

Towards restoring intestinal health after pelvic radiotherapy

Lessons from dietary fiber intervention in a novel mouse model

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Cover illustration: A picture depicting patient receiving pelvic radiotherapy by Mitusha Patel

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*“A dream doesn’t become reality through magic;
it takes sweat, determination and hard work.”*

Colin Powell

To my family

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ABSTRACT

Patients who have cancer in the pelvic region are at risk of developing gastrointestinal symptoms for weeks, months, or even years after receiving radiotherapy. These symptoms are triggered due to unwanted radiation-induced damage to surrounding non-cancerous tissues, resulting in the disruption of normal physiological functions. Worldwide, millions of cancer survivors suffer from reduced bowel health as a consequence of radiotherapy. Quality of life is reduced due to severe gastrointestinal symptoms, such as urgency, fecal leakage, blood and mucus discharge, and excessive production of odorous gases. Our goal was to identify the molecular mechanisms responsible for these symptoms and to prevent its occurrence. To address these issues, we have developed a novel mouse model in which mice were irradiated within a small restricted field that encompasses the distal bowel using a clinical linear accelerator that is used to treat patients. In **Paper I**, biomarkers to identify radiation-induced intestinal injury were studied, and elastase was found to be a good biomarker of radiation-induced injury to the distal bowel. In **Paper II**, the long-term progression of mucosal injury and repair mechanisms after radiotherapy were studied. It was observed that radiation-induced mucosal damage occurs through

persistent crypt loss and that repair proceeds through the crypt fission process. In **Papers III** and **IV**, we wanted to investigate whether the advice given to the patients to avoid fiber during radiotherapy is beneficial or detrimental. We found that mice that were fed the fiber-deficient diet and exposed to irradiation had high serum levels of pro-inflammatory cytokines, aberrant mucosal histology, high levels of mucus degradation, low levels of short-chain fatty acids, and signs of gut dysbiosis, as compared to mice that were fed the fiber-rich high oat bran diet and exposed to irradiation. This supports the notion that avoiding fiber during radiotherapy might not be beneficial to patients.

In conclusion, diet plays an important role in modifying the effects of irradiation on intestinal health. A fiber-rich high oat bran diet helps to reduce the harmful effects of radiation and to ameliorate radiation-induced intestinal damage, whereas a fiber-deficient diet exacerbates radiation-induced intestinal damage.

Keywords: Pelvic radiotherapy, radiation-induced gastrointestinal symptoms, oat bran.

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SAMMANFATTNING PÅ SVENSKA

Patienter som har cancer i bäckenområdet riskerar att utveckla symtom från tarmen i veckor, månader eller till och med år efter att de fått strålbehandling. Dessa symtom utlöses på grund av oönskad strålningsinducerad skada på omgivande icke-cancervävnader, vilket resulterar i störning av normala fysiologiska funktioner. Över hela världen lider miljoner canceröverlevande av nedsatt tarmhälsa som en följd av strålbehandling. Livskvaliteten reduceras på grund av allvarliga tarmsymtom som trängningar, tarmläckage, utsöndring av blod och slem och omåttlig förekomst av illaluktande gaser. Vårt mål är att identifiera de underliggande molekyllära mekanismer som orsakar symtomen och att förhindra att de uppstår. För att adressera dessa frågor har vi utvecklat en ny musmodell där möss fick strålning till ett litet begränsat fält som omfattar distal tarm med en klinisk linjär accelerator som används för att behandla patienter. I **Paper I**, studerades biomarkörer för att identifiera strålningsinducerad tarmskada, och elastas visade sig vara en bra biomarkör för strålningsinducerad skada på den distala tarmen. I **Paper II**, studerades den långsiktiga utvecklingen av slemhinneskada och reparationsmekanismer efter strålbehandling. Det observerades att strålningsinducerad slemhinneskada inträffar genom långvarig kryptförlust och att reparationen fortskrider genom kryptfission. I **Paper III** och **IV**, ville vi undersöka om råd som ges till patienterna att undvika fiber under strålbehandling är fördelaktiga eller skadliga. Vi fann att möss som fick en kost utan fiber och exponerades för strålning hade höga nivåer av pro-inflammatoriska serumcytokiner, avvikande slemhistologi, höga nivåer av slemnedbrytning, låga nivåer av kortkedjiga fettsyror och tecken på tarmdysbios jämfört med möss som fick den fiberrika havrekli-kosten och utsattes för bestrålning. Detta stöder uppfattningen att det inte är till nytta för patienter att undvika fiber under strålbehandling.

Sammanfattningsvis spelar kost en viktig roll för att modifiera effekterna av bestrålning på tarmhälsan hos möss. En fiberrik havrekli-

kost hjälper till att minska de skadliga effekterna av bestrålning och hjälper till att förbättra strålningsinducerad tarmskada, medan en kost utan fiber kommer att förvärra strålningsinducerad tarmskada.

Nyckelord: Bäckenstrålbehandling, strålningsinducerade tarmsymtom, havrekli.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

- I. Sjöberg F, Malipatlolla DK, Patel P, Wilderäng U, Kalm M, Steineck G, Bull C. **Elastase as a potential biomarker for radiation-induced gut wall injury of the distal bowel in an experimental mouse model.** Acta Oncol. 2018;57(8):1025-30.

- II. Malipatlolla DK, Patel P, Sjöberg F, Devarakonda S, Kalm M, Angenete E, Lindskog EB, Grandér R, Persson L, Stringer A, Wilderäng U, Swanpalmer J, Kuhn HG, Steineck G, Bull C. **Long-term mucosal injury and repair in a murine model of pelvic radiotherapy.** Sci Rep. 2019;9(1):13803.

- III. Patel P, Malipatlolla DK, Devarakonda S, Bull C, Rascón A, Nyman M, Stringer A, Steineck G, Sjöberg F. **Oat bran fiber reduces systemic inflammation in mice subjected to pelvic irradiation.** Manuscript.

- IV. Patel P, Jin C, Nookaew I, Malipatlolla DK, Devarakonda S, Bull C, Robeson MS, Rascón A, Nyman M, Karlsson N, Wold AE, Steineck G, Sjöberg F. **Oat bran fiber protects against pelvic radiation-induced intestinal damage by reducing gut dysbiosis and mucus degradation.** Manuscript.

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ABBREVIATIONS

aISC	Active intestinal stem cells
CBC	Crypt base columnar
rISC	Reserved intestinal stem cells
TLR	Toll-like receptor
DAMP	Damage associated molecular patterns
ROS	Reactive oxygen species
NOS	Reactive nitric oxide species
IL	Interleukin
IFN	Interferon
TGF- β 1	Transforming growth factor beta 1
G-CSF	Granulocyte colony stimulating factor
VEGF	Vascular endothelial growth factor
EGFR	Epidermal growth factor receptor
bFGF	Basic fibroblast growth factor
ECM	Extracellular matrix
SCFAs	Short-chain fatty acids
HDACs	Histone deacetylases
GPCRs	G-protein coupled receptors
TNF- α	Tumor necrosis factor alpha

PMN	Polymorphonuclear leukocytes
MUC	Mucin
BrdU	Bromodeoxyuridine
ESV	Exact sequence variants
OTU	Operational taxonomic unit
GLC	Gas-liquid chromatography
T-RFLP	Terminal restriction fragment length polymorphism
DGGE	Denaturing gradient gel electrophoresis
TGGE	Temperature gradient gel electrophoresis
HITChip	Human intestinal tract chip
NF-kB	Nuclear factor kappa-light-chain enhancer of activated B cells

INTRODUCTION

We all celebrate the great victories of cancer therapy with an overall cure rate approaching 65%. At least half of the cancer patients receive radiation therapy at some stage of the disease, and it plays an important role in 25% of cancer cures [1, 2]. With the introduction of new radiotherapy techniques such as brachytherapy, multileaf collimators, intensity-modulated radiotherapy, and volumetric modulated arc radiotherapy, the ratio between the wanted dose of ionizing radiation to the tumor and unwanted dose to surrounding normal tissue has increased dramatically. However, there is an increasing number of cancer survivors with radiation-induced gastrointestinal symptoms [3]. Worldwide, millions of cancer survivors suffer from decreased bowel health due to radiation-induced gastrointestinal symptoms [4]. Their quality of life is impaired and restricted due to severe gastrointestinal symptoms, such as diarrhea, fecal incontinence, leakage of blood and mucus into clothing, excessive production of foul-smelling flatulence and involuntary loud flatulence, may be socially isolating and embarrassing [5, 6]. The occurrence and severity of radiation-induced intestinal symptoms depend on the number of therapy-related and patient-related factors. Therapy-related factors include radiotherapy dose, the volume of bowel exposed to radiation, fractionation schedule used, irradiation technique used, and the use of concomitant chemotherapy or biotherapy [7, 8]. Patient-related factors include age at the time of radiotherapy, body mass index, previous abdominal surgeries, and occurrence of co-morbidities such as inflammatory bowel disease, diabetes vascular disorder, and collagen vascular disease [9-11]. Younger age at the time of treatment could result in increased intensity of the symptoms [12]. Patients with abnormal body mass index may be predisposed to radiation-induced intestinal symptoms, and previous abdominal surgery might increase the risk of developing radiation-induced intestinal symptoms [3]. Smoking and diet also appear to affect the intensity of the symptoms [12, 13].

Perhaps most remarkably, some cancer survivors will develop one or several of the symptoms, with vastly different intensity levels, while some will not develop them at all. Today, there is no satisfactory way to predict the outcome for a certain individual. Thus, it is imperative to investigate the pathophysiological processes which can cause symptoms in the cancer survivors.

Radiation-induced pathophysiological processes

Pelvic radiotherapy induces the number of pathophysiological processes in the intestine, which can be classified into acute response and late response. Acute radiation injury induces the production of free radicals, which damages DNA and other cellular components. There is an occurrence of transient mucosal atrophy and infiltration of plasma cells and polymorphonuclear leukocytes in the lamina propria within the tissue [14]. The loss of stem cells leads to epithelial denudation and mucosal ulceration [15]. The epithelial dysfunction then leads to nutrient and fluid loss, whereas decreased epithelial integrity exacerbates mucosal inflammation [16]. Acutely, radiation causes hyperemia and increases the permeability of small vessels, resulting in edema in surrounding tissues and thickening of basement membranes [17]. This leads to decreased gases and metabolite exchange, altering the extracellular milieu, and leading to tissue hypoxia [18].

The late response causes obliterative endarteritis (inflammation of the inner lining of the artery), resulting in chronic ischemia and necrosis. Other late responses include submucosal fibrosis, lymphatic dilatation, and transmural fibrosis due to mesenchymal cell activation and collagen deposition [19]. The cumulative effects of these pathophysiological processes may result in vascular degeneration, telangiectatic vessel formation, mucosal ulceration, fistula formation, intestinal wall necrosis, and serosal adhesion formation [20]. Some of the pathophysiological processes which were studied in this thesis have been described below.

Loss of intestinal stem cells

The intestinal epithelium is rapidly self-renewed every 3-5 days. The resident intestinal stem cells and progenitor cells present in the intestinal crypts are responsible for the self-renewal of the tissue [21]. The intestinal stem cells within the crypts are the most vulnerable cells to radiation due to its rapidly replicating property, because of which they are often in the G2 and M phase of mitosis. Loss of stem cells due to irradiation leads to a reduction in the production of epithelial cells, consequently leading to loss of mucosal integrity [16, 22]. Intestinal stem cells consist of at least two types of stem cell pools; the active intestinal stem cells (aISC) also called crypt base columnar (CBC) cells and the quiescent stem cells [23]. CBC cells are rapid cycling long-lived stem cells and are major contributors to epithelial renewal. However, they are very sensitive to external stimuli, such as radiotherapy or chemotherapy [24]. Markers of CBC cells are Lgr5, ASCL2, OLFM4, SMOC2, PROM1, and SOX9^{LO} [25-30]. In contrast, quiescent stem cells are slow-cycling and are activated only when the CBC cell proliferation is perturbed, thus named as reserved intestinal stem cells (rISC). Quiescent stem cells are believed to be resistant to stimuli such as radiotherapy. Markers of quiescent stem cells are BMI1, MTERT, HOPX, LRIG1, SOX9HI, and KRT19 [31-36].

Crypt loss and crypt fission

Intestinal stem cells are highly vulnerable to radiation-induced damage due to their rapid self-renewing properties. Radiation causes DNA double-strand break, thereby triggering p53 mediated response [37]. After DNA damage, the cell will undergo cell cycle arrest and/or apoptosis. If the DNA double-strand breaks are few, then the p53 may cause cell cycle arrest, allowing DNA-double breaks repair and thus allowing the cell to survive [38]. However, if the damage is beyond repairable, then the cell will undergo apoptosis. Radiation of 8 Gy or lower causes apoptosis in the crypts. Still, the damage is reversible, and

the full recovery is possible, suggesting that there is little or no permanent damage. However, at higher radiation doses (12 Gy or higher), there is a permanent loss of CBC cells, causing activation of radioresistant quiescent stem cells [23, 39]. Once all the stem cells get exhausted, the crypts will degenerate and will lead to crypt loss [40]. The loss of crypt stem cells has been suggested to cause radiation-induced intestinal damage [39]. The progressive loss of crypts leads to the mucositis that continues for several days after starting radiotherapy [41]. Studies using germ-free mice have shown that gut microbiota can also stimulate radiation-induced crypt loss [42, 43].

The crypt loss can be compensated by the process of crypt fission (a division of a parent crypt into two or more daughter crypts). During postnatal development, intestinal crypts multiply via crypt fission. It is believed that the crypt fission is driven by the increase in intestinal stem cells during development [44]. However, after reaching adulthood, the process of crypt fission occurs at a low frequency [45]. Even though under normal conditions, the occurrence of crypt fission is rare. However, it occurs more frequently under pathological conditions and certain mutations [46]. Irradiation has also been shown to trigger crypt fission [47]. In mice, the duration of crypt fission is thought to be completed in around one week [48]. The crypt fission process starts with a budding event in the base of the colonic crypt, where the crypt stem cells reside [49].

Microvascular damage and angiogenesis

Microvascular damage and hypoxia have been widely associated with the pathogenesis of radiation-induced tissue damage. Radiation damages blood vessels, causing changes in endothelial cell physiology, thereby contributing to late-radiation induced injury. Endothelial cell apoptosis, detachment from the basement membrane, increased endothelial permeability, increased fibrin deposition, and shifting of the thrombo-hemorrhagic balance toward coagulation are some of the

changes in endothelial cell physiology [1]. Out of all the vascular structures, capillaries are the most sensitive to radiation-induced injury. It is believed that microvascular endothelial apoptosis leads to stem cell dysfunction, eventually leading to the development of the gastrointestinal syndrome. However, the administration of basic fibroblast growth factor can reduce the endothelial apoptosis, indicating that the sensitivity of the microvasculature to radiation-induced toxicity may be dependent on basic fibroblast growth factor [50]. Basic fibroblast growth factor is an angiogenic growth factor that has been shown to increase endothelial proliferation and increases wound vascularity in an irradiated tissue [51]. Macrophages are the major source of angiogenic factors that stimulates the production of granulation tissue [52]. Hyperbaric oxygen therapy has been used to treat hypoxia and to promote angiogenesis and wound healing following radiotherapy.

Radiation-induced inflammation

From an evolutionary perspective, the doses of radiation received by cancer patients during radiotherapy far exceed any doses that humans would have endured naturally. Therefore, to cope with a large amount of damage caused by high doses of radiation, the body needs to utilize the repair mechanisms that are already in place to combat other damaging events such as oxidative damage and damage due to physical injuries. Even though DNA damage repair is the major response; however, due to high doses of radiation, there are a large number of cells undergoing apoptosis, eventually causing severe tissue damage. The response to this tissue damage invokes many aspects of the innate immune system. Most of the DNA double-strand breaks are repaired; however, incorrectly repaired breaks may lead to cell death, which can trigger a strong immune response aimed at tissue repair. This process involves recruiting various immune cells that secrete cytokines, chemokines, and growth factors to attract other immune cells, thus creating a cascade of strong inflammatory response. Thus, radiation

triggers immune response due to oxidative stress and tissue damage. Usually, the immune response is acute and stays only until the damaged tissue is resolved. However, if the damage is too great, then the immune response continues for an extended period, causing a chronic inflammatory state. The immune responses to radiation can be broadly classified into early immune responses and late immune responses.

Early immune responses to radiation

The initial response is the production of damage-associated molecular patterns (DAMPs) released from radiation-induced damaged cells, radiation-induced damaged DNA, RNA, or components of the extracellular matrix, radiation-induced reactive oxygen species (ROS) or ROS produced by activated immune cells. The secretion of DAMPs causes activation of the toll-like receptors (TLRs), which are involved in the immune signaling pathway. Activated TLRs can then mediate activation of a transcription factor, nuclear factor kappa-light-chain enhancer of activated B cells (NF- κ B) [53]. Activated NF- κ B induces secretion of pro-inflammatory cytokines and growth factors, which then recruits more immune cells and causes more secretion of pro-inflammatory cytokines, thus inducing a vicious circle of inflammatory responses [54].

The first immune cells to respond to the irradiation are the resident immune cells, producing pro-inflammatory cytokines and growth factors. The early responses include post-translational modifications such as phosphorylation, which then quickly activate the production and release of effector molecules such as cytokines. The early immune response to irradiation is characterized by the release of pro-inflammatory cytokines, such as interleukin 1 (IL-1), IL-6, IL-8, interferons (IFNs), granulocyte colony stimulating factor (G-CSF), tumor necrosis factor alpha (TNF- α), vascular endothelial growth factor (VEGF), and epidermal growth factor receptor (EGFR), which then activates resident immune cells, such as lymphocytes and macrophages

[55]. These cytokines are released within minutes to hours after exposure to radiation. The plasma levels of IL-1, IL-6, and TNF- α are elevated soon after irradiation [56, 57]. It is believed that IL-1 β , TNF- α , transforming growth factor beta 1 (TGF- β 1), and basic fibroblast growth factor (bFGF), may be associated with morbidity in the patients receiving radiotherapy [58]. Apart from cytokines, chemokines are also released, which attracts circulating neutrophils, macrophages, lymphocytes, and eosinophils to the radiation-damaged site. The recruited immune cells receive the signal from the resident immune cells, following which they release more cytokines and growth factors to perpetuate the damage signal. As the first line of defense, the recruited cells initially remove the damaged tissue matter and also releases a variety of cytokines and chemokines to augment the ongoing inflammatory response.

The detrimental effects of pro-inflammatory cytokines are countered by feed-back signaling, which causes the production of anti-inflammatory cytokines, such as IL-4, IL-10, and TGF- β to diminish the inflammation and to promote tissue repair. The anti-inflammatory cytokines activate M2 macrophages and increase the production of extracellular matrix (ECM) formation required for wound healing [59]. However, if the inflammatory process is not resolved through anti-inflammatory cytokines and persists to continue, then it would lead to late immune responses.

Late immune responses to radiation

Late immune responses occur if the DNA damage is not repaired, causing chronic inflammation. Chronic inflammation may occur in individuals where there is a deficiency in DNA repair [60]. The reactive oxygen species generated during chronic inflammation may also induce genomic instability, which in turn perpetuates the inflammation [61]. Occasionally, radiotherapy causes chronic inflammation leading to pathological fibrotic conditions. Radiation-induced fibrosis occurs at six

months or longer after receiving radiotherapy. For fibrosis, the activation of myofibroblast is very important. Myofibroblasts are derived from fibroblasts, fibrocytes, macrophages or epithelial cells (through epithelial to mesenchymal transition). Activated myofibroblasts cause deposition of extracellular matrix proteins and other signaling molecules required for the fibrotic process. TGF- β is the major regulator of fibrosis and is involved in the activation of myofibroblasts. Other profibrotic cytokines and signaling molecules which are also involved in the activation of myofibroblast include IL-1 β , TNF, IFN- γ , IL-13, and connective tissue growth factor [62, 63]. Radiation-induced fibrosis has been shown to alter intestinal function by diminishing its motility, eventually leading to loss of function [64, 65]. Radiation-induced fibrosis has also been shown to cause increased morbidity in patients receiving pelvic radiotherapy, thus might lead to decreased quality of life [58].

Fecal inflammatory markers

Fecal biomarkers include a biologically heterogeneous group of molecules that either leak from or are actively secreted by the inflamed mucosa. Fecal biomarkers offer promise as a simple, non-invasive, rapid, and low-cost option for disease monitoring. Traditionally, non-invasive biomarkers such as neutrophil elastase, calprotectin, lactoferrin, and myeloperoxidase have been used to identify intestinal inflammation. In this thesis, neutrophil elastase and calprotectin were evaluated to identify the radiation-induced intestinal injury.

Neutrophil elastase

Polymorphonuclear leukocytes (PMN), also called polymorphonuclear neutrophils, are the most abundant leukocyte cells in the blood. PMN comprise about 50-60% of the total circulating leukocytes and are critical components of the innate immune system [66]. Neutrophils play an important role in protecting the host against invading pathogens and

in maintaining the intestinal homeostasis [67, 68]. Their antimicrobial function is rendered through degranulation and phagocytosis [67]. Neutrophils are capable of producing a large amount of reactive oxygen species (ROS) and other toxic components responsible for destroying invading pathogens [68]. Other antimicrobial components of neutrophils include antimicrobial peptides (α -defensins and cathelicidins), myeloperoxidase, hydrolytic enzymes (lysozyme, sialidase, and collagenase), lactoferrin and proteases (Cathepsin G, azurocidin, and elastase), which are released upon contact with bacteria [69]. Neutrophils promote mucosal healing by producing growth factors, such as vascular endothelial growth factor and lipid mediators, such as lipoxins, resolvins, and protectins, that facilitate the healing [70, 71]. They also phagocytose cell debris accumulating at the injured site [72]. Apart from the above-mentioned beneficial responses, neutrophils are also involved in direct recruitment of immune cells and contributing to intestinal inflammation by secreting pro-inflammatory cytokines, such as interleukin (IL)-8 and IL-17 [73-76].

Neutrophil elastase is a neutral proteinase stored in the azurophil granules of polymorphonuclear leukocytes (PMN) and is released by the activation of these cells. It binds to the membrane of the bacteria and cleaves the virulence factors of bacteria, such as *Enterobacteria*, with high specificity [77]. Mice lacking neutrophil elastase has been shown to have decreased host defense against invading pathogens and is highly susceptible to develop infection [78, 79]. Apart from having beneficial roles, neutrophil elastase also has detrimental effects on the body. It is an essential mediator of inflammation and tissue damage [80-82]. It is also involved in the pathological inflammation in inflammatory bowel diseases. Treatment with anti-elastase therapy has been shown to decrease dextran sulfate sodium or trinitrobenzene sulfonic acid-induced colitis in rats [83].

Calprotectin

Calprotectin is a calcium and zinc-binding heterocomplex protein, which consists of two heavy chains and one light chain protein. It is a 36 kDa protein that belongs to the S-100 protein family (S100 A8/A9) and is expressed by neutrophils and monocytes [84]. It is presumed to be a protective protein that is distributed in myelomonocytic cells, epithelial cells, and keratinocytes and in various tissues and fluids in the body [85]. Calprotectin and its subunits seem to have regulatory functions in the inflammatory processes and have antimicrobial and antiproliferative activity [86, 87]. Due to its antimicrobial activity, it plays an important role in neutrophil defense against microbial infections [88]. It renders its antimicrobial activity by chelating divalent metal ions that are required for bacterial growth [89]. In acute inflammation, elevated levels of calprotectin have been found, which corresponds to increased numbers of neutrophil granulocytes [85]. The calprotectin measurement is clinically relevant in several conditions, such as inflammatory diseases, bacterial infections, and neoplastic conditions. Calprotectin is elevated in cystic fibrosis, rheumatoid arthritis, ulcerative colitis, Crohn's disease, and bacterial infections [90-95]. Calprotectin is also elevated in the fecal samples of the patients undergoing pelvic radiotherapy [96].

Dietary fiber and pelvic radiotherapy

Diet is an important factor in shaping the composition of the gut microbiota, as they rely on complex compounds, especially dietary fibers, to obtain energy for their growth. In 1972, Trowell suggested dietary fibers as those which consist of the remnants of edible plant cells, polysaccharides, lignin, and associated substances resistant to digestion by the alimentary enzymes of the humans [97]. The Codex Alimentarius Commission defines dietary fiber as carbohydrate polymers with ten or more monomeric units which are not hydrolyzed by the endogenous enzymes in the small intestine of humans [98]. The European Food

Safety Agency defines dietary fiber as “non-digestible carbohydrates plus lignin” [99]. Dietary fiber can be classified in several ways, such as structure and solubility. In terms of structure, polysaccharides can be classified into linear or non-linear molecules. On the basis of solubility, dietary fibers can be divided into soluble fiber and insoluble fiber. Soluble fiber mainly consists of noncellulosic polysaccharides (e.g., pectin, gums, and mucilage), while insoluble fiber consists mainly of plant cell wall components (e.g., cellulose, lignin, hemicellulose) [100].

Based on finding from our epidemiological study of over 600 irradiated gynecological patients, we observed that patients who consumed a higher amount of dietary fiber had a lower prevalence of radiation-induced gastrointestinal symptoms [13]. Also, in a prospective study with randomly allocated dietary advice indicated that a high-dietary intake results in a better intestinal health one year after radiation as compared to a low or habitual-fiber intake [101]. However, contradictory results have been found in a study where dietary fiber intervention had no significant effects on radiation-induced symptoms [102, 103].

Oat bran and microcrystalline cellulose fibers were used in this thesis. Oat beta-glucan is extensively fermented in the colon, producing short-chain fatty acids (SCFAs), whereas microcrystalline cellulose is resistant to colonic fermentation [104]. Oat bran contains beta-glucan, which is a linear polysaccharide composed of blocks of consecutive $\beta(1-4)$ linked D-glucopyranosyl residues that are separated by single $\beta(1-3)$ linkages [105]. On the other hand, microcrystalline cellulose is a linear polysaccharide that consists of $\beta(1,4)$ glycosidic linkages [106]. The exposed reducing ends of oat beta-glucan make it more soluble in contrast with microcrystalline cellulose, which lacks such reducing ends [107].

Dietary fiber has been known to influence the composition of the gut microbiota, thereby increasing the production of short-chain fatty acids

and colonic mucus barrier. The role of all these factors in radiotherapy is described in detail below.

Gut Microbiota

The intestine of a healthy adult human hosts up to approximately 10^{13-14} microbes, with approximately 300-500 species of bacteria. The two most dominant phyla are *Firmicutes* and *Bacteroidetes*. Gut microbiota has a profound impact on human health. The microbiota and the host interact in several ways. The microbiota stimulates the immune system, helps in the development of the epithelium, provide nutrients, and exerts colonization resistance against pathogens [108]. They synthesize vitamins and provide energy to the colonic epithelial cells by producing short-chain fatty acids (SCFAs). They also play an important role in host mucosal defense mechanisms. It is difficult to define a healthy gut microbiota as every individuals' gut microbiota comprise of the unique mix of bacterial species. However, certain bacterial family and class are related to gut health [109].

A study by Hibberd *et al.* has shown that patients who have pelvic cancer have altered gut microbiota [110]. Radiation also significantly alters the already perturbed gut microbiota composition in both humans and animals [110-112]. A study by Manichanch *et al.* showed that patients who developed post-irradiation diarrhea had distinct gut microbiota than patients who did not develop it [113]. Also, patients who developed diarrhea after radiotherapy had greater alteration in their microbiota profile compared to patients who did not develop it. Lastly, the patients who developed diarrhea had a high abundance of *Bacilli* and *Actinobacteria* and decreased in *Clostridia* compared to patients who did not develop diarrhea. Gram-negative *Bacilli*, along with abnormal motility, are important factors in the pathogenesis of late radiation enteropathy [114]. Similarly, a study by Wang *et al.* found that radiation caused decreased microbial diversity in the diarrhea group than the non-diarrhea group [115]. Germ-free mice are resistant to whole-body

irradiation compared to conventionally raised mice. All these results indicate that gut microbiota plays a vital role in the pathogenesis of radiation-induced enteropathy.

Probiotics are live microorganisms, that when consumed in an adequate amount, confers a health benefit on the host health. Several studies investigating the effects of probiotics in radiation-induced gastrointestinal symptoms have been conducted with contradictory results. Some studies have shown no effects, while some have shown beneficial effects of probiotics [116, 117]. Probiotics, such as *Lactobacillus rhamnosus*, have been shown to have beneficial effects in reducing radiation-induced diarrhea in patients undergoing radiotherapy [118]. A gavage of *Lactobacillus rhamnosus* has also been shown to increase crypt survival and reduce radiation-induced intestinal injury [119].

Short-chain fatty acids

Gut microbiota affects host health and disease by producing different types of metabolites. Certain metabolites have a beneficial role in the body, which would promote host health, while certain metabolites have detrimental effects in the body that would contribute to the development of diseases. One such class of beneficial metabolites are short-chain fatty acids (SCFAs). SCFAs are a subset of fatty acids with up to six carbon atoms. They are produced by gut microbiota during the fermentation of diet-derived, indigestible poly- and oligosaccharides and the carbohydrates that escape proximal digestion and adsorption [120]. Acetate, propionate, and butyrate are the major SCFAs released through fermentation of dietary fibers. However, when the dietary fibers are short in supply, then the gut microbiota will switch to energetically less favorable sources such as amino acids from dietary proteins to produce SCFAs, such as isobutyrate and isovalerate [121]. SCFAs are found at the highest levels in the proximal colon, where they are a major energy source for the residing colonocytes [122]. Thus, they are

essential for maintaining gut energy homeostasis. Butyrate is the major energy source for the colonocytes, while acetate and propionate reach the liver through the portal vein.

SCFAs effects are rendered through two signaling mechanisms, inhibition of histone deacetylases (HDACs) and activation of G-protein coupled receptors (GPCRs). GPCRs, such as GPR41, GPR 43, and GPR109A, have been identified as receptors for SCFAs and have been found to have an important role in the regulation of metabolism, inflammation, and disease.

SCFAs have been found to alter chemotaxis and phagocytosis, change cell proliferation and function, induce reactive oxygen species, have anti-inflammatory and anti-tumorigenic activity, and alter the gut integrity. The anti-inflammatory activity of SCFAs is majorly through butyrate, which suppresses the production of pro-inflammatory cytokines, such as IL-12, TNF- α and IL-1 β , upregulate the production of anti-inflammatory cytokine IL-10, and reduce NF-kB activity [123-125]. Butyrate also possesses anti-tumorigenic activity by inducing apoptosis, thus inhibiting the growth of colon cancer [126]. All these findings suggest that SCFAs plays a vital role in maintaining gut and immune homeostasis.

Diet has been shown to alter the composition of gut microbiota by which it also influences the production of SCFAs. A comparative study has shown that children living in a rural area, consuming a diet rich in fiber, have a high abundance of SCFAs-producing bacteria than children living in the urban area [127].

Colonic mucus barrier

The digestive tract has ten times as many bacteria as human cells that live in harmony to ensure homeostasis. This is possible because the intestinal epithelium is protected from bacterial invasion by two mucus

layers, the inner and outer mucus layer (Figure 1). These two layers have similar protein composition and are ~150 μm thick in mice [128]. Mucins (MUC) are the major components of mucus layers and are part of the innate immunity and are well preserved in evolution [129]. The gastrointestinal tract consists of 12 different types of mucin; MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, MUC6, MUC7, MUC12, MUC13, MUC16, and MUC17 [130]. MUC2, a gel-forming mucin, is the major component of both the inner and outer mucus layers. The inner mucus layer is densely packed, firmly attached to the epithelium, devoid of bacteria, and is renewed every hour by surface goblet cells. The outer mucus layer is loose, movable, has an expandable volume due to proteolytic cleavages of the MUC2 mucin, and is the habitat for commensal gut bacteria. The mucins are characterized by domains rich in the amino acids, proline, serine, and threonine (called the PTS domain). The PTS domains are heavily O-glycosylated by the glycosylation machinery in the Golgi apparatus. The numerous O-glycans present in the MUC2 serve as a nutrient and as an attachment site for the bacteria. Hence, MUC-2 O-glycans can contribute to the selection of species-specific gut bacteria [131]. Mice lacking MUC2 mucin have bacteria in direct contact with the epithelial cells, which can trigger the inflammatory response and cause inflammation [128]. Thus, maintaining the intact mucus layer is very important to prevent microbiota invasion and susceptibility to infections. Also, mice deficient in MUC2 displayed aberrant crypt morphology and are prone to develop colorectal cancer [132].

Diet and gut microbiota play an essential role in ensuring the integrity of the mucus barrier. A fiber-deprived diet causes severe mucus barrier degradation by promoting the expansion of mucus-degrading bacteria, such as *Akkermansia Muciniphila*, thereby increasing pathogen susceptibility [133]. A low-fiber diet can also lead to mucus degradation and can enhance the susceptibility to develop chronic inflammatory diseases [128, 134]. The increased inflammation due to pathogen invasion and immune reaction could be detrimental to the pelvic cancer

patients, considering the fact that they already have increased levels of pro-inflammatory cytokines due to radiotherapy. The augmented chronic inflammation, in turn, could facilitate lifelong radiation-induced gastrointestinal symptoms.

Dietary fibers can also mechanically induce the epithelial cells to secrete mucus [135]. Gut microbiota, such as *Bacteroides thetaiotaomicron*, increases goblet cell differentiation and upregulates the expression of mucin-related genes, thereby favoring mucus production [136]. SCFAs can also stimulate the production of mucus. SCFAs, such as butyrate, stimulates mucus production by increasing the concentrations of prostaglandin E1, thereby increasing mucus barrier functions [137].

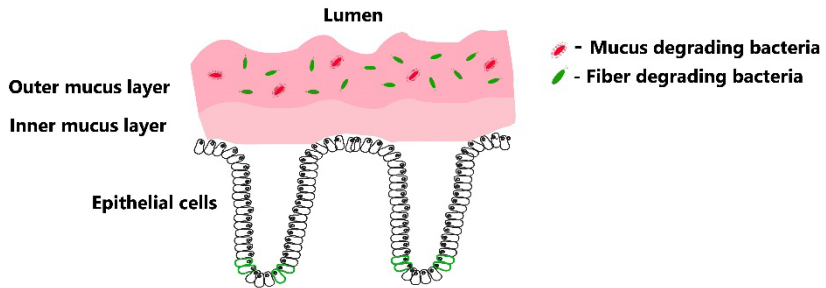


Figure 1. Colonic epithelial cells covered by two mucus layers, the inner and outer mucus layer.

AIM

The general aim of this thesis was to identify the mechanisms responsible for causing radiation-induced intestinal dysfunction. The thesis also aimed to provide knowledge that can be used to decrease the intensity and duration of mechanisms that may give lifelong impaired intestinal health after radiotherapy. The ultimate aim is thus to improve intestinal health in cancer survivors having received pelvic radiotherapy.

The specific aims are:

Paper I: To identify non-invasive biomarkers to detect radiation-induced intestinal injury of the distal colon.

Paper II: To study the long-term progression of radiation-induced mucosal injury and repair mechanisms that might help to decrease radiation-induced mucosal injury.

Paper III: To evaluate if consuming a fiber-rich high oat bran diet could help in reducing radiation-induced systemic inflammation.

Paper IV: To investigate whether consuming a fiber-rich high oat bran diet could decrease radiation-induced intestinal damage.

METHODOLOGY

In this chapter, I will briefly describe the materials and methods used in this thesis. I will also reflect on the rationale for using those methods and outline the possible pros and cons related to them.

For the detailed description of the methods, see the Materials and Methods section in the corresponding thesis papers.

Animals

In all the papers, C57BL/6J male mice from Charles River Laboratories (Sulzfeld, Germany) were used. All the mice were housed at a constant temperature and humidity. A 12-hr dark/light cycle was maintained, and food and water were available *ad libitum*. All the experiments were approved by the Gothenburg Committee of the Swedish Animal Welfare Agency (application number 22-2015 for the paper I and II and application number 1458-2018 for paper III and IV). All animal experiments were planned and performed based on the principle of the 3R, Refine, Reduce, and Replace.

Mouse model to study radiation-induced intestinal damage

The mouse model is one of the most commonly used experimental models to study molecular mechanisms responsible for causing radiation-induced intestinal damage. The C57BL/6J mice used in our model is one of the most widely used inbred strains in biology. It is a stable and homogenous strain that breeds easily and provides more reproducible results. All these characteristics make them suitable to study radiation-induced intestinal damage.

Both mice and humans have similar pathology and pathophysiology. Thus, the translation of the observations made in mice to humans is

possible. Another advantage of the animal model is that the radiation dose and fractions can be precisely and easily adjusted. Thus, making it easier to study the dose-response relationships in an animal model [1].

In our model, mice were irradiated with the same linear accelerator that is used to irradiate the human cancer patients (Figure 2). High-energy photons can be delivered to a specific area, encompassing the colorectal region of the mice. Thus, avoiding exposure of non-specific target tissues/organs of the mice to radiation. In paper II, we observe that the radiation-induced pathophysiological changes seen in the mice were identical to the changes observed in cancer survivors who underwent radiotherapy. We irradiate the mice with fractionation method, which mimics the clinical setting, where the patients receive the radiation dose divided into several fractions, delivered over a period of several weeks. The irradiated mice appear healthy and have a normal life span. Hence, our model is also suitable for long-term study, which is especially important, considering the fact that the patient can experience radiation-induced intestinal symptoms years or decades after receiving radiotherapy [138, 139].



Figure 2. A clinical linear accelerator used to irradiate our mice. Image courtesy of Varian Medical Systems, Inc. All rights reserved.

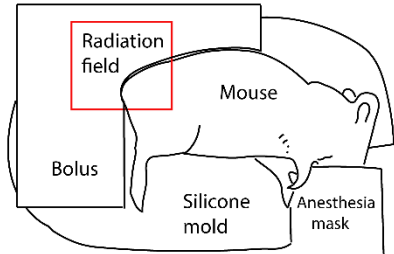
Despite having many advantages, the mice model has some disadvantages, as well. The sensitivity of the mice and the response to radiation varies depending on the strain. In a previous study, C57B1/6 mice were found to be more sensitive to radiation compared to C3Hf/Kam mice [140]. Also, the dose delivered to the animals might not be comparable to the dose given to patients [138]. Another disadvantage of the mice model is that the repair capacity of the intestine and the response to the treatment will not be the same in humans and animals. Finally, one of the most important delimiting factors is that many of the symptoms experienced by the patients are not observed in animal models and hence, cannot be studied.

Irradiation procedure

Mice were anesthetized with isoflurane and irradiated using a clinical linear accelerator. In paper I, Varian Clinac 600 CD (Radiation Oncology Systems LLC, San Diego, CA), a linear accelerator with 4 MV of nominal photon energy and a dose rate of 3.2 Gy/min was used. In paper II, III and IV, TrueBeam (Varian Medical Systems Inc., Charlottesville, Virginia, USA), a linear accelerator with 6 MV of nominal photon energy and a dose rate of 5.9 Gy/min was used. Mice were placed on a silicone mold used to ensure identical positioning of all the animals and were covered by a 5-mm tissue-equivalent bolus material, to ensure even distribution of irradiation throughout the irradiation field (Figure 3A and 3B). Approximately 1.5 cm of the distal bowel was irradiated with 2, 3, or 4 fractions of 6 or 8 Gy. In paper I and II, mice were irradiated with 2, 3, or 4 fractions of 6 or 8 Gy, whereas in paper III and paper IV, mice were irradiated with 4 fractions of 8 Gy. Each fraction was given with an interval of 12 hours. The sham-irradiated control mice were anesthetized but were not exposed to irradiation.

The advantage of using a clinical linear accelerator is that a clinically relevant high dose rate can be delivered to a small restricted area. Also, the dose variation within the target volumes are minimum ($\pm 5\%$).

A. 3x3 cm radiation field placement



B. 5 mm thick bolus covering

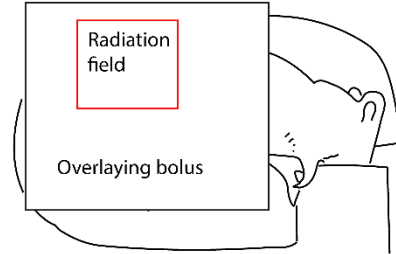


Figure 3. The mouse was anesthetized and placed in a silicone mold with the radiation field covering approximately 1.5 cm of the colorectum (A). The body was covered with 5-mm thick tissue equivalent bolus material to obtain even distribution of irradiation (B).

Fecal sample collection (Paper I and IV)

In paper I, fecal samples were collected at three time-points, i.e., at 1-, 3- and 6-weeks post-irradiation for analyzing the calprotectin and neutrophil elastase levels. In paper IV, fecal samples were collected every week, starting at 2 weeks before irradiation and continued until 6 weeks after irradiation. The fecal microbiota analysis and mucus degradation activity were analyzed at five different time-points, i.e., at 2 and 0 weeks before irradiation and at 1, 3, and 6 weeks after irradiation. The SCFAs concentrations were measured at 6 weeks after irradiation time-point. Lastly, the concentrations of neutrophil elastase were measured at four different time-points, i.e., at 1 week before irradiation and 1, 3, and 6 weeks after irradiation.

Measurement of calprotectin levels in the fecal samples of the mice using a sandwich ELISA technique (Paper I)

The concentrations of calprotectin levels were measured in the fecal samples of the mice using the S100A8/ S100A9 ELISA Kit (Immundiagnostik AG, Bensheim, Germany). The procedure was followed according to the manufacturer's instructions. In brief, fecal samples were diluted with extraction buffer, homogenized, and centrifuged. After that, sample supernatant, standards, and controls were added to the pre-coated wells and incubated. The plates were then washed and incubated with the lyophilized detection antibody (monoclonal mouse anti-calprotectin). After washing, the plates were incubated with the conjugate solution (anti-mouse peroxidase-labeled). The plates were washed again and incubated with the substrate (tetramethylbenzidine) in the dark. The color reaction was stopped by adding the stop solution, and the plate was read at 450 nm on an EMax microplate reader. The concentration of calprotectin was expressed as ng/ml feces.

Measurement of neutrophil elastase levels in the fecal samples of the mice using a sandwich ELISA technique (Paper I and IV)

The concentrations of neutrophil elastase were measured in the fecal samples by using the PMN-Elastase ELISA kit (Immundiagnostik AG, Bensheim, Germany). In brief, fecal samples were diluted with extraction buffer, homogenized, and centrifuged. Diluted samples, standards, and controls were transferred to the pre-coated wells and incubated. After washing, the plates were incubated with detection antibody (monoclonal mouse anti-PMN elastase). The plates were then washed and incubated with the conjugate solution. In paper I, instead of incubating with the conjugate solution, the plates were first incubated with anti-mouse IgG donkey biotinylated and then with streptavidin conjugated to poly-Horseradish peroxidase (Poly-

HRP (Sanquin, Amsterdam, Netherlands). The plates were washed again and incubated with the substrate in the dark. The color reaction was stopped by adding the stop solution, and the plate was read at 450 nm on an EMax microplate reader. The concentration of neutrophil elastase was expressed as ng/ml feces.

Sandwich ELISA is the most common ELISA technique used. In sandwich ELISA, the target antigen binds between a capture antibody and a detection antibody. The advantages of sandwich ELISA technique are that it has high specificity and high sensitivity. However, it may require a simple set of validation steps to remove false-positive results [141].

Fecal microbiota analyses (Paper IV)

In our study, fecal samples were analyzed to observe the gut microbiota composition of the mice belonging to different treatment groups. In brief, bacterial DNA was extracted from the fecal samples. Later, the V3-V4 region of the 16S rRNA gene was PCR amplified using primers adapted from Klindworth *et al.* [142]. The amplified DNA was then purified and quantified. The equal quantity of purified DNA was again amplified. The samples were again purified and quantified. DNA samples were also quantified to determine the amplicon size. After quantifying the concentrations, samples were diluted to equal concentrations and were pooled together. The V3-V4 regions of the 16S rRNA gene amplicons were sequenced using the Illumina MiSeq system with 2 x 300 bp setup. The raw sequenced data were quality checked with FastQC software and were processed with QIIME 2 [143]. DADA2 plugin was then used to receive the Exact Sequence Variants (ESVs), and the ESVs obtained were then clustered into 97% operational taxonomic unit (OTU) [144, 145]. The OTUs were then taxonomically assigned using the SILVA (v132) SSU rRNA reference sequence database [146, 147]. Later, the assigned OTUs reads were calculated to measure the abundance of various bacteria in the gut.

There are various methods to study the composition of gut microbiota. They can be broadly divided into culture-based methods and culture-independent methods. Historically, bacterial communities were cultured in appropriate growth media. Much of our knowledge of gut microbiota comes from culture-based methods. However, most of the bacterial species in the gut are strictly anaerobic and are difficult to culture [148]. Hence, culture-independent methods that target the 16S ribosomal gene (16S rRNA) of the bacteria are often exploited. The most common methods used for profiling the 16S rRNA gene includes, terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), human intestinal tract chip (HITChip), Phylochip and sequencing the 16S rRNA gene amplicon by next-generation sequencing [149-151]. Metagenomics is the most recent technique used in the analysis of gut microbiota. In this technique, all the DNA fragments are sequenced instead of sequencing particular DNA fragments.

The 16S rRNA gene is 1500 bp long and contains highly conserved regions in which the DNA sequences between different bacteria are very similar to each other. Apart from the conserved regions, it also has nine variable regions (V1-V9), the sequences of which differ among various bacterial taxa. The conserved regions are suitable for designing a universal primer, while the variable regions can be used to distinguish different taxa.

Nowadays, sequencing the 16S rRNA gene amplicon has become more and more common and have proven invaluable for advances in microbial ecology, as they are fast, facilitate high throughput, allow phylogenetic identification of the gut microbiota and have low sequencing cost [152, 153]. However, this method, like any other method, has its disadvantages. Even though the conserved regions are very similar between different bacteria, yet they are not completely identical to each other, and thus produces a bias in the primer-binding and PCR

amplification efficiency for sequences belonging to different taxa [154, 155]. Another problem in sequencing the 16S rRNA gene is that different bacteria contain different numbers of 16S rRNA gene. Around 40% of bacteria contain one or two copies, up to 7 copies are commonly found [156]. Another factor that influences the abundance and the detection of the bacteria is the efficiency of DNA extraction. Some Gram-positive bacteria, such as *Enterococcus* and *Staphylococcus*, have a thick cell wall; thus, it is difficult to extract the DNA from these bacteria. Also, Gram-positive bacteria such as *Actinobacteria* have a high GC content; thus, the universal primers are not capable of amplifying the 16S rRNA gene [157].

Fecal short-chain fatty acids (SCFAs) analysis by gas-liquid chromatography (Paper IV)

The concentrations of various SCFAs in the fecal samples of the mice were measured using the gas-liquid chromatography (GLC) method developed by Zhao *et al.* [158]. In brief, fecal samples were diluted with water and homogenized. The pH of the homogenized samples was then adjusted to 2-3 and homogenized again. The sample supernatant and internal standard (7.9 mM 2-ethylbutyric acid in 12% v/v formic acid) were then added in the glass vial and mixed by shaking. The samples were then analyzed using a Perkin-Elmer's autosystem gas chromatography with a flame ionization detector and an autosampler. SCFAs concentrations are expressed in $\mu\text{moles/g}$ feces.

Mucus degradation activity (Paper IV)

Mucus degradation activity was measured in the fecal samples of the mice belonging to different treatment groups. In brief, the mucus from the intestines of the mice fed the normal chow diet was scraped and extracted with guanidinium chloride, in the presence of protease inhibitor. The mucus was then reduced and alkylated using a method developed by Johansson *et al.* [159]. The mucus Mucin-2 (Muc2)

obtained was then transferred to the PVDF membrane using the dot-blotting technique. Fecal samples of the mice in different groups were diluted in PBS and incubated for 4 hours at 37°C with the Muc2 dot blots. The Muc2 O-glycans present on the dot blots were released by the technique developed by Schulz *et al.* [160]. The released O-glycans were then resuspended in water and were analyzed using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). The area under the curve of 23 dominant structures were selected and were compared to observe the mucus degradation activity between the different groups.

Blood collection and serum preparation (Paper III)

Mice were anesthetized, and the blood was extracted through cardiac puncture. Blood was then allowed to clot at room temperature. Later, centrifuged and stored at -80°C until further use. Blood was processed in a low protein-binding Eppendorf tube to avoid unspecific adsorption of proteins to the tube surface.

Serum cytokines and chemokines analysis by Luminex Bead-based Multiplex Assay (Paper III)

The concentrations of mice serum cytokines and chemokines were analyzed using the Bio-Plex Mouse Cytokine 23-Plex Assay (Bio-Rad Laboratories AB, Solna, Sweden). Initially, the plate was pre-wet with a wash buffer and was primed with a bead solution. After that, samples and standards were added to the plate and incubated. After washing, the plate was incubated with the detection antibody, followed by incubation with streptavidin-PE. The plate was then washed, and the beads were resuspended with assay buffer. The fluorescence intensity was measured using the Bio-Plex 200 system. The data were processed using Bio-Plex Manager software. The concentrations of serum cytokines and chemokines were expressed in pg/ml.

Luminex Multiplex Assay has many advantages over the standard ELISA assay, such as accuracy, flexibility, higher throughput, smaller sample volumes required, time-saving, and low-cost relative to equivalent ELISAs. In our study, concentrations of 23 cytokines were measured simultaneously in a single run.

Colorectal tissue collection (Paper II and IV)

In paper II, the mice were sacrificed at 24 hr and at 1, 6, 18, and 30 weeks after irradiation, followed by colorectal tissue collection. The tissues were then immediately fixed in Histofix, dehydrated, and paraffin embedded. In paper IV, the mice were sacrificed at 6 weeks after irradiation, and the colorectal tissues were immediately fixed in methanol-Carnoy's solution. Later, the tissues were dehydrated, and paraffin embedded.

Histochemistry (Paper II and IV)

In paper II, the mucosal crypts were visualized using the Alcian blue combined with Nuclear Fast Red or Verhoeff's Elastic stain. In paper IV, the mucosal crypts were visualized using the Verhoeff's Elastic stain. For the Alcian blue staining, the tissue sections were dewaxed, rehydrated, treated with acetic acid, and stained with Alcian Blue. The sections were then stained with Nuclear Fast Red, dehydrated, and coverslipped. For the Verhoeff's Elastic stain, the sections were dewaxed, rehydrated, and stained with Verhoeff's solution. The sections were then differentiated in a ferric chloride solution, followed by sodium thiosulfate solution. Later, the sections were washed and counterstained with Van Gieson's stain. Finally, the sections were dehydrated and coverslipped.

Immunohistochemistry (Paper II)

Immunohistochemistry was performed to quantify the number of proliferating cells in the crypts. Immunohistochemistry was also performed to measure the angiogenesis in the tissue by quantifying the number of blood vessels. For quantifying the number of proliferating cells, tissues were stained with the cell proliferation markers such as Ki-67 and BrdU. For BrdU labeling, the mice were injected intraperitoneally with BrdU 4 days before sacrifice, except for 24 hr time-point. BrdU is a synthetic thymidine analog that gets incorporated into the DNA of the proliferating cells during the S-phase of the DNA replication, thus labeling the proliferating cells [161]. For quantifying the number of blood vessels, tissues were stained with the CD31 marker.

The tissue sections were dewaxed and rehydrated, followed by antigen retrieval using the citrate buffer. For BrdU staining, the sections were incubated in hydrochloric acid and neutralized in borate buffer. The sections were then incubated with primary antibodies in blocking solution containing donkey serum and detergents. The antibodies used were as follows: anti-rabbit Ki-67 (1:150), anti-mouse BrdU (1:500), and goat anti-CD31 (1:150). After that, the sections were incubated with biotinylated secondary antibody (1:250), followed by an avidin-biotin solution. The staining was developed in a DAB-Peroxidase substrate solution. Finally, the sections were dehydrated and coverslipped.

Statistical Analysis

Data were processed in GraphPad prism software. In paper I, the statistical difference between the levels of neutrophil elastase and calprotectin in the irradiated and the sham-irradiated mice were calculated using the non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparisons test, to compare the difference between two groups. In paper II, for comparing two groups, a two-tailed student's *t*-test was used. In paper III, the data that were normally distributed, a

two-tailed student's *t*-test was used to compare the two groups. For the data that were not normally distributed, a non-parametric, Mann-Whitney test was used. The outliers in the data were identified and removed by using the ROUT method in the GraphPad prism software. In paper IV, if the data were normally distributed, a One-way ANOVA with Tukey's post hoc test was used to compare the differences between the irradiated groups. A non-parametric Kruskal-Wallis test was used if the data were not normally distributed. The statistical differences between the two groups were calculated using a two-tailed student *t*-test for the data that appeared to be normally distributed. Otherwise, a non-parametric, Mann-Whitney test was used. In the figures, statistically significant differences are shown with asterisks as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; and **** $p \leq 0.0001$.

MAIN RESULTS & DISCUSSION

Paper I

Traditionally, neutrophil elastase and calprotectin have been used as a diagnostic biomarker to detect intestinal inflammation in patients with inflammatory bowel diseases [95, 162, 163].

Calprotectin has also been evaluated as a biomarker to study intestinal inflammation induced by pelvic radiotherapy. Contradictory results have been found for calprotectin as a biomarker to study radiation-induced toxicity. Two studies showed that radiotherapy causes a significant increase in the calprotectin levels in the fecal samples of the patients suffering from pelvic cancer [96, 164]. One study exhibited that no changes in calprotectin levels were found in the fecal samples of the patients treated with pelvic radiotherapy [165]. One study showed that calprotectin levels increased in the non-rectal cancer patients but did not increase in rectal cancer patients receiving pelvic radiotherapy [166].

In this study, we wanted to investigate whether elastase and/or calprotectin could be used as a biomarker to study radiation-induced intestinal injury of the colorectal region in our mouse model. Neutrophil elastase and calprotectin levels were detected in the fecal samples of the mice exposed to 6 or 8 Gy irradiation with either 2, 3, or 4 fractions at three different time points, i.e., at 1, 3, and 6 weeks after irradiation and compared it with the sham-irradiated mice.

Irradiation causes increased neutrophil elastase levels

The elastase levels were found to be significantly higher in the fecal samples of the mice subjected to 3 fractions of 6 Gy irradiation than the sham-irradiated mice at 6 weeks after irradiation (Figure 4B). No

significant differences were observed in elastase levels between the mice irradiated with 2, 3, or 4 fractions of 6 Gy and the sham-irradiated mice at 1 week after irradiation (Figure 4A).

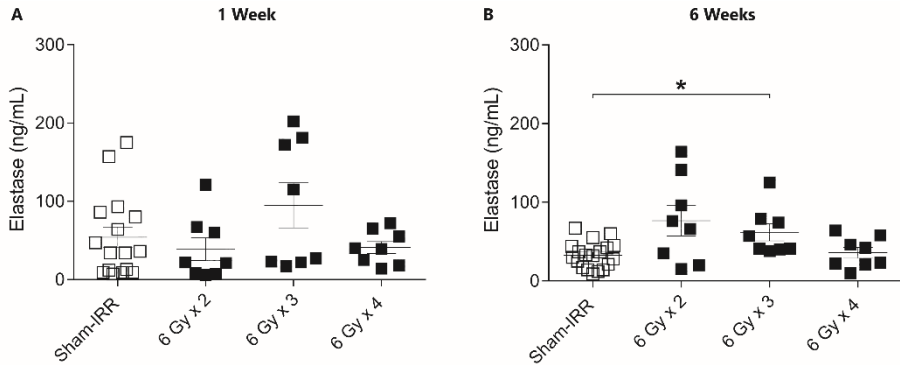


Figure 4. Elastase level in the fecal samples of the mice irradiated with 2, 3, or 4 fractions of 6 Gy and sham-irradiated mice at one (A) and six weeks post-irradiation (B). The figure is taken from Sjöberg F et al. *Acta Oncol.* 2018;57(8):1025-30.

The elastase levels were significantly elevated in the mice irradiated with 3 fractions of 8 Gy, as compared to the sham-irradiated mice at 1, 3, and 6 weeks after irradiation (Figure 5). Similarly, elastase levels were also significantly higher in the mice subjected to 4 fractions of 8 Gy than the sham-irradiated mice at 6 weeks after irradiation (Figure 5).

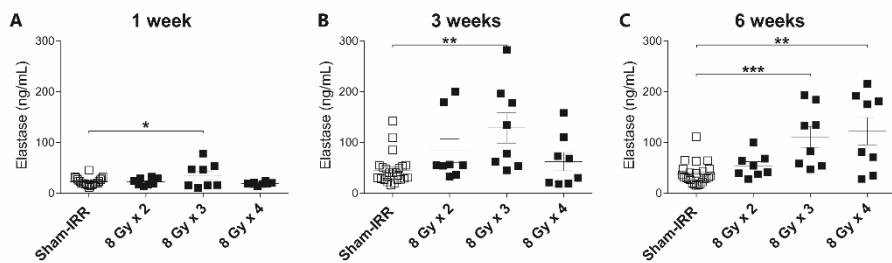


Figure 5. Elastase level in the fecal samples of the mice irradiated with 2, 3, or 4 fractions of 8 Gy and sham-irradiated mice at one (A), three (B), and six

weeks post-irradiation (C). The figure is taken from Sjöberg F et al. *Acta Oncol.* 2018;57(8):1025-30.

Irradiation did not influence calprotectin levels

Irradiation did not seem to alter the levels of calprotectin in the mice significantly. No significant differences were observed in the calprotectin levels between the mice irradiated with 2, 3, or 4 fractions of 6 Gy and the sham-irradiated mice (Figure 6).

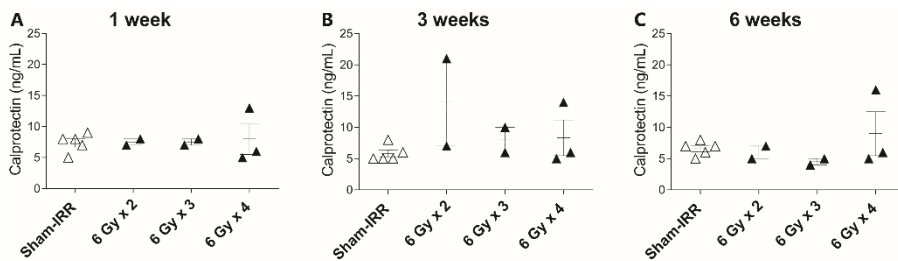


Figure 6. Calprotectin level in the fecal samples of the mice irradiated with 2, 3, or 4 fractions of 6 Gy and sham-irradiated mice at one (A), three (B), and six weeks post-irradiation (C). The figure is taken from Sjöberg F et al. *Acta Oncol.* 2018;57(8):1025-30.

Similarly, calprotectin levels were comparable between the mice subjected to 2, 3, or 4 fractions of 8 Gy irradiation and the sham-irradiated mice (Figure 7).

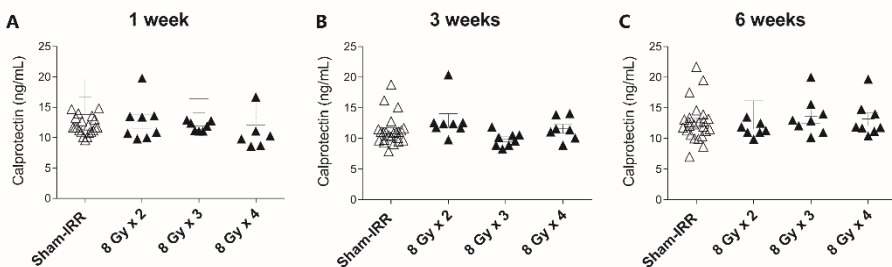


Figure 7. Calprotectin level in the fecal samples of the mice irradiated with 2, 3, or 4 fractions of 8 Gy and sham-irradiated mice at one (A), three (B), and six weeks post-irradiation (C). The figure is taken from Sjöberg F et al. Acta Oncol. 2018;57(8):1025-30.

These results indicate that irradiation causes increased neutrophil elastase levels. It also shows that neutrophil elastase is a sensitive biomarker to study radiation-induced intestinal injury than calprotectin. Neutrophil elastase has been associated with a wide variety of functions in the body. It has been shown to have antimicrobial activity rendered through cleaving bacterial virulence factors and outer membrane proteins, thereby causing increased host defense against invading bacteria [67, 77, 78]. Mice deficient in neutrophil elastase have been shown to have impaired immunity and are susceptible to fungal infections [79]. Neutrophil elastase has also been found to increase degradation of elastin fiber and to disrupt epithelial barrier function by disrupting cell-cell contact mediated through adherens junction proteins such as E-cadherin and β -catenin [167, 168]. Disruption of epithelial barrier function might lead to translocation of luminal content into the mucosa, thus might lead to activation of the inflammatory cascade. Thus, neutrophil elastase might be responsible for increased intestinal inflammation following pelvic radiotherapy.

Taken together, it seems that neutrophil elastase is a good biomarker to identify the radiation-induced intestinal injury. However, further studies need to be performed to understand the precise role of neutrophil elastase in the intestine following pelvic radiotherapy.

Paper II

Intestinal stem cells are the most vulnerable cells to irradiation due to its rapid proliferative property. Studies have shown that exposure to radiation causes apoptosis and a reduction in proliferating cells in the intestine [37]. The lost stem cells and progenitor cells are then compensated by increased cell proliferation [169, 170]. Each crypt must self-renew and sustain its own proliferation to survive [171]. Regeneration of the crypt is possible until the surviving stem cells will repopulate the crypt and the epithelium [172]. Once all the stem cells are reproductively sterilized, the crypt will degenerate and will lead to crypt loss [40, 173]. Apart from cell proliferation, crypts can survive through the second mechanism called crypt fission (a division of a single crypt into two or more daughter crypts) [47]. Crypt fission might compensate for the crypt loss and might restore crypt numbers [173].

In this study, we wanted to investigate the occurrence of mechanisms such as crypt loss, which is a sign of mucosal injury following irradiation. We also wanted to investigate the occurrence of repair mechanisms such as cell proliferation and crypt fission, which may restore the intestinal crypts damaged due to radiotherapy. Mice were irradiated with 2, 3, or 4 fractions of 8 Gy and sacrificed at five different time-points (24 hours and 1, 6, 18, and 30 weeks after irradiation), followed by colorectal tissue collection. Crypt loss, the total number of surviving crypts, cell proliferation, and crypt fissions were quantified in the colorectal tissue from both the irradiated and the sham-irradiated mice.

Irradiation causes crypt degeneration and increased crypt loss

Irradiation causes significantly higher crypt degeneration in the mice exposed to 4 fractions of 8 Gy radiation than the sham-irradiated mice

at 1-, 6-, 18- and 30-weeks post-irradiation (Figure 8A). However, at 24-hours post-irradiation time point, no significant differences were observed between the irradiated mice and the sham-irradiated mice, suggesting that crypt degeneration does not start immediately after irradiation. Radiation-induced crypt degeneration was similar in both mice and humans (Figure 8B and 8C). Radiation-induced crypt degeneration leads to persistent crypt loss, thereby causing a significantly decreased number of surviving crypts in the mice irradiated with 4 fractions of 8 Gy than the sham-irradiated mice at 6-, 18- and 30-weeks post-irradiation (Figure 8D). Similar results have been found in our previous study where the mice subjected to 4 fractions of 6 or 8 Gy irradiation had increased crypt loss and decreased crypt survival compared to control animals [174].

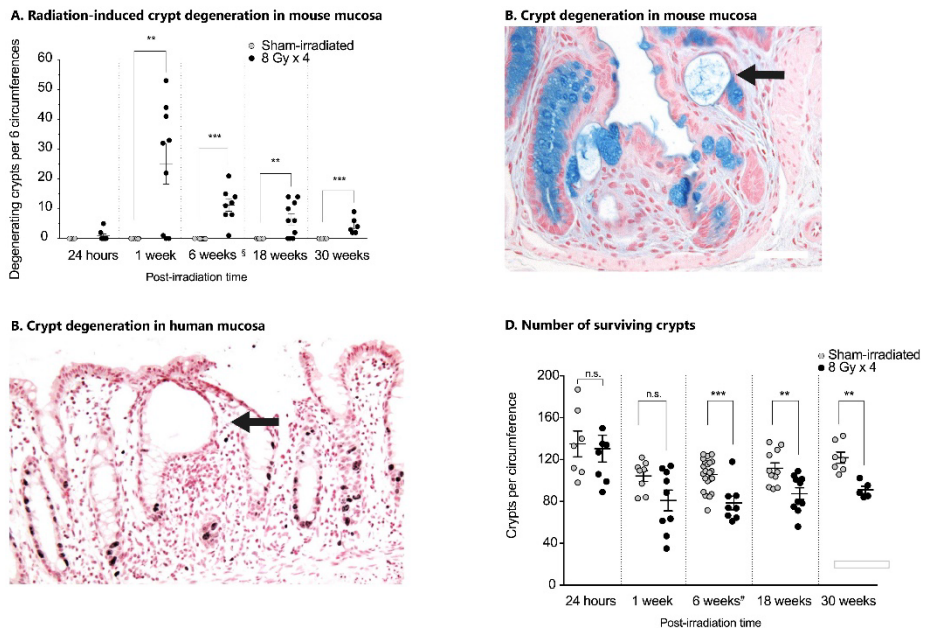


Figure 8. Radiation-induced crypt degeneration in mice mucosa at 24 hours, and 1, 6, 18, and 30 weeks post-irradiation (A). Radiation-induced crypt degeneration was similar in both mice (B) and humans (C). The number of surviving crypts per circumference (D). The figure is taken from Malipatlolla et al. *Sci Rep.* 2019;9(1):13803.

Irradiation causes increased crypt fission

Cell proliferative markers such as Ki-67 and BrdU were used to investigate whether irradiation causes changes in cell proliferation. No significant differences in Ki-67 positive cell numbers were observed between the mice irradiated with 4 fractions of 8 Gy and the sham-irradiated mice at all the time-points (Figure 9A). Similarly, no significant change in the number of BrdU positive cells was observed between the irradiated and sham-irradiated mice at all the time-points analyzed (Figure 9B).

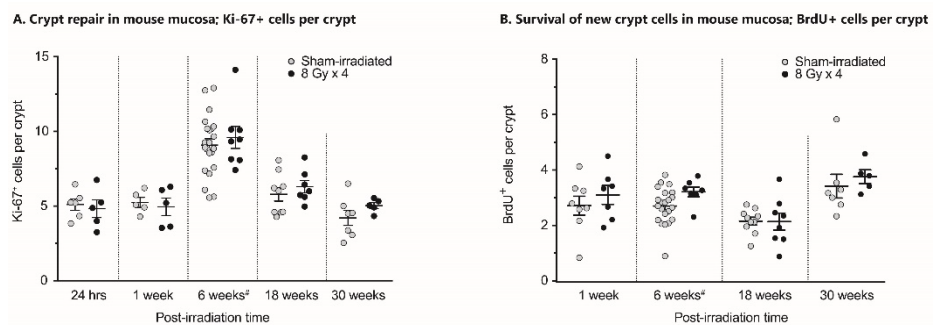


Figure 9. The number of Ki-67⁺ cells per crypt at 24 hours and 1-, 6-, 18- and 30-weeks post-irradiation (A). The number of BrdU⁺ cells per crypt at 24 hours and 1-, 6-, 18- and 30-weeks post-irradiation (B). The figure is taken from Malipatlolla et al. *Sci Rep.* 2019;9(1):13803.

However, irradiation did significantly increase the crypt fission process, were the mice subjected to 4 fractions of 8 Gy had significantly higher numbers of crypts undergoing fission than the sham-irradiated mice at 6 weeks post-irradiation (Figure 10A).

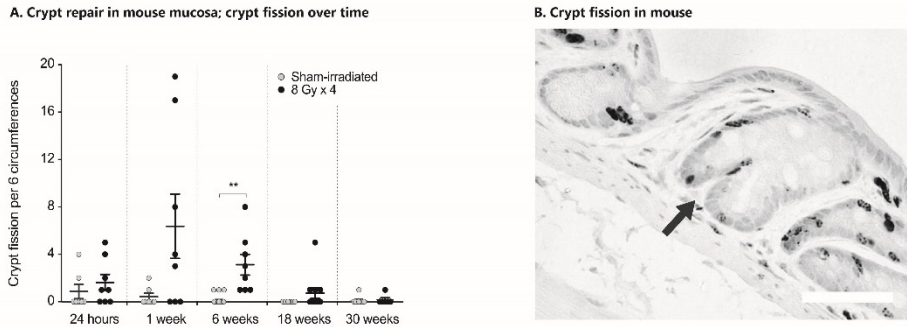


Figure 10. The number of crypt fission at 24 hours, and 1-, 3- 6-, 18-, and 30-weeks post-irradiation (A). Mouse colorectal mucosa showing crypt fission process (B). The figure is taken from Malipatlolla et al. *Sci Rep.* 2019;9(1):13803.

From the time-points we have studied, we can conclude that radiation-induced crypt loss is presumably compensated through the crypt fission process and not through cell proliferation.

Irradiation induces angiogenesis

Radiation proctitis is one of the major complications following pelvic radiotherapy. Radiation exposure may lead to ischemia, microvascular injury, and endothelial damage [175]. Angiogenesis has been found to be upregulated during radiation proctitis [176]. Angiogenesis is believed to be one of the mechanisms that repair radiation-injured tissue [177]. Therefore, we wanted to study if irradiation influences angiogenesis in our mouse model. It was found that irradiated mice had a significantly lower number of CD31⁺ blood cells than the sham-irradiated mice at 1-week post-irradiation. However, at 30 weeks post-irradiation time-point, irradiated mice had a significantly higher number of CD31⁺ blood cells than the sham-irradiated mice. (Figure 11).

Angiogenesis - Mouse mucosal and submucosal CD31+ blood vessels

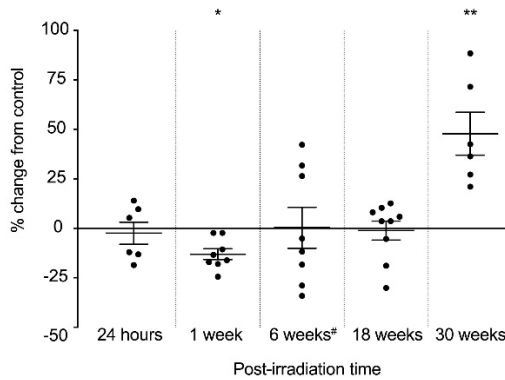


Figure 11. The number of CD31⁺ cells at 24 hours and 1-, 6-, 18-, and 30 weeks post-irradiation. The figure is taken from Malipatlolla et al. *Sci Rep.* 2019;9(1):13803.

These results indicate that initially, radiation causes loss of endothelial blood vessels. The endothelial blood vessels lost are compensated through angiogenesis. However, it takes around 30 weeks to observe pronounced effects. Thus, indicating that radiation-induced angiogenesis is a late phenomenon. Similar results have been found in a study showing that rectal biopsies taken during or shortly after irradiation from patients treated with pelvic radiotherapy did not show any changes in the blood vessel. However, the biopsies taken from patients four months or more after the irradiation showed severe vascular changes characterized by narrowing of the arterioles and endothelial degeneration, indicating that radiation causes late vascular injury [178].

Paper III

Irradiation causes DNA double-strand breaks. It also hydrolyzes water and other molecules, which leads to the production of reactive oxygen species (ROS) and reactive nitric oxide species (NOS). Most of the damaged DNAs are repaired; however, DNAs that are not correctly repaired may lead to cell death, which in turn can lead to a strong immune response. The radiation-induced damaged cells or dead cells secrete damage-associated molecular patterns (DAMPs). DAMPs lead to activation of the toll-like receptor (TLR) immune signaling pathway. Activated TLRs then leads to activation of a transcription factor, nuclear factor kappa-light-chain enhancer of activated B cells (NF- κ B) [53]. Activated NF- κ B induces secretion of pro-inflammatory cytokines and growth factors, which then recruits more immune cells and causes more production of pro-inflammatory cytokines, thus inducing a vicious circle of inflammatory responses [54].

Radiation-induced inflammation could lead to toxicity, which may then lead to the development of gastrointestinal symptoms. Radiation-induced acute inflammation occurs within a few hours of receiving radiotherapy, which subsides within a few weeks or months after the completion of radiotherapy. However, if the acute inflammation does not subside, then it develops into chronic inflammation.

Despite the known health benefits of fiber consumption, patients who are undergoing pelvic radiotherapy may be suggested to follow a strict low-fiber or fiber-deficient diet. The reason behind such recommendation is that a fiber restricted diet might help to curtail the occurrence of gastrointestinal symptoms such as diarrhea [179, 180]. However, there is no scientific evidence to back such advice.

The idea behind performing this study was to investigate the influence of fiber on systemic inflammation following radiotherapy. Mice were fed the fiber-rich high oat bran diet or the fiber-deficient diet, starting at

2 weeks before irradiation and ending at 1, 6, and 18 weeks after irradiation. At 1, 6, and 18 weeks after irradiation, mice were sacrificed, followed by measuring the concentration of serum cytokines/chemokines to observe acute, intermediate, and chronic radiation-induced inflammation.

Our results indicate that irradiation causes increased acute, intermediate, and chronic systemic inflammation. Mice fed the fiber-rich high oat bran diet and the fiber-deficient diet and irradiated with 4 fractions of 8 Gy had overall significantly higher levels of cytokines than their respective dietary control groups at all the time-points, i.e., at 1, 6 and 18 weeks after irradiation (data are shown in paper III figure 2, 4, and 6). Previous studies have shown results similar to our results, were irradiation caused increased levels of pro-inflammatory cytokines in both humans and rodents after irradiation [54, 181-183].

Our results also show that the mice fed the fiber-rich high oat bran diet had significantly lower levels of pro-inflammatory serum cytokines compared to the mice fed the fiber-deficient diet at all the time points (data are shown in paper III figure 2, 4, and 6). The possible reason could be that oat bran contains beta glucan fiber which has been shown to decrease the levels of pro-inflammatory cytokines in both mice and rats [184, 185].

Lastly, our data exhibited that the canonical pathways, biological functions and upstream regulators that were upregulated due to radiotherapy were downregulated in the mice fed the fiber-rich high oat bran diet compared to mice fed the fiber-deficient diet (data are shown in paper III figure S6, S7, and S8).

Taken together, our results illustrate that radiation induces long-term systemic low-grade inflammation and that the fiber-rich high oat bran diet would help to reduce both short-term and long-term systemic inflammation compared to the fiber-deficient diet.

Paper IV

Gut microbiota composition before and during irradiation plays an important role in the outcome of radiotherapy, meaning patients' susceptibility or protection against radiation-induced gastrointestinal symptoms could be linked to their gut microbiota composition [113]. Both human and animal studies have shown that irradiation causes significant alteration in the composition of the gut microbiota [111, 112, 186, 187].

Gut microbiota produces beneficial SCFAs, which have various roles in host health and disease [188, 189]. Gut microbiota also releases enzymes that would influence the colonic mucus degradation activity. In the presence of fiber, microbiota would utilize the carbohydrates present in the fiber. However, in the absence of fiber, gut microbiota will switch its mechanisms and would consume the carbohydrates present in the mucus layer as an energy source [190].

In this study, we wanted to investigate the influence of fiber and irradiation on intestinal health. Mice were fed the diet containing a varying proportion of readily fermentable fiber oat bran and/or less fermentable fiber microcrystalline cellulose or a fiber-deficient diet starting at 2 weeks before irradiation and ending at 6 weeks after irradiation. At 6 weeks after irradiation, mice were sacrificed, and the colorectal tissues were visualized for observing the gross intestinal histology. Fecal samples were analyzed for gut microbiota composition, SCFAs production, mucus degradation activity, and neutrophil elastase production.

Our results show that mice fed the fiber-rich high oat bran diet and exposed to irradiation had decreased intestinal damage, normal gut bacterial composition, high levels of SCFAs, low mucus degradation activity, and high levels of elastase. In contrast, mice fed the fiber-

deficient diet and exposed to irradiation had increased intestinal damage, a sign of gut dysbiosis with a high abundance of Gram-negative bacteria, low levels of SCFAs, high mucus degradation activity, and no elastase production (data are shown in paper IV).

Gram-negative bacteria are usually observed in gut dysbiosis, which might play a critical role in radiation enteropathy [191]. Hence, in our study, the mice fed the fiber-deficient diet might be more susceptible to develop radiation enteropathy.

SCFAs, such as butyrate, are utilized by the colonocytes as an energy source. Low levels of SCFAs cause intestinal starvation, which leads to mucosal malabsorption, mucosal hypoplasia, decreased gut barrier function, and increased bacterial translocation, eventually leading to an augmented inflammatory response [192, 193]. In our study, mice fed the fiber-deficient diet might be suffering from intestinal starvation, which might then lead to increased inflammation.

One of the mechanisms through which intestinal homeostasis is maintained is by keeping the mucus layer intact. If the mucus barrier is infringed, it will facilitate the translocation of bacteria from the lumen into the mucosa, and this would trigger a cascade of inflammatory events in the intestine [194]. Oat bran diet has been shown to decrease the permeability of the intestinal mucus [195]. In contrast, a fiber-deficient diet has been previously shown to increase the numbers of mucus-degrading bacteria, which would then lead to increased colonic mucus barrier degradation and enhanced pathogen susceptibility [133]. Therefore, in our study, the mice fed the fiber-deficient diet might be prone to increased mucus barrier disintegration and increased pathogen susceptibility.

As discussed earlier, neutrophil elastase has been shown to increase host defense against invading pathogens. Therefore, mice fed the fiber-rich

high oat bran diet might have enhanced host defense compared to mice fed the fiber-deficient diet.

Taken together, it seems that a fiber-deficient diet along with irradiation would severely decrease the host's intestinal health, whereas mice fed the fiber-rich high oat bran diet would help to decrease the harmful effects of irradiation and would help to restore the host intestinal health.

CONCLUSIONS

In conclusions, at the intestinal tissue level, irradiation causes prolonged crypt loss and late angiogenesis. The crypt loss due to irradiation was partly compensated by increased crypt fission in a dose-dependent manner, i.e., higher the irradiation dose delivered to the distal colon higher was the crypt fission observed. However, crypt fission failed to restore the crypts lost completely. Lastly, irradiation did not appear to alter crypt stem cell proliferation at any of the time-points we had analyzed.

Radiation induces both short-term and long-term systemic inflammation. However, the mice consuming a fiber-rich high oat bran diet exhibited decreased radiation-induced inflammation compared to the mice consuming a fiber-deficient diet. Also, the canonical pathways, biological functions and upstream regulators that were upregulated due to radiotherapy were downregulated in the mice consuming a fiber-rich high oat bran diet compared to the mice consuming a fiber-deficient diet. Thus, we can conclude that fiber-rich high oat bran diminishes radiation-induced inflammation, whereas fiber-deficient diet aggravates radiation-induced inflammation.

A combination of irradiation and fiber-deficient diet is detrimental to intestinal health as it causes gut dysbiosis with decreased SCFAs production, increased mucus degrading activity, possibly decreased host defense, and increased colonic tissue damage. In contrast, a fiber-rich high oat bran diet decreases the detrimental effects of irradiation by maintaining normal gut bacterial composition, increasing SCFAs production, decreasing mucus degrading activity, possibly increasing host defense, and by decreasing colonic tissue damage.

Taken together, we can conclude that the recommendation given to the patients to consume a low-fiber or fiber-deficient diet might be detrimental and might worsen radiation-induced intestinal symptoms.

SUMMARY OF THE FIELD OF INTEREST

Today millions of cancer survivors suffer from decreased intestinal health due to pelvic radiotherapy treatment. Cancer survivors suffer from lifelong gastrointestinal symptoms such as diarrhea, fecal incontinence, blood and mucus discharge from the anus, stool containing blood, excessive production of odorous gases, and involuntary loud flatulence. All these symptoms might be embarrassing to the patient and might lead to social isolation. The patient's work capacity, traveling, participation in family life, and sexual life could be greatly hampered by the need to visit the toilet 15-20 times a day. Below are the quotes from cancer survivors:

“It feels as though I have to be constantly thinking about being close to a toilet or that I have to control myself in order not to soil my clothing when I experience bowel spasms and flatulence. I always have to have extra underwear and sanitary napkins. As a 39-year-old woman, I experience this as emotionally degrading.”

“I have major problems with loose stools and gas, and this has affected my quality of life in a very negative way. I am really hoping now to be able to get some help. I beg you, please, HELP me.” – 43-year-old woman.

The health benefits of fibers have been known for decades. However, in the clinics, patients are advised to follow a low-fiber or fiber-deficient diet with the belief that it might help to reduce the gastrointestinal symptoms that occur due to radiotherapy. However, there is no scientific evidence to support this advice. To address this question, we have developed a novel mouse model in which mice were radiated to the distal bowel with the same clinical linear accelerator, which is used to treat the cancer patients. The results from this thesis show that the recommendation given to the patients to avoid fiber might not be fruitful to them. We found that mice fed the fiber-deficient diet and exposed to

irradiation had severe intestinal tissue damage, gut bacteria imbalance, low levels of beneficial short-chain fatty acids, high mucus degradation, and high inflammation compared to mice fed the fiber-rich high oat bran diet and exposed to radiation. We also found that a fiber-rich high oat bran diet helps to diminish the detrimental effects of radiation and to decrease radiation-induced intestinal damage. In contrast, a fiber-deficient diet aggravates the damaging effects of radiation and exacerbates radiation-induced intestinal damage. If it holds true in humans, then the practice of advising a low-fiber or fiber-deficient diet should be abolished.

FUTURE PERSPECTIVES

In this thesis, I have tried to identify the possible molecular mechanisms responsible for causing radiation-induced intestinal dysfunction in a mouse model. Although results have given some hints on the possible mechanisms, there is still a lot to discover. Moreover, studies should also be performed to check whether the findings in mice corroborates in humans.

In paper I, neutrophil elastase was found to be a good biomarker to study radiation-induced intestinal injury. However, in paper IV, it was found that neutrophil elastase was not associated with irradiation but was rather associated with oat bran diet and could be responsible for increasing the host defense mechanism against invading pathogens. Therefore, the role of neutrophil elastase in intestinal health after irradiation is not entirely understood and should be further exploited. This could be achieved by studying neutrophil elastase knock out mice and check its role in pathogen elimination activity during infection.

In paper II, it was found that repair mechanism such as crypt fission was elevated after radiotherapy in mice. The next step is to try to understand how we can stimulate these repair mechanisms.

In paper III, 23 serum cytokines/chemokines were analyzed to study the cytokine profiles of the mice in relation to irradiation and diet. Mice fed with a fiber-rich high oat bran diet had decreased radiation-induced inflammation compared to mice fed with a fiber-deficient diet. Also, the canonical pathways, biological functions, and upstream regulators that were upregulated due to irradiation were downregulated in mice fed with an oat bran diet compared to mice fed with a fiber-deficient diet. Similar experiments could be performed to evaluate if consuming fiber-rich high oat bran diet could decrease radiation-induced inflammation in patients undergoing pelvic radiotherapy.

In paper IV, we observed that mice fed with a high oat bran diet and subjected to irradiation had better intestinal histology, high levels of SCFAs, decreased mucus degradation, and normal gut flora, finally leading to improved intestinal health. In contrast, mice fed with a fiber-deficient diet and subjected to irradiation had worse intestinal histology, low levels of SCFAs, high mucus degradation and a sign of dysbiosis, eventually causing decreased intestinal health. Similar studies could be conducted in patients to observe whether consuming a fiber-rich high oat bran diet during and after radiotherapy could improve intestinal health and could eventually alleviate radiation-induced gastrointestinal symptoms.

Irradiation causes activation of many signaling pathways and mechanisms responsible for decreased intestinal health. Therefore, it is imperative to study the underlying molecular mechanisms responsible for causing the pathophysiological changes in the intestine after radiotherapy. Thus, it is crucial to identify the genes and proteins which are upregulated or downregulated due to radiation. The knowledge gained will help to determine the molecules responsible for causing radiation-induced gastrointestinal symptoms.

Colonic mucus layer thickness measurement could be performed on biopsies obtained from patients undergoing radiotherapy or healthy individuals. Later, the colonic mucus layer thickness could be compared between patients and healthy individuals to observe if irradiation could cause a decrease in mucus layer thickness. This might help in understanding the mechanisms which might trigger radiation-induced inflammation, as compromised mucus layer could allow the translocation of bacteria into the mucosa, which might trigger the immune cells to cause inflammation.

Fecal samples from patients (before, during, and after radiotherapy) and healthy controls could be analyzed to identify the metabolic profiles of the fecal samples in both groups. The most differing metabolites in both

groups could be used as a potential biomarker to identify radiation-induced intestinal injury and to gain more insight into metabolic pathways that are upregulated due to radiotherapy.

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