure 3. Example inverse FF-OCT images (left column) and corresponding histology images (right column) of ovarian metastases

An ovary containing a solitary metastasis originating from a primary invasive breast carcinoma is shown in panels A and B (indicated by arrows). An ovary in which disseminated breast tumor cells are dispersed throughout the ovary is shown in panels C and D. Micrometastases originating from a primary endometrial carcinoma are shown in panels E and F (indicated by arrows). Scale bars represent 500 µm (A-D) and 200 µm (E-F).

Viability of ovarian tissue measured using a glucose uptake assay

Next, we examined whether performing FF-OCT imaging on fresh cortical ovarian tissue affects tissue viability. Table 1 shows the mean glucose consumption per mg ovarian tissue per day over a 4-day culture period of the fresh cortical ovarian tissues that were imaged using FF-OCT for 0, 3, 10, 60, and 180 minutes. All values were adjusted for the number of ovaries used, and none reached statistical significance. Thus, cortical ovarian tissue remained viable — at least with respect to glucose uptake — up to three hours of FF-OCT imaging. No glucose uptake was measured in the cortical ovarian biopsies in which cell death was induced.

Table 1. Viability of cortical ovarian tissue after different FF-OCT exposure times

	Glucose uptake (mmol/mg tissue/day)	
	Mean	95% CI[a]
FF-OCT imaging time		
0 minutes	50.52	42.66 - 58.38
3 minutes	46.49	38.63 - 54.35
10 minutes	44.25	36.40 - 52.11
60 minutes	40.32	32.46 - 48.18
180 minutes	46.25	38.39 - 54.11

[a] CI; confidence interval

The mean glucose uptake over a 4-day culture period was determined in triplicate, as described in the material and methods section. All values were adjusted for the various ovaries, using a multivariate linear regression model, resulting in a standard error of the mean of 4.01.

Viability of preantral ovarian follicles measured using neutral red staining

We also examined the effect of different FF-OCT exposure times on the viability of preantral follicles in fresh cortical ovarian tissue fragments using neutral red staining. The percentage of viable preantral follicles in the samples that were not subjected to FF-OCT imaging was 86% (95% CI 0.81-0.92; Table 2). Although some variation was observed, this viability did not significantly decrease following FF-OCT imaging for up to 60 minutes. Cortical ovarian tissue fragments that were exposed to FF-OCT for 180 minutes had a significantly lower percentage (55%) of viable preantral follicles. As a negative control, no viable preantral follicles were observed in the cortical ovarian tissues in which cell death was induced. Figure 4 shows representative examples of preantral follicles in fresh cortical ovarian tissues that were not exposed to FF-OCT imaging (Figure 4A), preantral follicles that were subjected to FF-OCT imaging for 10 minutes (maximum time required to image a cortical ovarian fragment; Figure 4B) and preantral follicles that were exposed to FF-OCT imaging for 180 minutes (Figure 4C).

Table 2. Viability of preantral follicles after different FF-OCT exposure times

	Total number of follicles observed	Total number of viable follicles	Proportion	95% CI[a]
FF-OCT imaging time				
0 minutes	145	125	0.86	0.81 - 0.92
3 minutes	208	183	0.88	0.84 - 0.92
10 minutes	193	189	0.98	0.96 - 1.00
60 minutes	195	185	0.95	0.92 - 0.98
180 minutes	179	99	0.55	0.48 - 0.63

[a] CI; confidence interval

The proportion of viable preantral follicles was determined by a neutral red staining. Results are based on three different cortical ovarian fragments per condition.

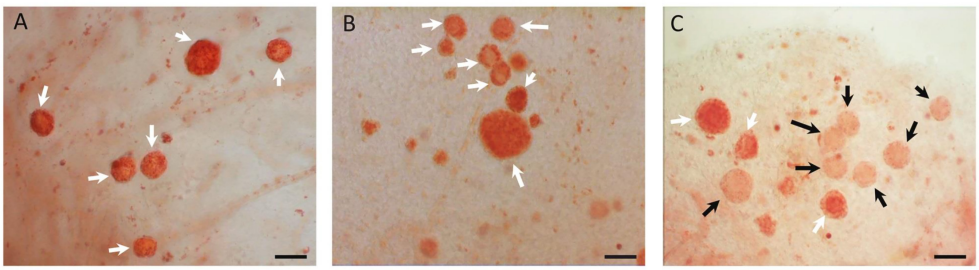

Figure 4. Viability of preantral follicles before and after FF-OCT imaging

Representative images of preantral follicles in fresh cortical ovarian tissues that were not exposed to FF-OCT imaging are shown in panel A. Preantral follicles in cortical ovarian tissues that were subjected to FF-OCT imaging for 10 minutes are shown in panel B. Preantral follicles in cortical ovarian tissues that were subjected to FF-OCT imaging for 180 minutes are shown in panel C. The white and black arrows indicate red-colored (i.e., viable) and uncolored (i.e., non-viable) follicles, respectively. Scale bars represent 100 µm.

Discussion

In this proof-of-concept study, we investigated whether FF-OCT can be used to detect metastases in ovarian tissue prior to autotransplantation, thereby reducing the risk of reintroducing ovarian tissue that might contain metastases. We found that FF-OCT can be used to visualize both normal structures and metastases — including micrometastases — in ovarian tissues derived from premenopausal women. These findings are particularly relevant from a clinical perspective, given that the pathologist must distinguish between benign and malignant lesions based solely on tomographic images. Routine pathology methods such as histology and immunohistochemistry cannot be used in this context, as these methods render the cortical ovarian tissue unsuitable for autotransplantation. Importantly, our results may serve to help pathologists use FF-OCT as a novel tool for detecting metastases in ovarian tissues.

In our study, we used deparaffinized FFPE tissue samples, as fresh ovarian tissues were not readily available for research purposes and ovarian metastases are relatively rarely encountered. However, we found no difference with respect to visual assessment of normal ovarian structures between the deparaffinized ovarian tissue samples and the fresh ovarian tissues that were used for measuring viability. Our finding is supported by a study of Wilson et al. in which the optical features of various non-paraffinized and deparaffinized tissues were compared and no substantial differences were found.[25]

Because of the relatively few number of ovarian tissue samples that contained micrometastases, we were unable to perform a blinded analysis in which two pathologists independently assessed the FF-OCT images without having access to the original H&E-stained tissue sections. Such an analysis would require a 'training set' in order to familiarize the pathologists with non-neoplastic and neoplastic ovarian tissues on FF-OCT, followed by a 'test set' for which the sensitivity and specificity of FF-OCT imaging could be determined.[14-15] Although this type of analysis was beyond the scope of this proof-of-concept study, it should be performed in future studies in order to confirm whether FF-OCT is a suitable diagnostic instrument for use in ovarian tissue. The maximum depth of FF-OCT imaging that provided high-resolution images in the cortical ovarian strips was approximately 100 μm, which is considerably lower than the imaging depth of up to 500 μm reported for other tissues.[10] This difference is due primarily to the large-scale extracellular matrix network in the ovarian cortex,[26-27] which greatly reduces tissue penetration.[28] Nevertheless, imaging depth could be increased somewhat by imaging the cortical ovarian tissue fragments from both sides, thereby effectively doubling the amount of tissue that can be imaged. Moreover, rapid advances in the field of optical imaging will likely enable clinicians to use this non-invasive approach to visualize even deeper structures in the near future.

Interestingly, previous studies that used conventional OCT to examine ovaries achieved an imaging depth of up to 2 mm.[29-30] However, the imaging resolution of conventional OCT is considerably lower than FF-OCT.[31] For our purposes, achieving ultra-high resolution is crucial, as patients with clinically diagnosed early-stage cancer who are eligible for cryopreservation of ovarian tissue can potentially have occult micrometastases in their ovarian tissues.[32-34] Importantly, FF-OCT imaging provides images with a resolution of approximately 1 μm.[31] In addition to the detection of occult ovarian involvement, this high-resolution imaging modality provides the opportunity to visually assess the density of primordial follicles in the ovarian autografts, which is highly relevant, given that successfully restoring ovarian activity depends critically on the number of primordial follicles present in the ovarian autograft.[3] Thus, FF-OCT provides the added value of selecting the cortical ovarian fragments with the highest potential for restoring fertility.

Because FF-OCT generates images at a rapid rate,[15] this technique is particularly suitable to the field of ovarian tissue cryopreservation, where ischemia and oxidative stress during a lengthy avascular period are known to cause loss of primordial follicles.[35] The average time required to image a cortical ovarian fragment measuring 5-10 mm in diameter was seven minutes using the

original FF-OCT system, and this time was reduced further to a mere one minute with the newly developed FF-OCT system. During these brief periods, the viability of both the ovarian tissue and the preantral follicles remained high. Even performing FF-OCT imaging for 180 and 60 minutes did not significantly reduce the viability of ovarian tissues and preantral follicles, respectively. These data support the notion that short-term FF-OCT imaging has little effect on either ovarian tissues or preantral follicles. Nevertheless, given that the most meaningful outcome in the field of fertility preservation is ultimately a successful pregnancy, future research will focus on evaluating pregnancy rates following exposure to FF-OCT.

With respect to using FF-OCT as a non-invasive means to detect metastases in the actual ovarian autografts, cortical ovarian strips can be imaged immediately following oophorectomy (see the proposed workflow diagram in Supplementary Figure 1). If the pathologist observes lesions that raise a suspicion of metastases, these fragments could then be analyzed further using histological examination and/or PCR analysis. If these further tests confirm the presence of metastatic tissue in the ovarian fragments, no autotransplantation will be performed. On the other hand, if the histological results are negative (i.e., a false positive result on FF-OCT), the remaining ovarian fragments—particularly those fragments that appeared normal on FF-OCT imaging—can still be transplanted. Moreover, as discussed above, the pathologist can also select the tissue fragments with the highest density of follicles, thereby maximizing the likelihood of restoring the patient's ovarian function. Importantly, FF-OCT imaging should be performed under sterile conditions in order to prevent bacterial contamination.

In conclusion, FF-OCT imaging is a promising new non-invasive method for rapidly detecting ovarian metastases in ovarian fragments prior to autotransplantation in patients whose ovarian function has been lost. Moreover, this method facilitates the selection of cortical ovarian tissues with the highest density of primordial follicles, significantly increasing the likelihood of restoring ovarian function. Although it is not yet possible to detect all ovarian metastases and/or follicles in the cortical ovarian fragments due to a limited tissue penetration depth, FF-OCT provides a large improvement over the current tumor detection methods as it is the only approach now available by which the actual ovarian autografts, used for transplantation, can be examined.

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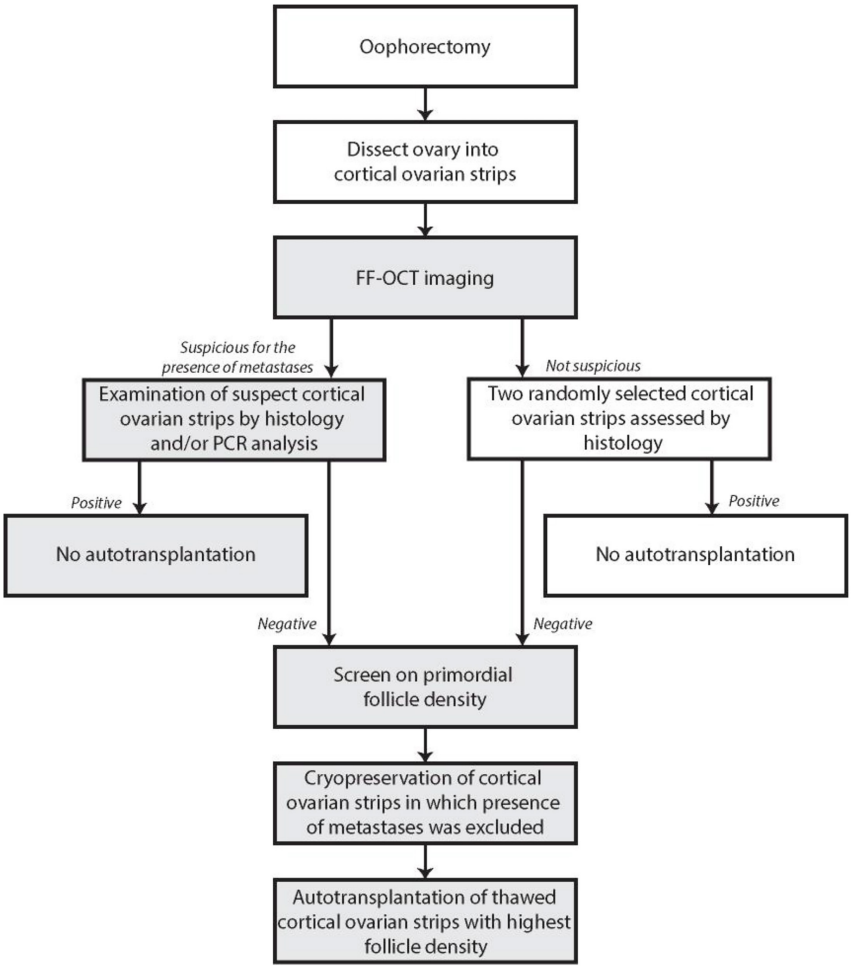

Supplementary Figure S1. Proposed clinical workflow for screening cortical ovarian strips prior to autotransplantation in order to minimize the likelihood of reintroducing tumor cells and maximize the likelihood of restoring ovarian function

The light-gray shaded boxes indicate steps in which FF-OCT imaging can provide added value.

Chapter 6

General discussion and future perspectives

Over the past years, cryopreservation and subsequent autotransplantation of ovarian tissue has become more prominent in the field of fertility preservation. Ever since the first successful autotransplantation,[1] much research has been performed on establishing patient selection criteria,[2] the efficiency of different freezing and transplantation techniques,[3-7] and the safety of this procedure in cancer patients.[8-10] Although a tremendous amount of knowledge has been gained from these studies, the safety of this procedure has not yet been fully elucidated. Through the studies in this thesis, we aimed to further determine the safety of ovarian tissue autotransplantation. Besides, we made an initial step toward the development of novel methods for the detection of ovarian metastases such as near-infrared fluorescence imaging and full-field optical coherence tomography, which may help overcome the limitations associated with the current tumor detection methods. Central in this thesis were studies on ovarian metastases derived from primary invasive breast cancer, as the majority of patients who undergo ovarian tissue cryopreservation are diagnosed with this condition and relatively much ovarian tissue is available due to prophylactic and/or therapeutic oophorectomies.

The likelihood of developing ovarian metastases

Recent reviews of the literature on the risk of reintroducing malignancy following ovarian tissue autotransplantation revealed that leukemia and lymphoma have the highest and lowest risk of developing ovarian metastases, respectively.[8,9] With respect to breast cancer, ovarian metastases were described to occur in 13-47% of patients.[9,11] Yet, the vast majority of published data were based on autopsies as well as therapeutic oophorectomies and therefore likely restricted to patients with advanced stage disease. In the study described in **chapter 2**, a prevalence rate of 2.4% of ovarian metastases was found among young breast cancer patients who underwent an oophorectomy for prophylactic or therapeutic reasons. Despite the fact that the prevalence of ovarian metastases in patients whose ovaries remained in situ could not be determined, these findings showed that the likelihood of encountering secondary ovarian involvement among young breast cancer patients is relatively low.

The majority of the ovarian metastases described in **chapter 2** were clinically indolent and diagnosed following prophylactic or therapeutic oophorectomy after a median time interval of 47 months since the diagnosis of breast cancer. Although some time has elapsed before overt metastases were formed, breast cancer cells may have spread to the ovaries early in the course of the disease. It is thought that tumor cells that are endowed with metastatic capacity soon escape from the primary tumor and transmit to secondary target organs, where they enter a quiescent state for an indefinite period.[12,13] Whether these cells subsequently cease dormancy and progress into metastatic lesions depends on molecular interactions between cancer cells and the microenvironment of the secondary organ.[12,14] These interactions rely on crosstalks with different host cell types such as endothelial cells, immune cells, fibroblasts and/or other stromal cells, which may favor angiogenesis and allow tumor cells to escape from immune surveillance.[13,15]

This implies that, although it remains uncertain whether disseminated tumor cells eventuate in overt ovarian metastases, tumor cells might be present at the time of ovarian tissue harvesting.

Risk factors for the development of ovarian metastases

According to the current selection criteria for ovarian tissue autotransplantation, patients should have a realistic chance of survival.[2,16] These criteria seem justified, as they may reduce the chance that a patient dies several months after the birth of her child.[17] Yet, these selection criteria do not specifically include criteria that may reduce the risk of developing ovarian metastases. In **chapter 2**, we therefore aimed to identify risk factors for the development of ovarian metastases in young breast cancer patients in order to more comprehensively define selection criteria for ovarian tissue cryopreservation in these patients. In this study, a strikingly high percentage of clinically evident metastatic disease at the time of oophorectomy was observed among the 57 cases of whom clinical data were available; nine patients (16%) had distant metastases outside the ovary at the time of breast cancer diagnosis. These results are in line with a previous study of Gagnon and Têtu who found that 12 out of 39 breast cancer patients who were diagnosed with ovarian metastases (20%), had stage IV disease at the time of breast cancer diagnosis.[18] Unfortunately, the presence of distant metastases as an independent risk factor for the development of ovarian metastases could not be confirmed in our study, as this factor could not be included in the multivariate logistic regression models due to empty categories in the matched control group. On the other hand, several risk factors for the development of ovarian metastases were identified in our study. We showed that the risk of ovarian metastases increased with the time elapsed since breast cancer diagnosis. Furthermore, a statistically significant association between the development of ovarian metastases and tumor stage was observed in the multivariate logistic regression analyses. Hence, particularly young breast cancer patients with tumors > 5 cm in diameter and/or inflammatory breast cancer are at risk of developing ovarian metastases.

The morphological features of ovarian metastases and the accuracy of the current tumor detection approach

In the study described in **chapter 4**, we found that 71% of the ovarian metastases included in the study manifested as a solitary metastasis or multiple distinct nodules separated by uninvolved ovarian tissue. Though, in patients who undergo ovarian tissue cryopreservation, an oophorectomy is much earlier performed than in the patients included in the study described in chapter 4. It is presumable that in this former group of patients, tumor cells manifest as micrometastases and/or single cells. Hence, the chance that disseminated tumor cells will be overlooked in the ovarian tissues from patients undergoing ovarian tissue cryopreservation will likely be even greater using the current tumor detection approach. The only option now available to corroborate this hypothesis would be to scrutinize cortical ovarian fragments from deceased patients who consented to the use of their excised tissue for research purposes. However, such

a study is limited by the fact that relatively little cortical ovarian tissue from deceased cancer patients is available. Besides, if such studies reveal that ovarian metastases appear as single tumor cells, it will remain difficult to interpret these results in terms of clinical significance. After all, what number of tumor cells within an ovarian autograft is needed to cause cancer relapse following ovarian tissue autotransplantation, remains to be questioned. A study conducted by Soares et al. showed that xenografting one hundred leukemic cells that were embedded in a fibrin matrix appeared to be insufficient to induce leukemia in immunodeficient mice after 20 weeks.[19] Nevertheless, conclusive evidence could not be provided, as cancer cells may outgrow differently in humans than in mice.[20]

With respect to the localization of metastases in the ovary, 70% of the ovarian metastases were localized in both the cortex and medulla. In addition, 19% seemed to be confined to the cortex. However, because we did not sequentially cut the entire ovary, it is possible that tumor cells elsewhere in the medulla may have been overlooked. It would be reasonable that disseminated tumor cells, once they have seeded to the ovary, spread from the hilum to the medulla and ultimately disperse in the cortex where they become trapped in the capillary bed. According to the 'seed and soil theory', tumor cells can only grow if they encounter a congenial microenvironment.[21] Factors that may possibly contribute to the development of metastases in the ovarian cortex are for instance the high amount of fibroblasts and the densely packed collagen bundles that make up the extracellular matrix in the ovary.[13,22,23] Furthermore, it is plausible that hormonal circumstances play a prominent role herein, since breast cancer metastases are more frequently found in ovaries from premenopausal women.[24]

Implications of our findings for routine patient care

What implications do our findings have for routine patient care? In case of ovarian tissue autotransplantation, the risk of reintroducing malignant cells needs to be discussed during counselling. Breast cancer patients should be informed about the fact that ovarian metastases occur in 2.4% of patients diagnosed with breast cancer. Based on the results from our risk factor analyses as described in **chapter 2**, we would discourage ovarian tissue autotransplantation in patients diagnosed with tumors > 5 cm and/or inflammatory breast cancer. Besides, since the time passed between the diagnosis of breast cancer and oophorectomy seems to play a role in the development of ovarian metastases, we recommend to perform an oophorectomy soon after the diagnosis of breast cancer and subsequently transplant the cortical ovarian fragments back to the remaining ovary. This approach provides the opportunity to completely remove the ovarian autografts at a later time, thereby minimizing the risk of developing ovarian metastases. Lastly, patients must be told that the current tumor detection approach presumably provides a false sense of security. Hence, as long as there is no accurate alternative to the current tumor detection approach, the desire to conceive and the likelihood of reimplanting malignant cells should be carefully balanced.[25]

Non-invasive tumor detection methods

It stands to reason that it would be far better to develop a tumor detection approach by which the presence of disseminated tumor cells in the ovarian autografts can be excluded with certainty. In this thesis, we focused on two different optical imaging techniques that could possibly be used for the detection of ovarian metastases without affecting the ovarian tissue viability, namely near-infrared fluorescence imaging and full-field optical coherence tomography.

Near-infrared fluorescence imaging

Near-infrared fluorescence (NIRF) imaging has the potential to revolutionize cancer surgery, as it has been proven to provide significant guidance in distinguishing malignant from healthy tissues as well as recognizing vital structures.[26] A NIRF probe consists of a fluorophore that emits light in the near-infrared spectrum (λ = 700-900 nm) conjugated to an antibody or peptide with high affinity for a protein marker that is specifically expressed at the cell surface of tumor cells.[27,28] In the study described in **chapter 4**, we tested a panel of cell-surface markers in primary invasive breast tumors and their corresponding ovarian metastases in order to examine whether primary invasive breast tumor tissue can be used to predict which target would be most suitable for the detection of the corresponding ovarian metastases by NIRF imaging. Interestingly, no correlation could be substantiated between the expression of the markers in the invasive breast tumors and their corresponding ovarian metastases. One explanation for this discrepancy could be that primary tumors deploy genetic and/or epigenetic alterations in order to successfully spawn metastases in distant organs.[15] Moreover, once these disseminated tumor cells have invaded a secondary target organ, they utilize several signal transduction pathways in order to survive in the foreign tissue microenvironment and foster metastatic colonization. As a result of these complex mechanisms, metastases may display a different phenotype than their primary tumor. Furthermore, this observation could be explained by the molecular and cellular heterogeneity of breast cancer.[29]

To ensure that an optimal tumor-to-background ratio can be achieved in the cortical ovarian fragments, the expression of the investigated markers was examined in ten normal ovaries from premenopausal women in **chapter 3**. Ovaries from women with a *BRCA* gene mutation or an unknown mutation status were excluded, as they could harbor primary ovarian carcinoma cells that express the markers investigated in this study and therefore potentially lead to false-positive results. Unfortunately, all markers (except CEA and uPAR) were expressed on epithelial cells in inclusion cysts. Consequently, administration of NIRF probes against these markers will not only illuminate disseminated breast tumor cells in the ovaries, but also inclusion cysts. One solution to this problem would be to seek markers that are exclusively expressed at the cell surface of these inclusion cysts. Antibodies or peptides against these markers could then be conjugated to a fluorophore with an emission wavelength beyond the NIR spectral range, making a clear distinction between these structures. Yet, since all potentially suitable markers that could detect

inclusion cysts are also abundantly present at the cell-surface of breast tumor cells,[30] this hurdle will not be remedied by this approach. Another possibility would be to use an additional imaging technique by which the distinct morphological features of these structures can be visualized. Such a technique might be for instance full-field optical coherence tomography.

Full-field optical coherence tomography

Full-field optical coherence tomography (FF-OCT) is a new imaging modality that can be used to generate high-resolution histology-like images within a short period of time.[31] A great advantage of this technique is that there is no need to fixate, freeze, or stain the tissue. In **chapter 5**, we investigated whether FF-OCT can be used to visualize metastases as well as normal structures in human ovarian tissue. In contrast to the study described in chapter 3 in which *BRCA* gene mutation carriers were excluded, normal ovaries were obtained from premenopausal women who underwent prophylactic bilateral oophorectomy because of the presence of a *BRCA* gene mutation. Although the absence of primary ovarian carcinoma cells could not be fully warranted, the chance that occult tumor cells would be found was on closer reflection deemed almost nil, as the tubal fimbria were free of malignancy in all cases.[32]

In this study, we found that the maximum tissue depth at which high-resolution could be retained for the detection of ovarian metastases and normal ovarian structures was limited to approximately 100 µm, whereas previous studies have shown tissue imaging depths up to 500 µm in other tissues.[31] The reason that we found a much lower tissue penetration depth presumably lies in the large-scale extracellular network in the ovarian cortex.[22,33] Yet, this limitation could partially be solved by imaging the cortical ovarian fragments from both sides, thereby doubling the amount of tissue that can be imaged. Reducing ovarian graft thickness to further circumvent this limitation is not an option, as it has shown to result in follicle activation and subsequent depletion of the primordial follicle pool.[34] Nevertheless, although the limited penetration depth does not yet allow visualization of all ovarian metastases and/or follicles in the cortical ovarian fragments, FF-OCT is the only approach now available that is capable of examining the actual ovarian autografts without compromising the ovarian tissue and follicle viability.

Feasibility of NIRF imaging and FF-OCT for the detection of ovarian metastases and future perspectives

To determine whether optical imaging is feasible for routine use in fertility preservation, a number of steps have yet to be taken. Preclinical studies should be designed that provide sufficient evidence to support or reject the notion that NIRF imaging and FF-OCT are safe and efficacious for use in human ovarian tissues. Although a combination of NIRF probes would ultimately be indispensable to detect all disseminated breast tumor cells in ovarian tissue, these studies should initially be tailored to one tumor-targeted fluorescent probe to establish proof of concept. As E-cadherin was abundantly expressed by 91% of the disseminated tumor cells in ductal ovarian

metastases (**chapter 4**) and the majority of breast cancer patients are diagnosed with infiltrating ductal carcinoma,[35] it would be beneficial to develop a clinically applicable probe that is specifically directed to E-cadherin. To this end, antibodies or peptides against E-cadherin could be conjugated to a fluorophore that is currently available for clinical use, for instance IRDye 800CW (LI-COR Biosciences, Lincoln, NE) or ZW800-1 (The FLARE foundation, Wayland, MA). Alternatively, clinically approved antibodies like trastuzumab (Herceptin; Genentech, San Francisco, CA) could be labelled with IRDye 800CW to detect tumor cells that express Her2/neu.[36,37] This probe might be particularly suitable for the detection of ductal and lobular breast tumor cells in the ovarian tissues, as described in **chapter 4**. Such antibodies have the further advantage that their safety and toxicity profiles have already been broadly investigated and approved for clinical application. After verification of the binding capacity of the NIRF-labelled antibodies using a cell-based plate assay, the probes can be used for preclinical in vivo imaging studies to test their suitability for the field of ovarian tissue autotransplantation.[38] In these studies, severe combined immunodeficient (SCID) mice or rats can be used that bear orthotopically implanted 'metastatic' ovarian tumors that are induced by injection of a breast cancer cell line. This breast cancer cell line should obviously be positive for the markers to which the probe is directed and preferably express green fluorescent protein (GFP), which could serve as a positive control during the imaging process.[39] Bochner et al. reported in vivo imaging of GFP-expressing human ovarian carcinoma cells that were xenotransplanted in the ovaries of nude mice.[40] After mounting an imaging window on the right dorsolateral side of the mouse, and subsequent exteriorization of the murine ovary from the abdominal cavity, tumor cell migration could be tracked using a multiphoton microscope. For our purpose, NIRF-labelled antibodies could be administered via tail vein injection. Following this, binding of these NIRF-labelled antibodies to proteins that are present at the cell-surface of the GFP-expressing breast tumor cells in the murine ovary can be imaged using a multiphoton microscope. An alternative to multiphoton microscopy might be photoacoustic imaging (PAI).[41] This emerging new imaging technique detects acoustic signals that are indirectly generated by photon absorption following tissue illumination, and possibly allows for deeper tissue imaging.

For optimal differentiation between the antigen-expressing tumor cells and the surrounding stromal cells, a sufficient tumor-to-background ratio is of utmost importance. Within this context, rapid elimination of fluorescent agents from the circulation by either the kidney or liver is crucial. To accelerate this clearance process, smaller targeting molecules such as $F(ab')_2$ and Fab fragments can be used.[42] These fragments retain the specificity and affinity of their parental antibody. Furthermore, they are usually less immunogenic due to lack of the Fc domain.[43]

For the objective to use NIRF imaging to detect malignant cells in cortical ovarian tissue prior to cryopreservation, a tumor-specific probe that has been approved for clinical use could be intravenously administered to the patient before oophorectomy. After oophorectomy, the ovary can be dissected into cortical ovarian strips and imaged using either multiphoton microscopy or PAI.[40,41,44,45]

With respect to FF-OCT, it is expected that this imaging technique can be relatively quickly implemented into daily clinical practice. Additional studies are needed to determine whether FF-OCT is a suitable diagnostic instrument for use in ovarian tissue. This could be established by performing a blinded analysis in which two pathologists independently assess FF-OCT images of human ovarian tissues without having access to the H&E-stained tissue sections of these samples. Cortical ovarian tissues from deceased cancer patients can be used to estimate the tumor detection limit. Furthermore, research should focus on the ability of having offspring following autotransplantation of ovarian autografts that have been exposed to FF-OCT imaging. To this end, cortical ovarian fragments, whether or not exposed to FF-OCT imaging, can be xenotransplanted to bilaterally oophorectomized SCID mice to assess follicle development, as previously described by Lotz et al.[46] Furthermore, murine ovarian tissue could be subjected to FF-OCT imaging and transplanted back to mice that previously underwent bilateral oophorectomy. Mature oocytes could then be harvested from the excised ovarian tissue, fertilized in vitro and transferred to surrogate mouse mothers to generate full-term offspring.[47] If these studies yield successful results, FF-OCT imaging could shortly thereafter be used as a non-invasive means to detect metastases in the ovarian autografts and ultimately replace the current tumor detection methods.

Other strategies to mitigate the risk of reintroducing cancer cells following ovarian tissue autotransplantation

In addition to optical imaging, other strategies are being developed that may potentially mitigate the risk of reintroducing malignant cells. These strategies can basically be subdivided into three categories. Firstly, strategies that aim to attenuate the harmful effects of the gonadotoxic treatments on the ovaries. Current research focuses on reducing the uptake of chemotherapeutic agents in non-targeting tissues by nano-encapsulation of the drugs,[48,49] and inhibiting the apoptotic effects of chemotherapy on actively growing ovarian follicles by co-treatment with either sphingosine-1-phosphate (S1P),[50] or trichloro(dioxoethylene-O,O') tellurate (AS101).[51] If these approaches turn out to be effective, fertility preservation may become even unnecessary. Secondly, strategies that are designed to eradicate tumor cells from the ovarian autografts, for instance by tumor cell purging.[52,53] Tumor cell purging aims to eliminate malignant cells from the cortical ovarian fragments, while leaving the ovarian reproductive function intact. Hence, as is the case with NIRF imaging and FF-OCT, purging of tumor cells has the intention to allow for ovarian function recovery upon ovarian tissue autotransplantation. Thirdly, strategies that focus on isolation of oocytes rather than eradication of tumor cells from ovarian tissue, for example in vitro maturation of immature oocytes.[54-56] Although in vitro maturation of human primordial follicles is still in its infancy,[54] two live births have been reported following in vitro maturation of prophase I oocytes that were aspirated from extracorporeal human ovarian tissue.[55,56] Besides, xenotransplantation of cryopreserved ovarian tissue might be a suitable

approach to obtain mature oocytes. Xenotransplantation of ovarian tissue from a 6-year old girl diagnosed with nephroblastoma to a bilaterally oophorectomized SCID mouse recently resulted in the spontaneous formation of an antral follicle, which subsequently matured in vitro to a metaphase II oocyte.[46,57] Furthermore, it is possible to transplant isolated preantral follicles in a fibrin scaffold, thereby creating a so-called artificial ovary.[58,59] In mice, this approach has recently led to viable offspring.[47] Elaborating on this, pluripotent cell-derived stem cells may constitute a success for fertility restoration in the long term.[60] Primordial germ cell-like cells derived from female embryonic stem cells recently produced meiotically competent oocytes in mice. These oocytes were subsequently matured in vitro, fertilized, and transferred to foster mouse mothers, eventually resulting in healthy descendants. Of note, although these latter strategies are very promising, they do not allow for ovarian function recovery.

Final conclusion

Over the past few years, major advances have occurred in both understanding the risk of reintroducing malignant cells by ovarian tissue autotransplantation and the development of novel approaches that all aim to reduce this risk to a negligible level. The rate of progress is certainly laudable, but serious challenges remain ahead before the current concerns can be permanently dispelled. Although compelling evidence is still lacking, our results indicate that the current tumor detection approach provides insufficient information regarding the presence of malignant cells in the actual ovarian autografts. With this in mind, and the fact that the vast majority of cancer-related deaths are due to metastatic tumor growth,[61] it is of utmost importance to continue scientific research in this field. Optical imaging needs to form part of this research, as it holds considerable promise for application in the field of fertility preservation. In contrast to other risk-reducing approaches that primarily aim at ensuring the ability of having genetic offspring, for instance in vitro maturation of immature oocytes, NIRF imaging and FF-OCT have the potential to preserve the ability of ovarian function recovery by ovarian tissue autotransplantation. Besides, due to their non-invasiveness, it may be possible to examine ovarian tissues by both NIRF imaging and FF-OCT, resulting in even higher sensitivity and specificity rates. Lastly, while the focus of this thesis was mainly on ovarian metastases derived from invasive breast cancer, both methods can be converted to other malignancies in which cryopreservation of ovarian tissue is performed. As a result, patients may also become eligible in whom ovarian tissue autotransplantation is currently explicitly discouraged because of the high risk of transferring malignant cells upon autotransplantation. Hence, optical imaging may ultimately extend the range of people to whom ovarian tissue autotransplantation can be applied.

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Appendices

The safety of ovarian tissue autotransplantation in cancer patients cannot yet be ascertained, as the actual ovarian autografts cannot be examined for the presence of ovarian metastases. This is due to the fact that the current tumor detection methods undermine the ovarian tissue viability. The studies described in this thesis focused on determining the risk of reintroducing malignant tumor cells following ovarian tissue autotransplantation using the current tumor detection approach, and novel detection methods by which metastatic disease can potentially be detected in the cortical ovarian fragments that are actually transplanted.

In **chapter 1**, a detailed review of the literature is given on the morphology and localization of ovarian metastases derived from primary neoplasms in which cryopreservation of ovarian tissue is performed. Based on this literature review, we found indications that metastases in the ovarian cortex can be exclusively present in a distinct area. These indications implied that the absence of disseminated tumor cells cannot be guaranteed in the actual ovarian autografts when the cortical ovarian fragments that were examined, do not contain metastatic tumor cells. The current tumor detection approach might therefore be considered inadequate.

Since breast cancer is one of the primary indications for ovarian tissue cryopreservation, we further focused on ovarian metastases derived from invasive breast cancer in this thesis. **Chapter 2** describes a nationwide retrospective cohort study in which the prevalence of ovarian metastases was explored among all patients with primary invasive breast cancer at age < 41 years in the period 2000-2010 in the Netherlands who subsequently underwent an oophorectomy. In this cohort, 63 out of 2642 patients (2.4%) had histologically confirmed ovarian metastases. Between these 63 cases and the 2535 controls without ovarian metastases in this population, a noticeable difference was found in the time interval between the diagnosis of breast cancer and oophorectomy, 47.0 and 32.0 months, respectively (p = 0.002). Based on these findings, we concluded that the risk of developing ovarian metastases may be reduced by performing an oophorectomy promptly after the diagnosis of breast cancer. Besides, grafting the cortical ovarian fragments to the ovary that remained in situ instead of, for instance, a peritoneal window may help to alleviate the risk of developing ovarian metastases, as it enables the complete extirpation of the cortical ovarian autografts at a later time. Multivariate logistic regression analyses performed in a case-control population that was matched on the time interval between breast cancer diagnosis and oophorectomy, showed that tumor stage was significantly associated with the development of ovarian metastases (p = 0.024). Hence, we should be careful with ovarian tissue autotransplantation in young breast cancer patients diagnosed with tumors > 5 cm and/or inflammatory carcinoma.

Near-infrared fluorescence (NIRF) imaging can be used to make a distinction between malignant and healthy tissues without modifying the examined tissues and can therefore potentially surmount the disadvantages of the current tumor detection methods. The first step towards developing tumor-specific NIRF imaging as a novel method for the detection of ovarian metastases is the identification of protein markers that are present at the cell surface of tumor cells, but absent on cells that compose the normal ovarian cortex. In **chapter 3**, we examined a panel of eight cell-surface markers in ten normal ovaries from premenopausal women by immunohistochemistry. We found that none of the ten ovaries were positive for any of the markers tested. However, all markers (except CEA and uPAR) were found on epithelial cells of inclusion cysts. Additionally, we studied the same panel in 24 primary invasive breast tumors from patients who were potentially eligible for ovarian tissue cryopreservation following the inclusion criteria of the Dutch Network of Fertility Preservation. We concluded that particularly E-cadherin could be suitable as a target for tumor-specific NIRF imaging, as E-cadherin was expressed by 94% of invasive breast tumor cells in these 24 breast tumors. However, our analysis was limited to a cohort that mainly consisted of ductal breast cancers. Moreover, these findings did not necessarily infer that metastatic breast tumor cells express these markers to the same extent at their cell surface.

In **chapter 4**, we therefore investigated the expression of the above-mentioned panel of cell-surface proteins in ovarian metastases from the previously described cohort of patients who were diagnosed with primary invasive breast cancer at age < 41 years in the period 2000-2010 in the Netherlands. The markers uPAR and FR-α were excluded from further analyses, as they were barely expressed by the primary invasive breast tumors tested in chapter 3. With respect to ovarian metastases from the ductolobular subtype, EMA resulted to be the most suitable marker, as it was present on the cell membrane of 99% of disseminated ductolobular breast tumor cells in the seven ovaries studied. An immunofluorescent triple staining revealed that in ductal ovarian metastases, a combination of the markers E-cadherin, EMA and Her2/neu led to the detection of 100% of metastatic breast tumor cells in 53 out of 58 ovarian metastases. By combining EMA, Her2/neu and EpCAM, 100% tumor cell detection could be reached in 7 out of 10 lobular ovarian metastases. In the remaining five ductal and three lobular ovarian metastases, the mean percentage of metastatic cells that could not be detected was 5% (no range) and 25% (range 10-40), respectively. These data showed that the diagnosis of the histological subtype could aid in selecting the most pertinent combination of markers for the detection of ovarian metastases by tumor-specific NIRF imaging. This is clinically relevant, as we found that the expression of the cell-surface markers in primary invasive breast cancer tissues cannot be used to predict the most suitable target for the detection of ovarian metastases in an individual patient.

In addition to the expression of cell-surface markers, **chapter 4** was also designed to strengthen the findings from our review of the literature on the localization and morphology of ovarian metastases, as described in chapter 1. To this end, we studied the distribution of disseminated breast tumor cells within ovarian tissues from breast cancer patients who were diagnosed with ovarian metastases. Considering the morphological features, 71% of the ovarian

metastases consisted of a solitary metastasis or multiple distinct nodules separated by uninvolved ovarian tissue. These findings confirm the results of the previously reported studies in the literature and suggest that in these ovarian tissues disseminated breast tumor cells might not have been detected if the current tumor detection approach was applied. Yet, in the patients included in our study, there was a median time interval of 42 months between the diagnosis of breast cancer and oophorectomy. By contrast, in patients who undergo ovarian tissue cryopreservation a unilateral oophorectomy is usually performed shortly after cancer diagnosis. It is therefore likely that in these latter patients, ovarian metastases manifest as micrometastases or perhaps even single cells. Thus, in patients who undergo ovarian tissue cryopreservation, there might be an even greater chance that disseminated tumor cells in the ovarian tissues will be missed using the current tumor detection approach. Additional research is required to validate this.

In addition to NIRF imaging, full-field optical coherence tomography (FF-OCT) might be an appropriate approach to detect ovarian metastases in a non-invasive manner. FF-OCT is a new imaging system by which high-resolution histology-like images can be produced. These images can be obtained without the need to fixate, freeze, or stain the tissue. In the study described in **chapter 5**, we investigated whether FF-OCT can be used to visualize metastases as well as normal structures in human ovarian tissue. We found that micrometastases measuring up to 0.9 mm in diameter derived from primary breast cancers and endometrial cancers could be clearly distinguished from the surrounding stromal cells up to a tissue imaging depth of 100 µm. Furthermore, follicles of all stages of development and inclusion cysts could be identified. Since FF-OCT enabled the assessment of the density of primordial follicles, this method can also be used to select cortical ovarian fragments that have great potential for restoring fertility. Besides, we evaluated whether ovarian tissues and follicles remained viable following FF-OCT imaging. We found that short-term exposure to FF-OCT imaging had no significantly different effect on ovarian tissues and preantral follicles. Using the original FF-OCT system, it took on average seven minutes to image a cortical ovarian fragment measuring 5-10 mm in diameter. With the newly developed FF-OCT system, this time was diminished to one minute. We concluded that, although the limited imaging depth should certainly be taken into account, FF-OCT is at present the only approach by which it is possible to examine the actual ovarian autografts and thus, has significant advantages over the current tumor detection methods.

In **chapter 6**, the general discussion, we concluded that both NIRF imaging and FF-OCT have high potential to exclude minimal residual disease in the actual ovarian autografts. Despite the fact that the studies described in this thesis were primarily dedicated to ovarian metastases in patients diagnosed with breast cancer, it is expected that both optical imaging techniques can similarly be used for the detection of ovarian metastases derived from other primary malignancies, making ovarian tissue autotransplantation accessible to a considerably broader patient population.

Appendices

Dankzij verbeterde screeningsmethoden en nieuwe behandeltechnieken is de kans op het overleven van kanker de laatste decennia enorm toegenomen. Tot deze nieuwe behandeltechnieken behoren onder andere chemotherapie en bestraling. Helaas hebben bestraling op het bekken en sommige vormen van chemotherapie een schadelijk effect op de eierstokken en kunnen zij leiden tot vroegtijdige uitval van de eierstokfunctie. Hierdoor ontstaan klachten die gerelateerd zijn aan een tekort aan oestrogenen, zoals osteoporose (botontkalking), een verhoogd risico op hart- en vaatziekten, vasomotorische symptomen (onder andere opvliegers en hartkloppingen) en verminderde emotionele gesteldheid. Daarnaast kunnen deze behandelingen resulteren in een verminderde vruchtbaarheid of onvruchtbaarheid. Het risico op een verminderde vruchtbaarheid en onvruchtbaarheid neemt toe met de leeftijd en is gerelateerd aan het type chemotherapeuticum, de dosering en de duur van de behandeling. Al deze gevolgen hebben een negatieve invloed op de kwaliteit van leven van jonge vrouwen met kanker.

Om jonge vrouwen met kanker de nadelige effecten van deze behandelingen te besparen, is een aantal vruchtbaarheidssparende behandelingen ontwikkeld. De huidige vruchtbaarheidssparende behandelingen zijn het invriezen van embryo's, eicellen, of eierstokweefsel. Het invriezen van embryo's en het invriezen van eicellen zijn erkende methoden die algemeen beschikbaar zijn. Zij kennen echter enkele nadelen. Ten eerste is hormonale stimulatie nodig om eicellen te verkrijgen. Dit heeft tot gevolg dat deze methoden noch geschikt zijn voor jonge meisjes bij wie de menstruele cyclus nog niet op gang is, noch voor vrouwen die onvoldoende tijd hebben om hormonale stimulatie te ondergaan. Ten tweede kan de eierstokfunctie door middel van deze methoden niet worden hersteld. Het invriezen van eierstokweefsel biedt mogelijk uitkomst.

Invriezen en terugplaatsen van eierstokweefsel

Voor jonge meisjes en vrouwen die direct moeten starten met chemotherapie en/of bestraling vanwege een agressieve vorm van kanker is het invriezen van eierstokweefsel de enige optie. Bovendien is het de enige vruchtbaarheidssparende behandeling waarmee de eierstokfunctie kan worden hersteld. Om in aanmerking te komen voor het invriezen van eierstokweefsel moet aan een aantal criteria worden voldaan. Deze criteria zijn onder andere: leeftijd < 35 jaar, een goede eierstokreserve, een realistische kans op overleving en een hoog geschat risico op uitval van de eierstokfunctie door de oncologische behandeling (> 50%). Overigens zijn deze selectiecriteria indicatief. Het invriezen van eierstokweefsel is in het verleden ook uitgevoerd bij vrouwen die iets ouder waren dan 35 jaar.

Het eierstokweefsel wordt bij voorkeur ingevroren voordat de behandeling met chemotherapie en/of bestraling plaatsvindt. Tijdens een kijkoperatie wordt één van de eierstokken verwijderd. Deze eierstok wordt vervolgens naar het laboratorium gebracht. Aldaar wordt de eierstok gehalveerd en het merg (de binnenzijde van de eierstok) verwijderd. De overgebleven schors (de buitenzijde van de eierstok die de rustende eicellen bevat) wordt dunner gemaakt

tot een dikte van 1-2 mm en in fragmenten gesneden van 5-10 mm doorsnede. Vervolgens worden de eierstokfragmenten ingevroren en opgeslagen in vloeibare stikstof (-196 °C). Als na de behandeling van de patiënte sprake blijkt te zijn van een verminderde eierstokfunctie en de patiënte een kinderwens heeft, kunnen de eierstokfragmenten worden ontdooid en worden teruggeplaatst in de achtergebleven eierstok en/of het buikvlies (orthotope terugplaatsing), of in de buikwand of de onderarm (heterotope terugplaatsing). Herstel van de menstruele cyclus na terugplaatsing van eierstokweefsel werd voor het eerst beschreven in 2001, gevolgd door de eerste levendgeborene in 2004. Tot op heden zijn wereldwijd meer dan 86 levendgeborenen gerapporteerd, deels na spontane conceptie en deels na in vitro fertilisatie (IVF). In Nederland is in 2015 de eerste baby geboren na terugplaatsing van eierstokweefsel in het Leids Universitair Medisch Centrum (LUMC). Bij 93% van de patiënten treedt na terugplaatsing van het eierstokweefsel herstel van de eierstokfunctie op.

Experimentele aard

Ondanks deze veelbelovende resultaten wordt terugplaatsing van ontdooid eierstokweefsel wereldwijd als experimenteel beschouwd. In ons land heeft Zorginstuut Nederland op 30 november 2016 een brief geschreven aan mevrouw Schippers, destijds minister van Volksgezondheid, Wetenschap en Sport, waarin werd geconcludeerd dat het invriezen en terugplaatsen van ovariumweefsel niet voldoet aan de stand van de wetenschap en praktijk. De achterliggende reden is tweeledig: in de eerste plaats is de effectiviteit nog onduidelijk, omdat zwanger worden ook mogelijk is bij een verminderde vruchtbaarheid en de eierstokfunctie na behandeling met chemotherapie soms spontaan herstelt. In de tweede plaats is de veiligheid van deze methode nog onvoldoende in kaart gebracht bij bepaalde vormen van kanker die naar de eierstokken kunnen uitzaaien. Het gaat hierbij om het risico op het herintroduceren van tumorcellen bij het terugplaatsen van eierstokweefsel.

Bezorgdheid omtrent de veiligheid van terugplaatsing van eierstokweefsel

De veiligheid van het terugplaatsen van eierstokweefsel kan tot op heden niet worden gegarandeerd. Dit komt doordat de huidige tumordetectiemethoden (histologie/immunohistochemie, PCR-analyse, xenotransplantatie) het eierstokweefsel ongeschikt maken voor terugplaatsing. Om die reden wordt op dit moment een beperkt aantal eierstokfragmenten onderzocht dat niet wordt teruggeplaatst. Men gaat er dan van uit dat deze onderzochte fragmenten representatief zijn voor de gehele eierstok. Of de eierstokfragmenten die wel worden teruggeplaatst tumorcellen bevatten, blijft echter onduidelijk. Het zou dus kunnen dat er met het terugplaatsen van eierstokweefsel tumorcellen worden geherintroduceerd, waardoor een patiënte mogelijk opnieuw kanker krijgt.

Evaluatie van de huidige tumordetectieprocedure

Om te bepalen of met het testen van één of twee eierstokfragmenten die niet worden teruggeplaatst voorspeld kan worden of tumorcellen afwezig zijn in het eierstokweefsel dat wel wordt teruggeplaatst, is het belangrijk om te weten hoe de uitgezaaide tumorcellen zich in het eierstokweefsel presenteren en waar zij zich exact bevinden. Dit kan worden geïllustreerd aan de hand van de volgende twee voorbeelden. Indien tumorcellen diffuus en homogeen verspreid zijn in het eierstokweefsel is het onderzoeken van één of twee eierstokfragmenten voldoende om na te gaan of tumorcellen aanwezig zijn in de eierstokfragmenten die worden teruggeplaatst. In dat geval kan de huidige tumordetectieprocedure als voldoende nauwkeurig worden beschouwd. Daarentegen is de huidige tumordetectieprocedure onbetrouwbaar als uitzaaiingen slechts in een beperkt gebied in de schors van de eierstok voorkomen. Dan bestaat de kans dat de eierstokfragmenten die onderzocht worden als tumorvrij worden afgegeven, terwijl eierstokfragmenten die worden teruggeplaatst uitgezaaide tumorcellen bevatten ('sampling error').

Non-invasieve tumordetectiemethoden

Een mogelijke benadering om eierstokfragmenten op een veilige manier terug te plaatsen is om methoden te ontwikkelen waarmee de aanwezigheid van uitzaaiingen kan worden uitgesloten zonder schade te berokkenen aan de vitaliteit van het weefsel. Twee nieuwe technieken zijn mogelijk voor dit doeleinde geschikt: nabij-infrarode fluorescente beeldvorming en full-field optische coherentietomografie.

Nabij-infrarode fluorescente beeldvorming

Nabij-infrarode fluorescente (NIRF-)beeldvorming kan worden gebruikt om 'real time' onderscheid te maken tussen kwaadaardig en goedaardig weefsel zonder het onderzochte weefsel aan te tasten. Het voordeel van NIR-licht ten opzichte van zichtbaar licht is dat het licht dieper in het weefsel dringt, waardoor het mogelijk is om dieper gelegen structuren te bekijken. Daarnaast geeft NIR-licht een lagere autofluorescentie van het normale weefsel waardoor voldoende contrast ontstaat. Omdat het humane oog ongevoelig is voor NIR-licht is een camerasysteem noodzakelijk om het fluorescente licht te kunnen waarnemen. Dit camerasysteem is uitgerust met een lichtbron dat een fluorofoor (een fluorescerende stof) aanstraalt, waarna een fluorescent signaal wordt uitgezonden.

Op het gebied van de oncologische chirurgie wordt NIRF-beeldvorming reeds gebruikt om met behulp van de fluorescente stoffen indocyanine groen en methyleenblauw weefsels te identificeren die moeten worden verwijderd, zoals kwaadaardige tumoren en aangedane lymfeklieren, en structuren te herkennen die moeten worden gespaard, zoals urineleiders en galwegen. Door de toegenomen vaatvorming en afgenomen lymfeafvloed bij kanker passeren deze fluorescente stoffen de haarvaten en hopen zij zich op in het weefsel rondom de tumor, waardoor tumorlokalisaties aan het licht kunnen worden gebracht.

NIRF-gelabelde tumor-specifieke probes kunnen worden gebruikt om tumorcellen nog nauwkeuriger op te sporen. Deze tumor-specifieke probes bestaan uit twee componenten: antilichamen of peptiden die met hoge affiniteit aan eiwitten op de celmembraan van tumorcellen binden en een fluorofoor dat licht uitzendt in het NIRF-spectrum (λ = 700-900 nm). Bij patiënten met eierstokkanker blijken uitzaaiingen beter te kunnen worden opgespoord na toediening van tumor-specifieke fluorescente probes. Dergelijke tumor-specifieke fluorescente probes zouden ook gebruikt kunnen worden om tumorcellen in eierstokweefsel te detecteren.

Om deze techniek te gebruiken voor de detectie van tumorcellen in eierstokfragmenten zou preoperatief een tumor-specifieke NIRF-probe intraveneus aan een patiënte met kanker kunnen worden toegediend. Nadat de eierstok verwijderd is, kunnen de eierstokfragmenten worden verkregen. Omdat deze fragmenten doorgaans een doorsnede hebben van 5-10 mm en een dikte van 1-2 mm is een beeldvormingssysteem noodzakelijk waarmee gedetailleerde opnamen kunnen worden gemaakt tot een weefseldiepte van ten minste 1 mm. Een multifotonmicroscoop lijkt hiervoor zeer geschikt te zijn, aangezien deze ontwikkeld is om een fluorescent signaal in de diepte te detecteren.

Full-field optische coherentietomografie

Full-field optische coherentietomografie (FF-OCT) maakt binnen afzienbare tijd histologie-achtige afbeeldingen van verse weefselfragmenten tot een weefseldiepte van 500 µm. Het beeldvormingssysteem bestaat uit een staande microscoop en een referentiearm in een Linnik interferometrische opstelling. FF-OCT detecteert teruggekaatst licht in weefsel. Het te onderzoeken weefsel wordt onder het objectief geplaatst. Om te bepalen op welke diepte in het weefsel het licht wordt teruggekaatst, wordt het licht zowel naar het weefsel uitgezonden als naar een referentiespiegel. Vervolgens wordt het licht gecombineerd ontvangen door een detector en wordt een afbeelding gemaakt. FF-OCT kan, in tegenstelling tot de conventionele detectiemethoden, worden verricht zonder het weefsel te fixeren, in te vriezen of te kleuren, waardoor het weefselbeschadiging kan voorkomen.

Dit proefschrift

Dit proefschrift richt zich op de veiligheid van het terugplaatsen van ontdooid eierstokweefsel. Het doel is om de nauwkeurigheid van de huidige tumordetectieprocedure in kaart te brengen en de noodzaak voor een alternatieve methode te bepalen waarmee het mogelijk is om ieder terug te plaatsen eierstokfragment te onderzoeken. Daarnaast heeft dit proefschrift tot doel na te gaan of NIRF-beeldvorming en FF-OCT mogelijk geschikt zijn als alternatieve detectiemethoden. Centraal in dit proefschrift staan studies gewijd aan uitzaaiingen in de eierstokken bij patiënten met borstkanker, aangezien de meeste patiënten van wie eierstokweefsel wordt ingevroren met borstkanker gediagnosticeerd zijn, en relatief veel eierstokweefsel van deze patiënten beschikbaar is voor onderzoek. Dit komt doordat bij borstkankerpatiënten de eierstokken vaak uit voorzorg

of in het kader van de behandeling bij hormoongevoelige borsttumoren worden verwijderd om hormonale stimulatie van de borsttumor te voorkomen.

In **hoofdstuk 1** wordt een gedetailleerd literatuuroverzicht gegeven van de lokalisatie en de vorm van uitzaaiingen in de eierstok. Hierbij lag de focus op uitzaaiingen die afkomstig zijn van de verschillende vormen van kanker waarbij eierstokweefsel wordt ingevroren. Deze vormen zijn borstkanker, baarmoederhalskanker, baarmoederkanker, darmkanker, leukemie, Hodgkin- en non-Hodgkin-lymfoom, en bot- en wekedelenkanker. Uit dit literatuuronderzoek bleek dat uitzaaiingen beperkt kunnen zijn tot een bepaald gebied in de buitenzijde van de eierstok. Dit impliceert dat de huidige tumordetectieprocedure niet nauwkeurig is door het te grote risico op een 'sampling error'.

Het onderzoek dat in **hoofdstuk 2** wordt beschreven had tot doel om na te gaan hoe vaak uitzaaiingen in de eierstok voorkomen bij jonge vrouwen met borstkanker. Hiertoe werd een landelijk cohort samengesteld van alle vrouwen die op de leeftijd < 41 jaar zijn gediagnosticeerd met borstkanker in de periode 2000-2010 in Nederland en bij wie de eierstokken na de diagnose borstkanker zijn verwijderd. Uit dit onderzoek bleek dat bij 63 van de in totaal 2642 vrouwen die aan deze criteria voldeden (2.4%) bij histologisch onderzoek uitzaaiingen in de eierstok werden aangetroffen. Dit percentage is mogelijk een onderrapportage, omdat alleen vrouwen werden bestudeerd die in het kader van de behandeling eierstokverwijdering ondergingen. Desalniettemin is dit percentage het meest exacte dat tot nu toe bekend is en geeft het een goede indruk van de ordegrootte van het probleem.

Daarnaast werden in dit onderzoek risicofactoren in kaart gebracht die een rol kunnen spelen bij de ontwikkeling van uitzaaiingen in de eierstok bij deze groep patiënten. Een opvallend verschil tussen de 63 cases en 2535 controles zonder uitzaaiingen in de eierstok in dit cohort was het tijdsinterval tussen de diagnose borstkanker en het verwijderen van de eierstok, namelijk respectievelijk 47.0 en 32.0 maanden (p = 0.002). Op basis van deze bevindingen concludeerden we dat het vroegtijdig verwijderen van de eierstok het risico op het ontwikkelen van uitzaaiingen in de eierstok aanzienlijk kan verminderen. Bovendien zou ons advies zijn om de eierstokfragmenten terug te plaatsen in de achtergebleven eierstok in plaats van, bijvoorbeeld, het buikvlies. Op deze manier bestaat de mogelijkheid om de eierstokfragmenten op een later tijdstip in het geheel te verwijderen, waardoor het risico op het ontwikkelen van uitzaaiingen in de eierstok eveneens kan worden verminderd. Multivariate logistische regressie analyses naar risicofactoren voor uitzaaiingen in de eierstokken werden verricht in een case-controle populatie die gematcht was op het tijdsinterval tussen de diagnose borstkanker en het verwijderen van de eierstokken. Deze analyses lieten zien dat het tumorstadium significant geassocieerd is met het ontwikkelen van uitzaaiingen in de eierstok (p = 0.024). Derhalve adviseren we een terughoudend beleid ten aanzien van het terugplaatsen van eierstokweefsel in jonge borstkankerpatiënten met tumoren > 5 cm en/of inflammatoire tumoren.

De eerste stap voor het ontwikkelen van tumor-specifieke NIRF-beeldvorming als nieuwe methode om uitzaaiingen in de eierstok te detecteren, is op zoek gaan naar eiwitten die specifiek tot expressie komen op de celmembraan van tumorcellen en afwezig zijn in het normale eierstokweefsel. Aangezien weefsel van borsttumoruitzaaiingen in de eierstok niet direct beschikbaar was in het LUMC hebben we in **hoofdstuk 3** in eerste instantie een panel van acht membraaneiwitten onderzocht in invasieve borsttumoren. Deze borsttumoren waren afkomstig van borstkankerpatiënten die op grond van de inclusiecriteria zoals opgesteld door het Nederlands Netwerk Fertiliteitspreservatie (NNF) in aanmerking hadden kunnen komen voor het invriezen van eierstokweefsel. Op basis van dit onderzoek lijkt E-cadherine de meest geschikte marker voor de detectie van borsttumorcellen door middel van tumor-specifieke beeldvorming, aangezien E-cadherine op 94% van de invasieve borsttumorcellen in de 24 borsttumoren tot expressie kwam. In dit hoofdstuk hebben we tevens gekeken naar de expressie van deze markers in normaal eierstokweefsel van premenopauzale vrouwen. Geen van de acht geteste membraanmarkers kwam tot expressie in de stromacellen van het normale eierstokweefsel. Echter, alle markers, behalve CEA en uPAR, bleken aanwezig op epitheliale cellen van inclusiecysten. Dit zou betekenen dat een NIRF probe die gericht is tegen een van deze markers, niet alleen borsttumorcellen maar ook inclusiecysten zou doen oplichten.

In **hoofdstuk 4** hebben we de expressie van het bovengenoemde panel van membraaneiwitten onderzocht in borstkankeruitzaaiingen in de eierstok. Dit weefsel was afkomstig van het eerder beschreven cohort dat bestaat uit patiënten met borstkanker op de leeftijd < 41 jaar in de periode 2000-2010 in Nederland. Uit dit onderzoek bleek dat uitzaaiingen in de eierstok afkomstig van invasieve ductolobulaire borsttumoren met één marker, namelijk EMA, kunnen worden opgespoord. Voor het opsporen van uitzaaiingen in de eierstok van invasieve ductale borsttumoren waren drie markers nodig, namelijk E-cadherine, EMA en Her2/neu. Ook voor de detectie van uitzaaiingen in de eierstok van invasieve lobulaire borsttumoren was een combinatie van markers gewenst; de combinatie EMA, Her2/neu en EpCAM bleek hier het meest geschikt te zijn.

Om te bepalen of de expressie van deze markers op de invasieve borsttumor kan voorspellen welke marker het meest geschikt is om de bijbehorende uitzaaiingen in de eierstok op te sporen, hebben we deze markers ook op de invasieve borsttumoren van deze patiënten onderzocht. Tussen de expressie van de markers op de invasieve borsttumoren en de bijbehorende uitzaaiingen in de eierstok kon geen correlatie worden aangetoond. Dit levert echter geen beperkingen op voor de klinische toepassing. De meest geschikte markercombinatie kan immers worden bepaald zodra de patholoog het histologische subtype van de invasieve borsttumor heeft vastgesteld.

Naar aanleiding van het literatuuronderzoek in hoofdstuk 1, hebben we in hoofdstuk 4 ook de lokalisatie en de vorm van de borsttumoruitzaaiingen in de eierstok onderzocht. Hieruit bleek dat 71% van de borsttumoruitzaaiingen in de eierstok bestaat uit een solitaire nodus of multipele

noduli die van elkaar worden onderscheiden door 'normaal' eierstokweefsel. Deze resultaten bevestigen de literatuurgegevens die werden beschreven in hoofdstuk 1. De bevindingen suggereren dat borsttumorcellen in de eierstok makkelijk kunnen worden gemist als de huidige tumordetectieprocedure zou worden toegepast. Er dient echter wel te worden vermeld dat de tijd tussen de diagnose borstkanker en het vaststellen van de uitzaaiingen in de eierstok in de patiënten in dit onderzoek gemiddeld 42 maanden was. Bij patiënten van wie eierstokweefsel daarentegen wordt ingevroren, wordt één van de eierstokken vlak na de diagnose borstkanker verwijderd. Het is derhalve aannemelijk dat uitzaaiingen in de eierstok van deze patiënten zich presenteren als micro-uitzaaiingen of losse tumorcellen. De kans dat deze uitzaaiingen worden gemist met de huidige tumordetectieprocedure lijkt derhalve nog groter. Aanvullend onderzoek is nodig om deze hypothese te bevestigen.

Uit het onderzoek dat beschreven is in **hoofdstuk 5** bleek dat FF-OCT uitzaaiingen in de eierstok zichtbaar kan maken. Bovendien bleek dat eicellen in verschillende ontwikkelingsstadia kunnen worden onderscheiden. Dit is gunstig, aangezien dit de mogelijkheid biedt om het aantal aanwezige rustende eicellen te bepalen. Dit aantal is sterk gerelateerd aan de kans op herstel van de eierstokfunctie. Met behulp van FF-OCT kunnen dus eierstokfragmenten worden geselecteerd die geen uitzaaiingen bevatten en bovendien een goede kans geven op herstel van de eierstokfunctie. Een kanttekening hierbij is dat uitzaaiingen en eicellen tot een weefseldiepte van maximaal 100 µm zichtbaar konden worden gemaakt. Deze beperkte weefselpenetratiediepte is zeer waarschijnlijk te wijten aan de grootschalige extracellulaire matrix in de eierstokfragmenten. De maximale weefseldiepte waarop uitzaaiingen en eicellen kunnen worden afgebeeld kan desalniettemin wel iets worden vergroot door de eierstokfragmenten van twee kanten te belichten. Bovendien is de verwachting dat het in de nabije toekomst mogelijk is om ook dieper gelegen structuren in de eierstokfragmenten af te beelden, aangezien FF-OCT sterk in ontwikkeling is. Een andere belangrijke bevinding van dit onderzoek was dat na kortdurende blootstelling aan FF-OCT de vitaliteit van zowel het eierstokweefsel als de eicellen behouden blijft. Ondanks het feit dat het ten gevolge van de beperkte weefselpenetratiediepte nog niet mogelijk is om alle uitzaaiingen en/of follikels met behulp van FF-OCT in de eierstok te detecteren, biedt FF-OCT een enorme verbetering ten opzichte van de huidige tumordetectietechnieken. FF-OCT is op dit moment immers de enige methode waarmee de eierstokfragmenten die daadwerkelijk worden teruggeplaatst kunnen worden onderzocht.

Toekomstperspectieven

In **hoofdstuk 6** worden de bevindingen van dit proefschrift samengevat en in een breder perspectief geplaatst. Tevens wordt ingegaan op de mogelijkheden voor toekomstig wetenschappelijk onderzoek. De haalbaarheid van NIRF-beeldvorming als nieuwe detectiemethode dient in eerste instantie in de preklinische setting te worden onderzocht. Hoewel het uiteindelijk noodzakelijk zal zijn om meerdere markers te combineren om alle borsttumorcellen in de eierstokken te kunnen detecteren, dienen deze studies zich in eerste instantie te richten op één tumor-specifieke probe om na te gaan of het principe werkt. Aangezien E-cadherine in 91% van de ductale borsttumorcellen in de eierstok tot expressie kwam (hoofdstuk 4) en de meerderheid van de borstkankerpatiënten gediagnosticeerd wordt met het ductale subtype, zou het zinvol zijn om een probe te ontwikkelen die specifiek gericht is tegen E-cadherine. Hiertoe kunnen antilichamen of peptiden tegen E-cadherine worden gebonden aan een fluorofoor die momenteel voor klinische toepassing beschikbaar is, bijvoorbeeld IRDye800CW (LI-COR Biosciences, Lincoln, NE) of ZW800-1 (The FLARE foundation, Wayland, MA). Daarnaast zouden antilichamen als trastuzumab (Herceptin; Genentech, San Francisco, CA) kunnen worden gelabeld aan IRDye 800CW om borsttumorcellen te detecteren die Her2/neu positief zijn. Dergelijke antilichamen hebben het voordeel dat zij reeds uitgebreid onderzocht en goedgekeurd zijn voor klinische toepassing. Nadat de bindingscapaciteit van de probes is vastgesteld, kunnen de probes in proefdieren worden getest. Voor dit doeleinde kunnen muizen worden gebruikt die geen afweersysteem hebben. In de eierstokken van deze muizen kunnen humane borsttumorcellen worden geïnjecteerd die positief zijn voor een van de markers waar tegen de tumor-specifieke NIRF-probe gericht is. Daarna kunnen de tumor-specifieke NIRF-probes intraveneus aan de muis worden toegediend en kan de binding van deze probe aan de membraaneiwitten op de borsttumorcellen zichtbaar worden gemaakt door middel van multifotonmicroscopie of eventueel fotoakoestische beeldvorming. Fotoakoestische beeldvorming is een nieuwe beeldvormingstechniek die ultrageluidsgolven combineert met licht, waardoor het mogelijk is om nog dieper in het weefsel te kijken.

Wat betreft FF-OCT dient de sensitiviteit en specificiteit te worden vastgesteld waarmee uitzaaiingen in de eierstok gedetecteerd kunnen worden. Daarnaast dient de mogelijkheid om nakomelingen te produceren te worden geverifieerd. Om dit te bewerkstelligen kan eierstokweefsel van een muis dat al dan niet aan FF-OCT is blootgesteld, worden teruggeplaatst bij muizen zonder afweersysteem waarbij beide eierstokken verwijderd zijn. Vervolgens kan worden bestudeerd of eicelrijping optreedt en kunnen rijpe eicellen worden verkregen en bevrucht door middel van IVF. De muizenembryo's kunnen tot slot worden teruggeplaatst in een draagmoedermuis. Indien dit succesvolle resultaten oplevert, kan FF-OCT als non-invasieve methode worden gebruikt om uitzaaiingen in humane eierstokfragmenten op te sporen.

Concluderend kan worden gesteld dat de huidige tumordetectieprocedure onvoldoende informatie lijkt te kunnen geven over de aanwezigheid van borsttumorcellen in de eierstokfragmenten die daadwerkelijk worden teruggeplaatst. NIRF-beeldvorming en FF-OCT zijn veelbelovende alternatieve technieken om uitzaaiingen in eierstokweefsel te detecteren. Aangezien beide methoden in opzet non-invasief zijn, kunnen deze methoden mogelijk gecombineerd worden toegepast om de sensitiviteit en specificiteit te vergroten. Hoewel de studies in dit proefschrift voornamelijk gericht zijn op uitzaaiingen in de eierstok die afkomstig zijn van borstkanker, kan zowel NIRF-beeldvorming als FF-OCT naar verwachting ook worden gebruikt om uitzaaiingen op te sporen die afkomstig zijn van andere typen tumoren. Dit zou betekenen dat in de toekomst ook patiënten in aanmerking kunnen komen voor terugplaatsing van eierstokweefsel die momenteel worden uitgesloten vanwege een te hoog risico op herintroductie van tumorcellen. Dankzij optische beeldvorming zouden dus mogelijk meer patiënten terugplaatsing van eierstokweefsel kunnen ondergaan.

Appendices

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ASRM	American Society for Reproductive Medicine
AS101	Trichloro(dioxoethylene-O,O') tellurate
BRCA	Breast cancer antigen
CAM 5.2	Cytokeratin CAM 5.2
CCCN	Comprehensive Cancer Center the Netherlands
CCD	Charge-coupled device
CEA	Carcinoembryonic antigen
CI	Confidence interval
CK-7	Cytokeratin-7
CML	Chronic myeloid leukemia
CLL	Chronic lymphoblastic leukemia
DAB	Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
EGFR	Epidermal growth factor receptor
EMA	Epithelial membrane antigen (MUC1; Mucin1)
EpCAM	Epithelial cell adhesion molecule
EPR	Enhanced penetration and retention
FCS	Fetal calf serum
FF-OCT	Full-field optical coherence tomography
FFPE	Formalin-fixed paraffin-embedded
FIGO	International Federation of Gynecology and Obstetrics
FMWV	Dutch Federation of Biomedical Scientific Societies
FR-α	Folate receptor alpha
GCDFP15	Gross cystic disease fluid protein-15
GFP	Green fluorescent protein
GnRH	Gonadotropin-releasing hormone
Her2/neu	Human epidermal growth factor receptor 2
H&E	Hematoxylin-and-eosin
Hz	Hertz
IgG1	Immunoglobulin G1
IgG2a	Immunoglobulin G2a
IVF	In vitro fertilization
LED	Light-emitting diode
LUMC	Leiden University Medical Center
MGB2	Mammaglobin B gene

MRI	Magnetic resonance imaging
NIRF	Near-infrared fluorescence
OCT	Optical coherence tomography
PAI	Photoacoustic imaging
PALGA	Dutch Pathology Registry
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Potential hydrogen
PT	Pre-treatment
Rpm	Revolutions per minute
SBR grade	Scarff-Bloom-Richardson grade
SCID	Severe combined immunodeficient
SD	Standard deviation
SEER program	Surveillance, Epidemiology and End Results program
SISH	Silver in situ hybridization
SPSS	Statistical Package for the Social Sciences
S1P	Sphingosine-1-phosphate
TNM stage	Tumor/node/metastasis stage
TMA	Tissue microarray
uPAR	Urokinase-type plasminogen activator receptor
VEGF	Vascular endothelial growth factor
WT1	Wilms' tumor antigen-1

Appendices

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Appendices

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Appendices

Inge Theodora Anne Peters was born in Arnhem on August 21, 1987. After graduating at the Thomas a Kempis secondary school in Arnhem in 2005, she started medicine at the Leiden University Medical Center (LUMC) in September of the same year. In 2009 she performed a scientific internship for a period of eight months at the department of Gynecology at the Sacro Cuore Don Calabria hospital in Negrar, Verona, Italy, under the supervision of Dr. M. Ceccaroni. During this internship, she participated in over 160 surgical interventions, mainly comprising treatment of gynecological cancers and nerve-sparing laparoscopic eradication of deep rectal and parametrial infiltrating endometriosis. After returning to the Netherlands, she began her clinical rotations and became involved in research on the significance of guest operations in early-stage ovarian cancer, which she conducted under the guidance of Prof. dr. J.B.M.Z. Trimbos. During this period, she also wrote a book chapter about the place of hormone replacement therapy in ovarian, endometrial and breast cancer in collaboration with Prof. dr. M. Hickey from the department of Gynecology at the Royal Women's Hospital in Melbourne, Australia. Inge achieved her medical degree cum laude in January 2012, after which she worked as a resident in Obstetrics and Gynecology at the Haga hospital in The Hague. In April 2013 she started as a PhD candidate on optical imaging in the field of oncofertility at the departments of Gynecology and Surgical Oncology at the LUMC (promotores: Prof. dr. J.B.M.Z. Trimbos and Prof. dr. C.G.J.M. Hilders; co-promotor: Dr. P.J.K. Kuppen). The results obtained are described in this thesis. Inge organized the national Conference on Experimental Research in Surgical Specialties ('Symposium Experimenteel Onderzoek Heelkundige Specialismen; SEOHS') in December 2015. Since March 2015, she has been a board member of the Special Interest Group Fertility preservation-Dutch Network of Fertility Preservation ('SIG Fertiliteitspreservatie-NNF'), which is part of the Pillar Reproductive Medicine of the Dutch Society of Obstetrics and Gynecology ('Nederlandse Vereniging voor Obstetrie & Gynaecologie; NVOG'). In this context, she organized a national conference on fertility preservation and endometriosis in January 2017. Inge started her residency training in Obstetrics and Gynecology at the Haga hospital (head: Dr. B.W.J. Hellebrekers) and the LUMC (head: Prof. dr. J.M.M. van Lith) in April 2016.