RE-IORT 01: Feasibility clinical trial of Intraoperative radiotherapy (IORT) and second breast-conserving-surgery after local recurrence of breast carcinoma

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PROTOCOL SIGNATURE PAGE

RE-IORT 01

Feasibility clinical trial of Intraoperative radiotherapy (IORT) and second breast-conserving-surgery after local recurrence of breast carcinoma

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Compliance statement

I undersigned Dr. .....

I have read the protocol, and agree to conduct the present clinical trial in accordance with applicable European Regulatory requirements, the Declaration of Helsinki and the principles of Good Clinical Practice Guidelines.

I agree:

- To obtain freely given written informed consent from each patient after she/he has been informed of all aspects of the trial relevant to his/her decision to participate,
- To declare all serious adverse events within 24 hours after having been informed of the event,
- To respect inclusion and non-inclusion criteria as well as the dates of onset and end of the study,
- To fully complete all sections of the electronic case report form (eCRF),
- To provide any clarification or correction related to the eCRF,
- To permit regular monitoring and auditing by the Sponsor,
- To archive and store all trial-related documents for a 15-year period.

Investigator's agreement:

Date: **Signature:**

SYNOPSIS

TITLE	**RE-IORT 01 :** **FEASIBILITY CLINICAL TRIAL OF INTRAOPERATIVE RADIOTHERAPY (IORT) AND A SECOND BREAST-CONSERVING SURGERY AFTER LOCAL RECURRENCE OF BREAST CARCINOMA**
PROTOCOL ACRONYM:	**RE-IORT 01**
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Design	Phase II single arm multicenter trial design to evaluate the feasibility of a second breast-conserving surgery combined with re-irradiation using IORT after local recurrence of breast carcinoma. 65 patients are scheduled.
Rational	Currently, the rate of ipsilateral breast tumor recurrence (IBTR) after breast-conserving surgery and radiotherapy remains at 10% at 10 years to 15% at 20 years, respectively. IBTR is an independent predictor of poor survival with a 3 to 4.6 increased risk of cancer-related death. In a heterogeneous population, local control remains a major therapeutic challenge for these relapses, especially those considered of better prognosis, namely occurring late and of low histological grade. Therapeutic de-escalation is possible for these relapses to avoid a mutilating and often traumatic mastectomy. However, this second conservative surgery has a high rate of second local

	relapse (19 to 50% at 5 years) due to the absence of a re-irradiation, rendered impossible by the problem of tolerance of previously irradiated tissues. Retrospective or prospective studies on partial breast irradiation (PBI) in adjuvant setting report promising results, both in terms of tolerance (saving healthy tissue) and local control (74% to 100% at 5 years). Used techniques include brachytherapy, external beam radiotherapy and intraoperative radiotherapy (IORT). IORT is now the subject of renewed interest in breast cancer. It has the advantages of high-precision ballistics on the operated area and of preservation of healthy tissue. To date, no prospective data, however, have been published in the indication of ipsilateral breast recurrence. We propose a prospective, multicenter, single arm Phase II design to evaluate the feasibility of repeated breast-conserving surgery combined with re-irradiation using IORT after local recurrence of breast carcinoma.
OBJECTIVES	**Primary objective** - To evaluate the tolerance of IORT delivered during a second breast-conserving surgery **Secondary objective** - Cosmetic outcome - Early Toxicities: Hematoma, Lymphorrhoea (punctures> 2). Breast Infectious Erythema. - Late Toxicities: Telangiectasia, Breast Pain, Skin hyperpigmentation, skin ulceration, cutaneous atrophy. - Quality of life - Local relapse–free survival (L-RFS) - Metastasis relapse–free survival (M-RFS) - Disease-free survival (DFS) - Overall survival (OS) - Mastectomy-free interval (MFI)
Assessment criteria	**Primary criterion:** - grade ≥ 2 late fibrosis, according to the NCI CTCAE v4.0 grading scale, occurring within 12 months following the IORT. **Secondary criteria:** Definitive cosmetic results: - assessed by a physician and the patient - Pictures will be taken before treatment, after 15 days, three, six, and twelve months then 1fs / year up to five years. Toxicities Evaluation : - assessed by a physician Quality of life: - assessed by a patient - EORTC QLQ-C30, QLQ-BR23 questionnaires delivered before treatment, and after 15 days, three, six, twelve months and twenty-four months. Local relapse-free survival (L-RFS): - L-RFS is defined as the time from surgery with IORT to any ipsilateral breast tumor recurrence, or death, whichever occurs first. Metastasis Relapse-free survival (M-RFS): - M-RFS is defined as the time from the surgery with IORT to any metastatic disease (excluding the ispilateral breast, contralateral breast, regional lymph nodes), or death, whichever occurs first. Disease-free survival (DFS): - DFS is defined as the time from the surgery with IORT to any recurrence, including local recurrence, metastasis, appearance of a

	second primary tumor, or death from any cause, whichever occurs first. Overall survival (OS): - OS is defined as the time from surgery with IORT to death from any cause. Mastectomy-free interval: - MFI is defined as the time from the surgery with IORT to a mastectomy for any reasons (all other events will be ignored). All patients without mastectomy will be censored at last follow-up.
ELIGIBILITY CRITERIA	**Inclusion criteria** Histologically proven non-lobular invasive breast recurrence (IBR) Time from whole breast radiation following the initial lumpectomy >5 years Unifocal tumor Recurrent tumor size ≤ 2 cm Adequate breast size for a second breast-conserving surgery with acceptable cosmetic result Bilateral breast mammogram within 90 days prior to study entry Breast MRI within 90 days prior to study entry Histological grade I-II Estrogen-receptor-positive tumor (ER+) Cerb2-negative tumor N0 status (ultrasound of the axillary area is required, with fine needle aspiration cytology, in case of doubt) M0 status determined by PET-CT or both thoraco-abdominal CT and bone scan within 90 days prior to study entry Prior radiotherapy delivered within a standard fractionation schedule Performance status (ECOG) 0-1 Women ≥ 50 years Absence of any psychological, familial, sociological, or geographical conditions with a potential to hamper compliance with the study and follow-up schedule Affiliated to the French Health Insurance regimen Written and signed informed consent form. **Exclusion criteria** History of non-invasive breast carcinoma (in situ) History of contralateral breast carcinoma Multifocal and/or multicenter recurrence Lobular carcinoma Estrogen-receptor-negative tumor (ER-) Cerb2 (her2) overexpressed - breast cancer Extensive intraductal component (EIC) on biopsy Lymph vessel invasion on biopsy N1-3 status: Regional cytological or histologically proven node recurrence M1 status: Metastatic disease cT4 (Skin or muscle involvement) or Paget's disease of the nipple Prior radiotherapy delivered within an accelerated or hypo-fraction schedule Prior malignancy other than non-melanoma skin cancer unless the patient has been disease free for at least 5 years Patients with a small breast volume, technically unsatisfactory for a second conservative surgery or intraoperative breast irradiation. Preoperative chemotherapy or hormone therapy for local relapse Connective tissue disease or scleroderma, contraindicating radiotherapy Age < 50 years Known BRCA1/2 gene mutation (genetic testing is not required) Failure to comply with medical monitoring test for geographical, social or

ANCILLARY STUDY	To prospectively identify patients with a personal intrinsic radiosensitivity, through a protein and genomic assay: • The proteomic approach (**mandatory**) will score the inherent individual risk of radiation-induced severe late effects and intrinsic radiosensitivity by dosage of proteins identified in the "radiosensitivity" program. • The genomic approach (**optional**) will evaluate the genomic acute changes on tumor bed after delivery of a single dose of 20 Gy.
TREATMENT	Surgery : Tumorectomy will be performed according to the current standards, obtaining "clear" margins. The axillary lymph node control will depend on the initial management (clinical and ultrasound) of these N0 patients, chosen by the teams. Intra Operative Radiotherapy (IORT): After the excision of the tumor, IORT will be delivered. A single dose of 20 Gy by 50 kV photons (Intrabeam™) will be administered in tumor bed. The addition of IORT does not modify the surgical procedure.
STATISTICAL CONSIDERATIONS	**Sample size calculation** The primary endpoint is the success rate at 12 months, the success being defined as no grade ≥2 fibrosis according to NCI-CTCAE V4.0 classification. A success rate of 95% is expected. The sample size calculation is based on: • The Fleming one-stage design with one-sided alpha=0.05 and beta=0.05. • p0= 80% (success rate at 12 months, threshold below which we will consider the Intraoperative Radiotherapy toxicity as unacceptable, assuming 20% failure) • p1= 95% (success rate target at 12 months) A total of 46 evaluable patients is required; taking into account un-evaluable patients, the number of subjects is adjusted to 65 It should be noted that the alpha level is set at 5% (one-sided), risk of concluding as to efficacy ($p > p0$) whereas there is no efficacy ($p \leq p0$), and the beta level is set at 5%, risk of concluding as to the absence of efficacy ($p<p1$) whereas there is noteworthy efficacy ($p \geq p1$) After inclusion of 46 evaluable patients (at least 3 assessments within 12 months), it will be possible to conclude that the study demonstrates inefficacy if the success number is less than or equal to 40, or efficacy if the success number is greater than or equal to 41. The patient size and the decision rule are presented here-below: *(N, n1, n2)

Hyp	Rate of success at 12 months	p0	p1	Fleming one stage design*	N total
Rate		80	95	(46,≤40,≥41)	65

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FLOWCHART

	Baseline	IORT	15 days after IORT	3 months after IORT	6 months after IORT	12 months after IORT	24 months After IORT	follow-up 36-48-60 months
Inclusion - Exclusion criteria	+							
Informed consent	+							
Weight – Height	+		+	+	+	+	+	+
Clinical evaluation	+		+	+	+	+	+	+
Breast skin evaluation	+		+	+	+	+	+	
Cosmetic evaluation- Breast photography	+		+	+	+	+	+	+
Mammography	+				+ puis fs/an	+	+	+
Breast ultrasound	+				+ puis fs/an	+	+	+
Breast MRI	+					**optional**	**Optional**	**optional**
Extension evaluation	+							
QLQ-C30	+		+	+	+	+	+	
QLQ-BR23	+		+	+	+	+	+	
Adverse events** (CTCAE V4.0)			+	+	+	+	+	+
Proteomic sampling	+							
Genomic sampling		+						

** Including all radiation local and general adverse events (induced fibrosis)

LIST OF ABBREVIATIONS

List of abbreviations	Explanation
ADL	Any Day Life
AE	Adverse Event
ASN	Authority for Nuclear Safety
BCS	Breast-Conserving Surgery
BCT	Breast-Conserving Treatment
CE	Capillary Electrophoresis
CLPA	Chemical Ligation Dependent Probe Amplification
EIC	Extensive Intraductal Component
ER	Estrogen Receptor
IBTR	Ispilateral Breast Tumor Recurrence
INCA	National Institute for Cancer
IORT	Intra-Operative Radiation Therapy
LR	Local Relapses
NP	New Primary Tumors
PBI	Partial Breast Irradiation
RILA	Radiation-Induced T-Lymphocyte Apoptosis
SAE	Serious Adverse Event
TLR	True Local Relapses
WBRT	Whole Breast Radiation Therapy

1-Background and rational

1.1. Introduction

Breast cancer is by far the most frequent cancer among women, with an estimated 1.38 million new cases diagnosed in 2008 (23% of all cancers in women). It is now the most common cancer for women, worldwide, both in developed and developing regions (1). In France, with 53 000 new cases diagnosed in 2011, breast cancer represents 33% of all feminine cancers (2).

Nowadays, thanks to perpetual progress in screening and early diagnosis, most of the cases can benefit from breast-conserving surgery and adjuvant radiation therapy, which is assessed in huge Phase III trials, to provide equivalent long-term survival (3,4).

The risk of ispilateral breast tumor recurrence (IBTR) is influenced by different factors, such as age, family susceptibility, pathological characteristics and systemic adjuvant treatments. In 2013, this risk of IBTR after lumpectomy and radiation therapy is still about 10% and 15 % at 10 and 20 years, respectively (5).

Actually, IBTR is a relatively frequent clinical situation occurring sometimes years or decades after the initial breast-conserving treatment (BCT). Today, clinicians face the delicate issue of determining the optimal therapy modalities for these patients. After conservative treatment, local recurrence is defined by the occurrence of a tumor in the mammary gland previously operated and irradiated.

Beyond the generated psychological trauma (with discouragement and loss of confidence in treatments), this local relapse constitutes a turning point in the evolutionary history of these patients with an increased risk of distant progression.

The clinical presentation and histopathological prognosis of these local recurrences are very heterogeneous. In some situations, IBTR are considered as the foremost marker of aggressive cancer, where the metastatic risk represents the main issue. In other situations, IBTR (i.e. indolent and late ones) are considered as less pejorative events, for which the optimal loco-regional treatment must be identified.

Indeed, for these patients, a therapeutic de-escalation must be considered, based on a different approach, such as a second conserving surgery combined with partial breast irradiation. A traumatic and mutilating mastectomy could, therefore, be avoided in some extremely selected situations.

1.2. Local recurrence and prognosis.

Local relapses (LR) present extremely heterogeneous characteristics. The difference between "True local relapses" (TLR) and "New Primary tumors" (NP) needs to be clarified. Generally, TLR are diagnosed earlier than NP. They are located in the same quadrant, close to the initial tumor bed, have similar or more aggressive histology and are said to be more radio-resistant. NPs appear later, are located in another quadrant of the breast, display different pathological types and are of lower grade. Given the complexity of each type of LR, each characteristic should be considered individually. In fact, the distance measured between the relapse and the tumor bed was not reported as a significant prognostic factor for overall and breast cancer specific survival in multivariate analysis (6).

The pejorative impact of most of the LR has been demonstrated by the Oxford meta-analysis (on 48 000 patients, with 15-year median follow-up) with a direct correlation between the rate of local recurrence and decreased specific and overall survival rates (a 20% increase in LR rate led to a 5% reduction in overall survival) (7). Indeed, 5 to 10% of these patients have already synchronous metastatic disease and the 5-year overall survival after LR is estimated around 60% with an important heterogeneity of published results (6,8–11). Women with invasive IBTR are at higher risk to develop distant metastases, with a relative risk of breast cancer-related death increased by a factor of 3 to 4.6 when compared to women who do not present IBTR (12–14). Most of the data assessing the correlation between clinical and histopathological prognostic factors of IBTR and survival are from retrospective studies (6, 15). Recent retrospective analyses were published on large randomized international Phase III trials (4, 12, 15, 16) which initially demonstrated the safety of the combination of conservative surgery and adjuvant radiotherapy.

After IBTR, the risk of metastases is first correlated with the precocity of the local relapse. Mortality is higher in early IBTR (6,8,15,17) probably due to different biology and tumor behaviour. 5-year disease-specific-free survival is around 75% for LR occurring within the first 2 (16) or 5 years (6,8), respectively after 5 years, to 40% for early LR diagnosed during the 2 first years (16). Therefore, 5-year delay after the initial treatment is considered as a critical point determining prognosis.

However, the role of young age remains unclear after IBTR, whereas this age is an important and strong predictive factor for local relapse. Indeed, IBTR rate for women aged 40 years and younger undergoing BCT remains 0.5-1% per year. After LR, few studies describe a higher risk of second local relapse (17) for young patients, especially under 40, with a shorter overall survival, whereas others show no difference (6). On the contrary, some studies found a longer time to distant failure and a decreased risk for patients under 60 (18,19) and it was recently shown that younger patients with a LR after BCT have a better prognosis than their older counterparts (14,16,20).

Pathological aspects of the recurrence are important tools and estrogen receptor (ER) negative status (5,6,15), Grade III histology (5,6,9,15), and lympho-vascular invasion (6) are described to be significantly associated with poorer overall survival. Subcutaneous or skin involvement should be considered as metastatic disease (6).

A large diameter of local relapse, especially larger than 20 mm, is associated with both an increased risk of second local relapse and distant metastases in several large retrospective studies (9,17,21).

No gene expression has been proved to be associated with higher further local relapse (22). No data are available about the impact of the cerb2 status of the local relapse. Further investigations are awaited on this question, since the cerb2 status has today an important role in risk-stratifying breast cancer cases.

1.3. Local recurrence and treatment

1.3.1. SURGERY

Standard salvage mastectomy

Salvage mastectomy represents the current standard of care for IBTR. This radical treatment is supposed to be the gold-standard, as a second conservative surgery may lead to unacceptable cosmetic outcome and given the second local relapse rate resulting from a purported impossible re-irradiation of the breast.

Nevertheless, the risk of second relapse is not eliminated by mastectomy and remains quite high: several single institutional studies describe rates of chest-wall recurrence around 10%, range from 2 to 32%, (23,25,35,36), principally influenced by the delay between the first treatment and IBTR (8).

Second conserving surgery alone

Although mastectomy remains the historical local treatment of LR and is still considered as a gold standard for local relapse, its benefits in terms of survival were not proved. Moreover, all the publications evaluating this mutilating treatment are retrospective evaluations.

No matched – pair analysis has been published and no randomized Phase III trial has ever prospectively compared mastectomy to lumpectomy when possible (with or without re-irradiation). Furthermore, for understandable psychological reasons, such trials appear difficult to conduct.

Few single institutional retrospective studies have been published with interesting results but difficult interpretation (26,36,37). Indeed, close or positive margins are also an important factor increasing both second local relapse and survival (6,23), achieving local control and

significantly improved survival (6). Independently of breast size, diameter of relapse, uni- or multifocality, the surgery has to obtain clear margins.

New examination of the specific association between margin widths and IBRT failed to demonstrate an association , when adjusted to age , use of systemic treatment, use of re-excision , use of an additional boost, or consideration of the study recruitment year (38).

Safe margins are today defined as "no ink on tumor" according to the recent 2014 Guidelines of the Society of Surgical Oncology and American Society of Radiation Oncology (38).

The margin status is often unknown in publications evaluating a second conservative surgery and radiological pre-operative assessments were only limited to a mammogram.

Voogd et al. (37) and Abner et al. (36) reported a 52-month and a 39-month local relapse rate of 38 % and 31 %, respectively. The rate of second IBTR (around 35%) is similar to the one observed in historical studies on lumpectomy alone performed without adjuvant irradiation and after a primary breast cancer.

Other retrospective studies evaluated a second conservative surgery to the standard salvage mastectomy (23,24,39–41). The difference between lumpectomy and salvage mastectomy (local recurrence rate of 38% and 25 %, respectively) was not statistically significant in the French (23) and Dutch (37) cohorts. In contrast, the Sweden (39) and Italian (24) cohorts showed a 5-year LR rate of 19% and 33% after breast-conserving surgery, versus 4% and 12% after mastectomy, respectively. These rates of second local relapse may be considered unacceptable.

Finally, all studies focused on survival, aiming to assess to what extent survival could be adversely affected by a second conservative surgery (24,40).

1.3.2. Systemic Treatment

The potential benefit of systemic adjuvant treatment delivered after local relapses is still unclear and has suffered, during decades, from a lack of large trials. The Phase III CALOR Randomized Clinical Trial (IBCSG 27-02, BIG 1-02, and NSABP B-37), which evaluates the impact of adjuvant chemotherapy for radically resected loco regional relapse of breast cancer (31), has been prematurely closed, due to a slow enrolment, recruiting only 162 patients between 2002 and 2010. Very recent results communicated at the San Antonio Breast Cancer Symposium (32) showed an improvement of the 5-year disease-free survival (DFS), from 57% to 69%, p = 0.046) and of the 5-year overall survival (OS), from 76% to 88%, p = 0.02. Results remained significant for both DFS and OS in multivariable Cox proportional hazards modelling controlling for Ispilateral local relapse location, disease-free interval, ER status and prior adjuvant chemotherapy.

However, if adjuvant chemotherapy was significantly effective for women with ER-negative tumors, its benefit has not been proved neither for ER positive relapses, nor for OS or for DFS.

Adjuvant hormone therapy reduces the risk of late local and distant failures (5). Tamoxifen improved the 5-year DFS in two Phase III trials (33,34). No statistically significant improvement of the OS was shown but hormone therapy is now considered as a standard for ER-positive local relapses.

These results underscore the need for individual tailoring of adjuvant systemic treatment after recurrence.

1.3.3. Second conservative surgery with additional radiotherapy

Several teams attempted to reduce the high rate of local relapse by adding a second adjuvant radiotherapy. However, delivering a second new whole breast irradiation is delicate, as normal tissue tolerance does not permit a second full-dose course of irradiation in the whole mammary gland.

Due to the impossibility to re-irradiate the whole breast, radiotherapy has not been really evaluated in this situation, until the birth of the concept of partial breast irradiation (PBI). According to the high rate (20% at 5 years, ranging from 7 to 32%) of the second local relapse observed after a second conservative surgery (23–26), an additional re-irradiation with various PBI techniques (interstitial brachytherapy, 3D external radiotherapy or intra-operative irradiation) were expected to bring interesting results.

Partial Breast Irradiation (PBI) is an innovative approach: PBI reduces both duration and volume of irradiation to the portion of breast that is at higher risk for recurrence and also spares the surrounding previously irradiated critical organs.

The concept of PBI is based on the fact that the majority of the local relapses occur close to the tumor bed (42–44). PBI has been developed during the last decade in an adjuvant setting after BCS. For selected patients with good prognosis tumors, PBI offers non inferior local-relapse rates at 4 years (45). Based on current published evidence and experts' opinion, the American and European Societies for therapeutic radiation oncology (ASTRO and ESTRO, respectively) provided in 2009 a Consensus Statement for the use of accelerated PBI in adjuvant setting (46,47).

This evaluation of PBI in the situation of local relapse has been performed mainly through single institutional retrospective or prospective studies using various techniques such as brachytherapy (29,30,48,49), external 3D conformal RT (27), or intraoperative radiation therapy (IORT) (50). The promising results showed a 5-year second local relapse rate of 10% (27–29).

No Phase III randomized trial has yet evaluated the benefit of the second course of irradiation. Nevertheless, retrospective or prospective single institution studies report a lower rate of 5-year second local relapse (from 10% to 20%) (27–30).

Partial breast re-irradiation by brachytherapy

To re-irradiate, interstitial brachytherapy was the first and most commonly applied technique, using low (29,30,48,49,51), pulsed (30,52), or high dose rates (53). Vectors were implanted intra- or post-operatively, and the median delivered dose was about 46 Gy (ranging from 30 to 50 Gy).

Most of the published data come from single institution studies and are either retrospective (48,51,53) or prospective (29,54). The rate of 5-year second local relapse is about 10% (ranging from 0 to 26%) and the 5-year DFS rate about 60% (ranging from 31 to 85%). The rate of late complications is considered acceptable, with a Grade 3-4 complications rate around 8% (ranging from 3 to 11%). Fat necrosis is observed in about 20% of patients. Finally, cosmetic results are reported as good to excellent for 70% of women (ranging from 53% to 100%) but should be considered cautiously for retrospective studies.

Partial breast re-irradiation by external 3D-Conformal technique

The main publication about adjuvant external radiotherapy for local relapse is a single institution retrospective study published in 2002 by the University of Pittsburgh using an external electron beam for a total dose of 50 Gy with conventional 2 Gy daily fractionation (27). With a median follow-up of 52 months, they experienced 20% of in-breast relapse despite 15% of positive margins.

The American Society of Radiation Oncology (RTOG) has just conducted a Phase II trial (RTOG 1014) (55), investigating, as primary endpoint, the tolerance (i.e. Grade 3 side effects as breast pain and skin fibrosis within 1 year) of 3D conformal PBI. The clinical target volume was defined as the post-operative cavity on CT scanner with an additive 25 mm margin submitted to twice-daily fractions of 1.5 Gy for a total dose of 45 Gy. With a planned quality assurance program, the investigators expected a Grade 3 toxicity rate as low as the one observed in the RTOG 0319 trial (evaluating the PBI in adjuvant irradiation for primary surgery, i.e. 4%). This trial, conducted in a large number of centers across the United States, recruited the expected 61 patients, and has just closed in June 2013.

2- Intra-operative radiation therapy (IORT)

The IORT was developed to allow a dose escalation in tumors known as being highly radio-resistant or in deep local recurrences occurring in previously irradiated areas such as pelvic

cancers. This dose escalation was rendered possible by saving the surrounding healthy tissue during the surgery procedure and IORT (Electrons or 50kV photons) (56).

Then, IORT was delivered by linac-accelerators located in the surgical rooms requiring dedicated equipment but only developed in some specific centers such as Milan, Salzburg, Montpellier and Madrid in Europe.

Its use in breast cancer started in the early eighties but was, however, of limited use.

The recent development of different small and mobile accelerators (Mobetrons™, Eliottt™ for mobile electrons and IntraBeam™ for mobile 50 kV x-rays) partly explained the increased interest and the recent large development of this technique. Due to their different radiobiological behavior, a comparison between these energies is not approvable (57) or must be read cautiously (58). Intrabeam™ is indeed known for presenting a very low-dose penetration (56,59). At a depth of 1 cm, the estimated delivered dose is only 5 to 7 Gy, which is substantially lower than the electron technique (60). Nevertheless, biological equivalence is difficult to determine precisely (58) whereas physical and biological aspects have not been proved to have significant consequences on local control.

Thanks to the recent development of this technique, ten clinical trials are open worldwide to evaluate IORT for breast cancer in adjuvant indications, but none in local relapses.

2.1. Advantages of IORT:

IORT has the advantage of a huge normal tissue sparing (61,62) and an excellent tumor delineation under visual control. This advantage allows minimizing potential geographical misses related to post-operative modifications generated by a delayed external irradiation.

IORT is described to have very low acute and late toxicity (45,63–68). Low-kilovoltage irradiation gives low side effects thanks to the spherical shape of the applicator, which avoids mobilizing the mammary gland in the post-lumpectomy cavity.

The spherical applicator allows therefore to irradiate the entire cavity very simply, thereby providing a low rate of fat necrosis (67,68) and good cosmetic outcome (67,69)

Moreover, the costs of these single fraction treatments seem lower than any other technique used for adjuvant radiotherapy; the saving represented by this new alternative is currently evaluated in a medico-economic randomized prospective trial conducted in France and supported by the National Institute for Cancer, INCa (70).

A consistent advantage of the technique is its unique attractive aspect of a one-day treatment. With both surgery and irradiation delivered during a one shot-procedure, IORT is a very simple treatment with maximal compliance and high benefits for quality of life.

2.2. Results of IORT:

The IORT experience in breast cancer started first in Montpellier in 1989 (62,64,71) and evaluated the role of a 10-Gy boost (delivered with electrons) associated with an additional 50-Gy post-operative whole breast radiation therapy (WBRT) (63,64). In this setting, very recent and mature results have been published in 2013, in a large multi-institutional ISIORT pooled analysis (63) with a cohort of 1100 patients and median follow-up of 72 months. The authors showed that a boost using IORT allowed an impressive local control rate of 99.2%.

IORT has been, more recently, evaluated in PBI setting for selected patients with a 20-21 Gy single dose:

A huge international Phase III trial, TARGIT A (45), including 2232 patients (pre-pathology arm) compared a single 20-Gy dose – delivered at the surface of the excision cavity - to a standard adjuvant whole breast radiotherapy. With a 4-year median follow-up (reached for only 20% of patients) the trial demonstrated the non-inferiority of the PBI approach to the traditional adjuvant WBRT. Fourteen percent of patients with definitive unfavourable histo-pathological prognostic factors received after PBI an additional external WBRT. Mature results are needed to reinforce the safety of this interesting approach.

The same trial was conducted with electrons in a large Italian Phase III trial for which mature results are still awaited.

In Montpellier, we published the results of a Phase II trial in highly selected patients with good prognosis tumors (older than 65, with ER-positive status, T1N0 unifocal non-lobular carcinoma resected with clear margins). The final results demonstrated the feasibility of this technique with very low acute and late toxicity and very good cosmetic results (65,72).

An international trial - Targit E (73) – has recently opened to evaluate the feasibility and results of this IORT in the elderly.

2.3. IORT for local relapse:

The only published study evaluating IORT for local relapse is a retrospective single-institution study conducted during 54 months and including 17 patients treated by heterogeneous treatments (50). Conducted in Mannheim, the results were published with a follow-up of 26 months and described no local relapse. A prospective multi-institutional trial is not yet available and is proposed in the current project.

3-Ancillary studies

3.1. Intrinsic hypersensitivity: Proteomic assay

Individuals vary widely in the susceptibility of the tissue to ionizing radiation damage. Current knowledge of individual variation is fragmentary; there is a need to monitor such adverse events and to predict their occurrence.

In this setting, our laboratory has developed in recent years a predictive assay based on flow cytometric assessment of radiation-induced T-lymphocyte apoptosis (RILA). This assay showed that no severe late effect was observed in patients with a high RILA level, with an excellent negative predictive value (NPV=99%). On the contrary, all patients with a severe toxicity had a low value of RILA but the positive predictive value of the test was low (PPV=20%) (74,75).

Based on these results and in order to improve the positive predictive value of the radiation induced late effect assay, we developed a quantitative proteomic approach to identify predictive radiobiological markers in patients with severe toxicity. First, we selected four patients with a low RILA value from the prospective studies mentioned above. Two patients had no toxicity at least four years after the end of treatment whereas two others patients developed a severe toxicity greater than Grade 2. T-lymphocytes have been isolated from whole blood and half of them have been irradiated ex-vivo. We then performed a quantitative proteomics workflow using an 8-plex iTRAQ labeling and after several fractionations to optimize resolution of analysis (off gel fractionation followed by nanoliquid chromatography), proteins were identified by tandem mass spectrometry (4800 plus MALDI TOF/TOF). More than 1300 total proteins were identified with high confidence (95%, one unique peptide). Among them, 5 proteins involved in several mechanisms including metabolism and energy production, apoptosis, calcium binding protein, and DNA damage repair are overexpressed and seem to be related with hypersensitive patients to RT (ongoing patent application). Biological predictive factors are warranted to identify the individual risk of development of severe late toxicity. As RILA has a low PPV, we propose here to prospectively identify patients with an intrinsic radiosensitivity through a protein assay. For that, blood samples (14 ml heparinized whole blood) will be performed before RT for each patient and the expression of five proteins will be assessed. This test will be mandatory in the RE-IORT 01 trial to validate our preliminary results of protein expression level in patients presenting with radiation-induced late effects and particularly of the positive predictive value of this test.

3.2. Genomics assay

Advances in molecular techniques have improved discovery of biomarkers associated with radiation exposure. A challenge still resides in characterizing signalling pathways involved in

molecular and cellular responses to radiological interactions in various biological fluids and tissues under different types of irradiation conditions (76). One of our collaborators (UA) is developing the devices, biomarkers and bioassays for the accurate determination of an individual's absorbed dose of ionizing radiation. The team has identified molecules in the bloodstream that could gauge the risk to predict the radiation level across species, from mice, non-human primates and human TBI patients, and for dose ranging from 0.1 to over 10 Gy and long periods post-exposure. These sets of samples show accurate dose predictions within 10 cGy by using a 30-genes CLPA detection panel on whole blood specimen with volumes less than 30 µl.

The team proposes to investigate a new set of biomarkers present among the miRNA in the pleural effusion and implement a test using a new assay chemistry or chemical ligation dependent probe amplification (CLPA) that uses a multiplex ligation assay with PCR amplification, and on chip capillary electrophoresis readout (CE). The CLPA has the tremendous advantage of being able to perform directly without the need of extracting the RNA, which simplifies the workflow and reduces noises introduced. In addition, the CLPA is a very fast assay (< 2 hour) in comparison to other commercially available kits (e.g qNPA, PCR (> 2-3 days) and very sensitive. The CLPA method is easily adapted to a variety of high-throughput platforms suitable for medical countermeasures, including generic planar microarrays, liquid bead arrays (e.g. the Luminex Map) or capillary electrophoresis systems

miRNAs are post-transcriptional regulators that bind to complementary sequences in the three prime untranslated (3'UTRs) regions of target mRNA, usually resulting in gene silencing. Nearly all miRNAs are conserved in closely related species and many have homologs in distant species. Other advantage in using miRNAs is the fact that many miRNAs are tissue specific and stable. Discovered just over a decade ago, miRNA is now recognized as one of the major regulatory gene families in eukaryotic cells. Through the profiling, miRNA expression has been identified as differentiation biomarkers, as well as cancer biomarkers. Recently, plasma miRNA have been identified as biomarkers for radiation exposure dosimetry. Several studies showed that miRNAs play important roles in cell signalling pathways, physiological processes, and human pathologies; and is believed to regulate more than 50% of the human genome by translational repression or target degradation and gene silencing. A few laboratories have applied complex genomic micro-array techniques to investigate how miRNA expression profile may change in response to ionizing radiation. Most of these studies have examined changes in miRNA expression profiles in response to ionizing radiation in both murine and human cells and tissues. One group also examined miRNA in peripheral blood cells of radiotherapy patients which is so far the only characterization of miRNA expression signature in humans irradiated in vivo (77).

We propose to identify the miRNA present in the serum of the resected cavity of the patients and investigate how miRNA expression profiles may change in response to ionizing radiation in these samples. The technology platforms will provide new tools to evaluate radiation

toxicity during the course of therapy, based on an individual's biology, identifying people at risk for radiation illnesses.

Finally, we propose a prospective and multicentre phase II trial to evaluate a new alternative approach combining a second breast-conserving surgery and intraoperative radiotherapy (IORT), based on the hypothesis that re-irradiation to a limited volume will be effective with an acceptable occurrence of side effects including fibrosis.

4- Objectives

4.1. Primary objective

To evaluate the tolerance of IORT delivered during a second breast- conserving surgery

4.2. Secondary objectives:

To evaluate the effect of IORT on:

- Cosmetic results,
- Early Toxicities :
    - Hematoma
    - Lymphorrhoea (punctures> 2).
    - Breast infection
    - Erythema.

- Late Toxicities
    - Telangiectasia.
    - Breast Pain
    - Skin hyperpigmentation
    - Skin ulceration
    - Cutaneous atrophy
- Quality of life according to EORTC QLQ-C30 and BR23
- Survival including:
    - Local relapse-free survival
    - Metastasis relapse-free survival
    - Disease-free survival
    - Overall survival
    - Mastectomy-free interval,

4.3. Ancillary objectives:

To prospectively identify, through a protein assay (blood sample) the individual risk of patients to develop severe late toxicity.

To prospectively identify, through a rapid genomic assay (effusion samples) the impact of the dose of radiation administered to the patient's tissue by identifying the molecular processes involved in the individual's response.

The correlation between the proteomic data and the fibrosis rate will be also evaluated according to ancillary studies.

5- Patient selection

5.1. Inclusion criteria

- Histologically proven non-lobular invasive breast recurrence (IBR)
- Time from whole breast radiation following the initial lumpectomy >5 years
- Unifocal tumor
- Recurrent tumor size ≤ 2 cm
- Adequate breast size for a second breast-conserving surgery with acceptable cosmetic result
- Bilateral breast mammogram within 90 days prior to study entry
- Breast MRI within 90 days prior to study entry
- Histological grade I-II
- Estrogen-receptor-positive tumor (ER+)
- Cerb2-negative tumor
- N0 status (ultrasound of the axillary area is required, with fine needle aspiration cytology, in case of doubt)
- M0 status determined by PET-CT or both thoraco-abdominal CT and bone scan within 90 days prior to study entry
- Prior radiotherapy delivered within a standard fractionation schedule
- Performance status (ECOG) 0-1
- Women ≥ 50 years
- Absence of any psychological, familial, sociological, or geographical conditions with a potential to hamper compliance with the study and follow-up schedule
- Affiliated to the French Health Insurance regimen
- Written and signed informed consent form.

5.2. Exclusion criteria

- Disease previous non-invasive breast carcinoma (in situ)
- Disease previous contralateral breast cancer
- Multifocal and/or multicenter recurrence
- Lobular carcinoma
- Estrogen-receptor-negative tumor (ER-)
- Cerb2 (her2) overexpressed - breast cancer
- Extensive intraductal component (EIC) on biopsy
- Lymph vessel invasion on biopsy
- N1-3 status: Regional cytological or histologically proven node recurrence
- M1 status: Metastatic disease
- cT4 (Skin or muscle involvement) or Paget's disease of the nipple
- Prior radiotherapy delivered within an accelerated or hypo-fraction schedule
- Prior malignancy other than non-melanoma skin cancer unless the patient has been disease free for at least 5 years
- Patients with a small breast volume, technically unsatisfactory for a second conservative surgery or intraoperative breast irradiation.
- Preoperative chemotherapy or hormone therapy for local relapse
- Connective tissue disease or scleroderma, contraindicating radiotherapy
- Age < 50 years
- Known BRCA1/2 gene mutation (genetic testing is not required)
- Failure to comply with medical monitoring test for geographical, social or psychological reasons

6- Methodology and study design

We propose a prospective, multicenter, single arm Phase II design to evaluate the feasibility of repeated breast-conserving surgery combined with re-irradiation using IORT after local recurrence of breast carcinoma.

The study is planned to enroll 65 patients.

The patient must provide a signed informed consent form prior to any study related procedure. The principal measured outcome will be the cutaneous fibrosis rate 12 months after treatment. We considered that a rate of 20% or more of Grade ≥ 2 fibrosis would be unacceptable.

All patients will be followed for:

- Survival and local breast control after this study treatment for an extended carcinologic follow-up.

- Toxicities during and after treatment (3, 6, 12 and 24 months after IORT and each year during 3 years)

- Survival follow-up will be done every 3, 6, 12 and 24 months after IORT and each year during 5 years by clinical visits unless death lost to follow-up or withdrawal of consent.

The survival follow-up period will continue for 3 years after the last patient was included in the study.

The end of study (last patient last visit) will occur after all patients have completed their last assessment.

Temporary interruption and definitive termination of the study

The study could be suspended or stopped by the sponsor after meeting with the principal investigator or under the request of the competent authority and/or the Committee for the Protection of Patients (CPP) for a high fibrosis rate

7-Treatment

7.1. Second breast surgery

The surgery is performed following a classic or ambulatory hospitalization scheme, according to the equipment and habits of the team.

7.1.1.Axillary management

For the patients with previous axillary surgery, there is no consensus about the management of the axilla. If the initial surgery has disrupted the lymphatic vessels draining, the lymphatic mapping with sentinel node is considered to be unsuitable.

For the node negative patients assessed by clinical and ultrasound examination, the surgical procedure will be adapted to the initial management:

- No axillary surgery will be performed after a previous standard axillary lymph node dissection
- Lymph node dissection can be discussed after an initial mapping with lymph node procedure.

Axillary node involvement, when preoperatively or intra-operatively proved, is a total contraindication for entering this trial.

7.1.2. Lumpectomy

Lumpectomy is performed with an incision, left to the surgeon's choice for a broad glandular resection, from deep dermis to face pectoral. Orientation of the specimen is then required.

The intraoperative evaluation of the margins will be realized according to the habits of the team, to ensure margins are free from tumor (through pathological frozen section when achievable). A safe margin is required to ensure the complete resection of the tumor.

Safe margins are defined as « no ink on tumors », as recommended on the New 2014 Guidelines on the Society of Surgical Oncology and the American Society for Radiation Oncology (38).

Then, the cavity is prepared to receive the INTRABEAM ™system, to make the gland stick around the collimator and the size of the Intrabeam™ radiation probe applicator will be chosen to fill the tumorectomy cavity.

7.2. Radiotherapy by IORT for re-irradiation

7.2.1.Equipment and physics

The IntraBeam™ device is a mobile light machine (1.6 kg) with a miniature system providing a source of 50-kV x-rays at the tip of a 3 mm diameter tube placed at the center of a spherical applicator. Accelerated electrons, aimed on a gold target, produce a spherical radiation field, with an isotropic dose-distribution around the tip of the probe.

These low energy X-rays are characterized by a sharper dose fall-off to the tissue depth than electrons or high-energy-photons. Spherical applicators are fixed at the end of the source and range in size from 2.5 to 5cm.

The X-ray source is combined with a small control consol. The operating room requires a simple shielding and, according to our national recommendations from the Authority for Nuclear Safety (ASN), a mandatory visit for official approval before any treatment. For radiation protection reasons, IORT is realized without any medical staff in the operative room.

Before each treatment, a medical physicist must control and validate the calibration of the processor (with PAICH: Probe Adjuster Ionization Chamber Holder and PDA: PhotoDiode Array), check the geometric alignment, the alignment of the electron beam in the "needle", the isotropy of the X-ray source, and the dose rate.

During treatment, the physicist programs the machine and waits for the end of treatment.

The nine French teams, involved in the National Cancer Institute network (INCa) and associated to this study, have obtained the required approvals and the experience of this specific technique.

The IORT treatment requires an optimal collaboration between the surgeon, the radiation oncologist and the medical physicist.

7.2.2. Modalities of IORT:

Prior to the insertion of the adequate applicator in the surgical cavity, the surgeon cautiously checks the hemostasis, the perfect vicinity (absence of blood or serum) of the excision cavity. The presence of blood or serum would seriously reduce the dose received by the surrounding mammary gland in this situation of important steep dose fall-off.

The diameter of the applicator, varying from 25 to 50 mm, is chosen under visual control, by the surgeon and the radiation oncologist, in order to be perfectly adapted to the size of the cavity left by the removal of the tumor. Thanks to its spherical shape, the applicator is easily covered by the gland and does not require extensive tissue mobilization, which is a source of late fat necrosis.

The cavity tissue will then be approximated around the spherical applicator with one or two purses string sutures with a harmonious thickness of mammary gland

The suture is then tightened. Sterile gauzes or two Beckmann orthostatic spacers are added to obtain adequate skin spacing between the applicator and the skin, defined as ≥ 1 cm, to avoid skin toxicity.

The course of the irradiation is monitored by the radiation oncologist and the medical physicist. A unique dose of 20 Gy is prescribed at the applicator surface and the time of irradiation depends on its diameter with a median of about 30 minutes (ranging from 18 to 40 minutes). The exact length of the radiation time is determined by the applicator size (ranging from 20 to 40 minutes) and the additional time for the entire IORT procedure will be around 40 to 60 minutes. Then, the surgeon removes the applicator. Surgical clips are placed in the tumor bed to facilitate further imaging follow-up. The cavity is closed and remodelled. A post-operative drainage could be necessary.

7.3. Additional treatments

Each case will be discussed during a multidisciplinary committee.

7.3.1.Local treatment

In case of involved margins on the definitive pathology report, a new surgery is required. New conservative surgery or mastectomy will be proposed according to histological and clinical presentation.

No additional external irradiation will be delivered.

7.3.2. Systemic treatment

An adjuvant hormone treatment will be prescribed, based on tamoxifen or an aromatase inhibitor in postmenopausal women, excepted for women with contraindications.

Adjuvant chemotherapy has to be discussed in a dedicated multidisciplinary breast committee.

7.3.3. Further treatments:

In case of second local relapse, further management of the patient must be decided in a multidisciplinary breast committee.

8- Clinical evaluation and follow-up procedures

8.1. Inclusion

The following procedures and assessments will be performed prior to the IORT:

- The patient must provide a signed informed consent form prior to any study related procedures
- Eligibility criteria
- Demographics
- Physical examination: body weight, height, body mass index, clinical stage
- WHO performance status

- **Diagnosis of isolated local recurrence:**

  All patients with breast cancer recurrence receive standardized staging:

  - Clinical Examination (palpation of the breast and axillary area)
  - Mammography and ultrasonography of the breast (tumor size and topography).
  - Breast magnetic resonance imaging to evaluate the size and unifocality of the recurrence.
  - **Biopsy**: Histological verification is mandatory, reporting pathology grade, ER, PR, HER 2 status, and Ki 67.
  - **Axillary area** assessment must be evaluated by physical examination and ultrasound, with a fine needle aspiration cytology in case of doubt (78,79).
  - A PET-CT or a thoraco-abdominal CT and bone scan will be performed to detect any metastatic disease prior to study entry.

- Complete medical history, symptoms, and ongoing medications
- Menopausal status
- Quality of life questionnaires EORTC QLQ-C30/BR23
- Photography of the breast
- Blood sampling for Proteomic assay

8.2. IORT

- Effusion tumour bed sampling for Genomic assay (optional)

8.3. 15 days after IORT

- Physical examination: body weight, local and systemic related IORT toxicities
- WHO performance status
- Clinical Examination (palpation of the breast and axillary area)
- Clinical Breast skin cosmetic evaluation
- Photography (cosmetic evaluation)
- Quality of life questionnaires EORTC QLQ-C30/BR23

8.4. Follow up period (3, 6, 12, 24, 36, 48, 60 months)

- Physical examination: body weight, local and systemic related IORT toxicities
- WHO performance status
- Clinical Examination (palpation of the breast and axillary area)
- Mammography and ultrasonography of the breast at 12 months only 6 months and 1 fs/year
- Breast magnetic resonance imaging at 12 months (optional)
- Clinical cosmetic evaluation
- Photography (cosmetic evaluation) (until 60 months
- Quality of life questionnaires EORTC QLQ-C30/BR23 (until 24 months)

9- Primary endpoint

The fibrosis grade will be scored following the NCI CTCAE scale v4.0 at 12 months after the IORT (73).

10- Secondary endpoints

Definitive cosmetic results:

- assessed by a physician

- Pictures will be taken before treatment, after 15 days, 3, 6, 12 and 24 1fs/year until.

Toxicities (excluding fibrosis):

- assessed by a physician
- Grade will be scored following the NCI CTCAE scale v4.0 at 15 days, 3, 6, 12 and 24, 36, 48 and 60 months after the IORT.

Quality of life:

- assessed by a patient
- EORTC QLQ-C30, QLQ-BR23 questionnaires delivered before treatment, and after 15 days, three, six and twelve and twenty-four months.

Local relapse-free survival (L-RFS):

- L-RFS is defined as the time from surgery with IORT to any ipsilateral breast tumor recurrence, or death, whichever occurs first.

Metastasis Relapse-free survival (M-RFS):

- M-RFS is defined as the time from the surgery with IORT to any metastatic disease (excluding the ispilateral breast, contralateral breast, regional lymph nodes), or death, whichever occurs first.

Disease-free survival (DFS):

- DFS is defined as the time from the surgery with IORT to any recurrence, including local recurrence, metastasis, appearance of a second primary tumor, or death from any cause, whichever occurs first.

Overall survival (OS):

- OS is defined as the time from surgery with IORT to death from any cause.

Mastectomy-free interval:

- MFI is defined as the time from the surgery with IORT to a mastectomy for any reasons (all other events will be ignored). All patients without mastectomy will be censored at last follow-up.

11- Toxicity and adverse events management

Definition

An Adverse Event (AE) is defined as "Any untoward medical occurrence in a patient or clinical investigation subject administered an investigational product or therapy and which does not necessarily have a causal relationship with this treatment/therapy". An AE can therefore be any unfavorable and unintended sign (for example: an abnormal laboratory

finding), symptom, disease, or worsening of a pre-existing medical condition temporally associated with the use of an investigational product/therapy, whether or not considered related to the investigational product/therapy.

An AE related to the investigational product or therapy is also called an adverse reaction or side effect.

Collection and Reporting

Every AE occurring during the clinical trial should be recorded on the corresponding page of the Case Report Form. Every AE should be documented, monitored and followed until the AE is recovered or until 12 months (3 years for radiation-induced fibrosis) after the intra-operative radiotherapy.

Severity Criteria

The severity criteria should not be mistaken with the seriousness criteria which determine the conditions of notification. The severity or grade of adverse events is evaluated by the Investigator following the NCI-CTCAE classification version 4.0.

The CTCAE displays Grades 1 through 5 with unique clinical descriptions of severity for each AE based on this general guideline:

Grade 1 = Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.

Grade 2 = Moderate; minimal, local or noninvasive intervention indicated; limiting age-appropriate instrumental ADL (Any Day Life)*.

Grade 3 = Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self-care ADL**.

Grade 4 = Life-threatening consequences; urgent intervention indicated.

Grade 5 = Death related to AE.

*Instrumental ADL refer to preparing meals, shopping for groceries or clothes, using the telephone, managing money, etc.

**Self-care ADL refer to bathing, dressing and undressing, feeding self, using the toilet, taking medications, and not bedridden.

Side effects of Special Interest: Radiation-induced Fibrosis

All cases of radiation-induced toxicities should be monitored closely. A radiation-induced side effect of special interest is radiation-induced fibrosis.

Acute toxicities could be observed during and after IORT:

- Hematoma
- Lymphorrhoea (punctures> 2).
- Breast infection
- Erythema.

Delayed toxicities after IORT (> 6 months):

- Fibrosis
- Telangiectasia.
- Breast Pain
- Hyperpigmentation
- Cutaneous atrophy.

The cases of radiation-induced toxicities will be recorded in the source documents and on the Case Report Form at 15 days, 3, 6, 12, 24 months then yearly during 3 years after the IORT. They will be evaluated according to the NCI-CTCAE classification version 4.0.

Serious Adverse Events

A Serious Adverse Event (SAE) is an adverse event which:

- results in death
- is life-threatening
- requires in-patient hospitalization (>24h) or prolongation of existing hospitalization,
- results in persistent or significant disability or incapacity
- is a congenital anomaly/birth defect, or
- medically relevant

Life-threatening in this context refers to an event in which the patient was at risk of death at the time of the event; it does not refer to a reaction that hypothetically might have caused death if more severe.

A hospitalization scheduled by the protocol (biopsy, chemotherapy...) is not considered a SAE. A hospitalization or prolongation of hospitalization for technical, practical, or social reasons, in absence of an AE is not considered a SAE. A hospitalization planned prior to patient enrolment is not considered a SAE, provided that his occurrence/outcome is clearly not aggravated by the investigational product or therapy. A hospitalization < 24 hours is not considered a SAE, provided that the AE does not meet any other seriousness criteria.

The terms "disability" and "incapacity" match with all physical/psychological temporary or permanent handicaps, clinically significant with consequences for the physical or mental functioning and/or the quality of life of the patient.

Medical and scientific judgment should be exercised in deciding whether other situations should be considered serious events, such as important medical events that might not be immediately life-threatening or result in death or hospitalization but might put at risk the patient or might require intervention to prevent one of the other outcomes listed above. Such events are considered serious with seriousness criterion "medically relevant". Examples of such events are allergic bronchospasm, torsade de pointes or convulsions.

The EudraVigilance Expert Working Group (EV-EWG) has co-ordinated the development of an Important Medical Event Terms (IME) list, which could help Investigators to determine whether an AE is serious or not:

http://eudravigilance.ema.europa.eu/human/textforIME.asp

The IME list is intended for guidance purposes only, and is not a mandatory requirement for seriousness assessment and regulatory reporting. Other examples of serious adverse events with seriousness criterion "medically relevant" are second primary malignancies and any suspected transmission via a medicinal product of an infectious agent. For every SAE the Investigator and the Sponsor evaluate separately the possible causal relationship to the investigational product or therapy. These evaluations might be different one from the other (for example: in the Investigator's opinion the SAE is not related to the investigational product/therapy and in the Sponsor's opinion the SAE is related to the investigational product/therapy).

SAE Notification Procedure

Every SAE occurring during the study period (from the signature of the informed consent form until up to 12 months after the intra-operative radiotherapy) should be notified to the Sponsor without any further delay, and within 24 working hours in all cases, using the "Serious Adverse Event Notification Form". This form should be completed following the completion instructions and be faxed to the Clinical Research Pharmacovigilance Unit of the ICM.

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Every SAE occurring beyond the 12-month period following intra-operative radiotherapy, judged by the Investigator to be related to this radiotherapy, should also be notified to the Sponsor in the same conditions as every other SAE. The "Serious Adverse Event Notification Form" should be completed in English and only one diagnosis or one symptom (except for linked symptoms) should be reported to enable MedDRA coding. If several symptoms are documented in the source documents, only the main symptom will be reported as verbatim on the notification form.

After the initial notification, a follow-up report should be completed and faxed every time complementary information on the SAE becomes available. Finally, when the case is closed, a final report with the complete information should be completed and faxed to the Pharmacovigilance Unit (Fax: +33 4.67.61.31.04). Complementary information or clarification could be requested by the Sponsor using Data Clarification Forms (DCFs). The Sponsor could also ask the site to send the anonymized medical records or laboratory findings corresponding to the SAE.

The risk-benefit balance of the study is evaluated continuously by the Clinical Research Pharmacovigilance Unit of the ICM and this risk-benefit balance will be discussed in the periodic safety reports. These reports will contain all required regulatory aspects and will be submitted to the competent authorities.

Trial Discontinuation

- Individual withdrawal :
- Withdrawal of informed consent
- Insufficiency skin-surface or unfavourable geometry
- Each withdrawal must be documented
- Investigator withdrawal

Scientific/Steering committee is allowed to close the study ahead of schedule (e.g. Insufficient recruitment, unexpected high toxicity, Ethical and medical considerations…).

12- Patient screening, inclusion and registration procedures

The assessments required as part of screening can be completed in one or more visits. Signed informed consent prior to any study related procedure is required. Enrolment in the study is defined as both the signing of the Informed Consent and validation of eligibility by the investigator. The inclusion and exclusion criteria should be checked during the screening period to ensure appropriate subjects are assigned to receive study treatment.

Registration is performed at the Biostatistics Unit of ICM (Montpellier, France). Investigators can use a dedicated registration form inserted into the CRF and fax the filled and signed form to:

Biostatistics Unit - ICM
From Monday to Friday (9.00 AM to 5.00 PM)
Fax: +33 4 67 61 37 18
Phone: +33 4 67 61 45 48 / 45 40

The investigator will automatically receive a notification form by fax with the patient's identification number to be reported on each CRF page.

13- Independent Data Monitoring Committee (IDMC)

The IDMC has an advisory role and provides his opinion concerning all safety issues related to the study or the study treatment to the Sponsor and the Coordinating/Principal Investigator of the study, and to the competent authorities (CPP and ANSM). If applicable, they can also provide their opinion about the efficacy data of the study. The IDMC can advise the Sponsor to continue, modify or stop the study. The Committee Members must be experts in clinical research and/or the study indication. They should not be implicated in the study protocol (no Principal or Co-Investigators) and are not allowed to have financial interests in the study. The Committee Members should sign a Financial Disclosure Form and a Confidentiality Agreement before attending the IDMC.

In this study, the IDMC will be constituted by 3 experts: a radiotherapist, a surgeon and a methodologist. The Committee Members will meet at the scheduled time point corresponding at the 12 months follow-up of the first 25 included patients.

The IDMC will meet to review safety of the therapeutic strategy, in term of toxicity and oncologic events (local and systemic), and other issues related to the appropriate conduct of the trial.

The IDMC will be regularly informed of the occurrence of death from cancer according to the a priori early stopping rule.

The data will be provided to the IDMC members by the Sponsors Biostatistics Unit, and / or the Clinical Research Pharmacovigilance Unit, and / or the Study Coordinator. The Sponsor can ask the IDMC for an ad hoc advice beyond the scheduled time points.

The ICM's Independent Data Monitoring Committee Charter is provided in Appendix 15.

14- Statistical considerations

14.1. Sample size calculation

The primary objective is to evaluate the success rate at 12 months, defined as the non-observation of fibrosis at 12 months after IORT.

A success rate of 95% is expected. The sample size calculation is based on:

• The Fleming one-stage design with one-sided alpha=0.05 and beta=0.05.

• p0= 80% (success rate at 12 months, threshold below which we will consider the Intraoperative Radiotherapy toxicity as unacceptable, assuming 20% failure)

• p1= 95% (success rate target at 12 months)

A total of 46 evaluable patients are required; taking into account non-evaluable patients (postoperative histological results that do not meet the inclusion criteria or less than 3 assessments before 12 months), the number of subjects is adjusted to 65.

It should be noted that the alpha level is set at 5% (one-sided), risk of concluding as to efficacy ($p > p0$) whereas there is no efficacy ($p \leq p0$), and the beta level is set at 5%, risk of concluding as to the absence of efficacy ($p < p1$) whereas there is noteworthy efficacy ($p \geq p1$).

After inclusion of 46 evaluable patients, it will be possible to conclude that the study arm demonstrates inefficacy if the success number is less than or equal to 40, or efficacy if the success number is greater than or equal to 41.

The sample size and the decision rule are presented here-below:

Hyp.	Rate of success at 12 months	p0	p1	Fleming one-stage design *	N total
Rate		80	95	(46, ≤40, ≥41	65

* (N, n1, n2)

N = total sample size required.

n1 = maximal number of success required to conclude => non-efficacy.

n2 = minimal number of success required to conclude => efficacy.

14.2. Statistical early stopping rule

The rate of deaths from cancer will be checked and an early stopping rule is defined on this criteria according to a sequential method (A Kramar, C Bascoul-Mollevi, 2009), used to recommend the stop of inclusions if the rate of deaths from cancer is significantly over 5% after 12 months of following, with a nominal alpha at 10% and gamma parameter at 4 for the inclusion of the 51 planned patients.

These statistical early stopping rule will be applied from the second cancer death until the 5th.

Simulations are summarized in the following table:

The maximum acceptable percentage of death from cancer at 1 year	Number of death from cancer leading to inclusion stop	Number of inclusions
4%	2	9
	3	20
	4	30
	5	39
5%	2	6
	3	16
	4	25
	5	33
6%	2	5
	3	13
	4	21
	5	29

The maximum acceptable percentage of deaths from cancer at 12 months was fixed at 5% according to literature data.

The interruption of inclusions is recommended if the second death from cancer occurred among the first 6 patients, the 3rd among the first 16 patients, the 4th among the first 25 patients and the 5th among the first 33 patients. Beyond the second death from cancer, regardless of the number of patients included a meeting of the Supervisory Committee of the trial will be scheduled to discuss how to continue this trial.

14.3. Statistical analysis

Statistical analyses will be detailed in the statistical analysis plan developed before the database is frozen.

Baseline characteristics, compliance to treatment, safety, and carcinologic events will be described.

Qualitative data will be described by frequencies and percentages and associated 95% confidence interval.

Continuous parameters will be described by mean, standard deviation, median and range.

The primary endpoint will be described by the percentage of patients without fibrosis at 12 months and its 95% confidence interval.

For secondary endpoints, EORTC QLQ-C30 and QLQ-BR23 will be described according to EORTC scoring manual at baseline and for each visit.

Time to event endpoints (L-RFS, M-RFS, DFS, OS and mastectomy-free interval) will be estimated using Kaplan-Meier method and described by their rates and 95% confidence intervals at 12 months, 24 months, and 3 years.

Statistical analyses will be performed with STATA software v13 (Stata Corporation, College Station, TX, USA).

14.4. Study population

*Intention to treat population (ITT) = all included patients analyzed, non-evaluable patients will be considered as failure.

*Per-Protocol population (PP) = all eligible (patients presenting a major deviation from the inclusion/exclusion criteria will not be selected) and evaluable patients.

*Evaluable population: all patients included, with postoperative histological results that meet the inclusion criteria and at least 3 assessments before 12 months.

*Safety population: all patients included in the ITT population, operated and treated with Intraoperative Radiotherapy.

All statistical analyses will be performed on ITT population, on evaluable population and PP population for primary endpoint and on Safety population for safety.

14.5. Modifications of the statistical analysis plan and initial strategy

Any modification to the initial statistical analysis plan (PAS) will be detailed with all the necessary arguments reported in an updated version of PAS. These modifications can include supplementary or exploratory analyses that were not initially planned.

15- Proteomics and IORT late-side effects (Mandatory)

15.1. Sample collection:

21 ml EDTA whole blood will be collected before starting radiotherapy.

15.2. Lymphocytes separation:

Immediately, lymphocytes will be isolated with ficoll density gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare) following manufactory recommendations. This protocol will allow recovering 7.5 to 15 million cells per patient. Purified lymphocytes will be cultured in two dishes containing RPMI 1640 medium (Gibco BRL Invitrogen) supplemented with 20% FCS during 24h. For each patient, one cell culture dish will be irradiated at 8 Gy and incubated for 48 hours, and one cell culture will be shamed irradiated and considered as control (0 Gy).

15.3. Western blot analyses:

Lymphocytes proteins will be extracted by RIPA buffer. Cell lysates will be quantitated using the BCA protein assay kit (ThermoFisherScientific, Rockford, IL) according to the manufacturer's protocol. Ten micrograms of proteins will be then loaded and separated on 12% SDS-PAGE and then transferred to a PVDF membrane. Nonspecific binding to the membrane is blocked for 1 hour at room temperature with 5% nonfat milk. Membranes will be incubated overnight at 4°C with the primary antibodies diluted as follows: AK2 (1/200, sc-28786; Santa Cruz Biotechnology, Inc., Santa Cruz CA), Annexin-1 (1/200, sc-11387; Santa Cruz Biotechnology, Inc., Santa Cruz CA), HSC70 (1/500, sc-7298; Santa Cruz Biotechnology, Inc., Santa Cruz CA), IDH2 (1/100, sc-134923; Santa Cruz Biotechnology, Inc., Santa Cruz CA) and Ref-1 (1/300, sc-5572; Santa Cruz Biotechnology, Inc., Santa Cruz CA). Membranes will be then incubated with secondary antibody (goat anti-rabbit IgG (H+L), G21234; Invitrogen for AK2, Annexin-1, IDH2, Ref-1 and goat anti-mouse IgG (H+L), 115-035-146; Jackson ImmunoResearch for HSC70) for 1 hour at room temperature. The immunoblots will be developed using the enhanced chemiluminescence detection system with the use of a SuperSignal West Pico Chemiluminescent Substrate kit (Pierce). Image analyses will be performed using ImageJ software (National Institutes of Health, Bethesda, MD).

15.4. Development of an ELISA assay for the five candidate proteins:

In order to propose a reliable, rapid and easy to used assay, an ELISA strategy will be developed. Two antibodies will be produced for each protein by Abnova against antigenic peptides (5 months). The company provides the antibodies already tested for ELISA. A sandwich-ELISA test will be established in a 96-well format, using the antigen used for antibody production. The latter will also serve as quantification standard. For each protein, one antibody serves to capture the target and will be used to coat the wells. The other antibody will be linked to biotin with the EZ-Link Sulfo-NHS-Biotinylation Kit from Pierce. Streptavidin-HRP together with an appropriate substrate buffer will be used for detection. The whole procedure should be achieved within one year. Concentration of the five candidate proteins will be measured in the cell extracts obtained above with this test.

16- GENOMIC and IORT (Optional)

The miRNA expression profiles are altered in a specific and radiation dose-dependent manner (an mRNA radiation exposure "signature") that provides a sensitive and effective, biodosimetry test.

16.1. Serum collection from resected cavity (Investigation centers, France):

In all investigation centers involved in the genomic assay, 5 ml of operative cavity serum will be collected from each patient before IORT and 5 ml after the IORT (one single dose of 21 Gy IORT). Five volumes of RNAprotect Cell Reagent (Qiagen) will be mixed with one volume of serum and immediately stored at -80C until shipping to the ICM. Samples will be regrouped at the ICM and send to the University of Arizona.

16.2. miRNA isolation (Phoenix, AZ, US):

After shipping the samples will be stored at -80°C until processing where miRNA will be isolated from the cells using the TaqMan miRNA ABC purification kit (Life Technologies) according to the manufacturer's instruction.

16.3. qRT-PCR and analysis (Phoenix, AZ, US):

Purified miRNA will be converted to cDNA and analyzed by quantitative PCR using the TaqMan array human MicroRNA (LifeTechnologies) according to the manufacturer's instruction to screen the miRNA for profile changes in response to irradiation.

16.4. CLPA analysis (Phoenix, AZ, US):

Once the miRNA signature will be optimized with the qRT-PCR, we will implement the CLPA assay using a customized panel with the selected miRNA from the qRT-PCR to follow the changes in expression to validate the assay chemistry. Future integration into a microfluidic platform will be explored for implementation in a rapid biodosimetry point of care system.

17- Study monitoring and data collection

17.1. Data collection

Data collected in this trial will be sent to the Coordinating Center in Montpellier for primary evaluation and follow-up.

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ICM (Institut régional du Cancer Montpellier)
Unité de Recherche Clinique, Bât. A
208, rue des Apothicaires, 34298 Montpellier

17.2. Study monitoring

A Clinical Research Associate (CRA) will be appointed by the Sponsor to monitor this study.

CRA activities include: Site initiation visit to collect and distribute essential pre-study documents; to instruct the investigator and site personnel about the protocol, study procedures and expectations; to obtain investigator's assurance to comply with study requirements and GCP guidelines and to inform the investigator and appropriate study staff about study materials.

Monitoring visits : according to the Good Clinical Practice Guidelines, the study CRAs involved in the present study are fully instructed concerning confidentiality and able to perform any necessary control on informed consent and CRFs, including cross-checking clinical and laboratory data with the patient's file. All observations and findings should be verifiable. During monitoring visits, the Sponsor CRAs will:

- check and assess the progress of the study;

- review study data collected;
- conduct Source Document Verification (hospital files);
- identify any issues and address its resolution;

This will be done in order to verify that the:

- Data are authentic, accurate, and complete.
- Safety and rights of subjects are being protected;
- Study is conducted in accordance with the currently approved protocol (and any amendments), GCP and all applicable regulatory requirements.

The investigator agrees to allow the CRA direct access to all relevant documents and to allocate his/her time and the time of his/her staff to the CRA to discuss findings and any relevant issues.

17.3. Case Report Form (CRF)

Study data will be recorded on the case report form (CRF) by the person designated by the investigator. A CRF is required and should be completed for each included subject. It is the investigator's responsibility to ensure the integrity of information transcribed on the CRF. CRFs must be signed by the investigator or by an authorized staff member. These signatures serve to attest that the information contained on the CRFs is true. The investigator agrees to keep records, including the identity of all participating subjects, all original signed informed consent forms, copies of all CRFs, serious adverse event forms, source documents, and detailed records of treatment disposition.

All data entered into the CRF should have source documentation available at the investigational site.

The Biostatistic Unit of ICM will enter into a database in an ongoing basis. Systems with procedures that assure the quality of every aspect of study data management will be implemented, using Clinsight® software v6.2.

The collection and management of data is carried out by the Biostatistic Unit of ICM. The conditions for data transfer of all or part of the study database are decided by the study sponsor and are the subject of a written contract.

18- Ethical and regulatory considerations

18.1. Responsibilities of investigators

The protocol and the proposed informed consent form must be reviewed and approved by the "Agence nationale de sécurité du médicament et des produits de santé (ANSM)" and the Comité de Protection des Personnes (CPP) before study start. A signed and dated statement that the protocol and informed consent have been approved by the ANSM and the CPP must be given to the sponsor before study initiation. Prior to study start, the investigator is required to sign a protocol signature page confirming his/her agreement to conduct the study in accordance with these documents and all of the instructions and procedures found in this protocol and to give access to all relevant data and records to the sponsor's monitors, and other representatives, and regulatory authorities as required.

18.2. Patient information and consent

Informed consent for each patient will be obtained prior to initiating any trial procedures in accordance with the statement entitled "Requirements for Informed Consent". A copy of the informed consent must be given to each patient and the signed original copy must be

retained in the investigator's trial records. The informed consent form must be available in case of data audits.

The "Declaration of Helsinki" (see Appendix I) recommends that consent should be obtained from each potential patient in biomedical research trials after the aims, methods, anticipated benefits, and potential hazards of the trial, and discomfort it may entail, are explained to the individual by the physician. The potential patient should also be informed of his or her right to not participate or to withdraw from the trial at any time. If applicable, the patient should be told that material from her/his tumor will be stored and potentially used for additional studies not described in this protocol.

If the patient is in a dependent relationship to the physician or gives consent under duress, the informed consent should be obtained by an independent physician. By signing this protocol, the investigator agrees to conduct the trial in accordance with the "Declaration of Helsinki".

18.3. Quality Assurance

Prior to the enrollment of any patient at a site, the investigator will review the protocol, the procedure for obtaining informed consent, and procedures for reporting adverse events.

The investigator is required to retain patient identification codes for a minimum of 15 years after completion or discontinuation of the trial. The investigator is required to retain all patient files and source documents for the maximum period of time permitted by the hospital, institution, or private practice, but for not less than 10 years in order to meet international registration requirements.

18.4. Confidentiality of information

The present materials (protocol, CRF) contain confidential information. Except as may otherwise be agreed to in writing with the study Sponsor, the investigator agrees to hold such information in confidence, and not to disclose it to others (except where required by applicable laws or regulations). All information from this study (excluding data from informed consent) will be entered into a computer by the sponsor in accordance with the French law, "Loi Informatique et Libertés" (art. 40, January 6, 1978) and with the European Directive 95/46/CE.

19- Dissemination policy

The data generated from this study are the property of the Sponsor and shall be kept in strict confidence along with all information provided by and to the Sponsor. Independent analysis

and/or publication of these data by the investigator(s) or any member of their staff are not permitted without prior written consent of the Sponsor.

19.1. General authorship rules

There should be a maximum of 12 authors unless stated otherwise in editor guidelines.

All authors must comply with the following ICMJE guidelines for authorship and all persons who qualify for authorship should be listed (81).

- Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
- Drafting the work or revising it critically for important intellectual content; AND
- Final approval of the version to be published; AND
- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

19.2. Specific authorship rules

The following additional authorship rules apply to any report of the major outcomes of the study in original articles or conference paper/abstracts.

Investigators who do not qualify for authorship should be listed in the *Investigator list* along with all investigators. Other collaborators may be listed in the *Acknowledgments* section.

Author position	
1	Study coordinator *(or investigator A)***
2	Investigator A* *(or B)***
3	Methodologist
4-5 *(or 4)***	Investigators C-D* *(or D)***
6+ *(or 5+)***	Thematic co-coordinators or experts (e.g. pharmacokinetics, genetics, pathology, etc.)
Second last	Investigator B* *(or C)***
Last	Expert** (*or study coordinator)*

* Each investigational center will designate one investigator. Investigators will be ranked from position A to D according to the number of patients included in their respective center in an increasing order (i.e. A = best inclusion number). If the study coordinator is among the five first investigators, he or she will keep the first position. Therefore, a maximum of five investigators may be listed as authors.

** The expert should not be an investigator. If no appropriate expert contributed to the study, the study coordinator may be last author of the publication, thereby up-ranking the position of the investigators A-D as indicated under brackets.

19.3. Publication quality, acknowledgment and conflict of interest

When preparing the manuscript, authors are encouraged to follow the guidelines of the EQUATOR network available at http://www.equator-network.org/.

All publications (when compatible with the editor guidelines, and, at least, in original articles) should acknowledge the contribution of the clinical research associate(s), the pharmacovigilance expert, the data manager(s) or data center and the medical writer (if applicable).

Funding sources should also be detailed in the *Acknowledgments* section when a separate funding section is not required by the editor.

As stated in the ICMJE guidelines, all acknowledged persons must give written permission to be acknowledged. If a specific acknowledgment consent form is not provided by the editor, a standardized form is available in Appendix 11.

All authors must also disclose any financial interest or personal relationship that may bias or be seen to bias their work. If the journal editor does not provide an appropriate conflict of interest disclosure form, a standardized and up-to-date form may be found on the ICMJE website (81).

20- References

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21- Appendices

Appendix 1: Fiche d'inclusion

Appendix 2: Note information patient

Appendix 3: Consentement eclairé

Appendix 4: ECOG performance status

Appendix 5: Procédure de Prélèvement Protéomique

Appendix 6: Procédure de Prélèvement Génomique Appendix

7: NCI Common Toxicity Criteria, Version 4.03

Appendix 8: Serious Adverse Event Notification Form with Instructions

Appendix 9: Word medical association – Declaration of Helsinki Appendix

10: Insurance policy

Appendix 11: Acknowledgement Consent Form

Appendix 12: EORTC QLQ-C30

Appendix 13: EORTC QLQ-BR23

Appendix 14: Evaluation cosmétique et de la satisfaction au traitement

Appendix 15 : CHARTE du COMITÉ de SURVEILLANCE INDÉPENDANT