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CICLO XXXI

« Study of the effects induced by high doses per fraction in radiotherapy: correlations between biological and clinical parameters – the case of intraoperative irradiation of prostate adenocarcinoma»

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1. Introduction

1.1 Definition of the problem and Rationale

Prostate cancer is the second most common cancer in men worldwide, with 1,276,000 new cases and 359,000 deaths estimated in 2018 [1]. In 2019, the incidence of prostate cancer in Italy was calculated as 37,000 new cases and 7,540 deaths [2].

In industrialized areas, prostate cancer is usually diagnosed when the tumor is still confined to prostate.

Radiotherapy represents a curative treatment option for prostate carcinoma, according to major medical guidelines [3,4], even if the definition of the optimal treatment for this tumor remains a controversial issue. According to the initial PSA and the clinical staging, we could classify patients into different risk-based classes.

Low risk prostate cancer has a favorable prognosis with disease-free survival rates of 80–92% at 5 years and 76–92% at 10 years, either after radical prostatectomy or curative radiotherapy, while intermediate and high-risk patients have worse outcome due to the occurrence of biochemical failure in 24–72% of cases after radiotherapy and hormone therapy [5,6].

Two randomized trials, the European Organisation for Research and Treatment of Cancer (EORTC) 22863 and the Trans-Tasman Radiation Oncology Group (TROG) 9601, demonstrated the advantage of combining radiotherapy with androgen suppression in intermediate and high-risk patients [7,8]. However, disease-free survival rates were not satisfactory: 47.7% and 36.0%, respectively for EORTC

22863 and TROG 9601. The TROG 9601 trial reports a biochemical failure and local progression rates of 52.8% and 13.3%, respectively. Surgery - radical prostatectomy - was adopted in several randomized trials in patients with high-risk prostate cancer, with improved outcomes when adjuvant radiotherapy was associated to. Extra prostatic disease extension and positive surgical margins led to a worse prognosis and, after radical prostatectomy, almost 50% of patients with locally advanced disease experienced local relapse.

Surgery can be considered a feasible treatment for high-risk prostate cancer in unfavorable features cases and could be associated with postoperative radiotherapy.

Intra-operative radiotherapy (IORT) for prostate cancer was proposed first by Abe et al. [9] and by Takahashi et al. [10] at the Kyoto University.

The rationale of using IORT is related to technical and biological aspects. Prostate exposure during surgical procedure may allow optimal target identification and sparing of surrounding structures so directing a higher dose on prostate and surgical bed.

Current meta-analysis suggests that prostate cancer cells may be particularly sensitive to radiation fraction size [11], representing the rationale for hypofractionation and dose-intensification. In this scenario IORT is a valuable dose-intensification modality, allowing the delivery of higher irradiation dose during surgery, so, reducing the risk of residual disease by sterilizing microscopic neoplastic cells. It is estimated that single-dose 12 Gy IORT irradiation is equivalent to 56.2 Gy according to biological equivalent dose and conventional radiobiology [12]. In the panorama of

scientific studies that explore the feasibility of IORT eventually combined with external beam radiotherapy, our institution published its clinical experience with the aim of improving clinical outcome and shortening overall treatment time. Our data showed that, during radical prostatectomy, IORT is feasible further allowing safe delivery of postoperative external beam radiotherapy to the tumour bed with no relevant toxicity [13,14].

Radiobiological studies also suggest that the use of a high single dose might increase treatment effectiveness by increasing the radio-induced intracellular death processes [15].

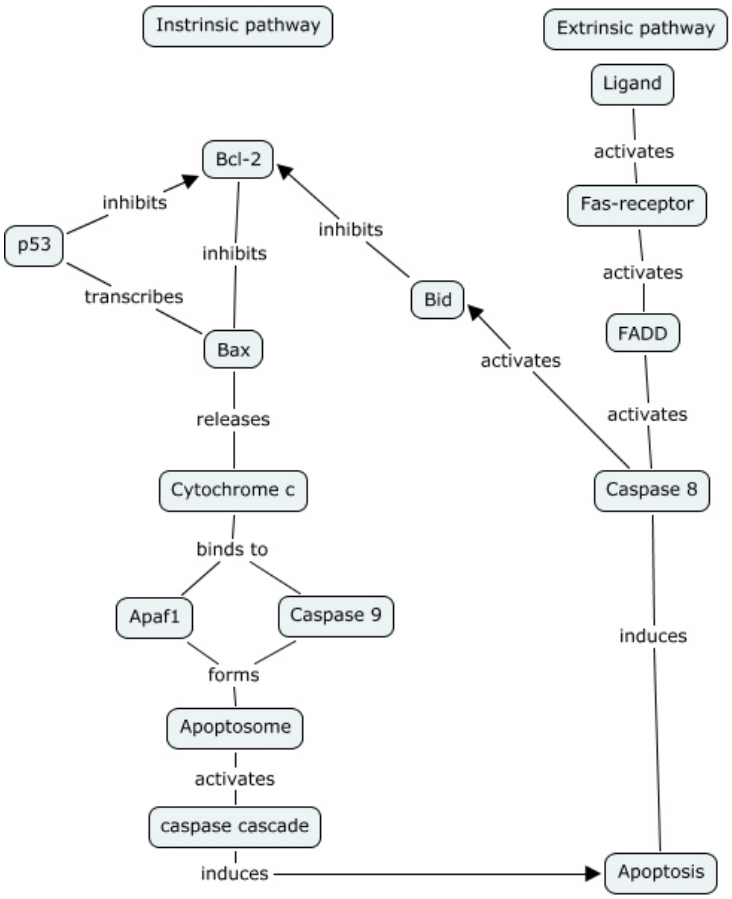
Cell death, particularly apoptosis or programmed cell death, is one of the most studied topics *in vitro*. Understanding the mechanisms of apoptosis in neoplastic disease is particularly interesting because it allows us to investigate the pathogenesis of disease and understand better how to cure neoplasia. Typically, in tumoral cells there is a loss of balance between cell proliferation, physiological cell death and signals that induce apoptosis.

During the apoptotic process, three types of biochemical changes can occur:

- activation of caspases and other pro-apoptotic proteins;
- DNA breaks;
- changes in membrane morphology and phagocytosis of apoptotic bodies.

One characterising element is the activation of a family of cysteine proteases, called caspases. These enzymes, when activated, can damage a series of essential cell survival proteins. They also activate DNAases that degrade intranuclear DNA.

Caspases could be activated by three different pathways: the intrinsic (or mitochondrial), the extrinsic, and the less known one, the intrinsic pathway of the endoplasmic reticulum.



The extrinsic pathway begins when proteins, such as Fas and TNF, bind to Fas-ligand or TNF-receptor. These receptors have an intracellular part that, when activated, recruits some proteins, including caspase 8.

The intrinsic pathway takes place completely inside the cell. Irreversible damages, such as irreparable DNA damage, hypoxia, intracytoplasmic hypercalcemia, oxidative stress, can trigger this pathway. Regardless of the stimuli that induce the apoptotic cascade, it results in an increasing of mitochondrial permeability, with the

release of pro-apoptotic molecules, such as cytochrome c. This pathway is closely linked to a group of proteins belonging to the Bcl-2 family, which takes its name from the BCL-2 gene. There are two main groups of proteins belonging to the Bcl-2 family:

- pro-apoptotic proteins (inhibit the release of cytochrome c): Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk
- anti-apoptotic proteins (blocking the release of cytochrome c): Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1

Both intrinsic and extrinsic pathways converge on the common pathway and on caspase 3.

Transformation of healthy cells into malignant one is linked to genetic alterations. Among the alterations acquired by neoplastic cells there is the reduction of apoptosis or the resistance to stimuli that should induce apoptosis [16], and uncontrolled stem cells growth is the basis of tumoral cells transformation. The loss of apoptotic control and the presence of anti-apoptotic genes could lead to the formation of resistant neoplastic cells [17,18].

Radiations cause a series of damage to cells and DNA, producing single and double breaks (direct damage) and ionizing the oxygen molecules forming free radicals (indirect damage). The cell dies for necrosis, apoptosis, or mitotic death. Mitotic death is the most common death mechanism induced by conventional fractional irradiation (1.8-2.0 Gy/fraction). Apoptosis occurs within 4-6 hours after high dose irradiation leading to an increase of apoptotic cells increases, as was observed *in vivo*

in the intestinal cryptic lymphocytes. *In vivo*, apoptotic bodies are quickly eliminated, so it is difficult to quantify them. Radio-induced apoptosis is intermediated by the activation of p53, Bax and subsequent activation of caspases, in particular caspases 3, 8 and 9 according to apoptotic cascade [19].

Neoplastic cells frequently acquire auto-survival mechanisms, resulting protected from apoptotic death.

Irradiation increases apoptosis selectively in some cellular neoplastic lines: for example, irradiated lymphoma cells would die for apoptosis, while the same is difficult for glioma cells [20-24].

No updated and solid data exists about apoptosis and prostate adenocarcinoma.

1.2 Aim of the study

As previously mentioned, the treatment with IORT for locally advanced prostate cancers has been adopted at our center for several years. According to our data, IORT would be a safe and a feasible treatment modality with a low complication rate after short-intermediate follow-up.

The purpose of this study is to analyze early activation of radio-induced apoptosis pathways in prostate cancer cells in IORT treated patients followed by radical prostatectomy for locally advanced prostate adenocarcinoma, in order to understand the biological rationale of this method. Consequently, we evaluated Bax and caspases expression before and after irradiation on healthy tissue fields, tumoral cells and areas of PIN (intraepithelial neoplasia).

Cell proliferation indexes (Ki-67), a proto-oncogene (p53) and an anti-apoptotic protein (Bcl-2) were also assessed in irradiated cells.

Then we correlated these biological factors with pathological staging and local control to further define a nomogram to select patients that could really benefit from adjuvant radiotherapy and IORT. A review article from our institution analyzed the role of IORT in same neoplastic setting, such as genito-urinary malignancies.

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Radiation Oncology

REVIEW Open Access

Intraoperative radiotherapy in gynaecological and genito-urinary malignancies: focus on endometrial, cervical, renal, bladder and prostate cancers

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Abstract

Intraoperative radiotherapy (IORT) refers to the delivery of a single radiation dose to a limited volume of tissue during a surgical procedure. A literature review was performed to analyze the role of IORT in gynaecological and genito-urinary cancer including endometrial, cervical, renal, bladder and prostate cancers.

Literature search was performed by Pubmed and Scopus, using the words "intraoperative radiotherapy/IORT", "gynaecological cancer", "uterine/endometrial cancer", "cervical/cervix cancer", "renal/kidney cancer", "bladder cancer" and "prostate cancer". Forty-seven articles were selected from the search databases, analyzed and briefly described.

Literature data show that IORT has been used to optimize local control rate in genito-urinary tumours mainly in retrospective studies. The results suggest that IORT could be advantageous in the setting of locally advanced and recurrent disease although further prospective trials are needed to confirm this findings.

Keywords: Intraoperative radiotherapy, Endometrial cancer, Cervical cancer, Renal cancer, Bladder cancer, Prostate cancer

Background

Intraoperative radiotherapy (IORT) refers to the delivery of a single large dose of radiation to a limited volume of tissue during a surgical procedure.

Radiotherapy (RT) has a major role in the management of most gynaecological and genito-urinary cancer as adjuvant or neoadjuvant treatment or as radical treatment in combination with chemotherapy or hormone therapy. IORT has the capability to increase the radiation dose with very limited or no increase of toxicity thanks to the target exposition during the surgical procedure. For this reason, IORT can be used in various settings of gynaecological and genito-urinary tumours aiming at dose intensification and consequently at increasing tumour control rate.

IORT can be delivered using dedicated linear accelerator producing electron beams, X-rays sources delivering low-energy radiation or high dose-rate brachytherapy units through catheters positioned in the tumour bed and loaded with iridium-192. In particular, electrons generated by linacs and brachytherapy sources can be conveniently used for IORT procedures in gynaecological and genito-urinary tumours.

Interestingly, the first IORT experience was indeed reported in 1905 for the treatment of a 33 year old woman affected by uterine carcinoma [1]. Over the following decades, IORT was increasingly used for several tumours including gynaecological and genitor-urinary malignancies.

In 1998, the International Society of Intraoperative Radiation Therapy (ISIORT) was founded in order to promote a scientific and professional approach to IORT activity. Among their other activities, ISIORT-Europe collected and

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recorded information regarding IORT treatments, including those of gynaecological and genito-urinary cancers, from the affiliated centres in a database registry [2, 3].

This review focuses on the use of IORT in genito-urinary malignancies, reporting tumour setting and outcome for endometrial, cervical, renal, bladder and prostate cancers.

Research criteria

Literature search was performed through Pubmed and Scopus databases by using the following key words: "intraoperative radiotherapy/IORT", "gynaecological cancer", "uterine/endometrial cancer", "cervical/cervix cancer", "renal/kidney cancer", "bladder cancer" and "prostate cancer". Eighty-four articles were found from 1981 to 2015. Reviews and case reports were excluded as well as clinical series presented as abstract at conferences proceedings. Forty-seven articles were finally selected for the review.

Endometrial and cervical cancers

Patients with endometrial and cervical cancer are usually treated with surgery and RT with or without chemotherapy depending on risk factors. After primary treatment, the risk of local failure is up to 60% [4] and the options for a new treatment are surgery, RT when a reirradiation is feasible, and chemotherapy. After such treatments, disease control has been reported in 25–50% and 18–47% in patients with recurrent endometrial and cervical cancer, respectively [5]. In these recurrent patients, IORT after surgical resection can been considered to increase the probability of local control, especially when a repeated course of EBRT is not feasible. This treatment approach including IORT is reported in the NCCN guidelines with an evidence of category 3 [6].

The use of IORT in the management of endometrial and cervical cancer was explored in 15 studies, most of them analysing retrospectively patients affected by locally advanced primary and recurrent disease. The majority of articles reported on the clinical experience from the Mayo Clinic and the University Hospital Gregorio Marañón in Madrid [7–21] (Table 1). In these clinical series, IORT was delivered to the tumour bed with electrons in the majority of cases and with low kV x-rays or brachytherapy through catheters implanted during the surgical procedure and uploaded with iridium wires in postoperative setting in selected patient series.

In endometrial cancer patients, limited loco-regional recurrences have a relatively high control rate of about 60% at 5 years either with pelvic exenteration or local EBRT in non-previously irradiated patients [22, 23]. In this tumour setting, the use of IORT was reported in retrospective studies [14, 15]. Dowdy et al. [14] found that radical resection of the pelvic sidewall with negative margins and IORT resulted in a relatively high overall survival rate (71%) (Table 1). Awtrey et al. [15] reported that the addition of IORT to cytoreductive surgery in 27 recurrent endometrial cancer patients resulted in a 2-year disease free survival (DFS) rate of 78% versus 67% when IORT was not used, although this difference was not statistically significant. Based on these retrospective data, the addition of IORT to surgery could be proposed in patients with isolated endometrial cancer recurrences, especially when margins might be close or microscopically positive.

Patients with a loco-regional recurrence of cervical cancer and candidates for salvage surgery can undergo also IORT with the intent to sterilize the possible residual disease and improve the outcome. This approach was described in three series from Mahe et al. [20], Barney et al. [10] and Martinez-Monge et al. [16] who reported globally the results in 188 patients with recurrent cervical cancer. Intraoperative radiation dose ranged from 6 Gy to 30 Gy, with higher doses in case of macroscopically positive margins (R2). Mahe et al. [20] reported a slightly higher local control, although statistically not-significant, in patients with radical resection versus those who received partial resection (27% vs. 11%), Barney et al. [10] did not observe any influence of margins status for local control and Martinez-Monge et al. [16] reported a risk of distant metastases of 38% in patients with negative margins (R0) and 100% in those with macroscopic residual disease (R2). From these studies, it emerged that the status of the margins is the most important risk factor for treatment and the association of IORT seems to improve the probability of local control.

As far as locally advanced primary cervical cancer is concerned, two series treated by IORT are reported in the recent literature [12, 16]. In both studies, patients underwent radical hysterectomy and 10–25 Gy IORT after neoadjuvant EBRT, concomitantly to chemotherapy, to a total dose of 50.4 Gy. In the Giorda's phase II trial, patients tolerated radio-chemotherapy quite well, but developed high incidence of toxicity (79%) after surgery and IORT [12]. In the Martinez-Monge's retrospective series, 15% of side effects were related to IORT [16]. The available data suggests that this aggressive strategy is not advantageous in particular for the risk of severe side effects and that concomitant radio-chemotherapy alone should be considered the best treatment strategy in this patient setting [6].

In conclusion, literature data supports the use of IORT in recurrent endometrial and cervical cancer to improve local control whereas its use appears more controversial in primary locally advanced disease. The potential benefit of this approach is mainly based on retrospective mono-institutional studies and should be further verified by prospective possibly randomized trials investigating the potential advantage compared to EBRT alone.

Renal cancer

Historically, the standard therapy for renal cell carcinoma is radical nephrectomy. Local control and survival

Table 1 IORT studies for endometrial and cervical cancer

Reference	N.pts	Type of cancer	Primary/recurrent	EBRT N. pts Dose (Gy)	IORT dose (Gy)	Technique	Median follow-up months(range)	Local Control	Overall Survival	Toxicity
Sole [7]	61	Uterus 18 Cervix 32 Other 11	Pelvic recurrent 35 (57%) Paraortic recurrent 26 (43%)	Mean 31 Gy (29–45)	R0: 10–12.5 Gy R1: 15 Gy	IOERT	42 (2–169)	5-years 65%	5-years 42%	RTOG acute ≥ G3: 23 RTOG late ≥ G3: GI 8 GU 3 Neuropathy 1
Foley [8]	32	Cervix 21 Uterus 6 Other 5	Pelvic recurrent 26 (81%) Primary 6 (19%)	NA	Mean 13.5 Gy (10–22.5)	IOERT	Median 26 (3–196)	5-years R1 73% 5 years R2 71%	5-years 70% R1 77% R2 55%	≥G3 47% 5 IORT-related GU 2 Bone 1 Lymphedema 2
Backes [9]	32 21 IORT	Cervix 21 Other 11	Recurrent 32 (100%)	6 pts, mean 26 Gy (10–40)	Median 17.5 Gy (10–20 Gy)	IOERT HDR IORT	NA	Median PE + IORT 10 months LEER + IORT 9 months PE 33 months	Median PE + IORT 10 months LEER + IORT 17 months PE 41 months	NA
Barney [10]	86	Cervix	Pelvic recurrent 73 (85%) Primary 13 (15%)	61 pts (71%) No prior RT: median 45 Gy Prior RT: median 39.6 Gy	median 15 Gy (6–25 Gy)	IOERT	32 (1–306)	3-years 62%: 70% primary 61% recurrent	3-years 25%	≥G3 GI 4 GU 1 Neuropathy 1 Other 4
Calvo [11]	35	Uterus 7 Cervix 20 Other 8	Pelvic recurrent 35 (100%)	16 pts: 45 Gy no previous RT 30.6 Gy previous RT	R0: 10–12.5 Gy R1: 15 Gy	IOERT	46 (3–169)	5-years 58%	5-years 42%	acute ≥3: 14 late ≥3: GI 5 GU 2 Neuropathy 1
Giorda [12]	35	Cervix	Primary 35 (100%)	neoadj 50.4 Gy	Mean 11 Gy (10–15)	IOERT	NA	2-years 89%	5-years 49%	Peri/post-surgery GU 10
Tran [13]	36	Cervix 17 Uterus 11 Other 8	Recurrent 32 (88%)	18 pts (50%) mean 44 Gy	Median 11.5 Gy (6–17.5)	Orthovoltage-IORT	Mean 50 (2–198)	5-years 44% Cervix 45% Uterus 58%	5-years 42%	≥G3 10 pts 28%
Dowdy [14]	25	Uterus	Recurrent 25 (100%)	21 pts 45 Gy	Median 15 Gy (10–25 Gy)	IOERT	Median 34	84%	5-years: 71% R0 47% R1 0% R2	Neuropathy 8 GU 5 Fistulas 5 Bone fractures 2
Awtrey [15]	27	Uterus	Pelvic Recurrent 27 (100%)	12 pts	NA	IOERT 9 pts	Median 24 (5–84)	NA	2-years 78%	NA
Martinez-Monge [16]	67	Cervix	Pelvic Recurrent 36 (54%) Primary 31 (46%)	36 pts : 45 Gy	Primary: 12 Gy median (10–25) Recurrent: 15 Gy (10–20)	IOERT	Primary: 58 (8–144) Recurrent 19 (1–138)	10-year 69%: 93% primary 47% recurrent	10-year 35%; 58% primary 14% recurrent	15% IORT related
Gemignani [17]	17	Cervix 9 Uterus 7 Other 1	Recurrent 17 (100%)	2 pts dose NA	Mean 14Gy (12-15Gy)	HDR-IORT	20 (3–65)	67	54	NA
DelCarmen [18]	15	Cervix 5 Uterus 3 Other 7	Pelvic Recurrent 14 (93%) Primary 1 (7%)	-	10-22.5 Gy	IOERT	(3–36)	54%	74%	Neuropathy 4 GU 3 Lymphedema 2

Table 1 IORT studies for endometrial and cervical cancer *(Continued)*

Garton [19]	39	Cervix 22 Uterus 10 Other 7	Pelvic Recurrent 36 (92%) Primary 3 (8%)	28 pts Median 45 Gy (1–67)	Median 17.3 Gy (10–25 Gy)	IOERT	Median 25 (6–125)	5-years 67%	5-years 32%	≥G3 14 (36%) IORT related 6
Mahè [20]	70	Cervix	Pelvic Recurrent 70 (100%)	30 pts (20–45)	R0 mean 18 (10–25) R1-biopsy mean 19 (10–30)	IOERT	Mean 15 (2–69)	21% R0 27% R1-2 11%	3-years 8%	10-IORT related GI 1 GU 4 Neuropathy 5
Stelzer [21]	22	Cervix	Pelvic Recurrent 22 (100%)	6 pts: 26–50 Gy 7 pts: 45–62.4 Gy	22 Gy median (14–27.8 Gy)	IOERT	Minimum 15 months	5-years 48%	5-years 43%	Neuropathy 7

Pts patients, *IORT* Intraoperative radiotherapy, *IOERT* intraoperative electron radiotherapy, *EBRT* external beam radiotherapy, *GU* genitourinary *GI* gastrointestinal, *NA* not available, *R0* negative margins, *R1* microscopic residual disease, *R2* macroscopic residual disease

rates after surgery alone are satisfactory for T1-T2 N0 with rates of 90-100% and 80-90% at 5 years, respectively. The results are less favourable for locally advanced and N+ disease, where the 5-year local control rate and overall survival rates are 70-80% and 0-40%, respectively. In renal cancer, the isolated local recurrence after radical nephrectomy is uncommon (0.7-3.6%) but it is associated with a poor prognosis. An aggressive surgical approach to local advanced or recurrent disease, possibly including the removal of the renal fascia and leading to negative margins, seems to improve outcome and prolong survival [24, 25].

Although renal cell carcinoma has traditionally been considered relatively radiation resistant, recent data using hypofractionation for primary or metastatic lesions suggest that this resistance can be overcome by high dose per fraction, as used in the IORT scenario [26].

The role of IORT in the management of renal cancer was explored in a number of retrospective studies with patients presenting with locally advanced primary or recurrent disease [27–33] (Table 2). IORT doses varied from 10 to 25 Gy depending on the amount of residual tumour after maximal resection and on the dose of the combined EBRT. All cases of these series were characterized by postoperative microscopic or macroscopic residual disease in the renal fossa. A more recent study [27] considered 98 patients with advanced or recurrent renal cell carcinoma treated with IORT at nine institutions. Preoperative or postoperative EBRT to a total dose of 40–50.5 Gy was administered to 27% or 35% of patients, respectively. The median radiation dose administered with IORT was 15 Gy (range: 9.5-20 Gy). Overall survival and disease free survival rates at 5 years were quite similar and only 24% of relapses were local whereas 76% were distant. This fact suggests the potential benefit in local control when IORT is added. Similar results in terms of local control rates were reported in previous studies from other institutions (Table 2). In these series, the acute and late toxicity profile seems acceptable. Many studies, however, are characterized by a limited description of late side effects.

From all published data, although from retrospective series, it emerges that the addition of IORT to surgery and EBRT is associated with high rates of local control with acceptable toxicity. The best candidates could be untreated patients with large tumour volume and high risk of positive margins after radical nephrectomy and patients with locally recurrent tumours. The long-term prognosis is mainly related to the risk of onset of distant relapse that is quite common, especially in patients with recurrent disease. This fact advocates the need for additional systemic effective therapy.

Bladder cancer

The goals of treatment for invasive bladder cancer are high long-term overall and disease-free survival rates with acceptable functional outcome, however, radical cystectomy, that is nowadays the standard, needs urinary diversion and results in erectile impotence and infertility. In order to avoid these adverse effects and preserve quality of life, bladder-preserving treatments have been proposed as a viable option in selected patients [34]. Bladder preservation strategies for muscle invasive bladder cancer evolved over time from single modality to multimodality treatment approaches, including transurethral resection and chemo-radiation protocols. The use of an intraoperative radiation boost by brachytherapy or electrons may be advantageous for intensifying the dose and obtaining local control without compromising organ function.

From the literature databases, 15 studies using IORT by brachytherapy implants or electrons were selected for this review [35–49] (Table 3). Brachytherapy was the most used intra-operative modality and was employed either as a single treatment or as a boost dose combined with EBRT. It may represent a curative treatment for selected high-risk superficial and solitary muscle infiltrating tumours. Clinical target volume (CTV) typically includes the macroscopic disease or the tumour bed with safety margin to full thickness of the bladder wall.

All the studies about brachytherapy were retrospective analyses of single or multiple co-operative centres. In 2012, a multicentre survey [36], assessed the role of brachytherapy in 1040 patients with early stage bladder carcinoma in a muldisciplinary setting. Patients were treated by pre-operative EBRT and limited surgery with brachytherapy implant. From this analysis, it emerged that this approach can offer adequate results in terms of local control and overall survival in selected patients suitable (Table 3). In this regard, a careful patient selection is particularly important in relation to the non-negligible probability of acute toxicity leading to fistulas or necrosis.

A recent systematic review with meta-regression analysis showed better results after brachytherapy than after cystectomy in terms of overall survival, but not in terms of cause-specific survival in patients with muscle-invasive bladder cancer. The authors commented that this discrepancy can be explained at least in part by the differences in tumour stage between the two groups [50].

The integration of an IORT boost to the whole bladder in a multidisciplinary protocol combining neoadjuvant systemic chemotherapy, preoperative RT, and planned cystectomy has proven to be feasible in the Pamplona's series [44]. The mean sterilization rate of invasive bladder cancer, confirmed in pathologic studies by the cystectomy specimen, was 65%, and seemed to be increased

Table 2 IORT studies for renal cancer

Reference	N. pts	Type of cancer	Primary/recurrent	EBRT	IORT dose (Gy)	Technique	Median follow-up	Local control	Overall survival	Toxicity
Paly [27]	98	Advanced or recurrent renal cell carcinoma	Pelvic locally recurrent 100%	26 pts: 45–40 Gy pre or post surgery	Median dose: 15 Gy (9.5-20 Gy)	IORT	3.5-years (3–169)	5-years 39% advanced disease 5-years 52% recurrent disease	5-years 37% advanced disease 5-years 55% recurrent disease	NA
Habl [28]	17	Locally recurrent disease	Pelvic locally recurrent 100%	-	Median dose: 15 Gy (10–20 Gy)	IORT	18 months	2 years 91%	2 years 73%	No late toxicities
Calvo [29]	25	Advanced or recurrent renal cell carcinoma	Pelvic locally recurrent 100%	15 pts: 44 Gy perioperative	Median dose: 14 Gy (9–15 Gy)	IORT	22.2 years (3.6-26)	5-years 80%	5-years 38% 10-year 18%	6 pts acute/ late toxicities ≥ 3
Hallemeir [30]	22	Advanced or recurrent renal cell carcinoma	-	21 pts: 41.5 Gy perioperative	Median dose: 12.5 Gy (10–20 Gy)	IORT	9.9 years (3.6-20)	NA	5-years 40%	5 ptsacute/ late toxicities ≥ 3
Master [31]	14	Local recurrent renal cell carcinoma	Pelvic locally recurrent 100%	-	Median dose: 15 Gy (12–20 Gy)	IORT	NA	NA	5 years 30%	NA
Eble [32]	14	Advanced or recurrent renal cell carcinoma	-	14 pts:40 Gy postoperative	15-20 Gy	IORT	24.3 months	NA	11.5 months	0%
Frydenberg [33]	11	Local persistence or local recurrent		11 pts: 45–50.4 Gy preoperative	10-25 Gy	IORT	NA	NA	NA	NA

Pts patients, *IORT* Intraoperative radiotherapy, *IOERT* intraoperative electron radiotherapy, EBRT external beam radiotherapy, *GU* genitourinary, *GI* gastrointestinal, *NA* not available

Table 3 IORT studiesfor bladder cancer

Reference	N. pts	Stage	EBRT	Treatment	Local control (5 years)	Overall survival 5-years	Toxicity
Hallemeier [35]	11	Local recurrence	Neoadjuvant	Surgery + IORT (12.5 Gy)	51%	16%	NA
Koning [36]	1040	T1-T2	Neoadjuvant	surgery, Ir-192 (25–40 Gy)	75%	62%	Fistula 24, ulcers/ necroses 144
van Onna [37]	111	T1-T2	Neoadjuvant	Ir-192 (40 Gy)	NA	70%	Fistula 5 GU 5
van der Steen-Banasik [38]	76	T1-T2	Neoadjuvant	Cs-137, Ir-192 (30–60 Gy)	70%	57%	NA
Blank [39]	122	T1-T2-T3	Neoadjuvant	Ir-192 (20–70 Gy)	76%	73%	GU 5
Nieuwenhuijzen [40]	108	T1-T2	Neoadjuvant	Ir-192	73%	62%	NA
De Crevoisier [41]	58	T1-T2-T3	Neoadjuvant	surgery, Ir-192 (60 Gy)	65%	60%	5 major late toxicities
Gerard [42]	27	T2, T3	No	Surgery + IORT	85%	53%	NA
Pernot [43]	82	T1, T2, T3, T4, Tx	Neoadjuvant	surgery Ir-192 (30–50 Gy	78%	73%	7 late toxcities ≥ G3
Calvo [44]	40	T2, T3, T4	Neoadjuvant	surgery + IORT (15 Gy)	NA	68%	NA
Rozan [45]	205	T1-T2-T3	Neoadjuvant	surgery Ir-192 (30–50 Gy	NA	77.4% T1, 62.9% T2, 46.8% T3	haematuria, fistula, chronic cystitis 29
Batterman [46]	85	T2	Neoadjuvant	Ra-226	74%	55%	NA
Mazeron [47]	24	T2	Adjuvant	surgery, Ir-192	92%	58%	NA
van der Werf-Messing [48]	328	T2	Neoadjuvant	Ra-226	77%	56%	NA
Matsumoto [49]	28	T2	Adjuvant	IORT	82%	62%	NA

Pts patients, *EBRT* External beam radiation therapy, *Ra-226* brachytherapy, radium needles, *Ir-192* brachytherapy, afterloading iridium, *IORT* intraoperative electron radiation therapy

by the addition of neoadjuvant chemotherapy. This finding can be of importance with respect to the development of new protocols aiming at bladder preservation. In the Lyon series [42], an excellent bladder preservation rate of 69% was achieved with the combination of preoperative chemo-RT followed by IORT. This is the only prospective study about IORT in bladder carcinoma. It could be of interest to attempt verifying these results in further studies using an IORT approach.

In conclusion, after a careful patients selection, IORT could be used within a bladder sparing multidisciplinary approach because of the favourable 5-year local control rates aiming at escalating the radiation dose. IORT might have a role also in case of radical surgery for locally advanced disease in order to improve local control rates, as performed in the Pamplona's series. Multicentric prospective studies could useful to confirm the role of IORT in this tumour setting.

Prostate cancer

The rationale for dose escalation with IORT in prostate cancer is based on the demonstration of a dose–response relationship and a low α/β value in the radiobiological linear quadratic model [51]. Likewise, the exploitation of this principle is being increasingly investigated in EBRT with hypofractionation [52].

Among 14 IORT literature studies, 9 clinical series and the ISIORT registry were selected and presented in Table 4 [2, 53–61].

Early data on IORT in prostate cancer came from the Kyoto University and the Saitama Cancer Centre in Japan, where the authors treated patients through a perineal IORT approach without prostatectomy [59, 61]. More recent experiences were reported by Italian authors using IORT in combination with radical prostatectomy and regional lymph node dissection before or after the surgical procedure [53–56]. A relevant percentage (81%) of patients was included in prospective institutional study protocols as described in the ISIORT data-registry [2]. From this analysis, it emerged that IORT was used as a boost dose prior to prostate removal in most cases. When a single-shot radiation strategy was adopted, a dose of 18–21 Gy was delivered, similarly to the breast cancer model. The diameter and bevel end angle of the applicators were selected based on target dimensions, considering a margin of at least 5 mm around the prostate and the necessity to reach the target underneath the pubic arch while sparing the bladder. The electron beam energy, between 9 and 12 MeV, depended on

Table 4 IORT studies for prostate cancer

Reference	N. pts	Patients' selection	Surgical approach	IORT dose (Gy)	Technique	Adjuvant EBRT	BRFS	Overall survival	Toxicity
Krengli (ISIORT) [2]	108	Intermediate-high risk[a]	NA	8-15 Gy with EBRT 18–21 Gy single shoot	IORT or 50-KV	NA	NA	NA	NA
Krengli [53]	38	Intermediate-high risk[a]	Retropubic approach IORT + Prostatectomy	10-12 Gy	IORT	46-50 Gy, 2 Gy/fx	82%	2-years 100%	Lymphocele 16% hematoma 6%
Rocco [54]	33	Intermediate-high risk[a]	Retropubic approach IORT + Prostatectomy	12 Gy	IORT	45 Gy, 1.8 Gy/fx	97%	2-years 100%	GU: 17% ≥ G2 GI: 10% ≥ G2
Saracino [55]	34	Intermediate risk[a]	Retropubic approach Prostatectomy + IORT	16-22 Gy	IORT	No	77%	NA	No GU/GI toxicities ≥ G1
Orecchia [56]	11	High-risk[a]	Retropubic approach IORT + Prostatectomy	12 Gy	IORT	45 Gy, 1.8 Gy/fx	NA	NA	No GU/GI toxicities ≥ G1
Kato [57]	54	Stage B2-D1[b]	Perineal/retropubic No prostatectomy	25-30 Gy	IORT	30 Gy, 2 Gy/fx	74%	NA	Early GI G3: 7%
Higashi [58]	35	Stage B-C [b]	Perineal/retropubic No prostatectomy	25-30 Gy	IORT	30 Gy, 2 Gy/fx	NA	5-years 87% (stage C) 5-years 92% (stage B)	NA
Abe [59]	21	Stage B2-days [b]	Perineal	28-35 Gy or 20–25 Gy (if combined with EBRT)	IORT	50 Gy	NA	5-years 72%	GU: 100% early ematuria 10% early pollakiuria
Kojima [60]	30	Stage B-C [b]	Perineal/retropubic No prostatectomy	–	IORT	NA	NA	5-years 43%	NA
Takahashi [61]	14	Stage B2-days [b]	Perineal No prostatectomy	28-35 Gy or 20–25 Gy (if combined with EBRT)	IORT	50 Gy	NA	NA	0%

pts patients, *GU* genito-urinary, *GI* gastro-intestinal, *BRFS* biochemical relapse-free survival, *NA* not available
[a]National Comprehensive Cancer Network (NCCN) guidelines NCCN [6]
[b]Whitemore-Jewett staging system [Whitmore 1956, Jewett 1975]

the depth of the target and the position of the rectum, which should be spared.

Patient selection varied widely in the various studies. The Japanese series included either early or advanced stage disease and in particular the Kyoto University included stages from A2 to C treated with curative intent and even stage D2 treated with palliative intent [59, 61]. The Italian studies accrued only non-metastatic locally advanced disease based on the identification of pre-operative risk factors.

In terms of post-surgical early and late side effects, IORT for prostate cancer resulted an acceptable procedure. In the Japanese series, toxicity resulted in early haematuria, pollakiuria but only very few cases of late chronic cystitis and urethral stricture. Interestingly, Kato et al. reported a reduction in rectal toxicity by using a spacer to reduce the dose to the anterior rectal wall [57].

In the Italian series, surgical complications, such as haematoma and lymphocele, occurred with a similar incidence to that of conventional prostatectomy [53–56]. No major surgical complications were described and patients had no significant difference of estimated blood loss and need of transfusion. In this regard, Rocco et al. reported post-surgical complications in 42% of patients after surgery and IORT and in 30% after prostatectomy alone [54].

Although the relatively short follow-up, the outcome in terms of biochemical disease free survival was quite promising resulting higher than 70% in both the Japanese and Italian series (Table 4). Of note, a recent update of our clinical series of 95 patients showed a 5-years biochemical disease-free survival rate of 78% in high-risk patients (oral presentation at ISIORT-ESTRO Forum, Barcelona, 24–28 April, 2015).

Clinical trials with long follow-up are needed to assess the real efficacy of IORT in locally advanced prostate cancer but preliminary results look quite promising. The best candidates for IORT possibly combined with EBRT, could be the patients staged T3N0 with high risk for positive margins. In the future, multicentre studies should be designed to better clarify the real role of IORT for dose escalation in local advanced prostate cancer patients.

Conclusions

The delivery of a high single dose of radiation to a limited volume during the surgical time, achievable with IORT, is

useful to avoid normal tissues not at risk of microscopic disease. For gynaecological and genito-urinary cancers, IORT is not a standard treatment but it may be considered a treatment option in selected patients.

In endometrial, cervical and renal cancers, IORT can be used mainly in recurrent disease, whereas in bladder carcinoma it may be part of an organ-sparing treatment approach aiming at patient quality of life preservation. In the case of prostate cancer, IORT can be used in locally advanced high risk disease possibly combined with EBRT to intensify the radiation dose in the attempt to improve long term local control and possibly increase biochemical disease-free and overall survival.

The available literature data are interesting but the present review shows that the majority of published clinical studies are mono-institutional, retrospective and often included a limited number of patients. In order to overcome these limitations, large multicentre collaborations should be established to design prospective clinical trials aiming at better defining the role of IORT in tailored multimodality therapeutic approaches for gynaecological and genito-urinary tumours. For this purpose, the ISIORT could serve as a basis for future collaboration and the ISIORT-Registry could be a platform for sharing data and promote clinical research.

Abbreviations

CTV: Clinical target volume; EBRT: External beam radiotherapy; IOHDR: Intra Operative high dose rate; IORT: Intraoperative radiotherapy; ISIORT: International society of intraoperative radiation therapy; RT: Radiotherapy

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Authors' contributions

MK developed the design of the review and contributed to draft and revise the manuscript. CP and LD performed the literature search and analysis, and contributed to draft the manuscript. DS, AV, NS and CT contributed to the study design and to revise critically the manuscript. All the authors read and approved the final version of the manuscript.

Competing interests

The authors declare no conflict of interest.

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Not applicable.

Ethics approval and consent to participate

Not applicable.

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As already emphasized, the treatment management in prostate tumor is still a controversial issue. Moreover, no studies have been performed about the current practice in prostate cancer management and only a limited number of clinical audits investigate the level of QA in the related procedures.

In this scenario, the international multi-institutional IROCA (Improving quality in Radiation Oncology through Clinical Audits; www.iroca.eu) project was born. The aim of the project was to compare radiotherapy processes among participating institutions - the Wielkopolskie Centrum Onkologii (WCO) in Poznan, Poland; the Institut Català d'Oncologia (ICO) in L'Hospitalet (Barcelona), Spain; the Instituto Português de Oncologia (IPO) in Porto, Portugal; and the Università degli Studi del Piemonte Orientale (UNIUPO) in Novara, Italy - using a core set of quality indicators.

The project included the analysis of qualitative aspects of radiotherapy procedures in particular in prostate cancer.

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Preliminary communication

Improving radiation oncology through clinical audits: Introducing the IROCA project

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ABSTRACT

As radiotherapy practice and processes become more complex, the need to assure quality control becomes ever greater. At present, no international consensus exists with regards to the optimal quality control indicators for radiotherapy; moreover, few clinical audits have been conducted in the field of radiotherapy. The present article describes the aims and current status of the international IROCA "Improving Radiation Oncology Through Clinical Audits" project. The project has several important aims, including the selection of key quality indicators, the design and implementation of an international audit, and the harmonization of key aspects of radiotherapy processes among participating institutions. The primary aim is to improve the processes that directly impact clinical outcomes for patients. The experience gained from this initiative may serve as the basis for an internationally accepted clinical audit model for radiotherapy.

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1. Background

In recent decades, the effectiveness of radiotherapy has increased considerably due to the advent of ever more powerful, more precise technologies, such as intensity-modulated radiotherapy (IMRT). The use of more sophisticated technologies has also increased the complexity of radiotherapy delivery. As a result, every step in the radiotherapy process has become more demanding and multifaceted, requiring strict attention to detail to assure that the high doses of radiation are delivered precisely to the treatment target. To ensure the quality of radiotherapy delivery and treatment, it is essential to monitor the process carefully and systematically, with routine and frequent checks and assessments. However, the development and implementation of quality control measures have not kept pace with the remarkable technological advances achieved in recent years.[1–4]

A common approach to quality control in cancer care involves the use of quality indicators. Ideally—given the wide variety of processes and techniques involved in treating different types of cancer—these indicators should be specifically designed (or adapted to) each tumour type. In this sense, the availability of a set of internationally recognized and standardized indicators to permit international comparisons among radiotherapy centres would be highly desirable. Yet experience in this area remains limited, with no consensus with regard to the optimal indicators for radiotherapy.[3] Indeed, although several different groups[3–7] have attempted to identify a core group of quality indicators for radiotherapy, no widely-accepted or internationally-recognized core set of indicators is available at present.

While quality indicators are important to ensuring quality control, to be of any real value these indicators must be applied to actual clinical practice—preferably by external evaluators. This process, known as a clinical audit, provides an opportunity to conduct an in-depth analysis of the procedures and processes governing patient care. To date, such clinical audits have been used only sparingly in radiotherapy,[2,3,8] although measures to increase their use have been taken, including a European Union directive requiring their use.[4]

Interest in developing and implementing a system of quality standards in radiotherapy has increased greatly in recent years.[9–12] Nevertheless, only a limited number of clinical audits, including one by our group,[2] have been conducted to date.[2,13–15] It is in this context that the multi-institutional, international IROCA (*Improving quality in Radiation Oncology through Clinical Audits*; www.iroca.eu) project was born. The aim of this project is to compare radiotherapy processes among participating institutions [the Wielkopolskie Centrum Onkologii (WCO) in Poznan, Poland; the Institut Català d'Oncologia (ICO) in L'Hospitalet (Barcelona), Spain; the Instituto Português de Oncologia (IPO) in Porto, Portugal; and the Università degli Studi del Piemonte Orientale (UNIUPO) in Novara, Italy] using a core set of quality indicators. To our knowledge, this is the first project of its kind and scope.

In the present paper, we provide an overview of this international project, which involves the design and implementation of a clinical audit to assess adherence to a set of core quality indicators to evaluate departmental/institutional structure, radiotherapy processes and procedures, and clinical outcomes among the five participating institutions. The overall aim of the project is to improve quality and safety in radiation oncology by promoting adherence to quality indicators and by harmonizing radiotherapy processes among the participating institutions. Ultimately, the main objective is to improve clinical outcomes for patients. The approach used in this project to harmonize radiotherapy processes among different institutions may serve to promote a greater use of clinical audits in radiotherapy in Europe.

2. Methods & discussion

This study was modelled on two previous studies. The first was performed jointly by the ICO (Catalan Institute of Oncology) and the WCO (Greater Poland Cancer Centre), with results published in 2014.[2] In that study, which was conducted—in part—to generate more practical experience in quality control, the clinical audit assessed adherence to seven quality indicators for preoperative rectal cancer treatment. This experience was invaluable, both in improving key elements of care at the audited institutions, and in learning about how to develop and conduct a comprehensive clinical audit, a challenging and highly complex task. In addition, in the year 2015, the ICO (Catalonia, Spain) performed an in-house clinical audit among their three radiotherapy centres (in Badalona, L'Hospitalet, and Girona). Results from that study have not yet been published. Nevertheless, the combined experience of these two previous studies has helped to guide us in developing the model described here.

2.1. *Organization of the project*

A Steering Committee (SC) consisting of senior members of the IROCA project was formed to guide the development of this project. The IROCA members held a series of meetings to establish the aims and protocol for the study, including selection of the target cancer types for the audit. After a careful review of the literature and based on previous experience, the committee selected the most appropriate quality control indicators for those tumour sites and for general radiotherapy processes. A Technical Committee (TC) was constituted to perform the statistical analysis and to develop the reports. A detailed study protocol, including the questionnaire and all other relevant data, has been developed. The project's key aims are summarized in Table 1.

2.2. *Cancer types for evaluation*

Two cancer sites, prostate (ICD-9:185.9 and ICD10: C61.9) and rectal cancer (ICD-9: 154.1; ICD-10: C20.9) were selected for the clinical audit.

These specific cancer types were chosen due to their high incidence rate,[16] the relevant role of radiotherapy in their treatment, our prior experience, and because all participating institutions treat large numbers of patients for these two cancer types. In the case of rectal cancer, the high incidence and mortality rates associated with this cancer make it an

Table 1 – Improving clinical outcomes of radiotherapy through step-by step standardization of key elements in clinical practice at participating institutions.

1. Identify key aspects within the radiotherapy process that impact clinical outcomes and treatment efficiency.
2. Determine the most relevant indicators to measure these key aspects.
3. Design a clinical audit procedure to determine adherence to these indicators at participating institutions.
4. Identify the areas amenable to standardization of the following:
   - treatment approach
   - treatment planning and execution
   - reporting the outcomes (results and side effects)
   - patient comfort
   - healthcare provider accountability and reliability
   - efficient use of resources
5. Develop a minimum dataset for benchmarking.

ideal candidate for auditing due to the large impact even small improvements in cancer care could have on clinical outcomes.

2.3. *Target population and sample selection*

Patients diagnosed with either prostate or rectal cancer who underwent curative-intent radiotherapy during the study inclusion period (calendar year 2014). Patients who did not receive neoadjuvant radiotherapy and those with recurrent disease will be excluded. To minimize the risk of bias, patients will be randomly selected as follows: all patients who meet the inclusion criteria will be assigned an identification number; next, a separate register will be created for these eligible patients and a computer program will randomly select 60 clinical cases per tumour site. All patients who meet the inclusion criteria will be included in the audit, with a minimum requirement of 40 patients per tumour site.

2.4. *Selection of quality indicators, standards and questionnaires*

After a review of the available indicators, including those proposed by other authors,[3] those used in our previous study,[2] and the indicators used in the ICO study, we selected a set of clinical indicators applicable to all radiotherapy processes (Table 2), plus indicators specific to prostate and rectal cancer radiotherapy (Tables 3 and 4).

After selection of the specific indicators, we proceeded to develop three questionnaires, including a general questionnaire, to assess all the variables relevant to the quality indicators to measure overall performance of the radiotherapy process. The aggregate data needed to complete the general questionnaire will be obtained directly from the radiotherapy department and include the following key dimensions:

1. Organization (protocols, sessions, tumour boards)
2. Radiotherapy equipment
3. Work team
4. Research (publications, projects, and clinical trials)
5. Radiotherapy activity
6. Patient experience

Table 2 – General indicators.

1. Existence of technical protocols for treatment
2. Existence of departmental clinical meetings
3. Existence of departmental technical meetings
4. Existence of an action protocol in case of unplanned treatment interruptions
5. Existence of quality control protocol for treatment-related imaging
6. Existence of an informed consent form specific to each cancer type and/or technique
7. Existence of a protocol for irradiating patients with ICD/pacemaker (PM)
8. Existence of tumour-specific treatment guidelines
9. Number of articles published in indexed journals by radiation oncology, physics and radiation biology staff members
10. Number of published articles in which either the three first authors or the last author is a member of the team
11. Total impact factor of the articles published during the year by staff involved in radiation oncology, physics and radiation biology in which either three first authors or the last author is a member of the team
12. Number of projects submitted for funding to national or international bodies/institutions excluding trials financed by pharmaceutical companies
13. Number of projects approved for funding to national or international bodies/institutions excluding trials financed by pharmaceutical companies
14. Participation in European Union grant
15. Leadership of European Union grant
16. Number of clinical trials specific to radiation oncology and % of patients included in these trials
17. Number of patients included in clinical trials involving radiotherapy treatment and radiatio oncologist is primary investigator
18. Existence of patient satisfaction survey
19. % of patients who completed satisfaction survey
20. Patients treated per year
21. Patients treated per accelerator
22. Up/down time of the accelerators (according to recommended calculation formula; otherwise, the specific formula should be provided)
23. Average number of patients treated per hour per accelerator
24. % of reports completed within 2 months of treatment finalization

7. Quality of care (safety, efficiency, accessibility, and treatments delays)

The other two questionnaires are specific to the two cancer types (prostate and rectal cancer) and are designed to assess the core indicators for each tumour type. All data (>100 variables) required to complete the questionnaire will be obtained from clinical records.

The key dimensions of these two questionnaires are as follows:

1. Diagnostic phase: multidisciplinary tumour board assessment, clinical profile (stage, etc.), diagnostic tests, and treatment delays.
2. Treatment phase: treatment planned and performed; radiotherapy dose, fraction and duration (prescribed versus performed); quality of care; adjuvant treatments
3. Clinical results and follow up phase: treatment-related side-effects; clinical status (recurrence; mortality);

Table 3 – Prostate indicators.

1. % of patients evaluated in the clinical session in the Radiation Oncology (RO) department before treatment
2. % of patients with stratification (including PSA, Gleason, TNM)
3. % of patients with MRI staging
4. % of patients presented to the tumour board
5. % of patients with tumour localized with fiducial markers
6. % of patients with tumour localized by CBCT
7. % of patients with tumour localized by ultrasound
8. % of patients who experience an interruption in treatment
9. % of patients completing treatment in the prescribed time
10. % of high risk patients receiving long-term hormonotherapy
11. % of high risk patients receiving boost brachytherapy
12. Time elapsed between first visit at RO department and initiation of any type of treatment
13. Time elapsed between first visit at RO department and start of radiotherapy (EBRT, BRT)
14. Time elapses between CT simulation and start of radiotherapy (EBRT, BRT)
15. % of patients treated using new technologies (IMRT)
16. % of patients treated using new technologies (VMAT)
17. % of patients treated using new technologies (SBRT)
18. % of EBRT sessions with imaging controls performed during the treatment (kV, MV, CBCT, MVCT)
19. % of patients with rectal mucositis (grade 2 or 3) (less than 6 months)
20. % of patients with rectal mucositis (grade 2 or 3) (more than 6 months)
21. % of patients with cystitis-urethritis (grade 2 or 3) (less than 6 months)
22. % of patients with cystitis-urethritis (grade 2 or 3) (more than 6 months)
23. Biochemical survival
24. Regular follow-up after the treatment (Yes/No)
25. Regular follow-up during the treatment (Yes/No)

Table 4 – Rectal indicators.

1. % of patients evaluated in the clinical session in the Radiation Oncology (RO) department before treatment
2. % of patients with TNM staging
3. % of patients with MRI staging
4. % of patients presented to the tumour board
5. % of patients with tumour localized with CBCT-IGRT
6. % of patients with tumour localized with kV-IGRT
7. % of patients completing treatment in the prescribed time
8. % of patients receiving concomitant chemotherapy
9. % of patients prescribed long course radiotherapy
10. % of patients prescribed short course radiotherapy
11. % of patients receiving intraoperative radiotherapy (IORT)
12. Time elapsed between biopsy and first consultation at RO department
13. Time elapsed between first visit at RO department and start of radiotherapy
14. Time elapsed between CT simulation and beginning of radiotherapy
15. % of patients treated using new technologies (IMRT)
16. % of patients treated using new technologies (VMAT)
17. % of EBRT sessions with imaging controls performed during the treatment (kV, MV, CBCT, MVCT)
18. % of patients with rectitis (grade 2 or 3) (less than 6 months)
19. % of patients with rectitis (grade 2 or 3) (more than 6 months)
20. % of patients with cystitis-urethritis (grade 2 or 3) (less than 6 months)
21. % of patients with cystitis-urethritis (grade 2 or 3) (more than 6 months)
22. Overall survival
23. Local control
24. Regular follow-up after treatment (yes/no)
25. Regular follow-up during treatment (yes/no)

The questionnaires were primarily based on those previously used in the aforementioned ICO study (data not published), which in turn were based on the questionnaires used in the Fundowicz study,[2] and the International Atomic Energy Association (IAEA) QUATRO model.[17] We elected to use an online questionnaire due to the numerous advantages: minimization of registration data errors, data centralization in a single database accessible to participant centres and located at ICO servers), and centralization of the statistical analyses (ICO), which will be performed with the SPSS statistical software (IBM, NY, USA).

2.5. *Implementation of the clinical audit*

The pilot study is planned for September, 2016 at the WCO in Poznan, Poland. The pilot audit will serve as a model for future audits once completed. Here, we describe the current plans for the pilot audit, but based on our actual experience, we may need to modify the timing and structure of the official clinical audit (tentatively planned for the 4th quarter of 2016). After the first pilot audit has been completed, a meeting will be held to discuss any issues that have arisen and to correct and/or improve the auditing procedure. This will involve all members of the participating institutions.

2.6. *Audit schedule*

A checklist will be created to organize the audit program and to ensure coverage of all relevant topics. The working language of the audit is English. The clinical audit will be performed as follows: a) audit preparation (appointment of auditing team, review of the background information prepared by the institution to be audited, and preparation of the audit program); b) entrance briefing: to introduce the auditors to various staff members and to discuss the methods, objectives and details of the audit; and c) assessment: on-site clinical audit.

2.6.1. *Two-day pre-audit training and verifications*

The role of the specialists from the SC is crucial to the successful outcome of the audit. For this reason, before the Local Team (LT) begins to conduct the actual clinical audit, two specialists from the SC team will meet with the LT (i.e., the clinical auditor[s] and local leaders) for 2 days for training and verifications to assure that all procedures are clear and that everything is in place to properly conduct the audit.

Specific tasks during this two-day pre-audit period include:

- Assure access to the database
- Train the LT and verify the forms by reviewing 2 cases (randomly selected from the sample) for each pathology while jointly (i.e., the local auditor and the SC specialists) completing the online questionnaires.

- Confirm the accuracy of the data reported on the general questionnaire (which contains details about the centre and procedures). This questionnaire will be sent to the audited centre well in advance of the audit.

During this two-day period, in addition to the aforementioned training and verifications, the SC team will interview staff members from the institution about work practices and approaches, inspect the facilities, and review all procedures and relevant documentation (including the treatment records of rectal and prostate cancer patients included in the study). In addition, the auditors will directly observe the practical implementation of working procedures during the audit, including as many aspects of the patient treatment process (initial patient examination, diagnosis, evaluation, staging, treatment planning and delivery, and follow up) as feasible.

The medical records are to be reviewed by an 'external team' (i.e., not dependent on the departmental heads) to assure a bias-free ("neutral") assessment of the data collected. The audit teams will consist of at least one auditor, who should be a nurse (or other qualified health care professional) specialized in health information management with >two years of experience in clinical reviews (ISO or similar). It is strongly recommended that the audit not be performed by a radiation oncologist or medical physicist from the audited departments.

2.6.2. *Clinical audit*

After the two-day training and verification period, the local team will carry out the clinical audit during which the auditor(s) will review 40 randomly-selected cases per pathology and complete a relevant questionnaire. Based on our previous experience, we estimate that the time required to perform the audit will be approximately 20–30 min per case. For this reason, the local auditor(s) will need at least 2 weeks to audit all 80 cases.

After the data collection has been completed, the TC will carry out a quality control analysis on the data and then conduct the statistical analysis. Once this has been performed, an exit briefing will be organized to provide the host institution with preliminary feedback.

The estimated duration of the entire process, including review of clinical records and statistical analysis, is approximately 45 days, as follows: 15 days to review the medical records and 30 days for quality control of the data, statistical analysis, and preparation of initial results. Technical support (video conference) will be available during this phase should any doubts arise.

2.7. *Expected results and study limitations*

The IROCA project was created to promote quality and efficacy in radiotherapy. The project will compare radiotherapy processes among the member institutions using a core set of quality indicators selected by consensus among the participating institutions. The main aim of this study is to determine institutional adherence to the consensus standards jointly established by the project members in accordance with the best available evidence, and to compare adherence to these standards across the various institutions to identify best practices. Our broader aims are to develop a clinical audit model for radiotherapy that can be easily adopted by other centres around the world, thus expanding the use of clinical audits to improve the quality of care. To our knowledge, this is the first project of its kind and scope.

There can be little doubt about the importance of quality control in any area of medicine. However, in the area of radiotherapy, already considered among the safest areas, the use of high-dose ionizing radiation is important to monitor quality because of the risk of patient harm.[18] Moreover, modern radiotherapy requires numerous procedures and processes involving a large number of health care professionals, including radiation oncologists, medical physicists, other physicians, nurses and technologists. As a result, quality control is essential to guarantee optimal quality throughout this complex process.

The importance of performing a clinical audit to ensure adherence to treatment protocols in medical care cannot be understated,[19] and this is especially true in radiotherapy, in which even small deviations from standards can have a large negative impact on treatment quality and outcomes. The benefits of using quality indicators to assess adherence to clinical protocols was recently demonstrated by Cheng et al.[9] Those authors evaluated 10 quality indicators to measure the quality of care in 1378 breast cancer patients. They found that most patients received good care (defined as reasonably good adherence to the quality indicators); however, they also found that 100% adherence to the entire set of quality indicators was significantly associated with better overall survival. This finding underscores the crucial importance of strict adherence to established clinical protocols; moreover, this result also demonstrates the value of performing a clinical audit to assess compliance: without a clinical audit, it is not possible to assess adherence. As the authors of that study conclude, "100% adherence to evidence supported quality-of-care indicators is associated with better survival rates and should be a priority for practitioners".

In recent years, there has been a surge in interest in quality control in radiotherapy. As Donaldson et al.[12] recently wrote, all health care practitioners share the goal of conducting "best practice" medicine but the obstacles to doing so are enormous given the vast amount of quality standards, guidelines, recommendations, and indicators currently available. For these reasons, Donaldson and colleagues argue that we need to identify the essential dimensions of quality. However, it can be challenging to select the appropriate indicators of quality, particularly given the wide range of procedures, processes and techniques in radiotherapy; moreover, treatment varies widely depending on the cancer type and location. Consequently, it is not possible to evaluate, for example, prostate cancer and rectal cancer, in exactly the same way. In other words, specific indicators are necessary, which is what we are attempting to develop with the IROCA project.

At the time of drafting this manuscript, we have not yet begun the IROCA pilot study; however, we believe that the groundwork laid thus far will be immensely valuable, not only for the current project but also for future clinical audits to be performed by other institutions. Importantly, in line with the study aims, we have developed detailed questionnaires that evaluate most (if not all) of the key aspects within the radiotherapy process that impact clinical outcomes and

treatment efficiency. In addition, we have identified and selected (by expert group consensus) the most relevant indicators to measure these key aspects. The selection of quality indicators in radiotherapy has been keenly debated in recent years.[3,20] In prostate radiotherapy, several groups have developed quality indicators[6–8] although it should be noted that many of these indicators have not yet been validated.

3. Conclusions

Although identification and selection of the most relevant quality indicators is essential, the design and implementation of the clinical audit is equally if not more important. Our experience with the previous audit has demonstrated the importance of establishing well-defined procedures for the clinical audit. In addition, we have learned that the selection and components of the audit team are critical to guarantee an unbiased audit. When this audit is completed, members of our group will have completed three separate radiotherapy audits: 1) the initial WCO/ICO audit[2], 2) the local audit performed by the ICO at its three centres, and 3) this multinational, multicentre audit involving five cancer care centres across Europe from Portugal to Poland. The experience gained in this project will provide knowledge that will be transferrable to other centres wishing to perform a similar clinical audit.

The primary value of this project is that it represents a step towards increased harmonization of radiotherapy processes among five large European radiotherapy departments. Although we do not expect to immediately unify all aspects of diagnosis, treatment, and follow up at these centres, we do believe that—upon completion of the project—we will have achieved a much more detailed understanding and appreciation for the need to compare clinical practice at the home institution to the practices observed at the audited institutions.

There is an inexorable and growing interest in improving quality control in radiotherapy and the role of clinical audits can only grow. The value of the present study is that the auditing protocol and quality indicators developed here to assess rectal and prostate cancer can be adapted to improve treatment of other tumour localizations at other radiotherapy centres worldwide. Although clinical audits are time-consuming and complex undertakings, the potential benefits in terms of identifying and rectifying deficiencies in quality control procedures are potentially enormous. External clinical audits can undoubtedly improve both patient safety and quality of care.

Conflict of interest

None declared.

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Results of a multinational clinical audit for prostate cancer radiotherapy: the IROCA project

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PURPOSE:

Despite the widely recognized value of clinical audits, relatively few have been performed in the field of radiation oncology. The IROCA project - Improving Quality in Radiation Oncology through Clinical Audits - is a multicentre collaboration among six European comprehensive cancer centres to conduct clinical audits to assess adherence to radiotherapy protocols for the treatment of cancer.

MATERIALS AND METHODS:

Multi-institutional retrospective cohort study of 240 randomly-selected patients who underwent radiotherapy for prostate cancer in 2015, at six European cancer centres located in Spain, Portugal, Poland, and Italy.

Participants were randomly selected from institutional databases, 40 patients each centre.

Clinical indicators were evaluated to assess three phases of care: 1) diagnosis and pre-treatment; 2) treatments administered; and 3) follow-up.

Specific indicators included (table 1): presentation to multidisciplinary tumour board (MTB) and departmental clinical session (DCS); clinical record keeping; diagnostic tests; clinical trials; time between first visit and starting of radiotherapy; type of treatment administered; dose/fractionation and treatment duration; treatment delays, interruptions, and compensations; radiotherapy technique and image guidance; adjuvant treatment; and registration of adverse effects (AE) and appropriateness of follow-up.

RESULTS:

The audits were conducted in 2017. We evidenced substantial inter-centre variability in clinical practice, particularly for the following indicators: 1) proportion of patients undergoing staging MRI (range, 27.5%-87.5%); 2) percentage of patients presented to the MTB (range, 2.5%-100%); days elapsed between the 1st visit to the radiation oncology department and radiotherapy initiation (range, 42-102 days); treatment interruptions ≥ one day (range, 7.5%-97.5%). The most common divergence from generally-accepted good clinical practice was inconsistent data registration.

CONCLUSION:

This multi-centre clinical audit reveals substantial inter-centre variability in clinical practice. Although overall adherence to clinical protocols and practices was strong, several areas amenable to improvement were detected, particularly with regard to data registration. These results show that external clinical audits are invaluable to identify areas of strength and weakness, which can then be used to improve radiotherapy practices. These findings underscore the value of conducting clinical audits and support the greater use of audits in the field of radiation oncology.

HOSPITAL		A	B	C	D	E	F	All	
		n	n	n	n	n	n	n (%)	P
Staging MRI		21 (52,5%)	35 (87,5%)	24 (60%)	30 (75%)	24 (60%)	11 (27.5%)	145 (60.4%)	<0.001
Case presented to the MTB		6 (15%)	10 (25%)	29 (72,5%)	38 (95%)	1 (2,5%)	40 (100%)	124 (51.7)	<0.001
Patients included in clinical trial		3 (7.5%)	1 (2.5%)	0 (0%)	0	0	6 (15%)	10 (4,2%)	0.003
Patient presented at RO department clinical session prior to treatment, n (%)		40 (100%)	40 (100%)	30 (75%)	0	40 (100%)	0	150 (62.5%)	<0.001
Median time (days) between initial visit to RO department and initiation of radiotherapy		102	83	66	42	77	70	78	<0.05
Treatment interruptions ≥ 1 day during EBRT		39 (97.5%)	39 (97.5%)	29 (72.5%)	16 (40%)	35 (87.5%)	3 (7.5%)	161 (67.1%)	<0.001
Compensation for treatment interruption		0	0	0	14 (87.5%)	0	2 (66.7%)	16 (9,9%)	0.011
Treatment-related AEs registered on the medical record	Yes	37 (92,5%)	33 (82,5%)	34 (85%)	18 (45%)	32 (80%)	3 (7,5%)	157 (65,4%)	<0,001
	No AE/missing	3 (7,5%)	7 ((17,5%)	6 (15%)	22 (55%)	8 (20%)	37 (92,5%)	83 (34,6%)	

Table 1: Specific indicators

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2. Methodology

2.1 Structures and departments cooperating in the project

This project involved multiple skills from both Hospital "Maggiore della Carità" - Novara and University "Università del Piemonte Orientale":

- Department of Translational Medicine – design of the study
- Department of Health Science – immunohistochemical reactions and specific antibodies
- Division of Radiation Oncology – IORT procedure, acquisition of data, follow up of patients
- Division of Pathology – pathological analyses
- Medical Physics – statistical analyses
- Division of Urology – biopsies and IORT procedure

2.1 Study population and IORT procedure

From September 2005 to May 2021, 132 patients were candidate to IORT + radical prostatectomy + lymphadenectomy, after specific informed consent in the framework of the study project.

Our local ethics committee, "Comitato Etico Interaziendale Novara – AASSLL BI, NO, VCO, AOU "Maggiore della Carità" di Novara", stated that no formal ethics approval was required in this case because all the analysis were performed on histological specimens with no changes in patients' pathway of treatment.

The policy of our institution is to allow investigations on patients' tissues for those who signed an informed consent for a surgical procedure.

All patients received and signed a specific informed consent before IORT and surgery.

The study was performed in accordance with the Declaration of Helsinki.

Inclusion criteria for IORT were the presence of at least two of the following factors:

- Gleason Score ≥ 7,
- clinical stage ≥ cT2c,
- initial PSA ≥ 10 ng / ml,
- more than 2/3 of bioptic samples positive.

Patients with diagnosis of intestinal inflammatory disease, evidence of lymph node involvement or distant metastasis, suspected extracapsular extension probability > 25% according to Memorial Sloan Kettering Cancer Center nomograms were excluded.

We prospectively selected 20 patients according to the quality of the data regarding the parameters to be investigated in the biopsy and in the surgical specimen.

In the following table, main patients' characteristics.

Table 1

Main clinical and pathological features of the 20 patients included in the in study

Characteristics	**Value (mean, IQR)**
Median age at diagnosis (min-max)	65 years (52-74)
Median performance status at diagnosis	90 (80-100)
Mean initial PSA (min-max)	17 ng/ml (4.47-41)
Neoadjuvant hormonal therapy	0
Pathological stage pT2c pT3a pT3b pT4	 2 (%) 4 12 2
Adjuvant external beam radiotherapy	18 patients

As described in Krengli et al [14], IORT procedure is performed after exposure of the anterior portion of prostate, section of the pubo-prostatic ligaments, and control of the deep dorsal vein plexus. First, the anterior–posterior prostate diameter and the distance from prostate surface to the anterior rectal wall was measured by intraoperative ultrasound (US). Based on clinical and US parameters, the appropriate collimator and beam energy were chosen to include the prostate gland and the surrounding soft tissues with a suitable margin for subclinical disease of 0.5 to 1 cm. The IORT was delivered by a dedicated linear accelerator (Mobetron, Intraop, Sunnyvale, CA) using an electron beam of 9 to 12 MeV and a total dose of 12 Gy. The dose was prescribed at the 90% isodose.

Use of IORT was followed by radical prostatectomy and regional lymph node dissection. Indication for postoperative radiotherapy and adjuvant hormonal therapy followed our institutional protocol. Postoperative external beam radiotherapy was delivered to the prostate bed about 3 months after surgery by three-dimensional conformal radiotherapy with four to six customized beams or dynamic arcs to a total dose of 46 to 50 Gy in 25 fractions (2 Gy/fraction).

From this pool of patients, we prospectively analyzed a homogeneous group of patients with high-risk disease who had not started neo-adjuvant hormonal therapy.

Immediately after surgery excision, prostate specimen was formalin-fixed and treated according to the routine procedures in pathology unit.

2.2 Pathological analysis

The expression of proliferation and apoptotic indexes was evaluated by immunohistochemical reactions and specific antibodies.

From paraffin blocks, 3-5μm-thick sections were cut with a microtome (Leica, mod. Histoslide 2000R, Germany).

The following antibodies were used:

- CONFIRM anti-Ki-67 Primary Antibody of Ventana Medical Systems (Ventana): a monoclonal rabbit antibody (IgG) specific for the C-terminal portion of Ki-67 antigen. This antibody is used to identify proliferating cells.
- CONFIRM anti-p53 Ventana®: a monoclonal antibody of the mouse (IgG1, kappa) specific for p53. This antibody is used to identify wild-type and mutated isoform. The wild type form has a short half-life, leading to a low concentration at cytoplasmic level. Most mutated proteins, however, increase the half-life of the protein itself and favor intranuclear accumulation.

Detection of specific antigens was achieved by incubating the slides with 10% normal goat serum (NGS; Vector Laboratories)–phosphate-buffered saline (PBS) to reduce non-specific binding, then with the following primary antibodies in 5% NGS overnight at 4C in a humid chamber: anti human cleaved caspase-3 (working dilution 1:200; Cell Signalling Technology Inc., Pero, Italy), anti-human caspase-9 and anti-Bax (working dilution 1:200; Santa Table 1. Cruz Biotechnology Inc., Santa Cruz,

CA, USA). Slides were counterstained with 4',6-diamidino- 2-phenylindole (DAPI), mounted with a medium for fluorescence (Vectashield; Vector Laboratories) and sealed with coverslips. Images were processed using a Leica fluorescence microscope equipped with a digital camera. The samples were then acquired with Pannoramic MIDI (3DHISTECH Ltd, Budapest). After immunofluorescent staining and acquisition, samples were opportunely treated and stained using haematoxylin and eosin.

Two operators analyzed immunofluorescence data. Bax, caspases 3 and 9 positivity were measured with 40x magnification, on two healthy tissue fields, four PINs fields and four neoplastic fields.

2.3 Statistical Analysis

Data were analyzed by a Medical Physicist expert in analyzing clinical data, with over 10 years of activity.

The results were analyzed using GraphPad Prism 4 software (GraphPad Software Inc., La Jolla, CA, USA). The apoptotic values highlighted with Bax expression in neoplasia and PIN areas with healthy tissue values were compared. Preliminarily, p53, Ki-67, and Bcl-2 correlations were evaluated. We used two different statistical tests: t-student parametric test and Wilcoxon non-parametric test. Results with p-value values <0.05 were considered significant.

3. Results

Characteristics of the patients including postoperative tumor staging are listed in Tab.1.

Median follow-up of the study cohort was 63.6 months ± 9 months.

The follow-up schedule consists of periodical three-months-visits with PSA dosages, urological evaluation, and ultrasound with transrectal probe.

Acute and late urinary and gastrointestinal toxicity were also evaluated.

Fourteen out of 20 patients (70%) experienced biochemical failure and no patient developed distant metastases. Bioptic specimens were withdrawn 32 days (mean 32 days, SD: 26–45) before surgery. By the use of the p53 antibodies of our study, a higher p53 expression is related to the presence of a mutated protein isoform, being the wild type protein quickly eliminated by intracellular systems.

Specimens from prostate biopsies showed that prostate cancer cells had a Bcl-2 mean value of 2.2% ± 1.9, Ki-67 of 4.5% ± 3.8, and p53 of 22.5% ± 6.8.

Table 2 shows the results of Bax analysis on neoplastic, pre-neoplastic and healthy tissue areas.

Table 3 shows the results of immunohistochemistry analysis, expressed as percentages of positivity of Ki-67, p53, and Bcl-2 in cancer cells following IORT.

No statistical difference was observed in terms of Ki-67, p53, and Bcl-2 expression levels between normal and neoplastic cells ($p > 0.05$).

Figure 1 shows a neoplastic (cancer 1), a PIN (PIN 1), and a healthy tissue field in

hematoxylin/eosin and immunofluorescence, and biopsy neoplastic fields in Bax immunofluorescence.

There were significant differences in Bax expression among healthy tissue, PIN and cancer fields, as resulted from Friedman ANOVA ($p < 0.0001$), comparing to the irradiated samples. The pairwise Wilcoxon test showed that Bax was significantly overexpressed in neoplastic ($p = 0.0001$), PIN fields ($p = 0.0001$) and healthy cells after IORT ($p = 0.003$) compared to biopsy specimens before IORT.

We found a significantly increase of Bcl-2 expression after IORT in neoplastic areas ($p = 0.0041$). No differences were found in p53 and Ki-67 expression before and after IORT in neoplastic cells.

From the multiple regression analysis, we did not find any correlation between p53, Bcl-2 and Ki-67 expression and Bax activation after IORT.

Furthermore, we observed a significant overexpression of Bcl-2 on cancer cells following IORT ($p = 0.004$), while no differences were found in p53 and Ki-67 expression prior and after IORT in neoplastic cells.

From the correlation between Ki-67, p53, and Bcl-2 values with the levels of expression of the Bax apoptotic protein, we observed that cancer cells receiving IORT had a greater trend towards apoptosis when Ki-67 levels were greater than 8.4% ($p = 0.064$). However, with multiple regression analysis, we did not find any correlation between p53, Bcl-2 and Ki-67 expression and Bax activation after IORT.

Interestingly, patients harboring p53 levels >18% and Ki-67 levels >8% on biopsy

specimens had an increased likelihood of being detected of extracapsular invasion ($p = 0.04$ for both parameters) and nodal positivity ($p = 0.042$ for p53 and $p = 0.0001$ at pathology for Ki-67). We chose the median value of 8% for Ki-67 to discriminate patients with high and low proliferative index. p53 value of 18% was chosen according to values distribution in our sample because it represented the median one.

Figure 2 show neoplastic (cancer 1), PIN (PIN 1) and healthy tissue field in the surgical specimen with hematoxylin/eosin staining and immunofluorescence for Caspases 3.

After IORT, average Caspase 3 and 9 expressions were 4.32 ± 0.89 in cancer fields, 6.46 ± 1.70 in PIN areas, and 3.27 ± 0.02 in healthy tissue cells (Table 4). There were no significant differences of expression of such proteins among neoplastic, pre-neoplastic, and normal tissue cells ($p > 0.05$). As far as Bcl-2 values are concerned, we observed that patients with levels of Bcl-2 prior IORT higher than 9% had an increased risk of biochemical failure ($p = 0.004$). The 9% threshold was chosen since it represented the median value in our patient sample. In Figs. 3–5, and Table 5, we reported box plots and the results to summarize our findings.

Table 2

Bax expression levels after and prior (last column) IORT expressed in table and box plot diagram

#case	Bax/DAPI (%) neoplasic fields	Bax/DAPI (%) preneoplasic fields	Bax/DAPI (%) healthy tissues fields	Bax/DAPI (%) Biopsy fields
#1	8.40	19.60	0.40	1.04
#2	8.81	21.09	4.84	3.4
#3	4.69	7.56	0.55	1.42
#4	6.74	17.80	3.10	1.16
#5	2.15	28.86	3.54	2.51
#6	17.02	24.42	4.41	0.46
#7	5.82	19.12	1.31	2.86
#8	2.50	34.73	2.17	1.91
#9	17.02	24.42	6.00	0.31
#10	8.38	17.67	2.57	0
#11	7.25	23.48	3.84	1.71
#12	12.08	10.85	0.41	1.12
#13	7.46	19.1	1.25	0.58
#14	8.42	31.56	1.98	0.96
#15	4.58	18.74	0.84	1.24
#16	3.21	21.48	2.74	0.98
#17	9.58	23.5	5.4	0
#18	6.47	9.15	2.96	0
#19	12.9	26.84	4.1	2.11
#20	7.25	23.9	3.84	1.90
Mean value ± standard deviation	8.04±4.15	21.19 ± 6.9	2.81 ± 1.69	1.28±0.96

Table 3

p53, Bcl-2, Ki67 expression (neoplastic areas after IORT) expressed in table and box plot diagram.

#case	p53 (%)	Bcl-2 (%)	Ki67 (%)
#1	7	4	4
#2	19	<1	17
#3	<1	23	9
#4	<1	18	5
#5	41	2	9
#6	86	19	16
#7	<1	<1	7
#8	39	<1	2
#9	18	<1	<1
#10	7	17	7
#11	20	19	7
#12	7	<1	18
#13	22	4	<1
#14	28	28	6
#15	10	<1	8
#16	25	10	21
#17	94	7	7
#18	<1	3	2
#19	32	15	<1
#20	45	2	7
Mean value ± standard deviation	24.9 ± 26.4	8.85±8.92	7.8±6.09

Figure 1: hematoxylin/eosin (H/E) in surgical specimen and immunofluorescence fields for Bax (DAPI/BAX) (pt #9) in surgical and biopsy specimens. In blue all DAPI (4',6-diamidino-2-phenylindole) positive cells (all nucleate cells), in red the cells that expressed Bax.

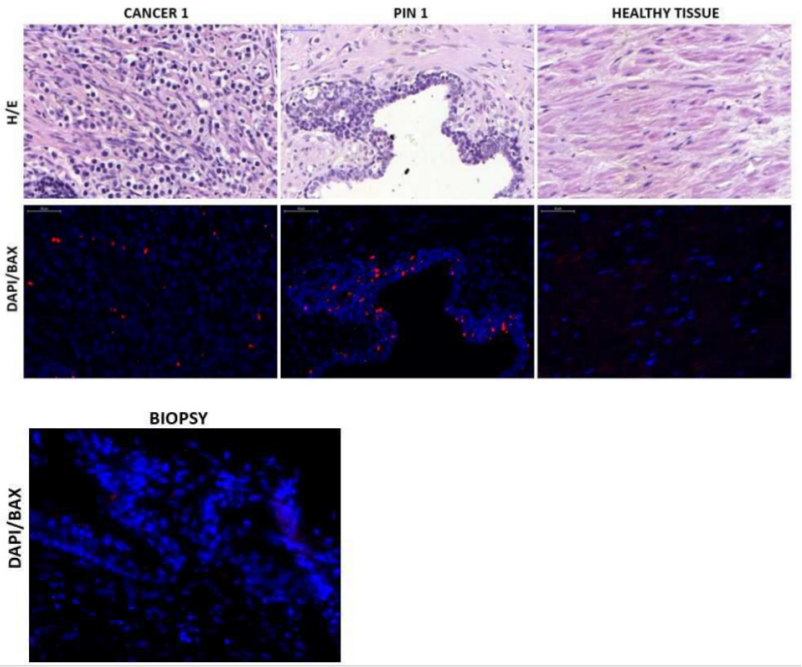

Figure 2: hematoxylin/eosin (H/E) and immunofluorescence fields for Caspase 3 (DAPI/BAX) (pt #9) in surgical specimen. In blue all DAPI (4',6-diamidino- 2-phenylindole) positive cells (all nucleate cells), in red the cells that expressed Caspases 3.

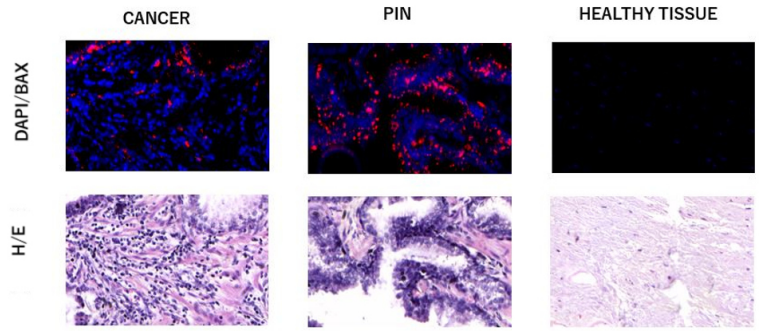

Table 4

Caspases 3 and 9 expression levels in the surgical specimen after IORT expressed in table and box plot diagram.

#case	Cas/DAPI (%) neoplasic fields	Cas/DAPI (%) preneoplasic fields	Cas/DAPI (%) healthy tissues fields
#1	4.12	6.49	3.24
#2	4.33	3.85	3.23
#3	6.49	11.85	3.28
#4	4.03	6.41	3.22
#5	4.98	6.44	3.26
#6	5.64	6.74	3.27
#7	4.12	6.11	3.27
#8	4.33	6.44	3.26
#9	2.66	2.1	3.29
#10	3.16	6.45	3.24
#11	4.31	6.72	3.28
#12	4.48	6.51	3.26
#13	5.01	6.43	3.24
#14	4.33	6.4	3.25
#15	3.64	6.25	3.27
#16	3.66	6.47	3.29
#17	5.64	5.98	3.26
#18	4.3	8.24	3.25
#19	4.79	6.45	3.25
#20	5.11	6.95	3.23
Mean value ± standard deviation	4.32±0.89	6.46±1.70	3.27±0.02

Figure 3

Box plot representation of Table 2 – Bax/DAPI expression in neoplastic (blue plot), preneoplastic (redplot), healthy tissue samples (black plot) after IORT and in bioptic specimen (yellow plot) before IORT.

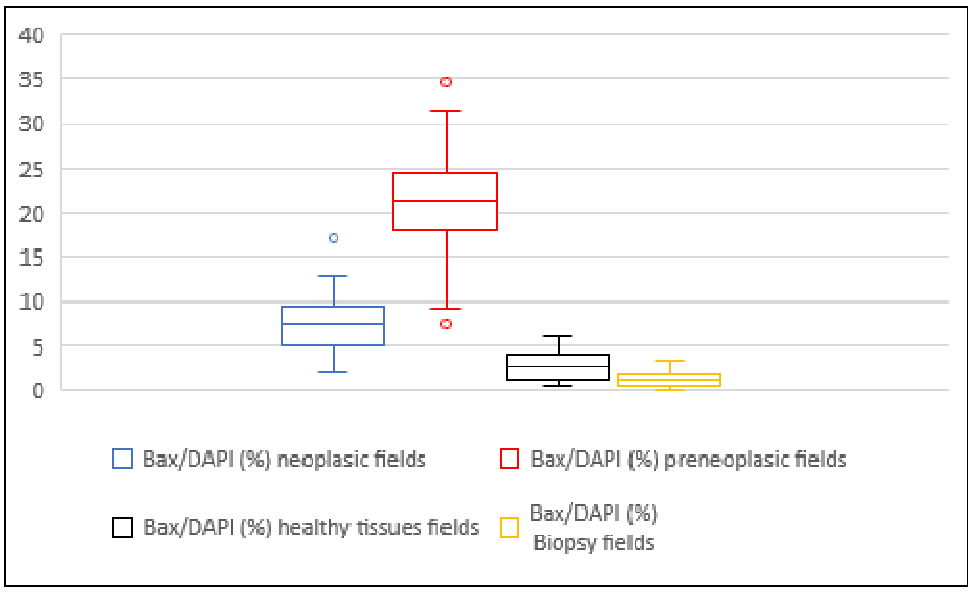

Figure 4

Box plot representation of Table 3 – p53 (blue plot), Bcl2 (red plot) and Ki-67 (black plot) expression in neoplastic fields after IORT.

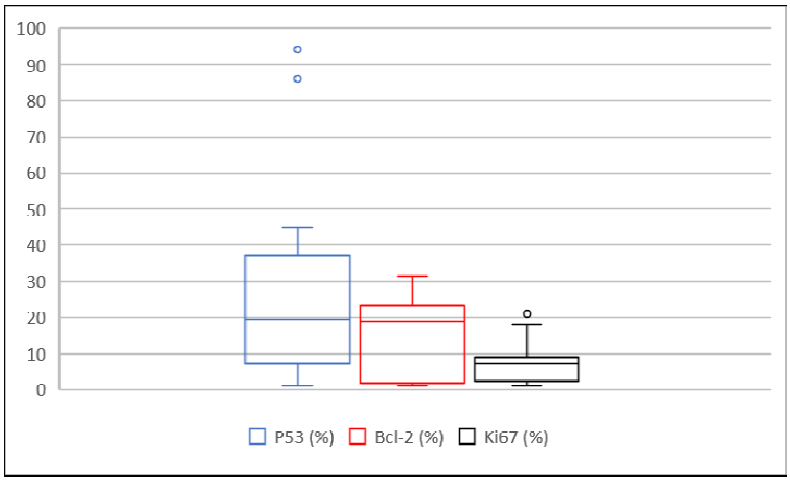

Figure 5

Box plot representation of Table 4 – Caspases/DAPI expression in neoplastic (blue plot), preneoplastic (red plot) and healthy tissue samples (black plot) after IORT.

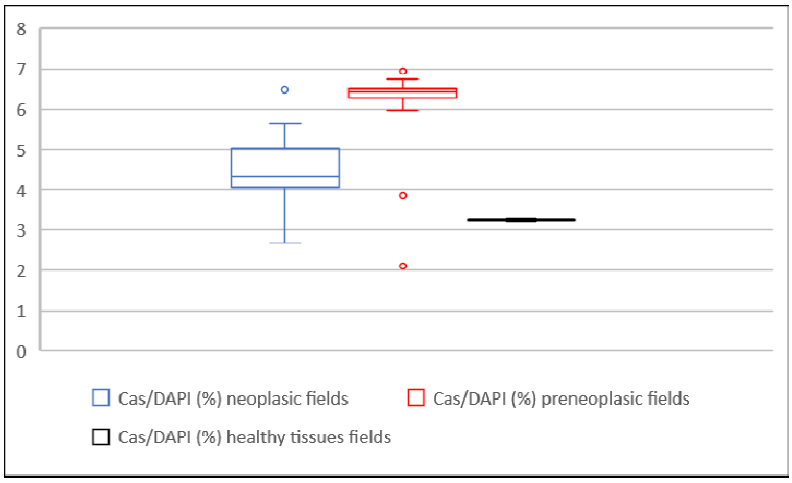

Table 5

Summary of results differentiated by protein values (% mean value ± standard deviation) and study time

Protein	% neoplasic fields after IORT	% preneoplastic after IORT	% healthy tissue fields sfter IORT	% before IORT (tumor area)
Bax	8.04±4.15	21.19±6.9	2.81±1.69	1.28±0.96
Caspases	4.32±0.89	6.46±1.70	3.27±0.02	/
p53	24.9±26.4	/	/	/
Bcl-2	8.85±8.92	/	/	/
Ki-67	7.8±6.09	/	/	/

4. Discussion

Patients with intermediate and high-risk prostatic cancer can experience biochemical recurrence after radical surgery or exclusive radiation therapy treatment.

Understanding the molecular pathways involved in apoptosis of prostate cancer cells in hypo-fractionated radiotherapy is still a daunting task for physicians. There is little evidence about radiobiological effects of single-shot radiation on prostate tissues, suggesting a possible endothelial damage to peritumoral vessels leading in turn to hypoxia and cellular death.

The interest of studying biomolecular changes after IORT resides in the possibility to better understand the mechanisms of cell death playing a role in extreme hypofractionation, which is a hot topic even for external beam radiotherapy of prostate cancer. IORT represents an ideal opportunity to investigate radiation related changes in tumor and healthy tissues just after irradiation and immediately before tissue withdrawn and pathology examination.

Some studies showed that hormonal therapy and a few chemotherapy drugs can induce apoptosis [25]. Starting from this premise, we included in the study only hormone-naïve patients.

We focused our analysis on the mechanisms related to the mitochondrial apoptotic pathway of cellular death following single-shot irradiation, evaluating the *in vivo* radio-induced damage received by tissues.

Prior to radiation, levels of Bax protein were significantly lower compared to PIN

and neoplastic cells treated with IORT ($p<0.05$). However, prior to a single-shot irradiation, neoplastic and pre-neoplastic cells do not express apoptosis proteins. This data suggests that IORT could be able to activate apoptosis in prostate cancer cells.

We observed that Bax protein is significantly increased in PIN cells ($p <0.0001$) and in cancer cells ($p = 0.006$) following IORT.

Interestingly, PIN areas appeared to be more sensitive to irradiation than normal prostatic tissue in our study population.

No significant correlation was observed between Bax expression and PSA at diagnosis or Gleason Score at histology, and no correlation between IORT and Caspases activation was noted.

Our data suggest that the activation of caspases occurs later than Bax pathway involvement. We did not investigate caspase expression in the biopsy specimen since there was no activation of apoptosis, according to the negative Bax results. In 1995, Raffo et al. first demonstrated that Bcl-2 oncoprotein could protect prostate cancer cells from apoptotic stimuli [26]. Now, there is evidence that proteins of the Bcl-2 family may play a role in the development of human malignancies and may act as key players in the process of programmed cell death.

Non-neoplastic prostate cells should express Bcl-2 levels of about 2-3% [27]. A review showed that Bcl-2 hyperexpression in tumor cells is associated with good prognosis in colorectal, breast, non-small cell, glioma, and gastric cancers. According to this review, measuring the levels of expression of Bcl-2 could be useful to stratify patients and understand the response to active treatments [28]. Other *in vitro* studies

demonstrated that Bcl-2 overexpression confers resistance to hormonal therapy among prostate cancer patients [29]. Our results are consistent with these literature data. Increasing expression of Bcl-2 following IORT in prostate cancer cells was associated with an increased risk of a local relapse. Based on our findings, it is reasonable to assume that the expression of Bcl-2 after IORT may activate intracellular mechanisms leading to radio-resistance.

Several studies investigated the predictive and prognostic role of Bax and Bcl-2 family proteins. [30, 31]. Clinical data from RTOG 86-10 and RTOG 92-02 showed that only Bax expression at a normal level was associated with significantly more favorable outcome [32]. *In vitro* data showed conflicting results with studies without significant differences in the expression of p53, Bcl-2 and Bax 2 and 4 hours after 10 Gy in to cell lines [33] and studies showing that single shot irradiation could induce Bax-mediated cell death *in vitro* [34. Our work seems to show that this process could happen *in vivo* as well.

To our knowledge, this study is the first describing that a single-shot irradiation may induce Bax-mediated cell death in patients receiving IORT, that represents an *in vivo* irradiation modality, allowing a rapid subsequent pathological examination of the irradiated tissue. PIN areas are closely related to the presence of prostate cancer.

At the time the manuscript is written, all literature data agree that neoplastic areas are related to intracellular mutations in pre-neoplastic areas and PIN morphological alterations have been shown to be associated with an amplified replication index [35]. In an animal model, Xie et al. demonstrated that pre-neoplastic cells with Bcl-2

hyperexpression have higher proliferative index, and increased expression of Bax.

An increased apoptotic rate in high grade pre-neoplastic cells probably implicates that apoptosis may accelerate cellular turnover in premalignant lesions of the prostate. According to this model, the well differentiated neoplastic cells possibly developed a genetic profile of natural resistance against apoptotic stimuli [36]. We could hypothesize that PIN cells are most susceptible to irradiation, since they already have a high turnover.

Ours is one of the first studies showing that *in vivo* pre-neoplastic cells are more susceptible to apoptosis after single dose irradiation than neoplastic prostate cells. Worthy of note, cancer cells present a significantly lower Bax positivity profile than PIN areas, most likely due to a relative radio-resistance induced by cancer transformation.

In some neoplasms, such as breast cancer, a correlation between Ki-67 value, and response to adjuvant treatments was observed. The literature about prostate cancer radiobiology is still poor. Most likely, Ki-67 values in prostate carcinoma would be extremely heterogeneous as observed by Mesko et al. who reported values ranging between 1.1 and 10.1% [37]. Ki-67 is higher among patients with locally advanced prostate cancer. *In vitro* studies showed that higher-proliferating cells were also those that tend to hyper-express apoptotic proteins after extracellular stimuli [28].

In our sample, patients had a mean Ki-67 value of 7.8% ± 5.1%. We chose the median value of 8% to discriminate patients with high and low proliferative index. In this regard, we observed that cells with Ki-67> 8% had an increased trend towards

apoptosis (p=0.0641). Therefore, even *in vivo*, there could be an increased sensitivity to single shot irradiation with the increase of the proliferation index.

In our biopsy samples, higher proliferation index and higher p53 expression were associated with worse pathological tumor stage, higher incidence of extracapsular extension, and higher risk of nodal disease. Our data are in concordance with those from previous studies by Saidi et al. [38] and Berlin et al. [39]. Relying on our results, we could hypothesize that p53 protein and Ki-67 could be used as prognostic factors. These data may be interesting in routine clinical practice, since there is no current cancer prognosticator of extra-prostatic extension.

In p53 mutated neoplastic cells, we observed a lower expression of Bax (p=0.47977), while there was a significant increase in expression of Bax in PIN areas (p=0.04239) and in healthy tissue areas (p=0.01941). p53 responds to radiation-induced damage in several ways, such as inducing cell cycle arrest and activating apoptosis [40]. Some *in vitro* studies highlighted that the activation of p53 protein increases the radio sensitivity of prostate cancer cells [41]. On the opposite, other studies concluded that p53 expression does not influence radiation sensitivity in prostate carcinoma [42, 43]. Our *in vivo* study confirms, indeed, that neoplastic cells with mutation in p53 are less sensitive to apoptosis induced by single dose irradiation than healthy cells and surrounding PIN areas. It can be reasonably hypothesized that in PIN and healthy cells p53 protein is still functioning and it is able to trigger the apoptosis after the radio-induced damage.

The high-dose-fraction radiobiology is a complex and foggy topic. Our study is only a small step towards understanding these mechanisms, in fact this issue must be at the basis of our studies, at a time when stereotaxic radiotherapy and immunotherapy are increasingly being used.

A better understanding of ionizing radiation effects will allow clinicians to optimize radiation therapy treatments, not only in prostate carcinoma.

By using IORT, we observed an increase of selective apoptotic death; on the other side, the biological mechanisms of other hypofractionation (HFRT) modalities (such as stereotactic radiotherapy SBRT and radiosurgery SRS) have been elusive.

In treating neoplastic disorders with SRS, the targets are irradiated with 15–25 Gy in 1–2 fractions or with SBRT, tumors are treated with 30–60 Gy in 2–5 fractions.

About hypofractionation in prostate cancer, the previously mentioned Zaorsky et al. [11] published an extensive review concerning the history of HFRT. The first experience was made at St. Thomas Hospital in London, where 200 patients were treated with a dose of 55 Gy in 12 fractions and later on with a dose of 36 Gy in 6 fractions, showing low rate of rectal and urological complications [44, 45]. The trial included men with early (T1-T2) and advanced (T3-T4) disease treated by external beam radiotherapy.

Scientific evidence increased over time and moderate hypofractionation regimens, 2.4–3.4 Gy per day over 20–30 treatment sessions, have been studied extensively. Three non-inferiority randomized clinical trials demonstrated efficacy and safety of this approach [46-48] and one superiority trial showed improved outcomes with no

increase in toxicity [49], establishing it as the preferred regimen for localized prostate carcinoma [50]. Extreme or ultra-hypofractionation radiotherapy regimens deliver more than 5 Gy per fraction and one randomized trial confirmed oncologic non-inferiority compared with conventional fractionation [51 and another randomized trial proved that SBRT has equivalent acute toxicity profile [52]. At the time the manuscript is written, ultra-hypofractionation is listed as a standard radiation option for all patients with localized disease in the NCCN guidelines [3].

Even if there are many clinical studies, only few analyzed the radiobiology of hypofractionation.

Literature studies showed that linear-quadratic model underestimates tumor control by hypofractionation [53], indicating that additional mechanisms could play a role, in addition to DNA strand breaks and chromosome aberrations; one of them may be significant vascular damage in tumors from hypofractionation, leading to indirect cell death [54,55]. Another study proved that ablative hypofractionated radiation therapy with dose higher than 10 Gy per fraction increases tumor-killing with the stimulation of apoptosis [56].

Not many data are available about intracellular modification induced by hypofractionation in prostate carcinoma.

Grellier et al [57] in a recent review, analyzed specific supposedly biological effects of high doses per fraction, such as vascular effects and anti-tumor immune effects. The vascular damage caused by high doses leads to degradation of the intra-cellular environment which in turn leads to secondary cell death. These phenomena are

accompanied by the release of tumor antigens and pro-inflammatory cytokines promoting anti-tumor immune response [54].

The major effect is unquestionably the apoptosis of the endothelial cells demonstrated by Garcia-Barros [58]. After transplanting fibrosarcoma cells into mice, the authors showed the apoptotic activation within the first hours after 11 Gy irradiation. Other mechanisms had been demonstrated: from 10 to 15 Gy, a collapse of the tumor vessels can occur, linked to eruption of plasma proteins and to increase in interstitial pressure [59]. Furthermore, we considered the role of tumor stem cells. There are different kinds of stem cell: some extremely hypoxic, in the center areas of the tumor, and others, perivascular, for which endothelial cells play an important role in maintaining tumor stem cells in their condition. The role of these cells during apoptosis has still to be assessed.

Anti-tumor immune effect begins as soon as the tumor cell dies. The release of tumor antigens allows the activation of dendritic cells. The three most important antigens are: calreticulin, release of ATP (adenosine triphosphate) in the tumor microenvironment, the release of high-mobility group protein B1 (HMGB1). All these elements activate dendritic cells via "Toll like receptor" TLR4 and allow optimal presentation of the antigen [60, 61].

Dentritic cells stimulate T lymphocytes through presentation of tumor antigen to T cell receptor. This will result in an acquired anti-tumor immune response [62, 63].

Preclinical studies reported an increase in the antigens released after irradiation at high doses, an improvement in the repertoire of T cell receptor after 6 to 8 Gy, as

well as an increase in the expression of the major histocompatibility complex of type I on the surface of tumor cells with 10 Gy doses, allowing better presentation of endogenic antigens to cytotoxic T lymphocytes [64,65]. Other studies have showed an accumulation of tumor DNA in the cytosol after 3-8 Gy irradiation allowing increasing level of interferon. This phenomenon participates in the activation of antigen presenting cells. In contrast, irradiation at higher fractional doses, greater than 12-18 Gy, induces Trx1 DNA-exonuclease which degrades cytosolic DNA, which may reduce the anti-tumor immune response [66].

5. Limitations and future perspectives

We acknowledge that our study has some limitations. This is a single center study based on a relatively small sample. Furthermore, we are conscious that in our study we have not investigated intracellular changes linked to hypoxia, which could be investigated. In the near future, we would like to study the expression of a transcription factor protein, hypoxia-inducible factor 1alpha (HIF-1alpha), to differentiate tissue changing related to surgery stress and IORT. However, the original design of our study, based on a translational research approach, has the strength to first report *in vivo* novel findings of molecular biology of mechanisms of apoptotic pathway in prostate cancer cells treated with single-dose radiotherapy.

6. Conclusions

From our study in the prostate cancer model, it emerges that mitochondrial apoptosis and Bax pathway is activated within 90 minutes from the irradiation. Apoptosis is significantly activated in neoplastic cells and in PIN area, while it does not appear in healthy cells and in surrounding stroma.

IORT-induced damage would therefore be specific in neoplastic cells, while healthy tissues would be spared from induced death, probably because of the preservation of anti-apoptotic mechanisms. PIN areas would then be more susceptible to radio-induced damage.

From our analysis, it emerges that neoplastic cells with higher proliferating index are more responsive to radio-induced damage. On the other hand, higher Ki-67 and mutated p53 cells are predictive for higher pathological staging, extra-capsular involvement and nodal disease. Mutated p53 is also predictive for radio-resistance. We also noticed that pre-operative and post-operative Bcl-2 could predict biochemical failure.

These elements might help stratifying patients, allowing the selection of patients who could benefit the most from intraoperative irradiation, and probably from all highly hypofractionated treatments.

Although an increasing number of cancer patients are treated with hypofractionation in recent years, the biological mechanisms of these new modalities have not been fully understood yet.

Further understanding of the biological mechanisms of death induced by higher doses per fraction could be a way to potentiate the anti-tumor efficacy. Only through a better understanding of how high doses of ionizing radiation act, we could refine our treatments in the future.

Data from this study were presented at ESTRO 35 in 2016 and ISIORT in 2018

Conclusion: Herein c-Myc acts as a key master regulator of in vitro migration, invasion and radioresistance. In fact, c-Myc depletion alone seems to be sufficient to block the in vitro pro-metastatic abilities and to radiosensitize ERMS cells. In addition, our data suggest c-Myc as important, but not essential, in controlling the molecular machinery responsible for cancer neo-angiogenesis. In conclusion these data strongly suggest that the targeting of c-Myc can be tested as a promising strategy for an anti-cancer therapy.

EP-2063

Apoptotic pathway activation in prostate neoplastic cells after 12 Gy-IORT

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Purpose or Objective: To evaluate apoptotic pathways involved in prostate cancer treated with intraoperative radiotherapy (IORT) with 12 Gy, studying the effects on cancer cells, prostatic intraepithelial neoplasia (PIN) and normal cells

Material and Methods: Since 2005, 111 patients treated at University Hospital of Novara, Italy with local advanced prostate adenocarcinoma were treated with radical prostatectomy and 12 Gy IORT followed by 50 Gy postoperative radiotherapy. In this setting, we selected a sample of 10 patients for a preliminary feasibility study. Selection criteria for this phase were: no neoadjuvant hormone therapy, Gleason score > 7. Proteins involved in the apoptotic cascade (Bax, Caspases -3 and -9) were studied before and after 12 Gy single shoot in neoplastic cells, high grade PIN areas and in normal prostate cells. Immunofluorescent detection of antigens (anti-Bax, anti-caspases-3 and -9), were performed on bioptic sample and on surgical specimens 5-mm slices. On surgical specimens there were also detected Bcl-2, and ki-67 with immunoistochimical analysis. A count of positive spots for immunofluorescence (Bax+, Caspases-3 and -9+/all nuclei, 40x magnification) was performed on tumor cells, PIN, healthy tissue areas. Bax and caspases immunofluorescent positivity was compared in different areas and in neoplastic areas before and after single shoot high dose

Results: A significant increase in Bax, Caspases-3 and -9 expression was detected in tumor and PIN areas comparing IORT treated and untreated samples ($p<0.05$). After 12 Gy-single dose, healthy areas expressed significantly lower level of Bax and caspases positive with respect to neoplastic cells ($p<0.0001$), while in PIN areas, Bax positive cells were significantly more present than in neoplastic areas ($p=0.0001$). Mean Bcl-2 in neoplastic cells is 17% (range: 1-23), mean ki-67 in neoplastic area is 4.5% (range: 1-17). With multivariate analysis, we find that cancer cells with Ki-67 ≥ 8% show a trend toward greater expression of Bax ($p=0.0641$)

Conclusion: After 12 Gy irradiation, Bax and caspases resulted overexpressed in tumor and PIN cells, in particular in prostate cancer with higher proliferation index. PIN areas seem to be more radiosensitive than neoplastic areas and healthy cells do not activate apoptosis after single shoot, showing an intrinsic radioresistence. This preliminary study represents the basis for an extensive work in which we would correlated clinical parameters with pathology and apoptotic factors. In fact, the comprehension of these relationships could allow to better understand the mechanisms of high dose per fraction and, radioresistence in order to personalize treatments

EP-2064

Radiation induces metabolic switch to lactate production to support tumour cell survival

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Purpose or Objective: Purpose: Radiation treatment of tumor cells resulted in a reduction of endogenous ATP levels. Aim of this study was to elucidate the molecular scenario standing behind this observation.

Material and Methods: Endogenous ATP-levels were dermined by ATP-ELISA. HIF1a, PDK1, LDH and PDH expressions were visualized by western blotting. Lactate production was quantified by lactate-assay. Cellular survival was proved by clonogenic survival assay.

Results: Results: Ionizing radiation induced expression of Hif1 alpha even at clinical relevant doses of 2 Gy. Hif1alpha induced activation of mitochondrial PDK1, which results in PDK1 dependent phosphorylation of pyruvate dehydrogenase (PDH). PDH is responsible for conversion of pyruvate to acetyl-CoA, which fuels the TCA cycle. Thus, irradiation blocks TCA cycle and mitochondrial activity. Simultaneously Hif1alpha induced expression and activity of lactate dehydrogenase (LDHA) to convert glucose to lactate. Indeed we observed a clear increase in lactate production in tumor cell lines in response to irradiation. Furthermore, inhibition of PDH activity was associated with mitophagy and ATP-depletion, which explains the radiation induced ATP drop down. In addition, this radiogenic switch to lactate production reduced production of mitochondrial derived radicals and increased cellular radio-resistance. Pretreatment with the Hif1 alpha inhibitor BAY87-2243 prevented the radiogenic switch to lactate metabolism and radio-sensitized the tumor cells. In addition, tumor cells are strictly dependent from high glucose supply after irradiation and can be radio-sensitized by blockage of radiogenic glucose uptake with glucose transporter SGLT inhibitor Phlorizin.

Conclusion: In summary, we could show, that tumor cells switch in a Hif1 alpha dependent manner to anaerobe glucose metabolism to generate ATP, which renders cells radio-resistant. Blockage of Hif1 alpha stabilization or blockage of glucose uptake radio-sensitized tumor cells.

EP-2065

Effects of spontaneous γH2AX level on radiation-induced response in human somatic cells

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Purpose or Objective: Phosphorylated histone H2AX (γH2AX) foci are well-known markers of DNA double-strand breaks in human cells. Spontaneous γH2AX foci form on unrepaired DNA double strand breaks, shortened telomeres and sites with altered chromatin conformation. The presence of such permanent γH2AX foci in cell is an important component of epigenetic background and potentially lead to the activation of DNA repair system. The objective of this study was to analyze the effects of spontaneous γH2AX level on radiation-induced response in human somatic cells.

Material and Methods: Spontaneous γH2AX foci and radiation-induced micronuclei were analyzed in peripheral blood lymphocytes of 54 healthy individuals after exposure to 2 Gy ionizing radiation in vitro. Further, a transcriptome analysis was performed using gene expression microarrays in lymphocytes of two sub-groups of individuals: 1)

Intraoperative radioterapy (IORT) in the multimodality treatment of locally advanced prostate cancer

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PURPOSE: The treatment for locally advanced prostate cancer is a controversial issue and multimodality treatment can lead to treatment optimization. The aim of this study is to describe technical and clinical aspects of intra-operative radiotherapy (IORT) in patients with high risk prostate cancer. **MATERIAL/METHODS:** A total of 136 patients were enrolled. The statistical analysis was performed in 112 patients with follow up > 12 months. Inclusion criteria were patients age < 76 years, KPS > 90 and at least 2 of the following preoperative risk factor: initial PSA (iPSA) > 10 ng/ml, Gleason Score ≥ 7, clinical staging > cT2c according with TNM, probability of organ-confined disease < 25%. Median age was 66.9 years (range 51-83), median iPSA was 14.8 ng/ml (range 2.0-154) and median Gleason Score (GS) was 8 (range 4-10). After surgical exposure of the prostate, IORT was delivered by a dedicated linear accelerator (Mobetron, Intraop, Sunnyvale, CA) with 30° beveled collimator, using an electron beam of 9-12 MeV to a total dose of 12 Gy. IORT was followed by radical prostatectomy and regional lymph node dissection. Rectal dose was measured “in vivo” by radio-chromic films placed on a rectal probe. All cases with pathological staging≥ pT3a, positive margins (R1) or metastatic lymph nodes (N1) received postoperative external beam radiotherapy (EBRT), delivered to surgical bed with 3D conformal technique or intensity modulated radiation therapy to a total dose of 46-50 Gy (2Gy/fraction). Patients with pT3 or pT4 disease and/or N1 received adjuvant hormonal therapy. **RESULTS:** IORT procedure lasted in average 30 minutes (range 15-50). No major intra- or post-operative complication occurred. Median dose to the anterior rectal wall was 4.32 Gy (range 0.06-11.3). Pathological stage was: 32 pT2, 97 pT3, 7 pT4. 83/136 (61,0%) patients were R1 and 45/136 (33,1%) patients were N1. Median post-operative PSA was 0.09 ng/ml (range 0-5.05). Post-operative radiotherapy was delivered to 106/136 patients (77.9%) with pathological staging ≥ pT3a or R1. Hormone therapy was prescribed to 88/136 patients (64.7%). Acute toxicity was: 22 G2 (12 GU; 10 GI), 3 G3 (2 GU; 1 GI). Late toxicity was: 11 G2 (5 GU, 6 GI), 4 G3 (2 GU; 2 GI). No G4 acute or late toxicity was observed. Twelve patients died of prostate cancer. With a median follow-up of 81 months (range 12-132), 34/112 patients experienced biochemical failure. Overall biochemical free survival (BFS) was 60% at 5 years. 5 years BFS was 81% and 55 % in high and very high risk classes according to NCCN classification. No macroscopic failure in the prostate surgical bed was observed. **CONCLUSIONS**: IORT during radical prostatectomy is a feasible procedure and allows to deliver safely post-operative EBRT to surgical bed without a significant increase of toxicity. With a median follow-up of 81 months, biochemical control seems to be optimal in particular for high risk patients.

Phase I/II trial of surface kilovoltage bra chytherapy in ocular conjuntival carcinoma: Preliminary results

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Purpose: To determine the safety dose and toxicity profile of adjuvant kilovoltage brachytherapy in post resected ocular conjunctival carcinoma. **Materials and methods:** Between October 2014 and June 2017, at the National Institute of Neoplastic Diseases from Peru, 39 patients with squamous cell carcinoma of ocular conjunctiva, T1 - T3, resected, were selected to adjuvant treatment. The portable accelerator of 50 kV INTRABEAM® (Carl Zeiss Meditec) was used, previous local anesthesia and blocking of ocular muscles movement. The doses used were 18 Gy for patients with free margins and 22 Gy for positive edges, according to calculation of equivalent dose of 2Gy per fraction of 46 and 66 Gy respectively, assuming a tumoral α/β ratio of 8 Gy. The prescription was done to 2 mm depth.

Measurement of peripheral dose to pelvic region during breast intraoperative electron radiation therapy

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OBJECTIVE: This study aimed to measure received dose to pelvic region of patients during breast intraoperative electron radiation therapy (IOERT). Furthermore, we compared the findings with these of external beam radiation therapy. Finally, second ovary and uterus cancer risks following breast IOERT were estimated. **METHODS:** In the current study, the received dose to pelvic surface of 18 female patients during breast IOERT boost were measured by thermoluminescent dosimeter (TLD-100) chips. All patients were treated by a 12 Gy as single fraction. Then, for estimation of the received dose to ovary and uterus of the patients, conversion coefficients of depth to surface dose were obtained in Rando phantom. Given the received dose to pelvic region of the patients, second ovary and uterus cancer risks following breast IOERT were estimated. **RESULTS:** The mean received doses to pelvic surface (ovary and uterus surface) of the patients for 8 and 10 MeV electron beam energies were 9.635 ± 7.286 mGy and 6.873 ± 5.244 mGy, respectively. Corresponding intra-pelvic (ovary and uterus) regional doses were 0.475 ± 0.341 mGy and 0.431 ± 0.331 mGy for 8 and 10 MeV electron beam energies. Findings demonstrated that the ratio of the received dose by pelvic surface to regional dose during breast IOERT was much less than that of external beam radiation therapy. The mean of the second cancer risks for ovary in 8 and 10 MeV electron beam energies were 1.054×10-4 and 1.427×10-4, as well as for uterus were 1.358×10-5 and 6.070×10-6, respectively. **CONCLUSION:** According to our finding, the use of breast IOERT in pregnant patients can be considered as a safe radiotherapeutic technique, because the received dose to fetus was lower than 5 cGy. Furthermore, IOERT can efficiently reduce the unnecessary dose to the pelvis region and lowers the risk of second ovary and uterus cancer following breast irradiation. ADVANCES IN KNOWLEDGE: As an alternative procedure for external beam radiation therapy, the use of IOERT technique in the breast cancer patients can be useful, especially in pregnant patients.

In vivo radiobiological analysis of prostate carcinoma treated with 12 Gy single-shot IORT.

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Purpose: To evaluate apoptotic pathways in prostate cancer treated with intraoperative radiotherapy (IORT), studying the effects on cancer cells, prostatic intraepithelial neoplasia and healthy cells. We evaluated correlations between p53, Bcl2 and ki67, pathological staging and local control. **Material and Methods:** We selected 20 patients. Proteins involved in the apoptotic cascade (Bax, Caspases -3 and -9) were studied before and after 12 Gy in neoplastic tissues, high grade PIN areas and in healthy prostate cells. Immunofluorescent detection of antigens (anti - Bax, anti - caspases - 3 and - 9), were performed on bioptic sample and on surgical specimens 5-mm slices. Before and after IORT, also Bcl2, p53, and ki67 with immunoistochimical analysis were detected. A count of positive spots for immunofluorescence (Bax, Caspases/all nuclei) was performed on tumour cells, PIN and healthy tissue areas. Bax and caspases immunofluorescent positivity was compared in different areas and in neoplastic areas before and after single shot high dose. **Results:** Before IORT, mean Bcl2 in neoplastic cells is 2.23% (range: 1-23), mean ki-67 in neoplastic area is 4.5% (range: 1 -17) and mean p53 is 22.5% (1 - 36). After IORT mean Bcl-2 in neoplastic cells is 8.85% (range: 1 - 28), mean ki-67 in neoplastic area is 7.8% (range: 1 - 18) and mean p53 is 24.9% (1 - 94). A significant increase in Bax expression was detected in tumour and PIN areas comparing treated and untreated samples ($p < 0.05$). After 12 Gy - single dose, healthy areas expressed significantly lower level of Bax positive with respect to neoplastic cells ($p < 0.0001$), while in PIN areas, Bax positive cells were significantly more present than in neoplastic areas ($p = 0.0001$). Results about Caspases 3 and 9 were conflicting and we did not find significant differences in expression between neoplastic and healthy tissue cells after IORT. With multivariate analysis, we find that cancer cells with ki67 ≥ 8% show a trend toward greater expression of Bax ($p = 0.0641$). We do not find correlations between ki67 and caspases activation. We

also found an increasing in Bcl2 expression after IORT in neoplastic areas ($p = 0.0041$); with multivariate analysis, we found that neoplastic cells with higher Bcl2 expression after IORT had a worsen local control with higher incidence in biochemical failure. Bioptic specimens with p53 higher than 18% and ki67 higher than 8% had worst post-operative staging with higher incidence in extracapsular invasion ($p < 0.05$) and nodal positivity ($p < 0.05$). **Conclusion**: After 12 Gy, Bax is overexpressed in tumour and PIN cells. PIN areas seem to be more radiosensitive than neoplastic areas and healthy cells do not activate apoptosis after single dose, showing an intrinsic radioresistance. Pre-operative ki67 and p53 definition could be use in clinical practice to predict patients with worsen pathological stage, while Bcl2 activation after IORT might be predictive factor for failure.

Outcomes and toxicity of electronic superficial Brachytherapy for Non-melanoma Skin cancer in Taiwan

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Introduction: The incidence of non-melanoma skin cancer (NMSC) is high and reaches the 8th rank among all malignancies in Taiwan. Although the mortality of NMSC is low, the incidence keeps on rising. While surgical approaches are the standard treatment, critical lesion sites such as nose, ears, eyelids and lips requires plastic reconstruction with great costs. Instead, a new treatment option for skin cancer is by using Axxent® superficial electronic brachytherapy (EBT) system. The Axxent EBT has surface applicators of four different sizes for delivering 50kV x-ray to the target region.The surface applicator is cone-shaped and also be used as irradiation shield. Therefore, the radiation protection is much easier than systems using radioactive isotopes and thus can be used in most outpatient treatment rooms. The radiation treatment is delivered by hypofractionated course as 8-10 fractions in 4-5 wks, which is far more convenient than the 35 fractions using conventional Linac-based treatment. Besides, the surrounding normal tissue toxicities are reduced due to the nature of brachytherapy. Since NMSC treatment using superficial brachytherapy is very new in Taiwan, we present our experiences (the First in Taiwan) employing Axxent device for cT1-T2 curative treatment. **Material and Methods:** 43 patients with 48 NMSC lesions were treated with EBT to a dose of 40 -50 Gy in eight-ten fractions, delivered twice weekly from June 2015 to December 2018 in Kaohsiung Municipal Ta-Tung Hospital. A commercial head mask fixation device based on thermoplastic materials was used to minimize head movement during radiotherapy. The target lesion depth were evaluated by combined methods of CT images and biopsy. An appropriate size of surface applicator was selected to provide best treatment coverage with acceptable margins. At follow-up, patients were assessed for cosmesis and local control. **Results:** 43 patients (mean age: 74.9years, range: 47-97) with 48 cutaneous malignancies were treated. Tumour reponses and complications were recorded at weekly basis. Most acute reactions were among Grade I-II and all wounds were healed by 6 wks after last treatment session. There has 1patient recurrence to date with a mean followup of 15.4 months (range: 2-31 months). Cosmesis were rated good to excellent for 100% of the lesions at follow-up. **Conclusions:** Treatment of local NMSC with EBT using surface applicators show great effectiveness with low recurrence and favorable cosmetic outcomes. The EBT provides a nonsurgical treatment option for NMSC patients.

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Apoptotic and predictive factors by Bax, Caspases 3/9, Bcl-2, p53 and Ki-67 in prostate cancer after 12 Gy single-dose

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Radio-induced apoptosis is mediated by the activation of tumor protein p53, Bax and caspases. The purpose of this study was to investigate the early activation of this pathway in men receiving *in vivo* irradiation immediately before radical prostatectomy for locally advanced prostate cancer. We also investigated cell proliferation index (Ki-67), proto-oncogene (p53) and anti-apoptotic protein (Bcl-2) levels as potential predictive factors. We selected a homogeneous sample of 20 patients with locally advanced prostate cancer and candidate to radical prostatectomy. To assess the apoptotic pathways, Bax, is studied through immunofluorescence assay, before and after 12 Gy single dose intraoperative radiotherapy (IORT) to the prostate, on bioptic samples and on surgical specimens. Moreover, before and after IORT, Bcl-2, p53, and Ki-67 were also detected through immunohistochemistry. A count of positive Bax spots for immunofluorescence was performed on tumor cells, prostatic intraepithelial neoplasia (PIN), and healthy tissue areas before and after IORT. We also analyzed Caspases 3 and 9 expressions after IORT. Before IORT, Bcl-2 mean value in neoplastic cells was 2.23% ± 1.95, mean Ki-67 in neoplastic area was 4.5% ± 3.8, and p53 was 22.5% ± 6.8. After IORT, Bcl-2 mean value in neoplastic cells was 8.85 ± 8.92%, Ki-67 in neoplastic area was 7.8 ± 6.09%, and p53 was 24.9 ± 26.4%. After the irradiation, healthy areas expressed significantly lower levels of Bax (2.81 ± 1.69%) with respect to neoplastic cells ($p < 0.0001$), while in PIN areas, Bax positive cells were significantly more present than in neoplastic areas ($p = 0.0001$). At statistical analysis, it was observed that cancer cells with Ki-67 ≥ 8% had a trend toward greater expression of Bax ($p = 0.0641$). We observed an increase of Bcl-2 expression after IORT in neoplastic areas ($p = 0.0041$). Biopsy specimens with p53 ≥ 18% and Ki-67 ≥ 8% had worse post-operative staging with extracapsular invasion ($p = 0.04$ for both parameters) and nodal positivity ($p = 0.04$ for p53 and $p = 0.0001$ at pathology for ki-67). No correlation between IORT and Caspases activation was noted. In conclusion, after 12 Gy IORT, Bax was overexpressed in tumor and PIN cells. Pre-operative Ki-67 and p53 definition could be used in future studies to predict patients with worse pathological stage, while Bcl-2 activation after IORT might be a predictive factor for loco-regional failure.

Intraoperative radiotherapy (IORT) is the ultimate expression of a dose-intensification treatment modality, with a high irradiation dose delivered during a surgical procedure. The rationale of hypofractionation and dose-intensification schemes of radiotherapy of prostate cancer is based on the particularly high level of sensitivity of prostate cancer cells to fraction size radiotherapy[1].

The IORT technique was described in a previous study from our institution[2].

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Radiobiological studies suggest that the use of a high single dose of radiations might intensify treatment effectiveness by increasing the radio-induced intracellular death processes[3]. Of note, some Authors observed that doses greater than 10 Gy may act through permeability alterations on endothelial cells, most likely causing apoptosis by caspases activation[4]. Caspases could be activated in 3 pathways: the mitochondrial pathway, the extrinsic, and the intrinsic pathway of the endoplasmic reticulum.

Radiation induced damages, such as DNA injury, hypoxia, intracytoplasmic hypercalcemia, oxidative stress, could trigger the intrinsic pathway, which is the objective of the current study. Regardless of the stimuli inducing the apoptotic cascade, an increasing mitochondrial permeability, with subsequent release of pro-apoptotic molecules such as cytochrome c, will happen. This pathway is closely linked to a group of proteins belonging to the Bcl-2 family, named from the BCL-2 gene. There are two main groups of proteins belonging to the Bcl-2 family: pro-apoptotic proteins (Bax family) and anti-apoptotic (Bcl-2). Both intrinsic and extrinsic pathways converge on the common pathway and on the activation of caspase-3, that is the protein activating the nuclear damage.

Radiations cause a series of damage to cells and DNA, and radio-induced apoptosis is intermediated by the activation of p53, Bax and subsequent activation of caspases[5]. Cancer cells usually acquire auto-survival mechanisms and are resistant to apoptotic death, albeit there is no solid evidence describing the modalities of radio-induced apoptosis in prostate adenocarcinoma cells.

The purpose of this study was to investigate the early activation pathways of radio-induced apoptosis in radical prostatectomy and ultrasound-guided prostate biopsy specimens from men receiving IORT followed by radical prostatectomy for locally advanced prostate cancer. We assessed cell proliferation index (Ki-67), proto-oncogene (p53) and anti-apoptotic protein (Bcl-2) levels in different irradiated tissues including prostate cancer, PIN, and benign cells. The IORT represents an *in vivo* modality of irradiation. We further conducted the assessment for prognosticators of disease progression by analyzing the relation between molecular data and clinical and pathological features. These biological factors were correlated with postoperative pathological staging and biochemical local control considering a prostatic specific antigen (PSA) values higher than 0.2 ng/ml for tumor recurrence.

Materials and Methods

We selected 20 men from a cohort of 132 patients treated by IORT, followed by radical prostatectomy and lymph node dissection for non-metastatic hormone-sensitive intermediate-high risk prostatic carcinoma as described in a previous article[2].

Case selection was performed upon the completeness of parameters to be investigated in the biopsy and in the surgical specimen, and upon the length of follow-up.

IORT was delivered by a dedicated linear accelerator (Mobetron, Intraop, Sunnyvale, CA, USA) using electron beams of 9–12 MeV to a total dose of 12 Gy. The dose was prescribed to the 90% isodose covering the tumor volume and the surrounding healthy tissue, including PIN, where biopsies had been performed.

Ethics approval and consent to participate. Our local ethics committee, "Comitato Etico Interaziendale Novara – AASSLL BI, NO, VCO, AOU "Maggiore della Carità" di Novara", ruled that no formal ethics approval was required in this particular case because all the analysis was performed on histological specimens with no changes in patients treatments.

The policy of our institution is to allow investigations on patients' tissues for those who signed an informed consent for a surgical procedure.

As a matter of fact, the informed consent for any surgical procedure includes a sentence in this regard.

All patients received and signed a specific informed consent before IORT and surgery. The study was performed in accordance with the Declaration of Helsinki.

Histological analyses on prostate samples. Prostatic specimens were sent immediately after the surgical removal sent to the Pathology Unit and fixed in 10% buffered formalin within 90 minutes (mean 80 minutes, SD: 74–90 minutes) from surgery and within 120 minutes (mean 102 minutes, SD: 95–120) from IORT procedure.

From paraffin embedded tissues, 3–5 µm-thick sections were cut with a microtome (Leica, mod. Histo Slide 2000R, Wetzlar, Germany). To study cell proliferation and cell cycle, immunohistochemistry with anti-Ki-67 (1:250, Ventana ® Medical Systems, Roche, Monza, Italy) and anti p-53 (1:250, Ventana® Medical Systems, Roche, Monza, Italy) antibodies was performed by using an automated immunostainer (Ventana, Roche, Monza, Italy).

For tissue immunofluorescence, rehydrated samples were incubated with the following antibodies: anti human cleaved caspase-3 (1:200; Cell Signaling Technology Inc., Pero, Italy), anti-human caspase-9 and anti-Bax (1:200; Cell Signaling Technology Inc., Pero, Italy).

Detection of specific antigens was achieved by incubating the slides with 10% normal goat serum (NGS; Vector Laboratories, Peterborough, UK)–phosphate-buffered saline (PBS), to reduce non-specific binding, then with the following primary antibodies in 5% NGS overnight at 4C in a humid chamber. Subsequently, they were incubated with a FITC-conjugated secondary antibody (1:500, Vector, CA, USA). Slides were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 1 microg/ml, Sigma-Aldrich, Milan, Italy), mounted with a mounting medium for fluorescence (Vectashield; Vector Laboratories, Peterborough, UK). Images were processed using a Leica fluorescence microscope (DM2500 Leica, Wetzlar, Germany) equipped with a digital camera. The samples were then acquired with Pannoramic MIDI (3DHISTECH Ltd, Budapest, Hungary), and analyzed with Pannoramic Viewer software (3DHISTECH, Budapest, Hungary).

After immunofluorescent staining and acquisition, samples were opportunely treated and stained using hematoxylin and eosin.

The urologist mapped the whole prostate and the intraprostatic dominant lesion with ultrasound guided prostate biopsy, and the pathologist reconstructed the site of the same lesion and surrounding tissues in the surgical

Characteristics	Value and IQR
Mean age at diagnosis (SD)	65 years (52–74)
Mean performance status at diagnosis (SD)	90 (80–100)
Mean initial PSA (SD)	17 ng/ml (4.47–41)
Neoadjuvant hormonal therapy	0
Pathological stage	**Absolute #**
pT2c	2
pT3a	4
pT3b	12
pT4	2
pN0	15
pN1	5
Adjuvant external beam radiotherapy	18 patients

Table 1. Clinical and pathological features of the 20 patients in study.

specimen to compare the expression of apoptotic factors in the corresponding areas. Two bioptic specimens in the dominant lesion were analyzed for the current study to consider inter-tumoral heterogeneity.

Bax, caspases 3 and 9 positivity were measured with 40x magnification, on two healthy tissue fields within the irradiated area, four PINs fields and four neoplastic fields. Laboratory analyses were performed by a PhD molecular biologist, supervised by an expert pathologist.

Statistical analyses. The results were analyzed using GraphPad Prism 4 software (GraphPad Software Inc., La Jolla, CA, USA). The apoptotic values highlighted with Bax expression in neoplasia and PIN areas with healthy tissue values had compared each other in the biopsy and in the surgical specimens. Apoptosis late pathway was assessed by Caspases 3 and 9 which were analyzed in surgical specimens in tumor, PIN and healthy tissue within the irradiated area. Friedman ANOVA and Wilcoxon tests were used to assess the differences of Bax expression among the samples. Results with p-values <0.05 were considered significant. Aware of the limited sample in our study, we evaluated the values of p53, Ki-67 and Bcl-2 as prognostic factors of Bax with a descriptive statistic.

Results

Characteristics of the patients including postoperative tumor staging are listed in Table 1. Median follow-up of the study cohort was 63.6 months $\pm$ 9 months. Fourteen out of 20 patients (70%) experienced biochemical failure and no patient developed distant metastases.

Bioptic specimens were withdrawn 32 days (mean 32 days, SD: 26–45) before surgery.

With p53 antibodies used in our study, higher p53 expression is related to the presence of a mutated protein isoform, being the wild type protein quickly eliminated by intracellular systems.

Specimens from prostate biopsies showed that prostate cancer cells had a Bcl-2 mean value of 2.2% $\pm$ 1.9, Ki-67 of 4.5% $\pm$ 3.8, and p53 of 22.5% $\pm$ 6.8.

Table 2 shows the results of Bax analysis on neoplastic, pre-neoplastic and healthy tissue areas. Table 3 shows the results of immunohistochemistry analysis, expressed as percentages of positivity of Ki-67, p53, and Bcl-2 in cancer cells following IORT. No statistical difference was observed in terms of Ki-67, p53, and Bcl-2 expression levels between normal and neoplastic cells ($p > 0.05$).

Figure 1 shows a neoplastic (cancer 1), a PIN (PIN 1), and a healthy tissue field in hematoxylin/eosin and immunofluorescence, and biopsy neoplastic fields in Bax immunofluorescence. There were significant differences in Bax expression among healthy tissue, PIN and cancer fields as resulted from Friedman ANOVA ($p < 0.0001$) comparing the irradiated samples. The pairwise Wilcoxon test showed that Bax was significantly overexpressed in neoplastic ($p = 0.0001$), PIN fields ($p = 0.0001$) and healthy cells after IORT ($p = 0.003$) compared to biopsy specimens before IORT.

We found a significantly increase of Bcl-2 expression after IORT in neoplastic areas ($p = 0.0041$). No differences were found in p53 and ki-67 expression before and after IORT in neoplastic cells.

From the multiple regression analysis, we did not find any correlation between p53, Bcl-2 and ki-67 expression and Bax activation after IORT.

Of note, we observed a significant overexpression of Bcl-2 on cancer cells following IORT ($p = 0.004$), while no differences were found in p53 and ki-67 expression prior and after IORT in neoplastic cells.

From the correlation between Ki-67, p53, and Bcl-2 values with the levels of expression of the Bax apoptotic protein. We observed that cancer cells receiving IORT had a greater trend towards apoptosis when Ki-67 levels were greater than 8.4% ($p = 0.064$). However, with multiple regression analysis, we did not find any correlation between p53, Bcl-2 and ki-67 expression and Bax activation after IORT.

Interestingly, we noted that patients harboring p53 levels >18% and ki-67 levels >8% on biopsy specimens had an increased likelihood to detect extracapsular invasion ($p = 0.04$ for both parameters) and nodal positivity ($p = 0.042$ for p53 and $p = 0.0001$ at pathology for ki-67). We chose the median value of 8% to discriminate patients with high and low proliferative index. p53 value of 18% was chosen according to values distribution in our sample because it represented the median one.

Figure 2 show neoplastic (cancer 1), PIN (PIN 1) and healthy tissue field in the surgical specimen with hematoxylin/eosin staining and immunofluorescence for Caspases 3.

#case	Bax/ DAPI (%) neoplasic fields	Bax/ DAPI (%) preneoplasic fields	Bax/DAPI (%) healthy tissues fields	Bax/DAPI (%) Biopsy fields
#1	8.40	19.60	0.40	1.04
#2	8.81	21.09	4.84	3.40
#3	4.69	7.56	0.55	1.42
#4	6.74	17.80	3.10	1.16
#5	2.15	28.86	3.54	2.51
#6	17.02	24.42	4.41	0.46
#7	5.82	19.12	1.31	2.86
#8	2.50	34.73	2.17	1.91
#9	17.02	24.42	6.00	0.31
#10	8.38	17.67	2.57	0
#11	7.25	23.48	3.84	1.71
#12	12.08	10.85	0.41	1.12
#13	7.46	19.1	1.25	0.58
#14	8.42	31.56	1.98	0.96
#15	4.58	18.74	0.84	1.24
#16	3.21	21.48	2.74	0.98
#17	9.58	23.5	5.40	0
#18	6.47	9.15	2.96	0
#19	12.90	26.84	4.10	2.11
#20	7.25	23.90	3.84	1.90
Mean value ± standard deviation	8.04 ± 4.15	21.19 ± 6.90	2.81 ± 1.69	1.28 ± 0.96

Table 2. Bax expression levels after and prior IORT: the first 3 columns show the percentage of Bax positivity in tumor, PIN and healthy tissue fields out of all cells (DAPI positive), while the last column shows the percentage of Bax cells positivity before IORT.

#case	p53 (%)	Bcl-2 (%)	Ki67 (%)
#1	7.2	4.1	4.2
#2	19.3	<1	17.2
#3	<1	23.2	9.6
#4	<1	18.2	5.4
#5	41.1	2.3	9.4
#6	86.2	19.3	16.2
#7	<1	<1	7.1
#8	39.2	<1	2.4
#9	18.3	<1	<1
#10	7.3	17.5	7.4
#11	20.3	19.2	7.1
#12	7.2	<1	18.3
#13	22.2	4.3	<1
#14	28.2	28.5	6.3
#15	10.1	<1	8.2
#16	25.3	10.4	21.2
#17	94.3	7.1	7.1
#18	<1	3.2	2.3
#19	32.2	15.4	<1
#20	45.2	2.3	7.1
Mean value ± standard deviation	24.9 ± 26.4	8.8 ± 8.9	7.8 ± 6.1

Table 3. The expression of proteins under investigations (p53, Bcl-2, Ki67) in neoplastic fields after IORT.

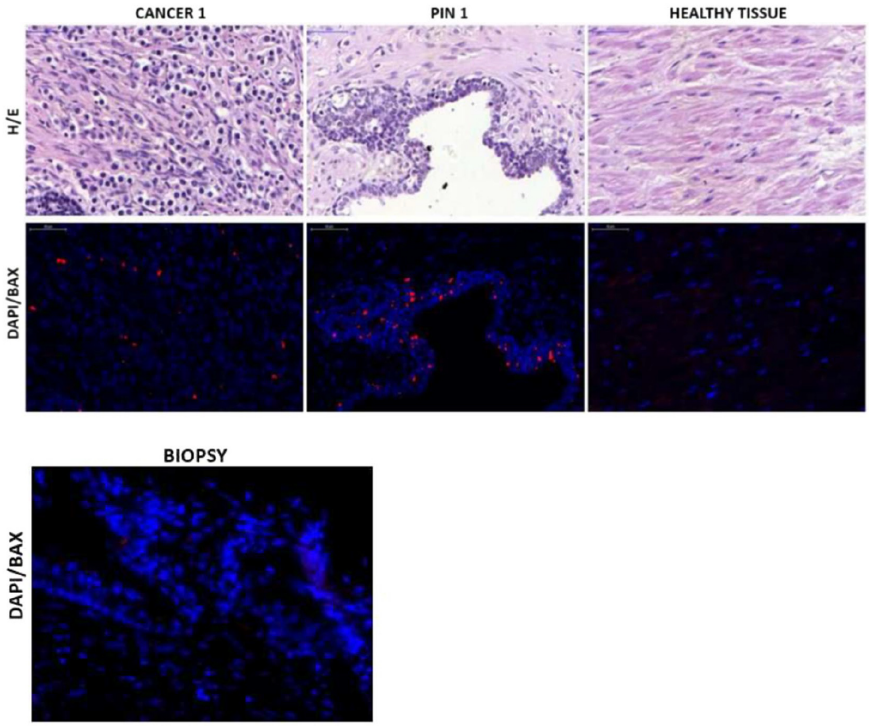

Figure 1. Hematoxylin/eosin (H/E) in surgical specimen and immunofluorescence fields for Bax (DAPI/BAX) (pt #9) in surgical and biopsy specimens. In blue all DAPI (4′,6-diamidino-2-phenylindole) positive cells (all nucleate cells), in red the cells that expressed Bax.

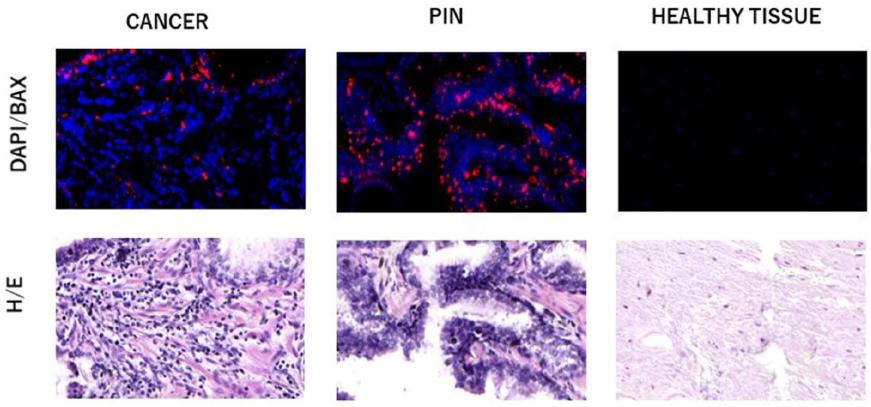

Figure 2. Hematoxylin/eosin (H/E) and immunofluorescence fields for Caspase 3 (DAPI/BAX) (pt #9) in surgical specimen. In blue all DAPI (4′,6-diamidino-2-phenylindole) positive cells (all nucleate cells), in red the cells that expressed Caspases 3.

After IORT, average Caspase 3 and 9 expressions were 4.32 ± 0.89 in cancer fields, 6.46 ± 1.70 in PIN areas, and 3.27 ± 0.02 in healthy tissue cells (Table 4). There were no significant differences of expression of such proteins among neoplastic, pre-neoplastic, and normal tissue cells ($p > 0.05$). As far as Bcl-2 values are concerned,

#case	Cas/DAPI (%) neoplasic fields	Cas/DAPI (%) preneoplasic fields	Cas/DAPI (%) healthy tissues fields
#1	4.12	6.49	3.24
#2	4.33	3.85	3.23
#3	6.49	11.85	3.28
#4	4.03	6.41	3.22
#5	4.98	6.44	3.26
#6	5.64	6.74	3.27
#7	4.12	6.11	3.27
#8	4.33	6.44	3.26
#9	2.66	2.1	3.29
#10	3.16	6.45	3.24
#11	4.31	6.72	3.28
#12	4.48	6.51	3.26
#13	5.01	6.43	3.24
#14	4.33	6.4	3.25
#15	3.64	6.25	3.27
#16	3.66	6.47	3.29
#17	5.64	5.98	3.26
#18	4.3	8.24	3.25
#19	4.79	6.45	3.25
#20	5.11	6.95	3.23
Mean value ± standard deviation	4.32 ± 0.89	6.46 ± 1.70	3.27 ± 0.02

Table 4. Caspases 3 and 9 expression levels out of all cells (DAPI positive) in neoplastic, preneoplastic and healthy tissue samples after IORT.

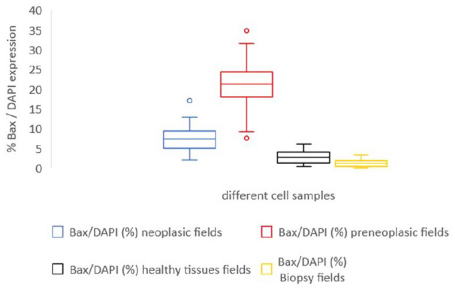

Figure 3. Box plot representation of Table 2 – Bax/DAPI expression in neoplastic (blue plot), preneoplastic (red plot), healthy tissue samples (black plot) after IORT and in bioptic specimen (yellow plot) before IORT.

we observed that patients with levels of Bcl-2 prior IORT higher than 9% had an increased risk of biochemical failure ($p = 0.004$). The 9% threshold was chosen since it represented the median value in our patient sample. In Figs. 3–5, and Table 5, we reported box plots and the results to summarize our findings.

Discussion

Patients with intermediate and high-risk prostatic cancer experience biochemical recurrence in 24–72% of cases after radical surgery or radiation[6]. Understanding the molecular pathways regulating apoptosis of prostate cancer cells due to hypo-fractionated radiotherapy is still a daunting task for physicians. There is very little evidence[7,8] about the radiobiological effects on tissues of single-shot radiation suggesting a possible endothelial damage to peritumoral vessels with consequent hypoxia and cellular death.

The interest of studying biomolecular changes after IORT resides in the possibility to better understand mechanisms of cell death related to the use of extreme hypofractionation which is of increasing interest for external beam radiotherapy of prostate cancer. In this regard, IORT represents an ideal opportunity to investigate radiation related changes in tumor and healthy tissues just after irradiation and immediately before tissue withdrawn and pathology examination.

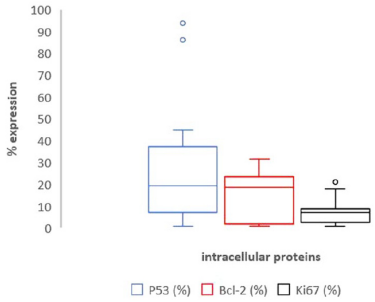

Figure 4. Box plot representation of Table 3 – p53 (blue plot), Bcl2 (red plot) and ki-67 (black plot) expression in neoplastic fields after IORT.

Protein	% neoplasic fields after IORT	% preneoplastic after IORT	% healthy tissue fields after IORT	% before IORT (tumor area)
Bax	8.04 ± 4.15	21.19 ± 6.9	2.81 ± 1.69	1.28 ± 0.96
Caspases	4.32 ± 0.89	6.46 ± 1.70	3.27 ± 0.02	/
p53	24.9 ± 26.4	/	/	/
Bcl-2	8.85 ± 8.92	/	/	/
ki-67	7.8 ± 6.09	/	/	/

Table 5. Summary of results differentiated by protein values (% mean value ± standard deviation) and study time.

Some studies showed that hormonal therapy and a few chemotherapy drugs can induce apoptosis[9,10]. Due to this evidence we included in the study hormone-naïve patients.

We focused our analysis on the mechanisms related to the mitochondrial apoptotic pathway of cellular death following single-shot irradiation, evaluating the "*in vivo*" radio-induced damage received by tissues.

Prior to radiation, levels of Bax protein were significantly lower compared to PIN and neoplastic cells treated with IORT ($p < 0.05$). However, prior to a single-shot irradiation, neoplastic and pre-neoplastic cells do not express apoptosis proteins. This data suggests that IORT could be able to activate apoptosis in prostate cancer cells.

We observed that Bax protein is significantly increased in PIN cells ($p < 0.0001$) and in cancer cells ($p = 0.006$) following IORT. Interestingly, PIN areas appeared to be more sensitive to irradiation than normal prostatic tissue in our study population. No significant correlation was observed between Bax expression and PSA at diagnosis or Gleason Score at histology, and no correlation between IORT and Caspases activation was noted. Our data suggests that the activation of caspases occurs later than Bax pathway involvement. In this regard, we did not investigate caspase expression in the biopsy specimen since there was no activation of apoptosis, according to the negative Bax results. In 1995, Raffo *et al.* first demonstrated that Bcl-2 oncoprotein could protect prostate cancer cells from apoptotic stimuli[11]. Nowadays, there is evidence that proteins of the Bcl-2 family may play a role in the development of human malignancies and may act as key players in the process of programmed cell death.

Non-neoplastic prostate cells should express Bcl-2 levels of about 2–3%[12]. A recent review showed that Bcl-2 hyperexpression in tumor cells is associated with good prognosis in colorectal, breast, non-small cell, glioma, and gastric cancers. According to such review, measuring the levels of expression of Bcl-2 could be used to stratify patients and understand the response to active treatments[13]. Other "*in vitro*" studies demonstrated that Bcl-2 overexpression confers resistance to hormonal therapy among prostate cancer patients[14]. Our results are consistent with these literature data. We observed that increased expression of Bcl-2 following IORT in prostate cancer cells was associated with an increased risk of a local relapse. Based on our findings, it is reasonable to hypothesize that the expression of Bcl-2 after IORT may activate intracellular mechanisms leading to radio-resistance.

Several studies investigated the predictive and prognostic role of Bax and Bcl-2 family proteins[15,16]. Clinical data from RTOG 86–10 and RTOG 92–02 showed that only Bax expression at a normal level was associated with significantly more favorable outcome[17]. *In vitro* data showed conflicting results with studies without significant differences in the expression of p53, Bcl-2 and Bax 2 and 4 hours after 10 Gy into cell lines[18] and studies showing that single shot irradiation could induce Bax-mediated cell death *in vitro*[19]. Our work seems to show that this process could happen "*in vivo*" as well.

To our knowledge our study is the first to describe that a single-shot irradiation may induce Bax-mediated cell death in patients receiving IORT, that represents an *in vivo* irradiation modality allowing for a rapid subsequent pathological examination of the irradiated tissue.

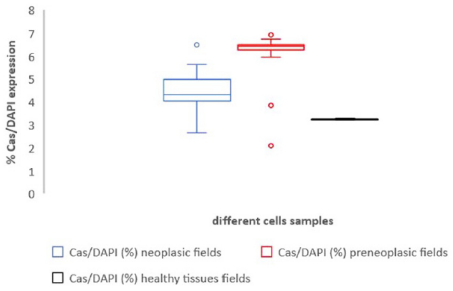

Figure 5. Box plot representation of Table 4 – Caspases/DAPI expression in neoplastic (blue plot), preneoplastic (red plot) and healthy tissue samples (black plot) after IORT.

It has long been known that PIN areas are closely related to the presence of prostate cancer. By now, all literature data agree that neoplastic areas are related to intracellular mutations in pre-neoplastic areas. Recently, PIN morphological alterations have been shown to be associated with an increased replication index[20]. Xie *et al.* demonstrated in an animal model that pre-neoplastic cells with Bcl-2 hyperexpression have higher proliferative index, and increased expression of Bax. An increased apoptotic rate in high grade pre-neoplastic cells probably implicates that apoptosis may accelerate cellular turnover in premalignant lesions of the prostate. According to this animal model, the well differentiated neoplastic cells possibly developed a genetic profile of natural resistance against apoptotic stimuli[21]. We could hypothesize that PIN cells are most susceptible to irradiation, because they have already a high turnover.

To our knowledge, this is one of the first studies that showed that "*in vivo*" pre-neoplastic cells are more prone to apoptosis to single dose irradiation than neoplastic prostate cells. Interestingly, cancer cells present a significantly lower Bax positivity profile than PIN areas, most likely due to a relative radio-resistance induced by cancer transformation itself.

In some neoplasms, such as breast cancer, a correlation between Ki-67 value, and response to adjuvant treatments were observed. Of note the literature is poor when prostate cancer is concerned. Most likely, Ki-67 values in prostate carcinoma would be extremely heterogeneous as observed by Mesko *et al.* who reported values ranging between 1.1 and 10.1%[22]. Of note, Ki-67 is higher among patients with locally advanced prostate cancer. *In vitro*, it was observed that higher-proliferating cells were also those that tend to hyper-express apoptotic proteins after extracellular stimuli[12].

In our sample, patients had a mean Ki-67 value of 7.8% ± 5.1%. We chose the median value of 8% to discriminate patients with high and low proliferative index. We observed that cells with Ki-67 > 8% had an increased trend towards apoptosis ($p = 0.0641$). Therefore, even *in vivo*, there could be an increased sensitivity to single shot irradiation with the increase of the proliferation index.

In our biopsy samples, higher proliferation index and higher p53 expression were associated with worse pathological tumor stage, higher incidence of extracapsular extension, and higher risk of nodal disease. Our data supports those from previous studies by Saidi *et al.*[23] and Berlin *et al.*[24]. Based on our results, we could hypothesize that p53 protein and Ki-67 could be used as prognostic factors. This data may be of great interest in routine clinical practice as there is no current prognosticator of extra-prostatic extension of cancer.

In p53 mutated neoplastic cells, we observed a lower expression of Bax ($p = 0.5$), while there was a significant increase in expression of Bax in PIN areas ($p = 0.04$) and in healthy tissue areas ($p = 0.02$). In this regard, p53 responds to radiation-induced damage in several ways, such as inducing cell cycle arrest and activating apoptosis[25]. Some *in vitro* studies underlined that the activation of p53 protein increases the radio sensitivity of prostate cancer cells[26,27]. On the contrary, other studies concluded that p53 expression does not influence radiation sensitivity in prostate carcinoma[28,29].

Our *in vivo* study seems to confirm, indeed, that neoplastic cells with mutation in p53 are less sensitive to apoptosis induced by single dose irradiation than healthy cells and surrounding PIN areas. On the contrary, non-mutated p53 cells (p53 < 18%) resulted more sensitive in tumor than in PIN and normal tissue cells. It can be reasonably hypothesized that in PIN and healthy cells p53 protein is still functioning and it is able to trigger the apoptosis after radio-induced damage.

We acknowledge that our study has limitations. This is a single center study based on small patient population. We are further conscious that in our study we did not investigate intracellular changing connected we hypoxia that could be matter of a further investigation. In the next future, we would like to study the expression of a transcription factor protein, hypoxia-inducible factor 1alpha (HIF-1alpha), to differentiate tissue changing related to surgery stress and IORT. However, the original design of our study, based on a translational research approach, has the strength to first report *in vivo* novel findings of molecular biology of mechanisms of apoptotic pathway in prostate cancer cells treated with single-dose radiotherapy.

Conclusion

Our study showed that mitochondrial apoptosis and Bax pathway is activated in a few minutes after irradiation in prostate cancer cells following a single high dose radiation.

In our study, the cell death program was significantly activated among cancer cells and PIN tissues, whereas this result could not be observed in benign cells. These findings support the role of radiations as a precise carrier of a cell damage specifically directing towards cancer cells, while sparing benign tissues most likely due to the preservation of anti-apoptotic mechanisms.

From our analysis, it emerges that neoplastic cells with higher proliferating index are more responsive to radio-induced damage. On the other hand, higher Ki-67 and mutated p53 cells are predictive for higher pathological staging, extra-capsular extension, and nodal disease. Mutated p53 is also predictive for radio-resistance. We also noticed that pre-operative and post-operative Bcl-2 might predict biochemical failure. These elements, if confirmed in larger cohorts, could help to stratify patients in clinical studies and to select which patients could benefit the most from highly hypofractionated regimens possibly including intraoperative irradiation.

Data availability

Information on data supporting the results reported in the article can be found in a dataset of the University Hospital "Maggiore della Carità", Novara, Italy.

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Author contributions

C.P., M.K., R.B. and F.B. designed the study; M.R. performed all the laboratory exams; R.B. supervised the laboratory results; C.P., R.B., M.R. and F.B. acquired and analyzed the data; M.K. and A.V. performed the intraoperative procedures; C.P. followed-up patients; C.P., F.B. and M.B. drafted the manuscript; M.K. and A.V. revised the manuscript; M.K., C.P. and G.L. performed statistical analysis; all authors approved the submitted version. All authors agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated, resolved, and the resolution documented in the literature.

Competing interests

The authors declare no competing interests.

Additional information

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