

Paper I

Faecal Calprotectin and Lactoferrin as Markers of Acute Radiation Proctitis: A Pilot Study of Eight Stool Markers

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Background: Non-invasive diagnostic tools to evaluate the severity of acute, radiation-induced proctitis are not readily available. The faecal excretion of eight markers of gut inflammation was therefore examined. Five proteins and three lipid derivatives were analysed in sequential stool samples taken before and during radiation therapy. **Methods:** Stool samples from 15 patients with prostate cancer scheduled for radiation therapy were examined. Pretreatment and in-treatment samples (2nd and 6th weeks) were measured by enzyme-linked immunosorbent assay (ELISA) (calprotectin, lactoferrin, transferrin, leukotriene B₄, prostaglandin E₂, thromboxane B₂ and TNF α) or nephelometry (α 1-antitrypsin). **Results:** Calprotectin and lactoferrin concentrations increased significantly during radiation treatment ($P = 0.0005$ and $P = 0.019$). Transferrin was detected in only 9 out of 45 samples. There were no changes in tumour necrosis factor α (TNF α), leukotriene B₄, prostaglandin E₂ and thromboxane B₂ during treatment. α 1-antitrypsin could not be detected in any sample. **Conclusions:** This study indicates that faecal calprotectin and lactoferrin concentrations could be markers of acute, radiation-induced proctitis. Patient compliance and stability of the markers make this a promising method for clinical research. Eicosanoids could be measured in stool samples, but the concentrations did not increase with increasing radiation dose.

Key words: α 1-antitrypsin; calprotectin; lactoferrin; leukotriene B₄; prostaglandin E₂; radiation proctitis; TNF- α ; transferrin; thromboxane B₂

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Acute normal tissue symptoms influence radiation therapy and may limit the total radiation dose that can be administered. Progressive gastrointestinal (GI) symptoms occur early in the radiation course, but only a minority of patients develop late radiation sequelae. Individual characteristics of this susceptibility are largely unknown. Patient-related factors account for the majority of delayed normal tissue responses to radiation (1). In animal experiments, prediction of late sequelae from the presence of acute intestinal effects could be achieved by measuring the faecal content of a surrogate marker of inflammatory cells (2). Three human correlates to this marker and five other markers of GI disease in man were tested for their possible usefulness for non-invasive diagnosis of acute radiation proctitis. The sequential, quantitative histopathology of acute radiation proctitis has previously been described, showing inflammatory changes early in the treatment course (3). The present study follows the same time schedule as our former study, with sampling of stool at repeated times before and during external pelvic radiation therapy in prostate cancer patients.

The aim of this study was to find candidate markers useful for non-invasive monitoring of acute radiation proctitis. Candidates must be validated as objective surrogate markers for clinical symptoms and histopathological mucosa changes in future studies.

Materials and Methods

Patients and radiation treatment

Fifteen patients (mean age 66 years, range 57–73) with prostate cancer (T1-3NxMo) were included in the study. These patients had no history of GI disease. No dietary limitations were given. The stool samples were taken within a week before initiation of radiation therapy, 2 weeks after start of treatment and 4 weeks later. The times of the stool examination corresponded to radiation doses of zero, approximately 20 Gy and 60 Gy, respectively. The radiation treatment was dose planned, box technique, 2 Gy per fraction, 5 days/week, with a planned target volume not cranial of the rectum-sigmoidal flexure. Four patients received 50 Gy to a

slightly larger volume of intestines. All patients completed the planned treatment, with 70 Gy to the prostate and vesicular glands.

Stool collection and sample preparation

The patients were instructed to collect at least 50 g stool in a plastic container and keep the sample in a cool place until it was delivered for examination. Each sample was divided in two equal portions.

One portion was suspended in an equal volume of sterile phosphate-buffered saline and centrifuged at 20000g for 1 min. Supernatant was stored at -70°C until assayed for TNF α , leukotriene B₄, prostaglandin E₂ and thromboxane B₂.

The other portion was frozen at -70°C as unprocessed faeces. The protein extraction was performed both according to the original and the new method described earlier (4, 5). Briefly, faeces was diluted in 1:50 of faecal extraction buffer (5), vortex mixed for 30 s, shaken at 1000 rpm for 30 min and centrifuged at 10000g for 20 min. The supernatant was frozen at -70°C in four aliquots until assayed for calprotectin, lactoferrin, transferrin and α 1-antitrypsin. A second portion of the faeces was also diluted in 1:50 of the faecal extraction buffer, mixed for 1 min, centrifuged at 10000g for 20 min and the supernatant frozen at -70°C until assayed for TNF α .

All the patients provided faecal specimens according to the schedule. On average, the samples were stored, prepared and frozen within 9 h after defecation (range 3–14 h).

Analyses

Calprotectin, lactoferrin and transferrin. Calprotectin was determined by an ELISA, as described previously (4). Briefly, standards and samples were incubated in microwells coated with a polyclonal rabbit anti-calprotectin. The amount of calprotectin bound was determined by enzyme-labelled immunoaffinity purified rabbit anti-calprotectin. Samples were tested in duplicates.

Lactoferrin was detected with the ELISA kit from TechLab, Blacksburg, Va., USA (IBD-CHEK). To quantify the results, a standard curve was prepared by serial dilution from normal serum. Samples were tested in duplicate.

Transferrin was determined by an ELISA very similar to that for calprotectin using reagents prepared in our laboratories. Transferrin was purified from human plasma and used for immunization of rabbits and preparation of an affinity column for isolation of anti-transferrin that was labelled with alkaline phosphatase. Samples were tested in duplicate.

TNF α . TNF α was assayed using ELISA kits from Amersham Pharmacia Biotech Limited, Buckinghamshire, England (BIOTRAK). Samples were tested in duplicate.

Leukotriene B₄, prostaglandin E₂ and thromboxane B₂. The three eicosanoids were assayed using kits from R&D Systems Europe, Oxon, England. Samples were tested in duplicate. The samples from four patients were excluded. These samples had the longest time-span before they were

frozen. Three of these four patients had only normal concentrations of calprotectin.

α 1-antitrypsin. α 1-Antitrypsin was measured by nephelometry using an automated instrument from Behring, Germany.

Normal concentrations and stability

The upper limit of normal faecal calprotectin is 50 mg/kg, and the protein is stable in faeces at room temperature for at least one week (4). Lactoferrin was considered abnormal above 1000 ng/g (6, 7), but was relatively stable at room temperature, with a decrease of 10% measured after 4 days (7). Transferrin at 0.4 $\mu\text{g/g}$ was used as the upper reference limit (8, 9). It was shown to be more resistant to degradation in faeces than haemoglobin (8). No upper reference limit has been established for TNF α (10, 11). TNF α is unstable in plasma and body fluids (12). In faeces, prostaglandin-like substances were measured in ulcerative colitis (1–75 ng/g PGE₂-equivalents) and absent in healthy controls (<0.6 ng/g) (13). Eicosanoids have been assayed in rectal dialysate with up to 0.64 ng/mL leukotriene B₄, 0.6 ng/mL prostaglandin E₂ and 0.88 ng/mL thromboxane B₂ in healthy controls (14, 15). α 1-Antitrypsin exceeding 620 $\mu\text{g/g}$ is considered abnormal (9). The amount of α 1-antitrypsin was decreased by less than 10% in faeces at room temperature for 24 h (16).

Statistics

The statistics were performed using the statistical packages SPSS 10.0 and StatXact 5. Concentrations at the three examination times were compared using exact permutation tests. *P* values less than 0.05 were considered statistically significant.

Ethics

The patients gave informed written consent to the study, which was approved by the Regional Committee of Medical Research Ethics.

Results

Calprotectin and lactoferrin

The mean concentrations of calprotectin were 56, 63 and 206 mg/kg at the three measurement times (Fig. 1). The difference between no radiation and 60 Gy was significant (*P* = 0.0005). Six of the 15 patients all had calprotectin concentrations in the normal range. One patient had a concentration of 340 mg/kg before radiation treatment, with an increase to the maximal concentration found, 1262 mg/kg at week 6. Two of the four patients with a slightly larger volume of intestines in the radiation fields had the two highest concentrations of calprotectin at week 6. Five patients had a normal concentration before radiation treatment and an elevated concentration at week 6.

The mean concentrations of lactoferrin were 1710, 1417 and 6823 ng/g at the three examination times, respectively

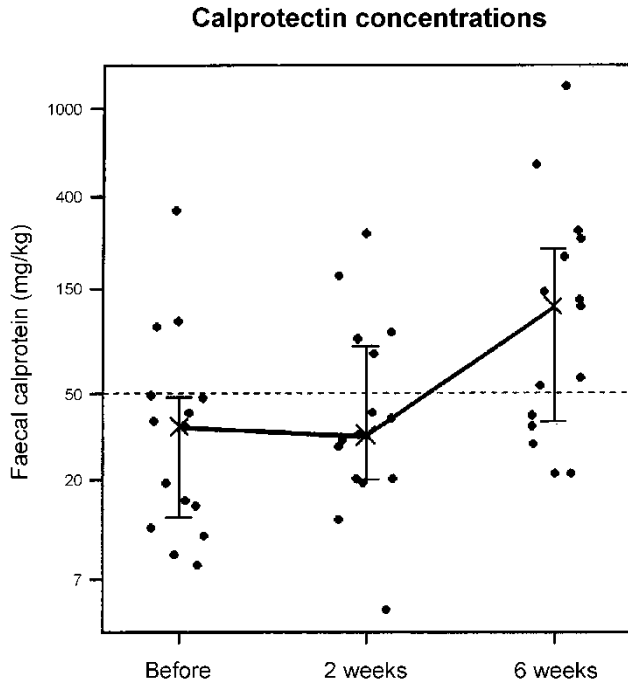


Fig. 1. Faecal calprotectin concentrations in patients irradiated for prostate cancer (median and quartiles). Vertical distances are logs. Broken line: upper reference value.

(Fig. 2). The difference between no radiation and 60 Gy was significant ($P < 0.02$). Six samples had concentrations below 200 ng/g, which was the detection limit of the assay. The highest concentrations at the three examinations were 7500, 6500 and 50 000 ng/g, respectively. Four patients had concentrations in the normal range. Five patients had a normal concentration before radiation treatment and an elevated concentration at week 6.

Transferrin

Transferrin could be detected in only 9 of 45 samples, with concentrations ranging from 27 to 118 $\mu\text{g/g}$. In one patient all three samples had increased concentrations. Four samples showed elevations at week 2 and two samples at week 6 of the treatment.

TNF α

Two different extraction methods were tested for the assay of TNF α . Of the 45 samples in phosphate buffered saline supernatant, 10 samples were zero (assay range: 0.1–10 pg/g and 25.6–1000 pg/g). The highest concentration found was 120 pg/g (at 60 Gy). Mean concentrations were 18, 45 and 44 pg/g at the three sampling times, respectively. In 33 protein extraction samples the mean concentrations were 19, 29 and 32 pg/g. No significant differences between the sampling times were found.

Leukotriene B4, prostaglandin E2 and thromboxane B2

The eicosanoids were measured in 11 of the 15 patients. Mean concentrations of leukotriene B4 were 1269, 1923 and 1896 pg/g at the three sampling times, respectively (measured concentrations from 240 to 4000 pg/g, assay range 47–12000 pg/g). Five of the 33 samples had concentrations below the detection limit, but other extraction methods were not explored. Mean concentrations of prostaglandin E2 were 9585, 7194 and 11384 pg/g, respectively (measured concentrations from 440 to 28000 pg/g, assay range 39–20000 pg/g). Two of the 33 samples had concentrations exceeding the detection limit. Mean concentrations of thromboxane B2 were 6656, 5875 and 10760 pg/g, respectively (measured concentrations from 1700 to 20000 pg/g, assay range 13.7–10000 pg/g). Six of the 33 samples had concentrations exceeding the detection limit. Fresh stool samples had been suspended in an equal volume of sterile phosphate buffered saline and centrifuged at 20000 g for 1 min. Supernatant was stored at -70°C until assayed for the three eicosanoids. Further dilutions of the thawed samples were omitted. No significant differences between the sampling times were found.

α 1-antitrypsin

α 1-Antitrypsin could not be detected in any of the samples.

Comparison of protein excretion in individual patients

Four patients had only normal concentrations of calpro-

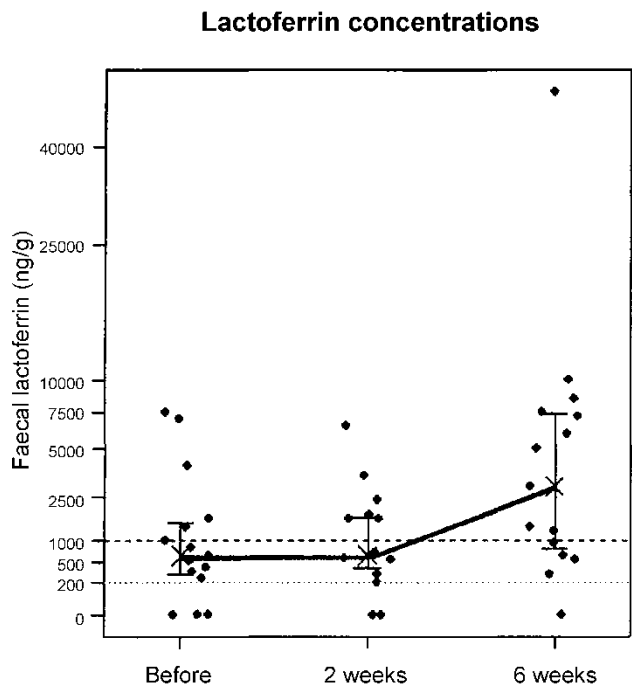


Fig. 2. Faecal lactoferrin concentrations in patients irradiated for prostate cancer (median and quartiles). Vertical distances are square roots. Broken line: upper reference value; dotted line: lower detection limit of assay.

tectin and lactoferrin in all samples. Four patients had an increase from normal concentrations of calprotectin and lactoferrin before the radiation treatment to elevated concentrations in the 6th week. In two patients both markers were above normal in all samples. Seven of the nine samples with elevated levels of transferrin also had elevated levels of calprotectin and lactoferrin.

Discussion

The aim of this study was to find non-invasive markers of acute radiation proctitis. Monitoring the inflammation that occurs in a group of patients will be useful in future intervention studies. All eight substances examined are known to be present in gut inflammation, and present in stool samples or by rectal dialysis (the eicosanoids leukotriene B₄, prostaglandin E₂ and thromboxane B₂). We are not aware of any reports on human faecal concentrations of inflammatory markers during radiation therapy.

This study demonstrates that faecal concentrations of calprotectin and lactoferrin increased from normal concentrations before radiation treatment to pathological concentrations in 5 out of 15 patients when the radiation dose reached 60 Gy. The two markers showed parallel results with respect to normal or elevated concentrations in 10 of the 15 patients. Transferrin was only detectable in 9 of 45 samples, and is therefore not considered useful in monitoring proctitis caused by radiation therapy. The concentrations of TNF α , leukotriene B₄, prostaglandin E₂ and thromboxane B₂ did not show any pattern similar to the increase of calprotectin and lactoferrin. We found that eicosanoids could be quantified in stool samples. Sampling of stool is less demanding for the patient than the rectal dialysate examination, and the laboratory method we used was not time-consuming. We did not find the previously described increase in the eicosanoids (LTB₄, PGE₂ and TXB₂) when measuring rectal dialysate before pelvic radiation therapy, at the end of radiation treatment and at least 4 weeks after radiotherapy (14). The eicosanoids might have been degraded during the time from defecation to freezing and analysis. We did not examine the patients with rectal dialysate, and can therefore not directly compare our results with the previously described increase during radiation therapy. TNF α , leukotriene B₄, prostaglandin E₂ and thromboxane B₂ are not considered useful in monitoring acute radiation proctitis. Since the α 1-antitrypsin levels were undetectable, we conclude that radiation-induced, acute proctitis is not a protein-losing enteropathy.

Calprotectin, lactoferrin and transferrin are abundant in macrophages and neutrophil granulocytes, and have been shown in increased amounts in faeces during inflammatory bowel disease (IBD) (6, 7, 9, 17, 18). Increased inflammatory cell transmigration to the gut lumen has been assessed by radiolabelling of granulocytes (19). There was a strong correlation between faecal excretion of labelled granulocytes and faecal calprotectin. Furthermore, calprotectin correlated

to disease activity in patients with IBD (20, 21). Lactoferrin concentrations in faeces were shown to be higher in active than in inactive IBD, and significantly higher than in healthy controls (6, 7, 22). Owing to differences in methodology, the cut-off value differs in the three articles referred to, from around 1000 ng/g to 4000 ng/g. We used a different method of extraction than the two research groups. The mean concentration we found in the 6th week of radiation lies above the highest of the cut-off limits. In acute radiation proctitis, both the extent and degree of inflammation are less pronounced than in IBD, explaining the moderately elevated concentrations of calprotectin and lactoferrin in our study. The stability of calprotectin and lactoferrin during sampling and extraction should be sufficient for the preservation of the markers in our samples. TNF α , a pro-inflammatory cytokine, has been found in faeces in children with active Crohn disease and ulcerative colitis (10). α 1-Antitrypsin reflects leakage of serum proteins to the intestinal lumen in Crohn disease (9).

Calprotectin is a member of the S100 protein family, which is important in regulating inflammatory reactions and non-specific defence (18). Calprotectin inhibits matrix metalloproteinases (MMPs) by sequestration of zinc (23). Two former studies with the same longitudinal design as this one showed increased activity of MMP-2 and MMP-9 in rectal mucosa during pelvic radiation therapy (24). The changes were associated with neutrophil infiltration in the biopsies and radiation-induced diarrhoea. The present results show elevated calprotectin concentrations by the end of radiation therapy, the time at which 86% of patients had GI symptoms (3). Calprotectin inhibits microbial activity via a zinc deprivation mechanism (25). Radiation-induced disturbance of the enteric flora could possibly escalate the inflammation and symptoms. Ingestion of live *Lactobacillus acidophilus* bacteria prevented diarrhoea during pelvic radiation therapy (26). Since TNF α is activated by a metalloproteinase, it is also possible that calprotectin reduces TNF α levels (27). Lactoferrin has antibacterial properties (reviewed in (28)) and has been shown to reduce the release of TNF α and other pro-inflammatory cytokines in a rat colitis model (29). Calprotectin might be the exclusive binding protein of arachidonic acid (AA) in neutrophils, and an important mediator of inflammatory effects from metabolites of AA (30). AA is the substrate for the cyclooxygenase-2 (COX-2) enzymatic pathway to pro-inflammatory eicosanoids, e.g. prostaglandin E₂. Radiation up-regulates the activity of COX-2 (31). Interventions to reduce the acute inflammation using PGE₁-analogue suppositories have been examined in a randomized study (32). The treated group had milder symptoms of late radiation proctitis. The mechanism is not understood. Acute radiation proctitis in patients with prostate cancer correlated with late GI symptoms (33, 34). The faecal content of transferrin could predict late radiation injury in an animal model (2). These studies support the assumption of consequential late effects for some aspects of late radiation injury. We did not correlate the faecal markers to symptom

scores, as the main aim of this study was to explore the presence of inflammatory markers in stool. In previous studies, clinical symptoms progressed toward the end of the treatment course (3, 35). This rise is similar to the elevation of calprotectin and lactoferrin in the last week of treatment. In contrast to this, histological changes were most pronounced in the 2nd week of treatment, and then showed fewer signs of inflammation at the end of treatment (3). The histologic changes, however, were mainly located to the superficial layers of the mucosa, while changes in the deeper parts could explain the discrepancies between histopathology and symptoms and possibly the excretion of faecal markers. The predictive value of the markers for late radiation proctitis should be studied. Individual differences in radiation response have not been thoroughly explored. Dietary and smoking habits should be evaluated together with predisposing diseases in order to predict susceptibility to radiation-induced proctitis. Our present results indicate that stool calprotectin and lactoferrin are present in increased amounts as a physiologic response to radiation-induced inflammation. We assume that the findings chiefly reflect changes in the rectum, but we cannot exclude a contribution from the small intestine.

In conclusion, this pilot study shows a significant increase in faecal calprotectin and lactoferrin when the rectal mucosa has been exposed to 60 Gy radiation therapy. No changes were found in transferrin, TNF α , leukotriene B₄, prostaglandin E₂, thromboxane B₂ or α 1-antitrypsin. Further studies should be undertaken to compare stool calprotectin and lactoferrin with symptom scores, biopsies and blood tests. The usefulness of monitoring acute radiation proctitis should be examined, and the possible role of predicting late radiation proctitis.

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