Safeguarding ovarian tissue autotransplantation in cancer patients

Peters, I.T.A.

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.