Safeguarding ovarian tissue autotransplantation in cancer patients

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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.

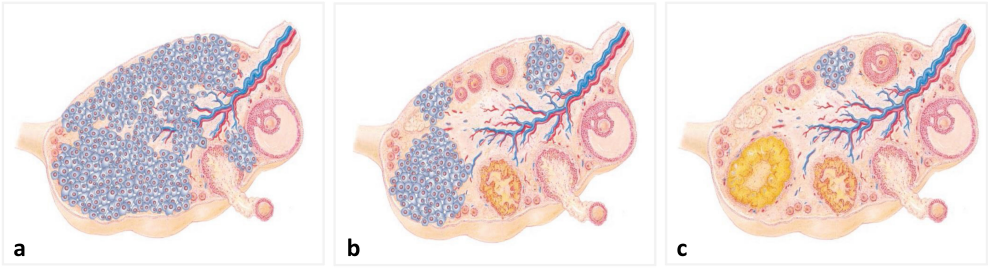

Figure 1. Morphological features of disseminated tumor cells within ovarian tissues

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.

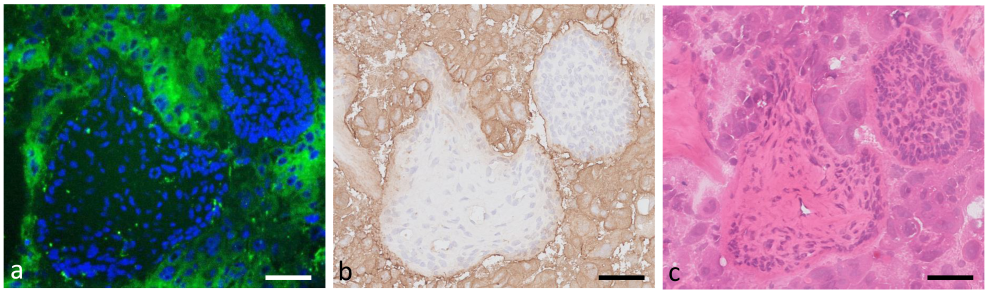

Figure 2. Histopathologic evaluation and fluorescence signal in ovarian cancer
Representative image of membranous and cytoplasmic accumulation of OTL38 in tumor cells visualized by fluorescence microscopy **(a)**. The fluorescence signal is indicated in green, and the nuclei were counterstained with 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.

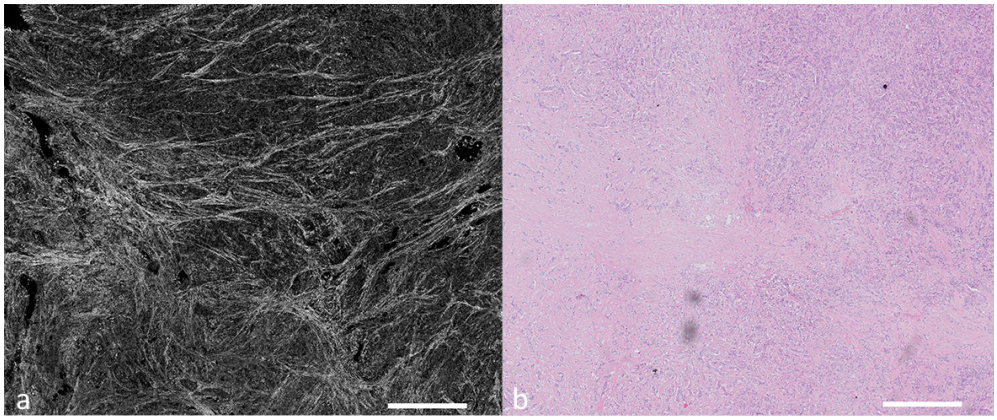

Figure 3. Representative FF-OCT image and corresponding histology image of primary invasive ductal breast cancer
Collagen bundles are clearly shown in the FF-OCT image **(a)**, corresponding to the stromal reaction in the haematoxylin-and-eosin staining **(b)**. Scale bars represent 500 µm.

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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