

ORIGINAL ARTICLE

Preoperative radiotherapy and extracellular matrix remodeling in rectal mucosa and tumour matrix metalloproteinases and plasminogen components

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Abstract

Background. Preoperative radiotherapy reduces recurrence but increases postoperative morbidity. The aim of this study was to explore the effect of radiotherapy in rectal mucosa and rectal tumour extracellular matrix (ECM) by studying enzymes and growth factors involved in ECM remodeling. **Materials and methods.** Twenty patients with short-term preoperative radiotherapy and 12 control patients without radiotherapy were studied. Biopsies from rectal mucosa and tumour were collected prior to radiotherapy and at surgery. Tissue MMP-1, -2, -9, TIMP-1, uPA, PAI-1, TGF- β 1 and calprotectin were determined by ELISA. Biopsies from irradiated and non-irradiated peritoneal areas were also analysed. **Results.** Radiotherapy increased the tissue levels of MMP-2 and PAI-1 in both the rectal mucosa and tumours while calprotectin and uPA showed an increase only in the mucosa after irradiation. The increase of calprotectin was due to an influx of inflammatory cells as revealed by immunohistochemistry. Prior to irradiation, the tumour tissues had increased levels of MMP-1, -2, -9, total TGF- β 1, uPA, PAI-1 and calprotectin compared to mucosa, while TIMP-1 and the active TGF- β 1 fraction showed no statistical difference. **Conclusions.** This study indicates a radiation-induced effect on selected ECM remodeling proteases. This reaction may be responsible for early and late morbidity. Interference of this response might reduce these consequences.

Key Words: Radiotherapy, extracellular matrix, rectal cancer, MMP-2, PAI-1, uPA, calprotectin

Surgical resection is a prerequisite to cure localized rectal cancer. In addition to surgery, preoperative radiotherapy is often administered to reduce local recurrence and possibly improve survival [1]. Radiotherapy is, however, associated with increased morbidity after surgery [2]. Further knowledge of the cellular and molecular events following irradiation is necessary to allow intervention and thus enable improved treatment.

The extracellular matrix (ECM) consists of collagens, laminins, proteoglycans and fibronectins and is remodeled by among others matrix metalloproteinases (MMPs). MMPs and tissue inhibitor of metalloproteinases-1 (TIMP-1) are a family of

zinc-dependent enzymes that are elevated in colorectal cancer tissue and associated with more advanced tumours [3] as well as with reactions in rectal tissue after radiotherapy [4–6]. It has been suggested that MMP-9 levels could be used to predict tumour response to radiotherapy [7].

Transforming growth factor- β 1 (TGF- β 1) also participates in the remodeling of the ECM, but has many other functions such as suppression of the immune system and regulation of cell growth. The physiological functions of TGF- β 1 are carried out after dissociation of the latency-associated peptide to form the active mature TGF- β 1. This activation can be elicited by endogenous agents, for example

plasmin or MMP-9 but also exogenous factors such as irradiation and radiation-activated TGF- β 1 may be involved in the mechanisms behind fibrosis [8]. However, results of clinical studies are conflicting: some studies have found a decrease or no effect on the TGF- β 1 levels after radiotherapy [9–11].

The fibrinolytic plasminogen system (PS) is closely linked to TGF- β 1 and MMPs through inhibition and activation. The PS consists of two activators; tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) as well as two inhibitors; plasminogen activator inhibitor -1 and -2 (PAI-1 and -2). PAI-1 is pivotal in fibrosis development [12] and the PAI-1 gene is activated by radiotherapy [13]. The plasminogen system is also involved in the response elicited by radiotherapy [14,15] and is associated with adhesion formation [16], which may result in small bowel obstruction.

The calcium-binding protein calprotectin is present in neutrophils, granulocytes and monocytes. It has been associated with acute and chronic inflammation [17] and is upregulated in cancers indicating a possible role in carcinogenesis [17] as well as postulated to be an inhibitor of MMPs [18]. Faecal calprotectin is elevated in faeces of patients with inflammatory bowel disease and studies of patients undergoing pelvic radiotherapy have evaluated its use as an indicator of radiation-induced proctitis [19,20]. No studies have examined the levels of calprotectin before and after irradiation in rectal cancer or adjacent mucosa.

The aim of this study was to prospectively study the effect of radiation therapy on MMP-1, -2 and -9, TIMP-1, TGF- β 1, uPA, PAI-1 and calprotectin in rectal mucosa and tumour. In addition the peritoneal response to radiotherapy in regard to MMP-2, -9, TGF- β 1, tPA, uPA and PAI-1 was studied. The major interactions of the different proteins described

above and analysed in this work are displayed in Figure 1.

Materials and methods

Study design and patients

This was a prospective study including consecutive patients with localized rectal cancer, scheduled for open surgery, with or without preoperative radiotherapy. Forty-two patients were included, of which 10 patients had to be excluded due to different radiotherapy protocol (two patients), transanal endoscopic surgery (two), neo-adjuvant chemotherapy (two), incomplete biopsies (three) and no surgical treatment (one). Thirty-two patients remained for analysis. Twenty patients received short-term preoperative radiotherapy with a three-field technique, 5x5 gray (Gy). Twelve patients not receiving radiotherapy, but otherwise treated equally served as controls. Inclusion criteria for preoperative radiotherapy were; rectal cancer confirmed by histology, preoperative tumour stage of T3 with possible lymph node involvement, and lowest tumour border less than 10 cm from the anal verge. Preoperative assessment was performed by clinical examination, rigid rectoscopy and, in selected cases, endoscopic ultrasound and/or magnetic resonance imaging (MRI). There was no difference in age (median age 67 (range 36–80) vs. 71 (range 45–88)) or gender (male 65% vs. 58%) between the irradiated and the control group. Surgery using total mesorectal excision (either abdomino-perineal resection or anterior resection) was performed within a median of 3 days (range 3–6) after completion of radiotherapy. No preoperative chemotherapy was given. The patients' tumours classified according to TNM of the surgical specimens are listed in Table I.

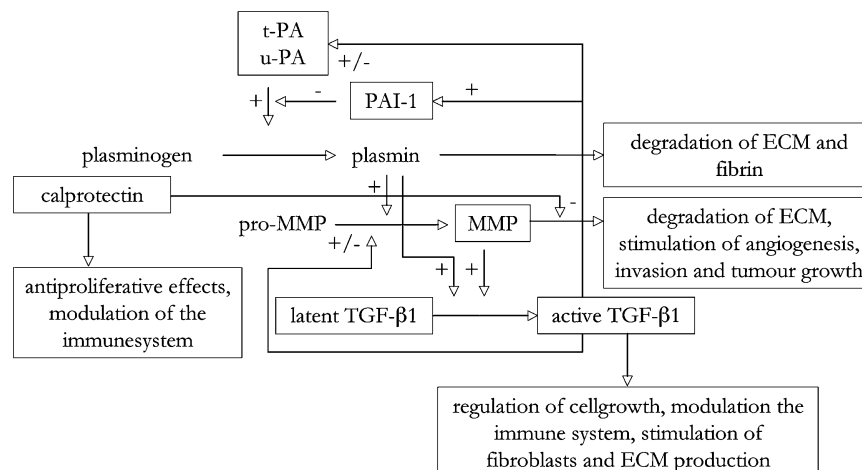


Figure 1. Schematic illustration of the main interactions of MMPs, TGF- β 1, the fibrinolytic plasminogen system and calprotectin.

Table I. TNM classification, total number of lymph nodes identified and number of lymph nodes with tumour growth in the intestinal specimens.

	Non-irradiated	Irradiated
pT classification		
T1	0	1
T2	5	7
T3	7	12
T4	0	0
Total	12	20
pN classification		
N0	8	6
N1	1	6
N2	3	8
Total	12	20
cM classification		
M0	11	19
M1	1	1
Total	12	20
Lymph nodes*		
Dissected	20 (10–37)	17.5 (5–29)
Tumour positive	0 (0–9)	2.5 (0–13)

*The number of lymph nodes identified in the resected specimens are given as median (range).

Tissue samples

Prior to radiotherapy and surgery baseline biopsies were obtained from both tumour and from adjacent macroscopically tumour-free mucosa using a rigid rectoscope. In the beginning of surgery, peritoneal biopsies from the upper non-irradiated abdomen and from the irradiated pelvic area were collected. Immediately after the rectal specimen was removed, biopsies were collected from the tumour and the rectal mucosa. The mucosal biopsies were obtained within 10 cm from the tumour, within the radiation field [6]. Tissue samples (approximately 20–40 mg wet weight) were immediately snap frozen in liquid nitrogen and stored at -70° until analysis. Biopsies collected from the same areas were immediately fixed in Bouin's solution (Sigma Diagnostic, St Louis, MO, USA) for histology and immunohistochemistry.

Protease and growth factor analysis

Biopsies were mixed in a PBS buffer (phosphate-buffered saline) with NaCl containing 0.01% Triton X-100 to a concentration of 40 mg of tissue/ml of buffer. After homogenization (Ultra Turrax; Janke & Kunkel GmbH, Staufen, Germany) and centrifugation (10 000 g/3 min) the supernatant was stored at -70° C until analysis. MMP-1, -2, -9 and TIMP-1 were analysed using ELISA-kits from Amersham Pharmacia Biotech (Buckinghamshire, UK) [6].

Total, as well as active, TGF- β 1, were analysed with ELISA kits from Promega Corporation (Madison WI, USA) [11]. The active fraction of TGF- β 1 was assayed directly in the ELISA plate using the kits provided. For measuring the total amount of TGF- β 1 additional samples were acidified to pH 3.0 using 1 mol/L HCL, followed by a 15 minute incubation at 22° C, resulting in activation of all TGF- β 1. To neutralize samples 1 mol/L NaOH was supplemented before application to the second ELISA plate. The fibrinolytic parameters uPA and PAI-1 were analysed using ELISA-kits from Biopool (Umeå, Sweden) [14]. Calprotectin was analysed using ELISA-kits from Bühlmann Laboratories AG (Schönenbuch, Switzerland). As the technique is not commonly used on homogenized tissue samples, samples with a known high inflammatory response were used as controls and several diluting series were performed.

All results were normalized to total protein content based on the method by Lowry [21], determined by an assay from Bio-RAD (Hercules, CA, USA). There was no difference in total protein content between irradiated and non-irradiated patients. In some patients limited biopsy material restricted the number of analyses. The number of patients included in each analysis is shown in the tables/graphs.

Histological examination and immunohistochemistry

Following fixation, biopsies were washed with PBS, pH 7.4, and dehydrated in increasing ethanol gradients and xylene prior to paraffin embedding. Four to six micrometer sections were deparaffinized and stained with Haematoxylin & Eosin (H&E) for morphologic assessment.

In addition to calprotectin ELISA quantification, the expression of calprotectin in mucosa was evaluated with immunohistochemistry in selected biopsies. Primary mouse anti-human Macrophage L1 protein/Calprotectin antibodies diluted 1:10 (Ab 62227, Lot No: 430744, Abcam, Cambridge, UK) were used together with the DAKO Envision system (DAKO Cytomation, Glostrup, Denmark) and detected with diaminobenzidine. Incubations of tissue sections with mouse IgG directed towards an enzyme neither present nor inducible in mammalian tissue instead of primary antibodies (X-0931, DAKO Cytomation, Glostrup, Denmark) at the same concentration, served as negative controls. Slides were counterstained with Haematoxylin prior to dehydration and mounting with cover slips. Evaluation with distribution and qualitative comparison was performed using Nikon Eclipse 800 microscope together with Nikon Coolpix 995 digital photo equipment (Nikon Instruments Inc, Melville, New York, USA).

Statistical analysis

The Kolmogorov-Smirnov test was used to confirm that most data was not normally distributed. Statistical analysis was performed using Wilcoxon signed rank test, χ^2 test, Fishers' exact test and the Mann-Whitney U analysis. The results are presented as median and interquartile range (IQR) as the distribution was non-parametric. The graphs are presented as box-plots showing the median (horizontal line), IQR (boxes) and 1.5 times IQR (error bars). Statistical analysis was carried out with SPSS 11.0.4 and SPSS 13.0.0 (SPSS Inc., Chicago, Illinois, USA).

Ethical aspects

The University of Gothenburg Ethical committee approved the study.

Results

The expression of ECM remodeling components analysed in irradiated and non-irradiated rectal mucosa, tumour and peritoneal tissues are listed in Tables II and III. The components analysed showing significant changes after radiotherapy are illustrated in Figure 2. Altogether 32 patients were studied of which 20 received preoperative radiotherapy and 12 were controls. There were no differences in the analysed components' levels in the baseline biopsies, prior to therapy, between the irradiated and non-irradiated patients.

Studies of rectal mucosa and tumour

MMPs and TIMP-1. In the baseline biopsies from both groups of patients MMP-1, -2 and -9 were higher in the tumour compared to mucosa, while no differences were seen in TIMP-1 levels (Table II). These findings did not differ between the two groups.

Radiotherapy significantly increased MMP-2 levels in both the rectal mucosa and the tumour tissue (Figure 2A). No significant changes in the levels of MMP-1, MMP-9 or TIMP-1 upon radiation were found (Table II). In control patients there was a significant reduction in TIMP-1 levels in the biopsies collected during surgery ($p < 0.05$, $n = 12$) compared to preoperative biopsies.

Total and active TGF- β 1. Total TGF- β 1 was higher in tumour compared to mucosa in the baseline biopsies. Neither the total TGF- β 1 nor the active form of this cytokine in the mucosa and tumour tissues was affected by irradiation (Table II).

uPA and PAI-1. Both uPA and PAI-1 levels were higher in the baseline tumour tissue compared to the mucosa (Table II). Irradiation resulted in a significant increase of both mucosal and tumour PAI-1 levels (Figure 2B) while only a slight increase in mucosal uPA and no effect on tumour uPA was seen (Figure 2C).

Calprotectin. The baseline biopsy levels of calprotectin were significantly higher in the tumour tissues compared to the mucosa (Table II). The levels were significantly increased in the mucosa after radiation treatment while the tumour tissues showed no significant difference (Figure 2D).

Immunohistochemistry (Figure 3) revealed that the high levels of irradiated mucosal calprotectin are due to an influx of inflammatory white blood cells, such as granulocytes (arrows in Figure 3) in lamina propria and submucosa.

As expected, the irradiated mucosa displayed increased cellularity of the lamina propria with fibroblasts, as well as granulocytes and lymphocytes compared to non-irradiated mucosa. There were also signs of architectural crypt distortion. In the non-irradiated tumour biopsies tumour cells and some inflammatory cells were found. After irradiation, tumour cells were swollen and there were apparent changes in the stroma with an abundance of fibroblasts, granulocytes and lymphocytes.

Expression of ECM remodeling factors in irradiated and non-irradiated peritoneum

Peritoneal biopsies were obtained during surgery from the pelvic region and upper abdominal region outside the radiation field. The results of analysed components are listed in Table III. No differences could be detected between the pelvic and upper abdominal biopsies in neither the non-irradiated control patients nor the irradiated patients. tPA was also analysed in the peritoneal biopsies since this factor has been shown to be of importance for adhesion formation [16]. The levels for non-irradiated pelvic peritoneum were 17.7 ng/mg protein (12.1–22.2) and upper abdomen 14.2 (6.6–20.5). In the upper abdomen peritoneum in irradiated patients the level was 11.4 (6.1–14.2). These figures are not significantly different. However, in irradiated patients, the tPA levels in the pelvic peritoneal biopsies was significantly lower; 7.8 (4.0–16.1; $p < 0.05$) compared to non-irradiated patients.

Discussion

This study shows that pre-operative radiotherapy for rectal cancer induces changes in tissue levels of several

Table II. Proteases and growth factors determined in rectal mucosa and tumour tissue of patients with or without preoperative radiotherapy. Biopsies were collected prior to therapy and at surgery. The results are reported as median with the range in parenthesis. The factors that showed a significant difference are indicated by asterisks (*) and the components showing statistically significant differences are shown as box-plots in Figure II. The reduced sample size in some of the measurements is due to insufficient biopsy material.

	Control patients (non-irradiated)					Irradiated patients				
	n	Preop mucosa	Postop mucosa	Preop tumour	Postop tumour	n	Preop mucosa	Postop mucosa	Preop tumour	Postop tumour
MMP-1 (ng/mg)	11	1.0 (0-5.2)	0.4 (0-1.9)	6.9 (0.9-60.3)	14.7 (0.1-41.3)	20	0.7 (0-3.0)	0.9 (0-12.9)	5.1 (0-48.7)	6.9 (0.4-161.3)
MMP-2 (ng/mg)	11	27.3 (19.9-47.3)	29.6 (15.6-245.4)	35.4 (10.2-84.7)	69.2 (19.5-168.4)	20	29.5* (19.1-71.5)	69.6* (16.7-538.3)	52.4* (17.9-354.4)	82.4* (1.6-389.6)
MMP-9 (ng/mg)	11	6.9 (2.2-22.8)	3.1 (1.6-19.6)	29.2 (6-281.1)	27.9 (1.9-42.7)	20	4.7 (1.6-29.0)	11.0 (1.5-78.7)	23.2 (1.5-190)	21.7 (2.1-139.8)
TIMP-1 (ng/mg)	11	8.3 (1.1-25.0)	5.2 (1.1-15.2)	8.1* (3.6-65.8)	5.0* (1.8-10.8)	19	6.3 (2.5-30.8)	6.8 (2.7-10.9)	12.0 (2.9-55.4)	9.8 (1.6-41.9)
Total TGF-β1 (pg/mg)	11	122 (98-263)	129 (95-193)	256 (52-681)	350 (144-704)	19	126 (32-447)	143 (74.2-312)	252 (32-927)	302 (126-582)
Active TGF-β1 (pg/mg)	11	44.2 (17.6-77.5)	31.4 (22.9-38.3)	38.4 (19.1-63.5)	33.0 (23.6-46.9)	19	33.4 (17.5-65.6)	29.4 (13.2-60.2)	35.6 (16.2-85.7)	30.5 (7.5-174.2)
uPA (ng/mg)	11	1.7 (1.1-4.0)	1.5 (1.0-2.8)	4.2 (2.0-17.8)	4.4 (0.9-9.6)	20	2.4* (0.7-14.7)	2.7* (1.1-14.5)	4.8 (0-14.8)	6.4 (2.2-18.0)
PAI-1 (ng/mg)	12	0 (0-1.0)	0 (0-0.4)	1.4 (0-5.5)	1.9 (0-11.7)	20	0* (0-1.5)	1.0* (0-10.8)	1.1* (0-9.4)	3.4* (0-26.6)
Calprotectin (ng/mg)	11	530 (125-5680)	399 (312-767)	4085 (557-22493)	3623 (2864-4634)	19	594* (184-4966)	2999* (271-17173)	2668 (509-2918)	3503 (1162-27194)

proteases involved in ECM remodeling. Of the MMPs, MMP-2 was increased in irradiated mucosa and tumour tissue while MMP-1 and MMP-9 were not affected. It has been suggested that higher levels of MMP-2 after radiotherapy would facilitate tumour cell dissemination [5]. This is supported by in vitro studies showing an increased invasive potential in tumour cell lines after radiotherapy [22]. Although radiotherapy reduces local recurrence there is a possibility that MMP-2 inhibitors could further improve these results especially in cases where the surgical margin is small. Higher levels of MMP-2 have also been associated with anastomotic dehiscence [23], wound-infections, fistula formation [6] and radiation-induced diarrhoea [4].

Preoperative radiotherapy has been indicated as risk factor for anastomotic leakage [24] although this has not been confirmed by large randomized trials. A limitation with this human study is that biopsies were taken from the removed specimen. Although it is probable that the levels of factors analyzed in the biopsies reflect anastomotic area levels, this cannot be certain. There are indications that timing of surgery after radiotherapy is important to reduce complication rate [25]. A sub group analysis, showed lower levels of MMP-2 in rectal mucosa in irradiated patients operated three days after radiotherapy compared with patients operated four days after treatment. It is possible that these biopsies reflect the increased remodeling taking place and may indicate the importance of reduced surgical delay after radiotherapy. We found no effect of radiation on MMP-1 or MMP-9 in this study, which is somewhat contradictory to previous findings [4-6]. MMP-9 mRNA seems to increase after a few days after radiotherapy, which could indicate that the reactions of MMP-9 due to irradiation are not fully present at the time of our biopsies [15].

Our study is in accordance with an earlier report that radiotherapy does not affect TIMP-1 in rectal tumours [7]. TIMP-1 has been found to increase shortly after short-term radiotherapy in mucosa in animal studies [15]. The timing of biopsies is important when interpreting TIMP-1 results but we hypothesize that the control group TIMP-1 decrease is attributed to the surgical trauma and that radiotherapy may alter this response. It has been shown that tissue TIMP-1 initially decreases after arterial injury [26], but later the TIMP-1 levels increased.

Faecal calprotectin levels have been found to be higher after radiotherapy in humans although studies are not conclusive [19,20]. We found increased levels of calprotectin in the irradiated tissue due to an influx of inflammatory cells. Further studies are required to see if mucosal biopsies analysed for

Table III. Proteases and growth factors determined in peritoneal tissue biopsies collected at start of surgery. Biopsies were taken from the pelvic area and the upper part of the abdomen outside the radiation treatment field. The results are reported as median and the range (in parenthesis).

	Control patients (n = 12)		Irradiated patients (n = 19)	
	Pelvic biopsy	Upper abdomen biopsy	Pelvic biopsy	Upper abdomen biopsy
MMP-2 (ng/mg)	37.2 (8.7–268.9)	43.8 (24.5–274.5)	65.6 (9.1–249.1)	75.8 (8–158.6)
MMP-9 (ng/mg)	9.3 (2.6–94.6)	5.2 (2.9–48.5)	16.0 (2.2–229.0)	7.8 (1.1–98.8)
Total TGF- β 1 (pg/mg)	207.0 (129.8–1552.4)	175.4 (64.0–298.6)	220.5 (20.6–2221.0)	177.1 (22.4–373.2)
Active TGF- β 1 (pg/mg)	87.3 (0–305.4)	65.8 (21.7–126.7)	72.6 (15.3–281.5)	88.4 (20.4–473.0)
uPA (ng/mg)	2.1 (0–18.2)	0.8 (0–3.1)	2.0 (0–15.0)	2.8 (0–10.4)
PAI-1 (ng/mg)	0 (0–1.0)	0 (0–0.8)	0 (0–0.8)	0 (0–1.1)

calprotectin can be used as an indicator of the extent of radiation reaction and predict subsequent response. Such studies could be performed on archived specimens from randomized trials with

patients randomized to surgery alone or addition of preoperative radiotherapy.

Evidence points toward a radiation induced activation of latent TGF- β 1 [8] but this activation may

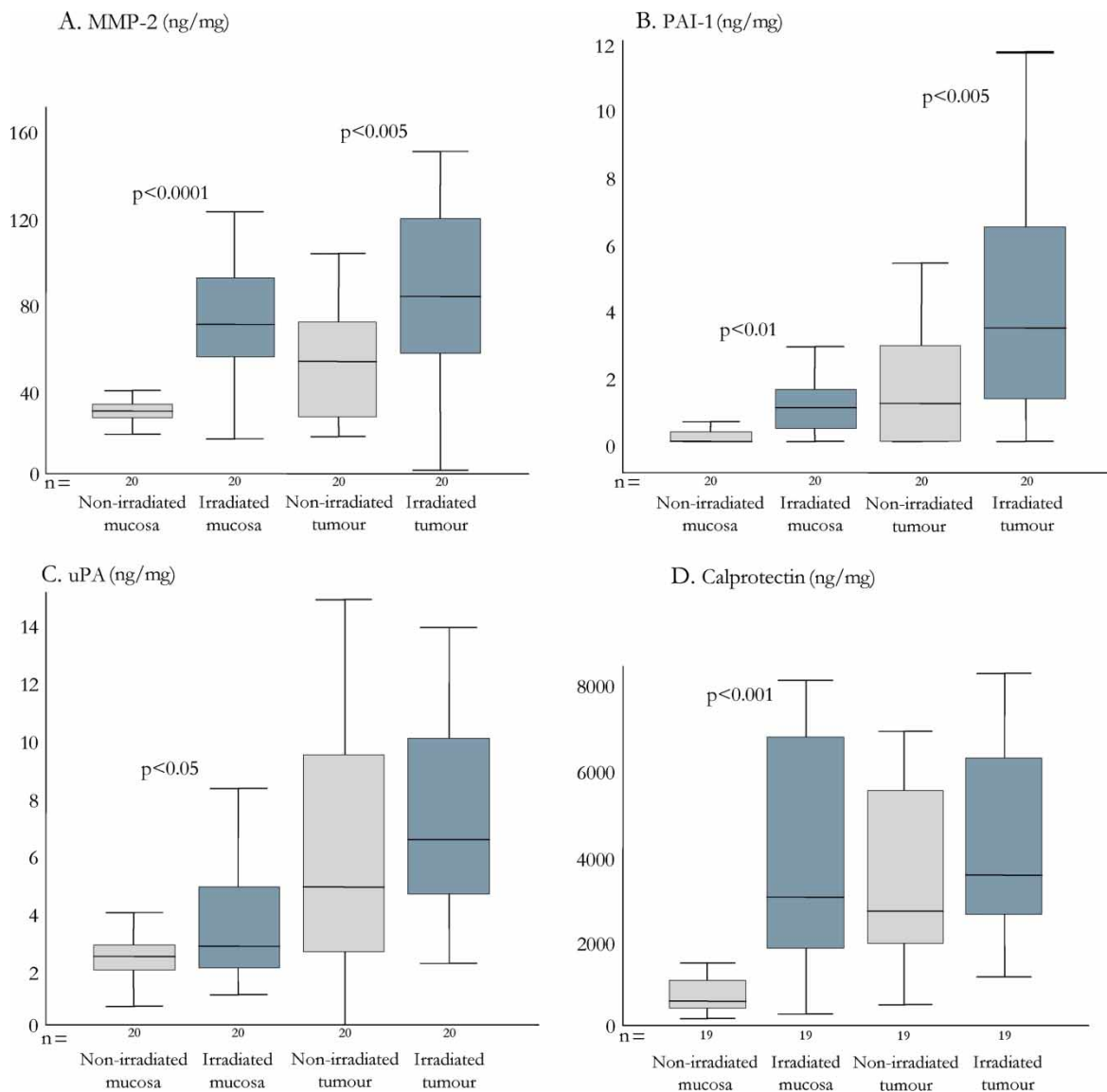


Figure 2. Tissue concentrations of MMP-2 (chart A), PAI-1 (B), uPA (C), and calprotectin (D) in irradiated and non-irradiated rectal mucosa and rectal tumours. The number of patients analysed are given below each graph and results are presented as box-plots showing the median (horizontal line), IQR (boxes) 1.5 IQR (error bars).

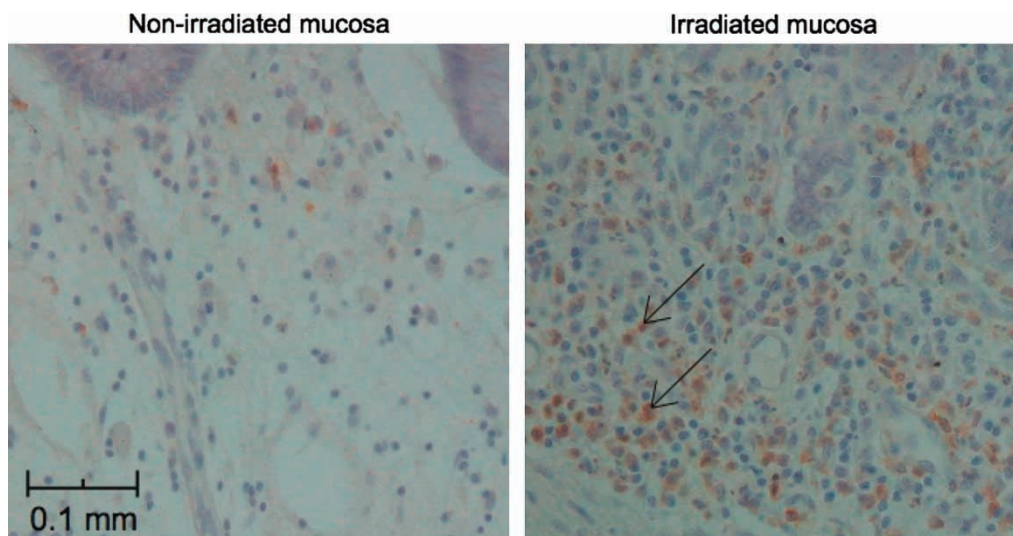


Figure 3. Immunohistochemistry of non-irradiated (left chart) and irradiated (right) rectal mucosa Calprotectin is located to inflammatory cells in lamina propria of the irradiated mucosa (arrows). The mucosal epithelial cells and submucosa are negative.

only be seen a limited time, which may explain why it was not seen in this study, as patients were operated up to six days after termination of radiotherapy.

The PAI-1 gene is activated by radiotherapy [13] and PAI-1 protein levels are increased in both rectal mucosa and tumour after radiotherapy [14,15], which is confirmed in this study. PAI-1 participates in the radiotherapy associated fibrosis development [12], and higher levels of uPA and PAI-1 are found in tumour tissue indicating their role in tumour development [14].

We hypothesized that the increased risk of bowel obstruction after surgery combined with radiotherapy [2] could be related to an unfavourable reaction of the peritoneum. We found lower levels of tPA in irradiated peritoneum compared to controls, which could indicate a reduced fibrinolytic activity signifying an increased risk for adhesions [16].

This is one of few human studies on this topic. A difference in TNM-classification between irradiated and non-irradiated patients could be expected due to radiotherapy treatment selection, but our material does not differ significantly in this regard. The use of MRI in the pre-operative setting has improved estimation of tumour stage and selection of patients to radiotherapy, but during the time of this study MRI was not available for all patients, and perhaps this can in part explain this lack of difference. One interesting finding was that the baseline biopsies displayed the same differences between mucosa and tumour as previously shown in operative biopsies except for active TGF- β 1 [6,11,14], indicating that operative biopsies in many cases can be used reflecting the preoperative setting.

Values are normalized to the total protein content but the biopsies are heterogeneous. However, each of the irradiated patients constitutes its own control, which is the strength of this study. The data from the non-irradiated patients make a reference for the possibility of systematic differences due to anaesthesia, surgery procedures and possibly differing biopsy procedures at diagnoses and at surgery. Since the expression of the various markers change over time post irradiation, the results of this study may over-look early and late changes, which could be studied in patients having other radiotherapy schedules.

In conclusion, although radiotherapy reduces recurrence rates in rectal cancer treatment, it contributes to the increased morbidity following surgery. This study supports our previous data pointing to effects on the levels of MMP-2, PAI-1, uPA by radiotherapy in rectal mucosa and tumour. It is clear that radiotherapy affects remodeling of extracellular matrix and that it also has an effect on the rectal mucosal levels of calprotectin, which hasn't previously been demonstrated in radiotherapy of rectal cancer.

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