

CRYOSURGERY OF PRIMARY BREAST CANCER EN BLOC COMBINED WITH INTRAOPERATIVE ULTRASOUND-GUIDED TRACER INJECTION

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Purpose. The objective is to trap the tumor cells into the frozen mass, momentarily deprived from blood flow, so as to prevent the intraoperative dissemination of cells during the tumor manipulation (34). The surgery is conventional and consists in complete excision of the frozen block with negative surgical margins. For the treatment of unresectable tumor we use the cryoprobe as an ablative and palliative tool in situ (30,34). The operated breast is always treated with radiotherapy (RT), and when indicated with chemotherapy (CH), or endocrine therapy (ET) in the adjuvant setting (35). For the surgical management of the regional lymph node we usually perform an axillary exploration and a lymph node dissection (ALND) or a pre-operative sentinel lymph node mapping with a radioactive tracer and a sentinel lymph node dissection (SLND). Due to the cost and constraints of the lymphoscintigraphy we have elected to use the intraoperative injection of blue dye for the sentinel lymph node mapping during our cryo-assisted procedures. **Methodology.** The tumors were treated as follows: A cryo-assisted tumor injection and resection (CATIR) had their tumor frozen and injected with the selected blue dye during the freezing process; after a few minutes the frozen tumor and a breast margin were resected in a conventional manner, marked with sutures for spatial orientation and for the tumor characterization and the margin evaluation on frozen samples. The intraoperative sonography (IOUS) is used to assess the tumor contours along with palpation to position the needle tip at the tumor deep margin, and its shaft at least 20 mm away from the probe tip edge. During the freezing sequences the operator could visualize the icing zone and the ice margin that developed about the CP tip, laterally and in depth. The goal was to engulf the tumor into an ice block including 3 mm to 12 mm of breast tissue. The ice block along with an unfrozen breast tissue layer thirty mm \pm 5 mm thick determined the palpable limits of the resection plane. The IOUS helped with evaluating the location of the frozen margin into the breast. Additionally, a color Doppler flow imaging was performed during the freezing process to observe whether blood flow could be seen in the tissue surrounding the ice ball. **Results.** Targeting the lymphatic system with various diagnosis or therapeutic agents is of great interest in the oncologic community. An intra-lymphatic chemotherapy can likely be effectuated with our cryo-assisted procedure, as it has been shown that cytotoxics injected at the breast tumor margin and in the peritumoral tissue can migrate into the tumor lymphatic drainage. Such strategy could address the loco-regional minimal residual disease and the infra-clinic disease on a selected population of patients. A lymphatic chemotherapy that injects free or carrier-bound cytotoxic(s) into the peritumor area has shown its safety and efficacy on disease free survival (DFS) and overall survival (OS) in short series of BC patients stage II-III. **Originality.** Our original technique consisted in injecting under intraoperative ultrasound guidance the deep aspect of the tumor with the dye during the freezing process; the resulting solid ice block was immediately resected and the lymph nodes cleared. **Conclusions.** Our cryosurgical techniques of tumor deep repeated freezing for an en bloc resection or for a in situ ablation of various breast cancer can be associated with the simultaneous injection of two dye tracers during a conventional surgery including the lymphatic mapping. The intraoperative ultrasound monitoring facilitated the procedure. The frozen-and-thawed mass behaved as an exclusion-and-trapping zone for the tracer(s). The margin status determination was not affected by the procedure. References 23.

Keywords: primary breast cancer, cryosurgery with surgery, intratumor tracer injection, intraoperative ultrasound imaging, lymphatic mapping.

**КРІОХІРУРГІЯ ПЕРВИННОГО РАКУ МОЛОЧНОЇ ЗАЛОЗИ
КОМБІНОВАНА З ІНТРАОПЕРАЦІЙНОЮ ІН'ЄКЦІЄЮ МЕТИЛЕНОВОГО ФАРБНИКА
ПІД КОНТРОЛЕМ УЛЬТРАЗВУКУ**

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Хірургічні технології є одними з найпоширеніших і найбільш ефективними у лікуванні раку молочної залози. Зважаючи на специфіку фізичної дії, кріохірургічні методи лікування первинного раку молочної залози можуть застосовуватись, як самостійно, так і у поєднанні з традиційними методиками резекції пухлини молочної залози. Кріоабляція, як метод, дозволяє самостійно здійснювати видалення злоякісної пухлини молочної залози. Кріодевіталізація злоякісної пухлини проводиться з подальшим хірургічним видаленням попередньо замороженої пухлинної тканини, яка переведена з активної фази в неактивну фазу життєдіяльності злоякісних клітин. Враховуючи специфіку розвитку первинного раку молочної залози проблема необхідного але не надмірного видалення тканин з застосуванням кріохірургічних технологій є надзвичайно актуальною. Метою даної роботи було дослідження можливості застосування кріохірургічних методик для лікування раку молочної залози самостійно чи у поєднанні з традиційними хірургічними методиками з використанням метиленового фарбника для отримання карти лімфатичного дренажу чи циркуляції лімфи в молочних залозах та сусідніх до них ділянках людського організму.

Ключові слова: первинний, рак, молочної залози, кріохірургія, хірургічне, втручання, внутрішньо пухлинне трасируючий, інтраопераційне, ультразвукове, зображення, лімфатичне.

PROBLEM STATEMENT. The therapy of breast cancer (BC) in stage II, III or IV is multimodal, associating systemic chemotherapy (CT), surgery and radiotherapy [1] a combination that has demonstrated a lower rate of local recurrence and an improvement of the disease free survival (DFS) and overall survival (OS). Surgery is considered a central method but the rate of post-surgical recurrence is an ongoing issue [2] and the first site of recurrence is associated with a different survival [3]. Various pre-, intra-, or post-operative therapeutic strategies aim at improving these results such as neo-adjuvant therapies, intraoperative lymph node mapping, intraoperative tumor margin evaluation and immunotherapy [4].

Cryoablation (CA) for primary and secondary breast cancer (BC) is a well-known palliative or curative focal ablative for tumors of various sizes [5]. The surgical resection of a frozen tumor was also proposed for primary and secondary BC based on the hypothesis that the procedure could prevent or decrease the loco-regional and systemic dissemination of tumor cells [6].

Percutaneous cryoablation (PCA) is an efficient method on advanced or recurrent BC, when use alone or associated with surgery, loco-regional therapy, adjuvant systemic chemotherapy (ACH), endocrine therapy (ET), and/or immunotherapy [7]. Cryosurgery is an adjuvant tool during the surgical resection of solid tumors [8]. The cryoprobe (CP) can grasp the frozen tumor mass, ease the tumor manipulation and dissection, and trap the tumor cells in the frozen state during the resection [9]. Cryosurgery can also be associated with the intra operative local injection of various fluids: fluids buffers are used to prevent the unintended freezing of risky or sensitive body structures during image-guided cryoablation (CA), e.g. the skin for BC [7]; therapeutic fluids like mixtures and suspensions of tracer and active drugs have also been interstitially co-injected during cryoablation to increase the local efficacy of cytotoxic(s) at the tumor margin on transplanted tumor models [10]. However, the timing of the injection of the drug (s) relative to the freeze-thaw sequences and the route of injection are the subject of many debates [11].

At our institute we are using various cryosurgical procedures on patients presenting with primary breast tumor metastatic or not, or with recurrent breast cancer. These procedures are based on the tumor resectability: when the resection is possible we freeze the tumor and a safe breast margin to make an ice block that will be resected with negative surgical margin [4]. This cryo-assisted tumor resection (CAR) is similar to that described for the management of liver tumors [7] or for the recanalization of airway structures [8]. (There is no DOI for some of your references, making them impossible to read. Usually the reviewers are very critical at this point. It's better to have a few strong references to which the reader can rely on.

The objective is to trap the tumor cells into the frozen mass, momentarily deprived from blood flow, so as to prevent the intraoperative dissemination of cells during the tumor manipulation [13]. The surgery is conventional and consists in complete excision of the frozen block with negative surgical margins. For the treatment of unresectable tumor we use the cryoprobe as an ablative and palliative tool in situ [12]. The operated breast is always treated with radiotherapy (RT), and when indicated with chemotherapy (CH), or endocrine therapy (ET) in the adjuvant setting [13]. For the surgical management of the regional lymph node we usually perform an axillary exploration and a lymph node dissection (ALND) or pre-operative sentinel lymph node mapping with a radioactive tracer and a sentinel lymph node dissection (SLND). Due to the cost and constraints of the lymphoscintigraphy we have elected to use the intraoperative injection of blue dye for the sentinel lymph node mapping during our cryo-assisted procedures.

In this paper we report our initial experience with the procedure. To the best of our knowledge there is no clinical study on the transport and loco-regional distribution of a dye injected into a breast tumor submitted to a simultaneous cryo-assisted resection. This observational study was conducted at a private institution on fourteen breast cancer patients stage IIA-IV. The primary objective was to define the conditions and to evaluate the feasibility of injecting a dye tracer into the tumor undergoing the freezing procedure. The flow and transport of the dye, either the Patent blue vital (PBV), or the methylene blue (MB) was evaluated intraoperatively under ultrasound (IOUS) imaging. The second point was to determine whether and how the procedure affected the normal surgical workflow, in particular regarding the lymph node mapping, the tumor characterization and the margin status determination.

EXPERIMENTAL PART AND RESULTS OBTAINED. *Patients.* This study was conducted at the Rudolfinerhaus Hospital, Vienna, Austria. All the patients gave their informed consent. 14 Patients aged 50 ± 14 y (mean \pm SD) presenting with primary breast tumor, stage II, III or with de novo stage IV BC(13) or locally recurrent BC (1) were randomly selected and

treated between August 2013 and October 2015 (Table 1). 8/14 patients were locally advanced (LABC), but estimated resectable without neoadjuvant chemotherapy. None was inflammatory. One patient presented with a large matted axillary nodal metastasis. No patients except one had any previous chemo-radiotherapy before the procedure. The tumors were all invasive ductal carcinomas (IDC), with histological grades G2 (2) and G3 (3) according to the Nottingham histologic score. Two of the four disease staged II had "aggressive" tumor characteristics, i.e. G3 and triple negative receptor status (TNBC) (6). One of the invasive ductal BC was from the medullary type, which is histologically G3 but with better prognosis.

Table 1 – Baseline characteristics of patients and tumor lesions

Characteristics	Data (n= 14 Patients)		
Age (mean \pm SD)	50 y \pm 14, range 24-57 y		
Stage (TNM)	Stage II ¹ (4)	Stage III (4)	Stage IV ² (6)
T	T1 (1/4); T2 (3/4)	T2 (1/4); T3 (1/4); T4 (2/4)	T2 (2/6); T4 (4/6)
N	N0 (4/4)	N1 (3/4); NX (1/4)	N1 (6/6)
M	M0	M0	M1 (6/6)
Tumor Size (mean \pm SD)	5,4 cm x 3,1 cm \pm 4,6 x 2,7 (range, 1,9 cm x 0,9 cm to 17 cm x 10 cm)		

Treatment Summary. The tumors were treated as follows: Acryo-assisted tumor injection and resection (CATIR), 11 patients (group A), had their tumor frozen and injected with the selected blue dye during the freezing process; after a few minutes the frozen tumor and a breast margin were resected in a conventional manner, marked with sutures for spatial orientation and for the tumor characterization and the margin evaluation on frozen samples. An axillary exploration and lymph node (LN) clearance was done as follows: 11 patients had a sentinel lymph node mapping and dissection (SLND); frozen sections of the resected node(s) were examined. A completion axillary lymph node dissection (ALND) was conducted on 3 patients. The 3 patients of the cryo-assisted tumor injection and ablation (CATIA) had a palliative tumor freezing and intratumor injection of blue dye without any surgery (group B). The intraoperative therapies are summarized in Table 2.

Table 2 – Summary of breast intraoperative combination therapy: cryosurgery-injection-resection

GROUP	PROCEDURE		
CRYO-ASSISTED TUMOR INJECTION + ABLATION (CATIA) n=3	Tumor Frz	(60-90%)	+ Dye IT Injection
CRYO-ASSISTED TUMOR INJECTION + RESECTION (CATIR) n=11	Tumor Ice Block (100%) + Breast Margin	+ Dye + Injection	Lumpectomy (1)
			Quadrantectomy (8)
			Mastectomy (2)
	+ Axillary Clearance		Resection-ALND ² (1)
			SLND ¹ (8)
			ALND (2)

Keys: Frz: Volume percentage of freezing coverage; IT: intratumor; Dye: patent blue V or methylene blue; Ice Block: the frozen tumor and breast margin; ¹ SLND: Sentinel Lymph node dissection; ² A massive matted lymph node was frozen-and-injected before axillary lymph node dissection (ALND).

Cryoprobe and injection needle. The cryogenic console and the reusable metal cryoprobe (CryoPulse, Ky-iv, Ukraine) use liquid nitrogen as the refrigerant; the cryoprobe (CP) comes in different round-shaped freezing tips diameters: 20mm, 25mm, and 50mm. A single CP is used per procedure; the tip is selected to best fit the tumor surface on which it is centered while insuring a good contact with moderate pressure. The lowest temperature of the tip -180 °C is reached after about 2 minutes of continuous cooling.

The injection system is a 19GChiba or spinal type needle (1,1mm) or a 25G (0,5mm) needle, connected directly or through an extension line to a 3ml or 5ml plastic syringe. The needle and the fluid line is debubbled and primed before insertion into the tumor.

Ultrasound Imaging. Two ultrasound systems (US), a Sonosite M-Turbo (www.Sonosite.com), a Voluson 730 Expert (www.gehealthcare.com), and linear array transducers were used for 2D or 3D real-time intraoperative needle guidance, ice ball visualization and flow imaging of the dye during the injection. The intraoperative sonography (IOUS) is used to assess the tumor contours along with palpation to position the needle tip at the tumor deep margin, and its shaft at least 20mm away from the probe tip edge. During the freezing sequences the operator could visualize the icing zone and the ice margin that developed about the CP tip, laterally and in depth. The goal was to engulf the tumor into an ice block including 3mm to 12mm of breast tissue. The ice block along with an unfrozen breast tissue layer thirty mm ± 5mm thick determined the palpable limits of the resection plane (38). The IOUS helped with evaluating the location of the frozen margin into the breast. Additionally, a color Doppler flow imaging was performed during the freezing process to observe whether blood flow could be seen in the tissue surrounding the ice ball [14].

Drug dye, dosing and site of injection. We have selected the patent blue vital (PBV, Guerbet, France) 2,5 %, a small molecule (MW, 582,7) that has a known tracing ability for lymphatic mapping of breast cancer

(40); another dye the methylene blue (MB) 1 % aqueous solution was used in three cases (41, 42). Either one is prepared at full or half strength diluted in 0,9 % saline solution. A fixed 2 ml dose is manually injected at a single deep tumor site, continuously over one to two minutes-period. All the tumors are receiving the same dose regardless of their size. It is in the discussion and shouldn't be inserted here.

Tumor freezing and dye injection. Briefly, for the patients of the CATIR group A (n=11) the IOUS guided procedure (Fig. 1) consisted in a freeze-thaw-refreeze of the tumor to create an ice block that will be resected according to our published protocol (11). The additional step was to directly inject a blue dye into an unfrozen zone of the tumor before it was engulfed with the ice. For the patients of the CATIA group B (n=3) a similar freezing-injection procedure was conducted without any associated surgery. The tumors larger than 5cm were treated at 2 to 4 contiguous locations. A single CP was used for each patient; the CP was repositioned whenever necessary.

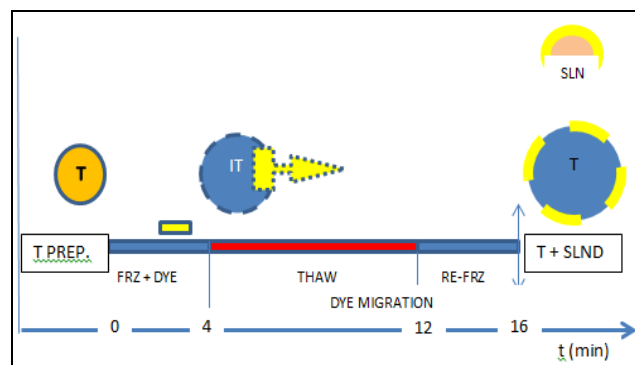


Figure 1 – Procedural diagram of the tumor surface (T) surgical approach (PREP.) and the ice block formation (FRZ, blue color) with intratumor (IT) dye injection (yellow arrow) before resection

Figure 1. Procedural diagram of the tumor surface (T) surgical approach (PREP.) and the ice block formation (FRZ, blue color) with intratumor (IT) dye injection (yellow arrow) before resection. This freeze-inject-thaw-refreeze method takes about 16 minutes from start to completion. The blue dye injected IT during the end of the first FRZ migrates in the tumor and breast interstitium (yellow patterns). After the 2nd FRZ the ice block and an unfrozen breast margin is resected along with the sentinel lymph node(s) (SLND).

The procedural steps of the CATIR patients consisted in (Fig. 2): a conventional surgical approach to the tumor surface; a careful, gentle dissection of the tumor surface to minimize the disruption of the deep lymphovascular draining pathways; the selection of the appropriate CP that best fitted the tumor size and the placement of its tip centered onto the tumor surface; the positioning of the US transducer for the visualization of the tumor contours; the US guided insertion of the needle tip at the tumor margin most distal from the probe and in a direction that set the needle shaft or tip at least 20 mm distant from the probe tip; the verification of the needle lumen patency with injecting a few microliters (ul) of the selected tracer; the initiation of the first tumor freeze (FRZ) for 3 minutes and of the injection during the 2nd minute into FRZ (Fig. 2A).

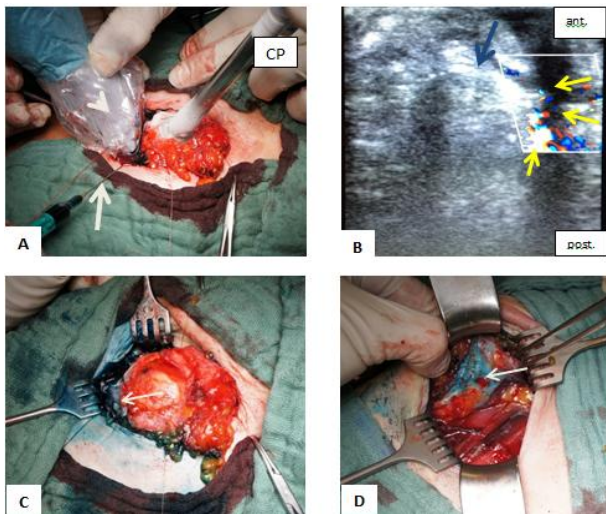


Figure 2 – Intraoperative sequences showing a cryo-assisted tumor injection and resection (CATIR) of a breast tumor located in the right upper outer quadrant (RUOQ)

The drop of the tip temperature to -180°C took two minutes. This temperature was maintained for the following minute. The diameter of the ice ball was measured with a cm scale in surface and US imaged in the anteroposterior plane to insure that it engulfed the whole tumor along with a margin of breast tissue. Our rule is to get 5 mm to 12 mm of frozen margin exceeding the tumor contours. The continuous delivery of the injected dye, intervening in the third minute of the cooling process, was also imaged with the color flow Dop-

pler. After the completion of the injection and the first FRZ the cryoprobe was set to thaw; this heating was active for 45 seconds and passive for 8 minutes, allowing us to image the ice front extending to the posterior aspect of the tumor and to evaluate the migration of the dye about the tumor (Fig. 2B). A second FRZ of similar 3 minutes duration was set without any dye re-injection, immediately followed by the resection of the ice block (Fig. 2C). The resection line was located in the unfrozen breast tissue at $30\text{mm} \pm 5\text{mm}$ from the ice block margin. The lymph node(s) surgery was conducted according to the intraoperative SLN mapping (Fig. 2D) and/or the pre-operative plan. Overall about 14 min to 18 min were left for the tracer to migrate away from its site of injection, counted from the initiation of freezing until the surgical procedure disrupted the interstitial lymphovascular network.

Gross examination and microscopic analysis. The resected sample was tagged with sutures for orientation and photographed. Tumor sections made in the operative room allowed an evaluation of the stain distribution at gross examination. Frozen sections allowed for the immediate pathological characterization of the tumor and determination of the resection margin status as well as that of the resected node(s). The tumor fixation and staining was conducted as usual for further pathological evaluation, and for the determination of the ER, PR and HER receptors in formalin-fixed paraffin-embedded (FFPE) sections. Our pathologist was aware of and familiar with the tissue alterations of the cryo-assisted extirpation procedure. The inked resection margin was considered free of tumor for a distance $\geq 1\text{mm}$.

Post-operative follow-up and therapy. It was conducted without any deviation from that of the cryo-assisted extirpation only patients. After the operation a pressure dressing was applied to decrease the risk of hematoma formation. Specific attention was given to signs of allergic reaction to the dye(s). 9/11 patients had breast radiation therapy (RT); adjuvant chemotherapy (CH) and/or endocrine therapy (ET) were performed on 12 of the 14 patients.

Results. The blue dye injection was feasible and imageable with the IOUS during the ice block forming process. The delivery of the dye into the unfrozen part of the tumor was uninterrupted by the concomitant first freezing, regardless of the needle size. Both the energy delivery and the dye delivery were conducted to their intended dose and duration for all cases. For both groups the IOUS monitoring facilitated the needle placement and demonstrated the presence and progression of the ice margin within the tumor and the surrounding parenchyma in the anteroposterior direction. The flow of either dye during its delivery was imageable at all times; it did not permeate the ice block margin and migrated away during the cooling process. The estimated proportion of ice in the tumor at the time of injection, i.e. after two minutes of continuous freezing, was different for the CATIR group A and the CATIA group B, respectively about 50 %, and 75 % (range, 60 %-90 %) of the tumor volume. For the

CATIR group A, upon the completion of the re-freeze sequence the tumor and 5mm to 1 mm breast tissue margin was frozen as intended, before the probe detachment and the initiation of the resection. For the first patients an anesthesiologist skilled with percutaneous US guided needling procedures assisted with the transducer manipulation so that the operator could focus on the cryo-assisted injection process. The use of an extension line between the needle and the syringe was helpful but didn't always prevent the needle to move a little, although the move was inconsequential. The injection step didn't add time to the cryo-assisted resection procedure; however, the proper needle tip positioning at the deep part of the tumor under IOUS took an additional 5 to 10 minutes for the first patients, and took under 5 minutes for the last. The refreeze period of 3 minutes intervening after the first freeze injection and thaw sequence did increase the size of the ice block of a few millimeters without affecting the dye distribution observed after the first freeze (43). The presence of blood flow wasn't detected by IOUS flow imaging before cryosurgery [15].

The surgical exposure of the tumor anterior surface was carefully made to spare as much as technically possible the peritumorallymphatic drainage and minimize the creation of paths of least resistance and the risk of dye spillage and loss. During the freezing a little amount of dye surfaced at the operative field in the tumor vicinity for 2 cases (Fig. 2C), but wasn't related to a reflux in the needle tract, or to the needle size. The loss of dye was estimated negligible, likely less than 200 microliters (ul), and wasn't compensated for.

The dye was transported at the ice margin, within the tumor and the lymphatic drainage. Our cryoprocure that contacts and freezes the tumor (Fig. 1A) from its surface to its depth resulted in a lateral and anterior-posterior progression of the ice block whose hyperechoic margin was readily visualized at US imaging. The flow of either dye was imageable by the colour-Doppler at the ice margin during the injection for either group.

Table 3 – Surgical group (CATIR) node staining, pathological status and axillary surgery

Axillary clearance n=11	Stained LN (n)	Unstained LN (n)	Meta- static
SLND 8	(1)3; (2)1; (3)2; (4)1	(1) 1	7
ALND 3	(1)1*	(1) 1; (1) 1	1

*One patient had a large matted nodal mass that was directly injected and resected. ALND: Axillary Lymph Node Dissection Level I & II.

The pathological characterization of the tumor and evaluation of the resection margin were realized. The breast tissue containing the hard tumoral ice block was

easily dissected along this template and it was resected during the melting following the cryoprobe removal. The gross examination of the resected samples after full thawing revealed a constant and partial staining of the tumor and the breast parenchyma in the vicinity of the injection site. Such staining wasn't an obstacle to the pathological characterization of the tumor, to the resection margin evaluation or to any parenchymal lymph node identification.

The gross examination shows a variable tumor staining more intense in the deep part of the tumor (arrowhead) along with the breast tissue staining in the direction of the breast tail (arrow). B, another sample evidences a light staining of the anterior tumor side (white arrow) where the cryoprobe was located, and an intense staining of the posterior deep part of the tumor (arrowhead) where the needle was located. In this region the breast parenchyma is also stained.

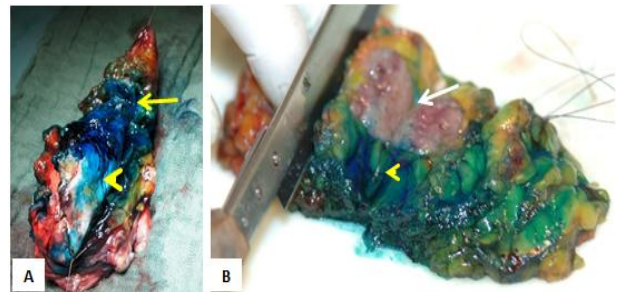


Figure 3 – Gross examination of the resected samples.

A, after thawing the sample is sectioned along the anteroposterior axis corresponding to the freeze margin progression

The intraoperative examination of the frozen sections allowed the detection of a single R1 margin in one of 10 patients. The positive margin was immediately re-resected. The resection cavity was at times stained. Our freeze-thaw-refreeze procedure didn't affect the post-operative histological (Fig. 4) or hormonal receptor status evaluation on FFPE tissue sections.

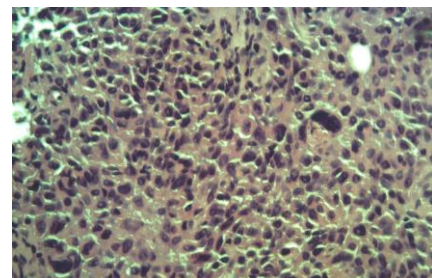


Figure 4 – Typical microscopic alterations observed within the frozen core of a resected tumor.

H&E staining, magnification: 200 X.

The tumor tissue is distinguishable, evidencing early signs of apoptosis with enlarged cells, swollen eosinophilic cytoplasm and shrunk nuclei.

Isolated giant tumor cells are also visible

The procedure was well tolerated and uneventful for all the patients. There was no unintended freezing of the breast structures –skin, fascia or pectoralis muscle– and the cryoprobe/tumor target was maintained in the same position from start to finish. We did not observe any allergic or anaphylactic reactions during the injection procedure, or in the postoperative period. The wound healing was uneventful.

Discussion. The cryoprobe-assisted resection of small breast tumor has been found useful for the localization and the containment of the tumor cells during the surgical manipulation of the frozen mass [12]. The injection of fluids [6] allowing a safer [7] or more effective [11] tumor kill during cryoablation is in use during image guided interventions. A tracer can be injected in the cryoablated area of a breast cancer up to 2 weeks after cryoablation, making the lymphatic mapping possible during the resection of the necrotic tumor [16]. It is not known whether a tracer could be injected into a breast tumor during the freezing and whether its transport would allow the lymphatic mapping. It is not known whether the architectural or molecular alterations of the tumor would still be distinguishable and measurable. Our short clinical study aimed at investigating these points during the performance of a conventional surgical procedure.

Our technique consisted in injecting under intraoperative ultrasound guidance the deep aspect of the tumor with the dye during the freezing process; the resulting solid ice block was immediately resected and the lymph nodes cleared. Our short study demonstrates that the tracer delivery and the intraoperative lymphatic mapping, an essential component of an effective SLND were feasible. The injection of fluid(s) during cryosurgery is common practice [14]. Therefore, it was not surprising that a blue dye injected into an unfrozen part of the tumor did distribute within the interstitial compartment, where it could be taken up by the lymphovascular drainage. However, many parameters had to be controlled for the successful delivery of the dye at a single intratumoral location during the freezing sequence. The primary goal of the CATIR procedure was to start the tumor freezing and to perform the injection into an unfrozen region of the tumor before it was entirely engulfed by the ice. The precise needle positioning at the deep part of the tumor and the injection of the dye intervening before the needle cooling could be adjusted for each case thanks to the IOUS [17]. Remarkably the dye was excluded from the ice block confirming the findings of previous pre-clinical studies using a similar freeze-plus-injection strategy [6, 10]; an exclusion evidenced by the IOUS imaging. The dye did migrate from its site of deposition into the interstitial fluids of the unfrozen tumor towards the lymphatic drainage and the lymph node(s). Such drug delivery strategy was preferred to an injection and freeze delivery sequence since the dye distribution volume, i.e. the unfrozen tumor, would have been larger. A previous clinical study has shown [15] that drug-dye-radiocontrast agent lipophilic or hydrophilic mixtures

deposited before cryoablation (CA) are engulfed by the frozen zone and therefore are no longer available for any lymphovascular uptake. Alternatively, the injection could have been done immediately post-freeze but it would have been only peritumoral about the frozen breast tissue. We purposely didn't select this option given that our strategy seeks to use the intratumor lymphatics for an improved targeting of the actual lymphatic drainage of the tumor [17]. The successful migration of either dye, the MB or the PBV, to the axillary lymphatic drainage from a single intratumoral site of injection confirms previous observations [18]. The staining of 1 to 4 lymph nodes in the axillary region for 8/11 patients of the CATIR demonstrates the detection effectiveness of our technique. This 72 % *detection rate* should improve considering our inexperience and the small number of cases [19]. We couldn't evaluate the *accuracy* of the lymphatic mapping due to the limited number of ALND.

The axillary dissection was performed after the resection of the tumor-breast ice block [i.e. about 18 ± 2 minutes (range 16 to 20 minutes)]. The time left for the dye migration, about 20 to 25 minutes, was sufficient for the migration of the dye as compared to literature [19].

Previous studies have demonstrated that a dye can be injected intratumoral for BC localization (53) and sentinel lymph node (SLN) mapping [18, 19]. An intratumoral rather than multiple peritumoral site(s) of injection was chosen based on the premises that the presence of intratumoral lymphatics and their role in lymphangiogenesis may probably better reflect the tumor lymphatic drainage. Additionally, a single deep site of injection was hypothesized to be less prone to leakage and loss of dose during the surgical approach, compared to a more conventional peritumoral multi-site injection (20) or to a superficial plus deep injection [17]. Our results seem to confirm the above observations, since the dye leakage was minimal. The tracer dose, 2 ml, was fixed regardless of the tumor volume and estimated sufficient to achieve a lymphatic mapping. Our results show that the PBV or the MB was staining at least one node in 8/11 CATIR patients. A success rate that is quite in the range of other studies injecting a similar dose of methylene blue dye fractionated at 4 points of the subareolar area 10 minutes before surgery. Would a larger dose or higher dye dilution have brought a higher success rate at the lymphatic mapping during the cryo-assisted resection is an open question? The usual dose for SLN mapping with a blue dye is 3ml to 5ml and the detection rate is better with diluted dye (MB) [19].

The preserved ability to do the pathological evaluation of the specimen is of note. Indeed, the frozen sections of the resected specimen allowed the immediate pathological evaluation of the frozen-thawed tumor and of the healthy breast resection margin. This resection margin was found positive in one of eleven cases. The use of IOUS was very helpful to delineate the tumor margin and to make sure that the target volume had

been properly frozen according to our criteria; whether the occurrence of this positive margin was operator-, or disease-related is unclear. However, the multifocality of apparently localized breast tumor is well-known [(20)]. Based on this study the probability for the resection line to reach the residual cancer foci is <50%. Therefore, a whole breast RT was administered like for any breast surgery made on such stage tumor. The tumor was easily measured and characterized on the frozen sections but the nuclear grade wasn't clearly identifiable confirming the observations of others (56). It looks like our double freeze procedure does not affect the histological parameters significantly. Our freezing source located at the tumor margin does generate asymmetrical isotherms and therefore various degrees of cell and tissue structural alterations compared to the central freezing source of Sahoo [21]. It is likely that our pathological sample made at the tumor margin, and therefore remote from the cooling source, revealed lesser alterations than those of a core tumor sample. Although it is noteworthy that the tumor markers cannot be reliably assessed after cryoablation our CATIR procedure allowed the pathological and hormonal receptor status evaluation. This is understandable given the relatively good preservation of the sample in the absence of the cellular damages and necrosis due to lasting vascular alterations that were present in the above study [6]. However, we didn't evaluate the level of the markers.

We are aware of the many limitations of the study. First, the limited number of patients and the absence of comparison group with the intratumoral injection of tracer without any tumor freezing. Although the lymphatic mapping is possible with the direct intratumor injection of tracer(s), we couldn't evaluate the influence of the extruding effect of the ice volume on the transport of the dye to the lymphatic drainage due to the large variation of the tumor volume in which the dye could disperse. Second, the tumor cell containment and the prevention of cell shedding theoretically permitted by our freezing technique couldn't be evaluated. We didn't measure the presence or variation of the circulating tumor cells before and after the procedure. It has been demonstrated on cryoablated bronchial tumors that cryoablation increases the number of CTCs [21]; but its decrease is related to the efficacy of cryoablation of liver metastases from colorectal cancer [18]. On a technical viewpoint we did extirpate the tumor during the thaw period following the refreeze sequence, using the palpation to assess the location of the frozen edge. The regression of this edge during the surgical resection may have somehow shrunk a little the frozen volume thus reducing the thickness of the resection line made in the healthy breast. It is a palpation and visualization guided method that sets the resection rim at a distance from the frozen mass. We could have used the IOUS to guide our resection line [22] but still the ice edge would have regressed in the absence of sustained cooling.

We do recognize that we have been conservative with our use of the procedure. Our ability to conduct a conventional surgical procedure, lymphatic mapping

and pathological examination during and after our cryo-assisted technique paves the way to therapeutic strategies that aim at preventing or treating the dissemination of tumor cells. Two strategies emerge as they are already used during image-guided or surgical interventions; namely the targeted lymphatic therapy and the surgical cavity shaving [23]. Combining the intraoperative and post-operative effects of cryosurgery with cytotoxics and radiotherapy bring several potential advantages in terms of tumor cell kill or sensitization. For instances a zone of tissue hypothermia develops about the frozen mass; this zone contained within the isotherm +12°C is about twice as large as the frozen mass; such thermal stress induces a selective sensitization of tumor cells to radiation therapy [23]. Such effect, if proven true for breast cancer, could be advantageously used on small or less advanced breast tumors. Although we didn't use our cryoprobe to treat the positive margin observed in one patient of our study, we could have done so rather than doing the re-resection or the surgical cavity shaving. Targeting the lymphatic system with various diagnosis or therapeutic agents is of great interest in the oncologic community. An intra-lymphatic chemotherapy can likely be effectuated with our cryo-assisted procedure, as it has been shown that cytotoxics injected at the breast tumor margin and in the peritumoral tissue can migrate into the tumor lymphatic drainage [23]. Such strategy could address the loco-regional minimal residual disease and the infra-clinic disease on a selected population of patients. A lymphatic chemotherapy that injects free or carrier-bound cytotoxic (s) into the peritumor area has shown its safety and efficacy on disease free survival (DFS) and overall survival (OS) in short series of BC patients stage II-III [22].

CONCLUSIONS. Our cryosurgical techniques of tumor deep repeated freezing for an *en bloc* resection or for a *in situ* ablation of various breast cancer can be associated with the simultaneous injection of two dye tracers during a conventional surgery including the lymphatic mapping. The intraoperative ultrasound monitoring facilitated the procedure. The frozen-and-thawed mass behaved as an exclusion-and-trapping zone for the tracer(s). The margin status determination was not affected by the procedure.

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**КРИОХИРУРГИЯ ПЕРВИЧНОГО РАКА МОЛОЧНОЙ ЖЕЛЕЗЫ
КОМБИНИРОВАННАЯ С ИНТРАОПЕРАЦИОННОЙ ИНЪЕКЦИЕЙ МЕТИЛЕНОВОГО КРАСИТЕЛЯ
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Хирургические технологии являются одними из самых распространенных и наиболее эффективными в лечении рака молочной железы. Учитывая специфику физического воздействия, криохирургические методы лечения первичного рака молочной железы могут применяться, как самостоятельно, так и в сочетании с традиционными методиками резекции опухоли молочной железы. Криоабляция, как метод, позволяющий самостоятельно осуществлять удаление злокачественной опухоли молочной железы. Криодевитализация злокачественной опухоли проводится с последующим хирургическим удалением предварительно замороженной опухолевой ткани, переведена из активной фазы в неактивную фазу жизнедеятельности злокачественных клеток. Учитывая специфику развития первичного рака молочной железы проблема необходимого но не чрезмерного удаления тканей с применением криохирургических технологий является чрезвычайно актуальной. Целью данной работы было исследование возможности применения криохирургическим методик для лечения рака молочной железы самостоятельно или в сочетании с традиционными хирургическими методиками с использованием метиленового красителя для получения карты лимфатического дренажа или циркуляции лимфы в молочных железах и соседних к ним участках человеческого организма.

Ключевые слова: первичный рак, молочной железы, криохирургия, хирургическое, вмешательства, внутренне опухолевое трассирующей, интраоперационное, ультразвуковое, изображения, лимфатическое.

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