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Safeguarding ovarian tissue autotransplantation in cancer patients

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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 ($\lambda = 700\text{-}900\text{ nm}$) 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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