Intestinal health after pelvic radiotherapy

Towards understanding persisting pathophysiological mechanisms

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Cover illustration:

Courtesy of Dr. George Birchenough. Confocal images of intestinal biopsies that were incubated with bacteria-sized fluorescent red beads, six years after treatment for anal cancer. Biopsies were taken at 25 cm and 5 cm from the anal verge. Beads are shown in red, epithelial surface in grey. The mucus layers are not visible but represented by the transparent space between the beads and epithelial surface.

Intestinal health after pelvic radiotherapy - towards understanding persisting pathophysiological mechanisms

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I was taught that the way of progress was neither swift nor easy.

-Marie Curie

Intestinal health after pelvic radiotherapy

Towards understanding persisting pathophysiological mechanisms

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ABSTRACT

Pelvic cancer survivors struggle with life-long symptoms caused by radiotherapy. Many pelvic cancer survivors live with a constant fear of leaking faeces and the need to always have a toilet within reach. Some of these survivors are forced to retire early and live in social isolation. Radiotherapy saves lives, but radiation-induced damage to intestinal tissue close to, or within, the radiation field causes permanent changes in the cancer survivor's bowel habits. Our goal was to understand the pathogenic mechanisms that continue to cause symptoms even decades after the treatment.

By irradiating the mouse colorectum using the clinic's linear accelerator for radiotherapy, we have developed a novel model that mimics the human pathophysiological condition in the irradiated colorectal mucosa. The model was used to investigate whether the intake of dietary fiber, known to promote intestinal health, could modulate radiation-induced normal-tissue damage (Paper I). Our findings revealed that dietary fiber modified several intestinal pathophysiological processes and repair mechanisms following irradiation, most notably preventing a late, possibly chronic, bacterial invasion and reducing signs of inflammation. Dietary fiber appeared to also modulate parameters of neurogenesis in the brain following radiotherapy-induced injury to the colorectum, suggesting a connection between intestinal health after radiotherapy and brain health (Paper II). To translate the preclinical findings on inflammation to clinic, a biopsy-study was performed in cancer survivors up to 20 years after pelvic radiotherapy (Paper III). A low-grade chronic intestinal inflammation was observed after pelvic radiotherapy, as evidenced by an elevated neutrophil presence and activity. Inadequate mucus protection was also found, possibly leading to increased pathogen infiltration. These

results challenge the notion that pelvic radiotherapy causes acute intestinal inflammation that either heals, or causes tissue to become fibrotic without further inflammation.

In conclusion, this thesis demonstrates that the mucosal resilience to radiotherapy may be modulated by quite simple means, such as dietary approaches. Moreover, protecting intestinal health after radiotherapy may also protect brain health. The thesis also demonstrates the presence of a chronic, low-grade inflammation in the intestinal mucosa after pelvic radiotherapy, possibly driven by infiltrating bacteria due to a poor mucus protection. The findings can have implications for future approaches to safeguarding intestinal health while providing effective pelvic cancer therapy.

SAMMANFATTNING PÅ SVENSKA

Många av de som överlever en cancer i bäckenområdet kämpar med livslånga symtom efter att ha fått strålbehandling mot nedre delen av buken för att utrota cancern. Det kan vara att tvingas leva med en ständig rädsla för att läcka avföring och ett behov av att alltid ha en toalett inom räckhåll. Besvären ökar risken för förtidspensionering, och stigmat kring symptomen kan leda till social isolering. Symptomen orsakas av strålningsinducerade skador på tarmvävnaden, men få studier har genomförts för att förstå de skademekanismer som resulterar i långvariga och ibland kroniska symptom. Vårt mål var att förstå dessa mekanismer, både på cellulär och molekylär nivå.

Vi utvecklade en modell av bäckencancerstrålning genom att bestråla kolorektum hos mus med hjälp av de linjäracceleratorer som används vid cancerbehandling. Tillvägagångssättet genererar en patofysiologi i tarmen som är mycket lik den man ser hos bestrålade canceröverlevare, utan att djurens viktkurva eller livslängd påverkas. I artikel I undersökte vi med hjälp av modellen om den strålningsinducerade skadan i tarmen kunde påverkas av en kost rik på fiber. Våra resultat avslöjade att närvaron av kostfiber modifierade flera patofysiologiska processer och reparationsmekanismer efter strålning, påverkade förekomsten av inflammationsmarkörer i blodet och förhindrade en sen, möjligen kronisk, bakteriell invasion i den bestrålade tarmens mukosa. I artikel II visade vi att bestrålning av kolorektum även påverkade processen som ger upphov till nya neuron i hjärnans hippocampus, ett område viktigt för minne och inlärning. Dieten spelade också här en roll för utfallet. I artikel III undersökte vi, genom att ta biopsier från canceröverlevare som strålats mot en tumör i bäckenområdet, huruvida det förekom en långvarig inflammation i tarmen efter avslutad strålbehandling. I vår studie observerades en låggradig kronisk inflammation i upp till minst 20 år efter strålbehandlingen, vilket framgick av en ökad närvaro av immunceller i mukosan och ökad immunologisk aktivitet. Vi fann också att de lager av mukus på insidan av tarmen som skyddar tarmväggen från patogener i lumen var av låg kvalitet, och det fanns tecken på bakterieinfiltration i kryptorna. Dessa resultat utmanar den gängse uppfattningen att strålning mot tarm orsakar en akut inflammation som antingen läker ut, eller ger en fibrotisk tarm utan ytterligare pågående inflammation.

Sammantaget har denna avhandling stärkt evidensen för kostens betydelse för tarmhälsan efter strålbehandling mot bäckenområdet, och föreslår även att hjärnhälsan indirekt kan gynnas av en förbättrad tarmhälsa hos dessa canceröverlevare. Vidare visar avhandlingen att det i den bestrålade tarmen förekommer en låggradig och kronisk inflammation, som kan bero på att tarmens skyddsbarriär mot patogener i lumen är otillräcklig. Fynden kan komma att få betydelse för utvecklingen av strategier som skyddar tarmhälsan under och efter strålbehandling utan att minska strålbehandlingens effektivitet.

LIST OF PAPERS

The thesis is based on the following studies that are referred to in the text by their Roman numerals:

I. Malipatlolla, D. K., Devarakonda, S., Patel, P., Sjöberg, F., Rascón, A., Grandér, R., Skokic, V., Kalm, M., Danial, J., Mehdin, E., Warholm, M., Norling, H., Stringer, A., Johansson, M., Nyman, M., Steineck, G., and Bull, C. (2021). A Fiber-Rich Diet and Radiation-Induced Injury in the Murine Intestinal Mucosa. *International Journal of Molecular Sciences,* 23(1), 439.

II. Devarakonda, S., Malipatlolla, D. K., Patel, P., Grandér, R., Kuhn, H. G., Steineck, G., Sjöberg, F., Rascón, A., Nyman, M., Eriksson, Y., Danial, J., Ittner, E., Naama Walid, R., Prykhodko, O., Masuram, S., Kalm, M., and Bull, C. (2021). Dietary Fiber and the Hippocampal Neurogenic Niche in a Model of Pelvic Radiotherapy. *Neuroscience*, 475, 137–147.

III. Devarakonda, S., Thorsell, A., Hedenström, P., Rezapour, A., Heden, L., Johansson, M.E.V., Birchenough, G., Toft, A., Gustavsson, K., Skokic,.V., Petterson,.V., Sjöberg, F., Kalm, M., Al Masri, M., Yrlid,.U., Bergmark, K., Steineck, G., Bull, C. Low-grade intestinal inflammation two decades after pelvic radiotherapy. *Manuscript.*

TABLE OF CONTENTS

ABBREVIATIONS

PRD	Pelvic radiation disease
CNS	Central nervous system
DNA	Deoxyribonucleic acid
SGZ	Subgranular zone
SVZ	Subventricular zone
GCL	Granular cell layer
DG	Dentate gyrus
GC	Goblet cells
SCFA	Short-chain fatty acid
Gy	Gray
NeuN	Neuronal nuclear protein
MPO	Myeloperoxidase
DAPI	4′,6-diamidino-2-phenylindole
DCX	Doublecortin
Iba1	Ionized calcium-binding adapter molecule 1
IL	Interleukin
MIP	Macrophage inflammatory protein
MUC2	Mucin 2
FISH	Fluorescence in situ hybridization
TMT	Tandem Mass Tag
DAB	3,3′-Diaminobenzidine
TBS	Tris-buffered saline

INTRODUCTION

Cancer is a disease where transformed cells divide excessively, and radiotherapy is one of the cornerstones in the treatment to eradicate cancerous cells. Radiotherapy can be used along with other treatment modalities such as chemotherapy, surgery, immunotherapy, and hormonal therapy. More than half of patients diagnosed with cancer undergo radiotherapy, and in some cases radiotherapy can be used as the sole treatment[1].

During pelvic radiotherapy, damage to organs located in the pelvic cavity is common. The intestines are radiosensitive, hence damage to intestinal tissue located in, or nearby, the radiation field is inevitable[2]. Normal-tissue damage due to radiotherapy leads to both early and late symptoms in pelvic cancer survivors[3, 4]. According to Globocan, an online database with global cancer statistics from The International Agency for Research on Cancer, a total of 19.3 million new cancer cases were recorded in 2020 worldwide and Europe accounted for 22.8 percent of the total cancers. Calculated with the help of Globocan statistics, nearly one fifth of all new cancer cases worldwide were pelvic cancers[5]. Most pelvic cancer patients undergo radiotherapy and at least 90%, and perhaps all, have some kind of permanent change in their bowel habits[6, 7]. These changes affect their daily duties and quality of life. The intensity of injury in healthy tissue depends on various factors including type of treatment, dosage, size of the area and site of the tumor. Additional factors that are known to influence, or that may influence on the injury and symptoms, include lifestyle[8, 9], other treatments such as past surgical procedures[10] and diseases and the medications used to treat these. Even genetic factors and psychological conditions, and the composition of the individual's microbiota may play a role[11, 12].

Pelvic radiation disease (PRD) is a term coined to refer to radiation-induced injury to non-cancerous tissue and also to the collection of symptoms that arises following pelvic radiotherapy[3]. Pelvic cancer survivors with intestinal PRD struggle with a range of symptoms including urgency, loose stools, abdominal cramping, bloating and nausea. Steineck *et al.* identified 28 symptoms in gynecological survivors up to 15 years after completed radiotherapy and then categorized these 28 into five syndromes (clusters of symptoms) as follows: fecal leakage syndrome, urgency-tenesmus syndrome, blood discharge, excessive gas discharge and excessive mucus discharge[13].

RADIOTHERAPY

Radiotherapy is established as one of the two most used treatments for cancer, with surgery being the oldest treatment that dates back to 1500-1600 BC. The first documentation of a cancerous tumor was found on ancient Egyptian papyri, the Edwin Smith Papyrus, which is a copy of a 5000-year-old textbook on trauma surgery[14]. The text states that tumors were removed by cauterization using a tool called a "fire drill" and the text does note that the disease has no treatment[15]. X-rays were discovered much later in 1895 by Wilhelm Conrad Röntgen in Germany[16]. This discovery was followed by the discovery of radium in the early 1900s by Marie Curie[17]. We have come a long way in the development of radiotherapy as treatment for cancer since then. Today, there are two **main** types of radiotherapy[18]:

1) External beam radiotherapy: External beam radiotherapy is characterized by using radiation beams generated outside the human body. Example of these beams are photon beams, electron beams, 60Co γ-beams, proton beams and heavy ions.

The most commonly employed treatment techniques in external beam radiotherapy include:

- Three-dimensional conformal radiation therapy
- Volumetric-modulated arc therapy

2) Brachytherapy: Brachytherapy makes it possible to give high doses of radiation in small areas while sparing the surrounding tissue. Needles, seeds, wires, or catheters containing radioactive material are implanted into the tumor or near it to treat cancer. They are two types:
    - Permanent implants
    - Temporary internal radiation therapy

Other examples of treatment types that uses radiation are intraoperative radiation therapy, radioimmunotherapy, radionuclides, radiosensitizers and radioprotectors.

HEALTHY TISSUE DAMAGE AND PATHOPHYSIOLOGICAL PROCESSES INDUCED BY RADIOTHERAPY

The onset of tissue injury following radiotherapy involves a series of events that begin with the emergence of damage in rapidly proliferating cell populations, such as in the crypts of the intestinal mucosa[12]. Ischemia, microvascular injury, and endothelial damage may result from radiation exposure[19]. Acute intestinal damage becomes visible in the microscope within days

after radiation therapy and is caused mostly by cell death in the rapidly growing crypt epithelium. At this time, an acute inflammatory response in the lamina propria can also be seen. The epithelium of the intestines is a single-layered, self-renewing tissue[20]. The regeneration of the epithelium following damage is dependent on the presence of stem cells near the base of the crypt[21]. During cell division, the exposed single-stranded DNA chains are more easily disrupted by radiation, and they are less likely to repair themselves effectively once broken. Because the cell cycle is disturbed by radiotherapy, this is most likely to result in cell death or cell cycle arrest[22]. Crypt stem cell loss causes inadequate epithelial replacement, a disruption of the mucosal barrier, and mucosal inflammation[23, 24]. The loss of stem cells is also believed to cause the crypts to degenerate, with fibrotic tissue ultimately taking over[19]. Hypoxia, vascular damage, loss of tight junctions, and loss of gut wall integrity are all radiation-induced effects that may exacerbate crypt degeneration[25, 26]. The epithelial layer acts as a wall protecting against pathogen invasion, however disruption of the integrity of the layer may increase the permeability of contents from the lumen into the mucosa and crypts. This stimulates a variety of immune cell responses to release pro- and anti-inflammatory mediators, which also play important roles in repair and damage of the tissue[12]. If the pathophysiological mechanisms involved in acute injury are severe, persistent or late damage may result.

Response to radiotherapy and inflammation

Shortly after irradiation, the healthy tissue responds by launching processes related to wound-healing[27]. During normal wound healing, vessels increase the expression of adhesion molecules to allow immune cells like neutrophils and macrophages to extravasate into the extravascular region[28]. The immune system and inflammation are regulated by cytokines through a complex and well-coordinated signaling cascade[29]. The first immune cells to respond to irradiation are resident innate immune cells, which produce pro-inflammatory cytokines, chemokines, and growth factors. Most of the studies on radiation-induced inflammation focus on acute radiation effects, partly due to a lack of animal models where the animals survive past the acute phase after experiencing clinically relevant doses of radiation[30]. Studies on biopsies from pelvic cancer survivors more than a few months after completed cancer therapy are very rare; a literature search returned only one article describing a study performed on nine prostate cancer survivors up to 10 years after pelvic therapy[31]. Thus, there is quite extensive knowledge concerning the acute effects of radiotherapy on the

intestinal mucosa, but much less is known about the long-term effects and the dynamics of the immune response over time.

Mucus protects the intestinal wall

Mammals have evolved several mechanisms that prevent the direct contact of bacteria in the lumen with the epithelial barrier and the immune cells in the lamina propria. Goblet cells (GC), which account for roughly 10–30% of human colonic epithelial cells, release mucins which builds a mucus hydrogel on the surface of the epithelium[32]. In contrast to the small intestine, which has a single unattached mucus layer, the mucus in the colon consists of a dense, adhering inner layer and a looser outer layer which is formed from the inner layer[33]. The secreted colonic mucus layer consists mainly of mucin-2 (Muc2), which is highly glycosylated. About 80% of the molecular weight of mucins is made up of O-glycan carbohydrates[34]. Despite the comparatively stronger mucus barrier, the colonic mucus hosts many more bacteria than the small intestine, and certain microbes may even penetrate the dense inner mucus layer [35]. Both mice and humans have a varied microbiota in their colonic crypts, which includes both aerobic and anaerobic organisms that may also be found in the mucus layers[36, 37]. This crypt-specific core microbiota has a high number of Proteobacteria in addition to Bacteroidetes and Firmicutes, particularly in the mouse cecum and proximal colon. Apoptotic epithelial cells release nutrients that facilitate Proteobacteria growth and expansion, showing that host-derived factors are also important for bacterial adaptation[38]. The human and mouse colon's inner mucus layer has consistently been proven to be impenetrable to bacteria-sized particles[39, 40]. However, some commensal bacteria are also capable of penetrating the thick inner mucus layer right above the epithelium. Certain bacteria are tolerated and will even prevent an immune response while others trigger an immune response[41]. Figure 1 shows a schematic representation of the mucus layers' protection against pathogens in healthy, and diseased conditions and the supportive role of immune cells in the protection. It has emerged that intestinal macrophages reside within the subepithelial lamina propria and are crucial to maintaining intestinal homeostasis[42, 43]. In the case of pathogen infiltration, neutrophils migrate from the blood through the intestinal mucosa via transendothelial migration as well as transepithelial migration in response to multiple chemoattractant gradients[44].

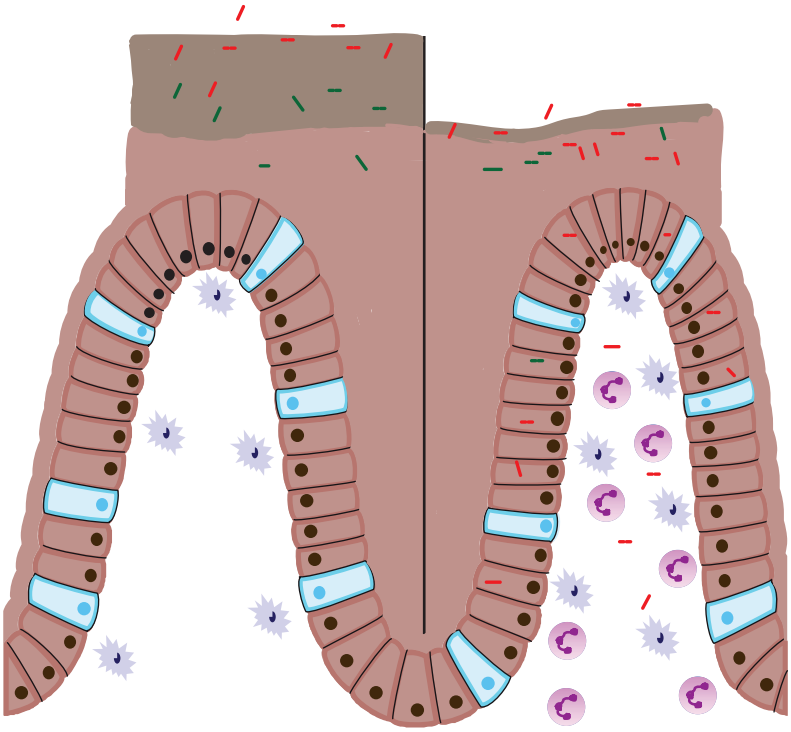

Figure 1. *The colonic mucus barriers and crypt protection by immune system in healthy (left) versus injured (right) state.*

IMPACT OF LOCALIZED RADIOTHERAPY ON DISTAL ORGANS – THE BRAIN

Pro-inflammatory mediators or antigens released into the bloodstream from a site of injury can cause severe and long-term behavioral changes, including the development of cognitive impairment and depression. This effect is seen during both acute and chronic inflammation. Interestingly, there are long-term symptoms associated with the central nervous system (CNS) following pelvic cancer therapy, such as impaired cognitive function, depression, and mental fatigue[45]. This has traditionally been attributed to the impact of chemotherapy, and not radiotherapy, hence the expression “chemobrain”, which describes cognitive issues during and after cancer treatment. But in inflammatory bowel disease, attention and executive function are impaired, particularly working memory, indicating that cognitive dysfunction may be a side effect of the disease[46]. A brain structure of particular interest in the field is the curve-shaped hippocampus in the temporal lobe, where adult neurogenesis take place. Neurogenesis is a dynamic process that is believed to be vital for cognitive functions including learning and memory[47]. The hippocampus sub-granular zone (SGZ), lining the dentate gyrus (DG), is one

of the adult mammalian brain's stem-cell-containing niches (Figure 2). Numerous intrinsic and extrinsic variables, such as environmental enrichment, stress, learning, nutritional treatments, blood brain barrier dysfunction and leaky gut, all influence hippocampal neurogenesis[48-50]. The relationship between intestinal inflammation and cognitive dysfunction has been postulated to be found in cytokines released from the inflamed intestine that penetrate the blood–brain barrier[51]. Studies on patients with gynecological carcinoma who had their gut microbiota observed before, during, and after pelvic radiotherapy showed that there was a difference in the composition of the gut microbiota before radiotherapy between those with postradiotherapy diarrhea and those without diarrhea[2]. Among other effects of radiation, dysbiosis can affect local and systemic immune responses, resulting in gastrointestinal damage[52]. An altered gut microbial composition has been linked to neuroinflammation, reduced hippocampal neurogenesis, and behavioral disorders such as depression[53]. Notably, there are also long-lasting effects related to the CNS after pelvic cancer treatment, such as reduced cognition, depression, and mental fatigue[54].

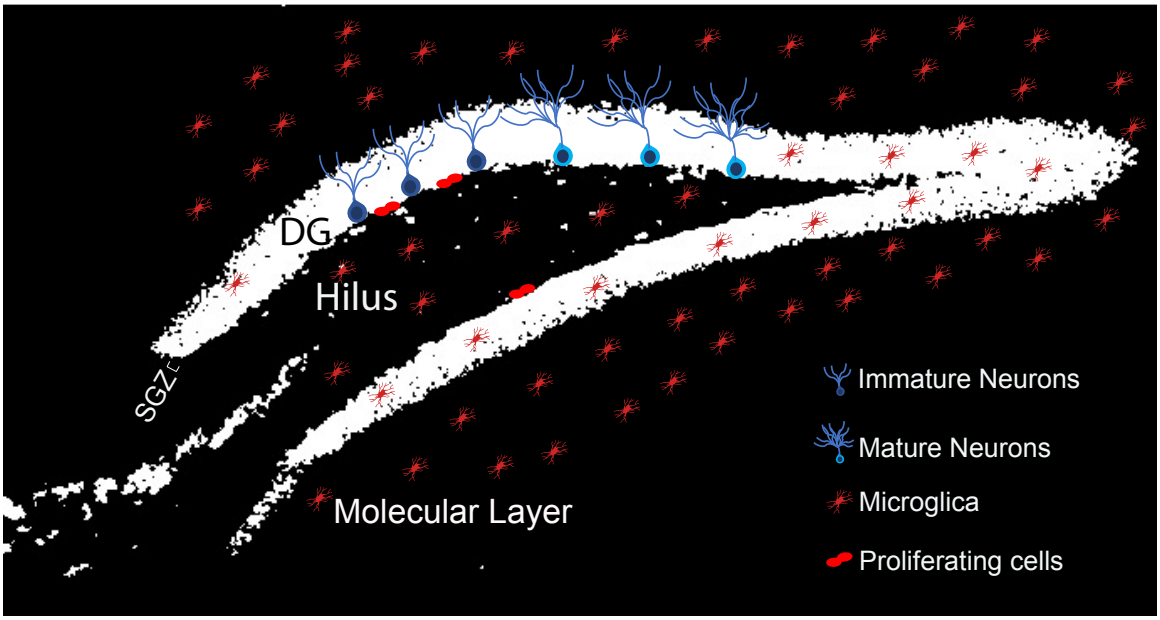

Figure 2. *Schematic representation of the hippocampal neurogenic niche showing the cell types that were quantified in Paper II; proliferating cells, immature neurons, mature neurons and microglia.*

DIETARY FIBER AND ITS BENEFICIAL EFFECTS

The cancer survivors who turn to healthcare for issues with their bowels are, at best, offered help to ameliorate the symptoms, as the underlying conditions of pelvic radiation disease in general are considered incurable[55]. One of the suggested approaches to managing symptoms

such as fecal urgency and excessive gas discharge includes dietary changes. Dietary fiber has been gaining grounds for its importance in the maintenance of crypt homeostasis, the production of short-chain fatty acids (the main source of energy for colonocytes) by fiber-degrading bacteria, and promotion of intestinal barrier integrity[56]. Consumption of fiber is also known for its protective benefits against colorectal cancer, which may be due to the immunomodulatory effects of microbes and their metabolites in the intestine[57]. However, pelvic cancer survivors with pelvic radiation disease are usually suggested to eat a low-fiber diet. This advice probably stems from clinical experience where fiber possibly increase bowel discomfort during radiotherapy[58]. The advice to avoid fiber long after completed pelvic radiotherapy is thus given based on individual clinician experience, not scientific evidence, since there is a lack of well-designed, large studies focusing on optimal diet regime during and after radiotherapy.

Dietary fiber consists of carbohydrate polymers and oligomeric materials that pass undigested into the large intestine where they are fermented, either partially or completely. “Insoluble” and “soluble” fibers refer to their solubility in water. Most fibers are a mixture of both. Fiber can also be described as fermentable or non-fermentable, based on whether they are fermented or not by bacteria in the large bowel, forming short-chain fatty acids mainly acetate, propionate, and butyrate. Different beneficial effects from dietary fiber consumption have been shown previously in animal models of radiotherapy[59, 60]. For an example, soluble pectin consumption has been shown to increase stem cell survival in intestinal crypts and animal survival after total body irradiation[61]. Other studies have shown that the absence of fiber can be detrimental, as it leads to an increase in mucus-degrading bacteria and reduces the growth of fiber-fermenting bacteria. This leads to the thinning of the two protective mucosal layers. In addition, as butyrate from fermented fiber is used for ATP production in colonic cells, a loss of fiber-fermenting bacteria is suggested to cause cell starvation[62]. Soluble fiber, with its capacity to produce ample amounts of butyrate when fermented by the microbiota, was the preferred choice in several studies and have the strongest evidence of a beneficial effect[63]. It is notoriously difficult in humans to implement dietary interventions due to factors like adherence and accurate estimation of dietary intake, so overall, the results from these studies are inconclusive[64, 65]. There is also less possibilities to collect samples in humans, for that nutritional studies performed in animals have clear advantages.

AIM

The overarching aim of this PhD thesis was to elucidate persisting effects of pelvic radiotherapy on the intestinal mucosa, and the possible consequences for the cancer survivor's health. The thesis consists of following three specific aims:

Paper I: To determine whether the enrichment of dietary fiber in the diet, known to promote intestinal health, can modulate the long-term outcome concerning radiation-induced mucosal injury and repair.

Paper II: To determine whether radiation to the colorectum can affect parameters of hippocampal neurogenesis and, if so, a fiber-rich diet can modify these effects.

Paper III: To determine if there is a chronic low-grade inflammation in the intestinal mucosa after pelvic radiotherapy.

MATERIALS AND METHODS

Paper I and II:

Model of pelvic radiotherapy

Gothenburg Ethical Committee of the Swedish Animal Welfare Agency authorized the experiments (application number 22-2015, with additions 124-2016 and 5.8.18-03774/2018). The mice were males belonging to the C57BL/6J strain from Charles River Laboratories and were 8 weeks old at the start of the experiments. Mice were housed five per cage with a constant temperature at 20 ºC and relative humidity 42 % and were provided food and water *ad libitum*.

Note: Many human diseases can be modelled in mice. An ideal animal model would mimic the human disease conditions perfectly, but this is never the case. Nevertheless, by taking into consideration the difference between mice and men, important knowledge can still be retrieved from mouse models. Mice can be engineered genetically, turning on and off genes to better understand the role they play in heath and disease, and much is already known concerning the mouse as a model system. In addition, the use of mice models has economic advantages over larger animals, due to less demanding housing conditions and the fast generation of large numbers of offspring. The use of *in vitro* models would not have been appropriate in this thesis, where studies on the organism level was required to adequately reflect the complex interactions and synergistic effects between different cell types and organs over an extended period of time.

Irradiation procedure

For the studies leading to Paper I and II, mice were sedated with isoflurane and irradiated using a linear accelerator (Varian Clinic 600 CD; Radiation Oncology System, San Diego, CA) at Jubileumskliniken at the Sahlgrenska University Hospital. While sedated, mice were placed on a silicon mold to ensure ideal position with the nose in a nose cone delivering 2.5% - 3% isoflurane with an airflow of 300mL/min during the procedure. On top of the mice, a thick skin-like 5-mm bolus was placed for even distribution of irradiation to the colorectum. Using a linear accelerator with 6MV of photon energy producing a dose rate of 5.9 Gray (Gy)/min, approximately 1.5 cm of the distal colon was irradiated with 4 fractions of 8 Gy. Sham-irradiated mice were subjected to sedation with isoflurane under the linear accelerator, but no radiation was delivered (Figure 3)**.**

Note: The irradiation procedure was done with a linear accelerator used to irradiate human cancer patients. Using the linear accelerator, we were able to deliver high doses of radiation with the same dose rate the pelvic cancer patients receive, and the radiation field could be focused to the colorectal area. Each fraction was given with a 12-hour interval during a weekend. The major reason for choosing fractionated radiation over one single dose was an earlier finding showing that the number of fractions, rather than dose, determined the extent of crypt degeneration. Four fractions of 8 Gy were required to mimic the tissue pathophysiology of patients in our model, but the regimen did not sterilize the stem cell niche in the majority of the crypts, allowing for regeneration. Other parts of the mice, particularly bone marrow and testes, were avoided by using a radiation field of 3 x 3 cm^2, placing only the lower quadrant over the caudal-dorsal part of the mouse. The localized radiation injury did not affect the general health of the mice, and they had a normal weight curve and life span. As it is difficult to avoid irradiating the reproductive organs of female mice, only male mice were used for these initial studies. To evaluate if scattered radiation could reach the brain, a diode was placed close to the head of a mouse during the irradiation procedure. Scattered radiation near the head did not exceed 20 mGy. 20mGy given for 300 consecutive days to mouse brain did not affect parameters of hippocampal neurogenesis[66].

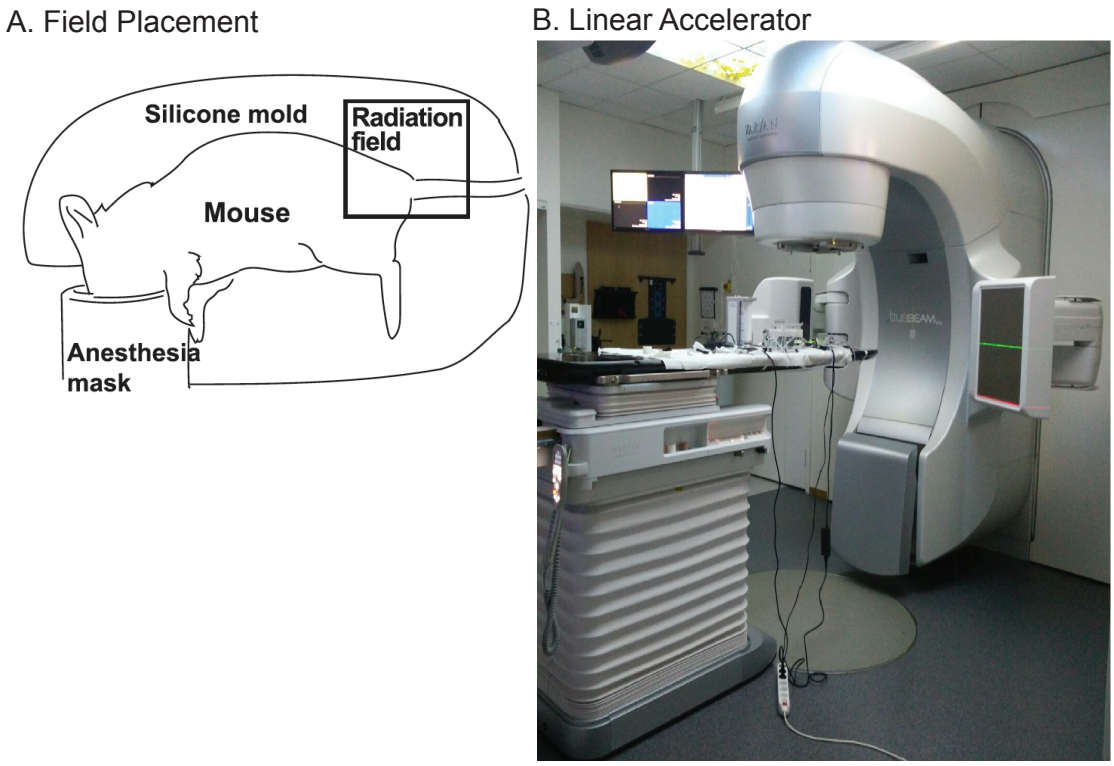

Figure 3. *A. Irradiation procedure with small-field irradiation. Modified from S. Devarakonda et. al. Neuroscience 475 (2021) 137–147. B. Linear accelerator used in the experiment.*

Dietary intervention

For each diet and time point, 8-weeks old male C57BL/6J mice were divided into four groups with ten mice in each group. Mice were placed five per cage and fed a fiber-rich diet (bio-processed oat bran) or a fiber-free diet for 1, 6 and 18 weeks after radiotherapy. The mice were fed the two diets starting two weeks before radiotherapy and continued after radiotherapy until the day of sacrifice. The fiber-rich diet contained 15% fiber and the fiber-free diet 0% fiber. Diets were prepared using bioprocessed oat bran and starch (fiber-rich diet), or starch only (fiber-free diet), added to a custom-made basal mixture in powdered form (Table 1). The starch was used to ensure that the two diets were somewhat isocaloric. Sham-irradiated control groups were fed with the same diets. Both diets were provided to the mice in a porridge-like consistency.

High-oat diet, 15% fiber	No-fiber diet, 0% fiber
Bioprocessed oat bran (28.8%)	Bioprocessed oat bran (0%)
Corn starch (4.7%)	Corn starch (33.5)
Basal mixture (66.5)	Basal mixture (66.5)
Total Diet Composition (100%)	Total Diet Composition (100%)

Table 1. *Dietary compositions, Paper I and II.*

Tissue preparation

Mice were deeply anesthetized, and blood was drawn through cardiac puncture. Mice were then decapitated, brains excised with the left hemisphere snap frozen and stored at -80°C, and the other hemisphere placed in 6% formaldehyde (Histofix; HistoLab Products, AB) overnight and next morning transferred and stored in 0.1 M phosphate-buffer with 30% sucrose, pH 7. The intestines were washed with ice-cold PBS and a thin, soft silicone tube was inserted rectally. Seven millimeters of the distal colorectum was carefully excised and immediately placed in methacarn and left overnight before dehydration and embedding in a paraffin block. The right hemispheres were cut into 25µm thin sagittal sections in a 1:12 series using a sliding microtome (SM2010R, Leica Microsystems), and these sections were stored in cryoprotective solution (25% glycerin, 25% ethylene glycol, and 0.1 M PO4) at 4°C. The paraffin blocks with the colorectal samples were cut into 4-µm thin sections using a microtome (Leica RM2235; Leica Biosystems), with the sections mounted in a 1:6 series on the slides.

Note: To evaluate histological changes, the sections were cut using serial sectioning; the sections were placed one after the other, such that each sixth section was on the same slide (1:6 series). This method ensures enough space between the sections analyzed to avoid quantifying the same object (e.g. cell or crypt) twice. Perfusion of the brains with a fixative normally increases histochemical staining quality but was not performed as multiple organs were collected for other analyses.

Immunohistochemistry and histochemistry

For colorectal tissue, sections were fixed in methacarn and later embedded in paraffin. Sections were deparaffinized at 60°C for one hour followed by rehydration using xylene and graded ethanol washes. Antigen retrieval was done by immersing the sections in sodium citrate pH 6 and boiling them in a pressure cooker for 3 minutes followed by blocking of endogenous peroxidase activity using 0.6% peroxidase blocking solution for 10 minutes on the sections. Sections were then washed and placed in blocking buffer (TBS containing 0.1% TritonX-100 and 3% donkey serum) for one hour in room temperature (RT) and incubated in primary antibody overnight (anti-Ki67 or anti-Iba1) at 4°C. The next day, sections were rinsed in TBS and incubated in secondary antibody for 1 hour at RT. Sections were rinsed again, and the antibody target was visualized by developing in DAB (Saveen Werner AB, Malmö, Sweden). Sections were then dehydrated using graded ethanol washes and xylene and mounted using X-TRA kitt mounting medium (Medite GmbH, Burgdorf, Germany). To perform Verhoeff's elastic stain, after dewaxing the sections were rehydrated and immersed in Verhoeff's solution followed by washing in tap water and distilled water. The sections were differentiated in a 2% ferric chloride solution and then by a 5% sodium thiosulfate solution and rinsed in tap water and distilled water and later counterstaining with Van Gieson. Finally, sections were dehydrated in alcohol series and xylene before mounting with mounting media X-TRA kitt (Medite GmbH, Burgdorf, Germany).

For brain tissue, sections were free-floating in well plates and washed with TBS and then incubated in blocking solution (TBS containing 0.1% TritonX-100 and 3% donkey serum) for 30 mins in RT to avoid unspecific binding. Later, they were incubated in primary antibody (1, 2 and 4 from Table 2 below) over night at 4°C in 3% donkey serum in TBS containing 0.1% TX100. The next day the sections were rinsed in TBS three times and incubated in fluorochrome-coupled secondary antibody for 1 h at room temperature. The sections were then

rinsed in TBS and mounted onto slides and coverslipped with 4',6-diamidino-2-phenylindole (DAPI). For immature neurons, the sections were first rinsed in TBS three times for 10 mins each and were then treated with sodium citrate (pH 6) 10 mM at 80°C in a water bath for 30 minutes. After the antigen retrieval, the sections were cooled down and washed with TBS. Endogenous peroxidase activity was blocked using a 0.6% peroxidase blocking solution for 10 minutes followed by incubation in blocking solution for 30 mins in RT. The sections were then incubated in primary antibody (3 from table below) overnight at 4°C. The next day they were rinsed and incubated in secondary antibody (biotinylated donkey anti-rabbit antibody, 1:1000, Jackson ImmunoResearch Laboratories Inc) for 1 hour at RT. After incubation in avidin–biotin-peroxidase (Vectastain Elite ABC Kit, Vector Laboratories) for 1 hour in RT, sections were rinsed and developed with DAB (Saveen Werner AB, Malmö, Sweden) for visualization. Sections were then dehydrated using graded ethanol washes and xylene and mounted using X-TRA kitt mounting medium (Medite GmbH, Burgdorf, Germany).

Primary Antibody	**Dilutions Colon**	**Dilutions Brain**	**Detecting**
1. anti-rabbit Ki-67	1:150, Merck Millipore	1:500, Abcam	Cell proliferation
2. anti-rabbit Iba1	1:2000, Wako Chemicals	1:2000, Wako Chemicals	Macrophages
3. anti-rabbit DCX	N/A	1:500, Abcam	Immature neurons
4. anti-rabbit NeuN	N/A	1:1000, Merck Millipore	Mature neurons
Secondary Antibody	**Dilutions Colon**	**Dilutions Brain**	**Type**
5. donkey anti-rabbit Alexa Fluor 555	N/A	1:1000, Invitrogen/Molecular Probes	Fluorochrome
6. biotinylated donkey anti-rabbit	1:250, Jackson ImmunoResearch Lab. Inc.	N/A	DAB Chromogen
7. ProLong Gold DAPI	N/A	50-100ul/slide (Invitrogen/Molecular Probes)	Nuclear stain mounting media

Table 2. *Antibodies and nuclear stain mounting media used in Paper I and II.*

Cell quantification

In colorectum, Ki67-positive cells were quantified using a Leica DMi6000 microscope. Only crypts that had a clear opening towards lumen and were cut perpendicularly along their entire axis were included to quantify cell proliferation. All Ki67-positive cells in a total of 24 crypts from two sections approximately 72µm apart were counted per mouse. The average number of

proliferating cells per crypt was then calculated. Iba1-positive cells were quantified using a Leica DM6000B microscope equipped with a semi-automated stereology system and the Stereo Investigator software (MBF Bioscience, Williston, VT, USA). The mucosal area was traced in 5X and a grid with 150 x 150 µm was placed over the traced area. Using 40X magnification, cells were counted within a 50 x 50 µm counting frame placed at all grid intersections landing within the traced area. Three circumferences, separated by approximately 48 µm, were analyzed per mouse, and around 50 counting frames per section were analyzed. Using the Stereo Investigator software, the total number of Iba1-positive cells in the traced area was estimated. The average number of Iba1-positive cells per three circumferences was calculated.

Sections stained with Verhoeff's Elastic Stain were used for the assessment of crypt degeneration, fission, and survival of crypts. All crypts per circumference in three sections 48 µm apart were counted per animal for estimation of surviving crypts. Six sections 24 µm apart were analyzed per mouse at 40X magnification to assess crypt fission and degenerating crypts. The criteria to be fulfilled for crypt fission was a crypt that divided at the bottom to form two or more daughter crypts with a common opening to the lumen. For degenerating crypts, the crypt should be closed towards the opening of the lumen and dilated, with a thin columnar epithelium.

In brains, quantitative assessments were done using a Leica DMi6000 microscope with a semi-automated stereology system and the Stereo Investigator software. The area of the granule cell layer (GCL) in each section was traced at 20X magnification, starting at where the GCL was separating into a dorsal and ventral part, and ending where the dorsal blade disappeared. The area was then multiplied by the thickness of the section (25 µm) and the series they were cut in (1:12) to get an estimation of the total GCL volume. In all sections, the cells labelled with Ki67, Iba1 or DCX were exhaustively counted.

NeuN-positive neurons in the GCL were counted using the Stereo Investigator software and a modification of unbiased stereology. The outline of the GCL was traced in very 12^{th} section, and a grid (80 mm grid size) with a counting frame (20x20 mm) at each grid intersection was placed over the traced area. NeuN-positive cells falling within or touching the green line of the counting frame (but not the red) were counted. The total number of cells per section was

estimated by the software and the total number of NeuN-positive cells per volume between the first and last section was assessed by multiplying with the series (1:12).

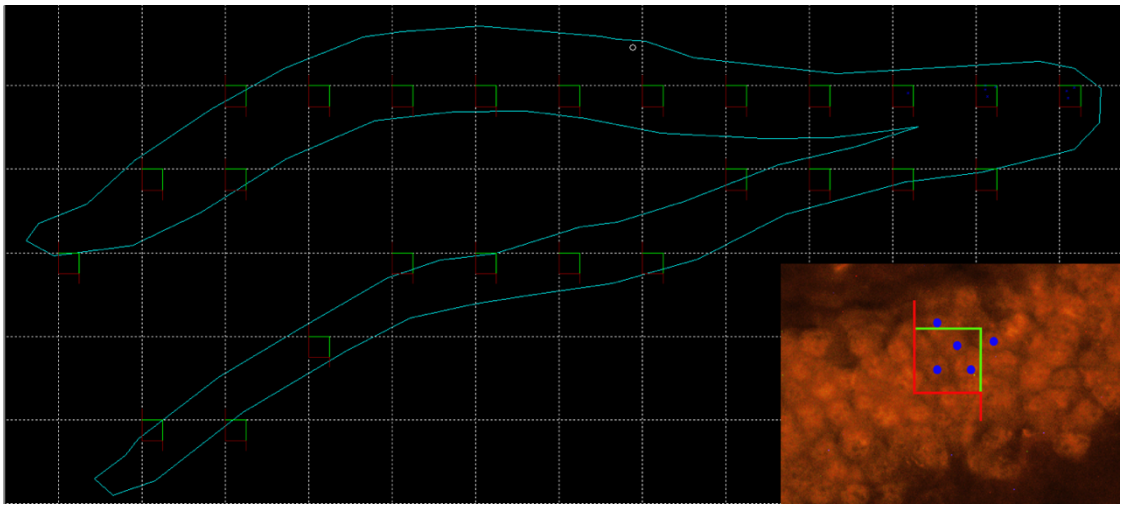

Figure 4. *The number of NeuN-positive cells (insert, in red) in Paper II were estimated using a method based on stereology with a grid placed randomly over the traced area of the GCL and counting frames in the intersections.*

Note: stereology is a method based on estimation to quantify objects, volumes and shapes with high accuracy. In this thesis, we used a stereology-based method which is describe more in detail in [67].

Fluorescence *in situ* hybridization to detect and quantify bacteria in mouse colorectum

The slides were dewaxed at 60 °C for 10 mins followed by xylene, rehydration in an EtOH and then air dried. EUB338, an Alexa 546-conjugated universal probe for bacteria (16S rRNA: 50-GCTGCCTCCCGTAGGAGT-30, Eurofins Genomics, Ebersberg, Germany) was added to pre-warmed hybridization solution (20 mM Tris-HCl pH 7.4, 0.9 M NaCl and 0.1% SDS). A total of 500ng/100 µL (probe/hybridization buffer) was added to each slide and the slides were covered with a cover glass and placed in a humidity chamber at 50°C overnight. The next day, slides were rinsed with H_2O and PBS for 1 min each and coverslipped with ProLong™ Gold Antifade Mountant with DAPI (Molecular Probes, Eugene, OR, USA). Strong and visibly rod- or sphere-shaped bacteria were quantified at 63X magnification in both mucosa and submucosa. A total of three sections 48 µm apart were analyzed and the average number of bacteria per section was calculated by dividing the total number of bacteria by the number of sections counted.

Note: In situ hybridization using rRNA-targeted oligonucleotide probes has become a popular technique for analyzing the microbial population structures of diverse natural and engineered systems. In such research, the probe EUB338 is regularly utilized to quantify bacteria with a sufficiently high cellular ribosome concentration[68].

Serum cytokine analysis

A Bio-plex Mouse Cytokine kit (Bio-Rad Laboratories AB, Solna, Sweden) was used to measure 23 cytokines in the mouse serum, a detailed description of the method was published previously[69]. The cytokines included in the 23-plex were: (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A, eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), interferon (IFN)- γ, keratinocyte-derived chemokine (KC), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated on activation normal T-cell expressed and secreted (RANTES) and tumor necrosis factor (TNF)-α.

Note: Apart from multiplexing ability, Luminex has other advantages such as superior accuracy, flexibility, higher throughput, small sample quantity, time savings, and low cost when compared to ELISA. Unlike ELISA that depends on a flat bottom to capture targets, Luminex uses beads to capture the target.

Statistical analysis

Statistical tests were performed using the Graph-Pad Prism 8 software (GraphPad Software LLC, San Diego, CA, USA), data were expressed as mean ± S.E.M and a p-value equal to or below 0.05 was considered statistically significant. For the cytokine analysis, R statistical software was used. The data sets were subjected to normality test and the data set that passed the Shapiro-Wilk test for normal distribution was subjected to two-way analysis of variance (two-way ANOVA) followed by a Bonferroni post-hoc test. Data sets that did not pass were subjected to a Kruskal-Wallis test on ranks followed by Dunn's post-hoc. Three groups (diet and irradiation as between-subjects variables, and time as within-subjects factor) were compared using a three-way mixed ANOVA with a Geisser-Greenhouse adjustment for the absence of sphericity.

Paper III:

Study participants inclusion criteria and biopsy collection

The study received approval from the Swedish Ethical Review Authority (686-10, addition T804-18 and addition 2020-03788) and all study participants gave informed written consent. Study participants were chosen by an oncology nurse at BäckencancerRehabiliteringen, a specialized clinic for cancer survivors at Sahlgrenska University Hospital. BäckencancerRehabiliteringen has data from more than 1000 cancer survivors and the oncology nurse selected, with the help of medical records, possible study participants on the basis of two inclusion criteria: the subject had received pelvic radiotherapy 2-20 years ago and had in an earlier questionnaire reported that he/she suffered from the urgency-tenesmus syndrome. Exclusion criteria were ongoing cancer treatments, not being able to read the Swedish language or having an intestinal stoma. Non-irradiated controls were identified via the endoscopy department when visiting the clinic for other reasons than the specific study. They were not to have any inflammatory bowel disease, or to have previously received any type of radiotherapy.

Five biopsies were taken at 5 cm from the anal opening, five at 15 cm and five at 25 cm. We biopsied the non-irradiated controls with an identical approach. The biopsies were either fixed in 6% formaldehyde (Histofix; HistoLab Products, AB) for histology and fluorescence-*in situ* hybridization, frozen in liquid nitrogen for protein expression studies, or transferred to RNAlater for future metagenomic studies. In a subset of participants, biopsies were stored in physiological buffer for the measures of mucosal permeability or immune cell quantification with flow cytometry.

Note: During pelvic radiotherapy, the lower part of the rectum is exposed to higher doses of radiation than areas further up in the colon. Thus, in Paper III, biopsies taken at 25 cm from the anal verge served as internal controls (low-dose area) to the biopsies taken at 5 cm (high-dose area). To verify that differences between the two biopsy areas were not due to morphological differences, non-irradiated controls were biopsied and analyzed in identical ways.

Almost all the participants were older Swedish women with Caucasian background, thus, the cohort was quite homogenous. The dose regimen differed between the participants and the

majority of the irradiated subjects, but not all, had received chemotherapy together with the radiotherapy.

Proteomics and Tandem Mass Tag (TMT) labeling

Global quantitative proteomics is a tool that uses systematic identification and quantification of a tissue's total proteins (the proteome) at a given point in time. TMT is a chemical labeling method for measuring relative protein abundances in multiple samples at once. It is possible to quantify a large number of proteins, usually between 75,000 and 155,000 proteins, within cells or tissues. The technique involves chemically labeling multiple peptide samples with isobaric chemical tag variants that have the same mass and structure. After digesting samples with trypsin, peptides with the same molecular mass and chemical structure are labeled with different tag variants and samples are multiplexed or combined into one. When analyzed in their intact form, identical peptides derived from different samples cannot be distinguished. When fragmented in a mass spectrometer, however, each peptide variant produces a unique reporter ion that is used to quantify the peptide and its associated protein. Prior to analysis of each fraction with nanoLC Mass Spectrometry, methods such as pre-fractionation (pH 10) are applied. Identification and quantification rates are directly related to sample complexity, hence reducing sample complexity is critical. The intensities measured in the MS2 spectra obtained reported the relative quantification of the peptides.

Database matching, statistics, and bioinformatics

There are many software packages designed to process TMT data for protein identification and quantification. We used Proteome Discoverer version 2.4 (Thermo Fisher Scientific) and the MASCOT search engine (matrixscience.com) against SwissProt Human database (UniProt) for identification and relative quantification of the proteins. Only unique peptides were used for quantification where the reference samples were used as denominator for calculation of ratios. Perseus, Microsoft Excel, and R statistical tools were used for statistics and bioinformatics analyses. Annotations from the Gene Ontology database were obtained from the Uniprot database.

Note: An advantage is that multiplexing minimizes technical variation between samples to less than 10%. A disadvantage is that the quantification is skewed toward proteins with molecular

weight greater than 20 kDa. The results must be confirmed using alternative methods and/or samples.

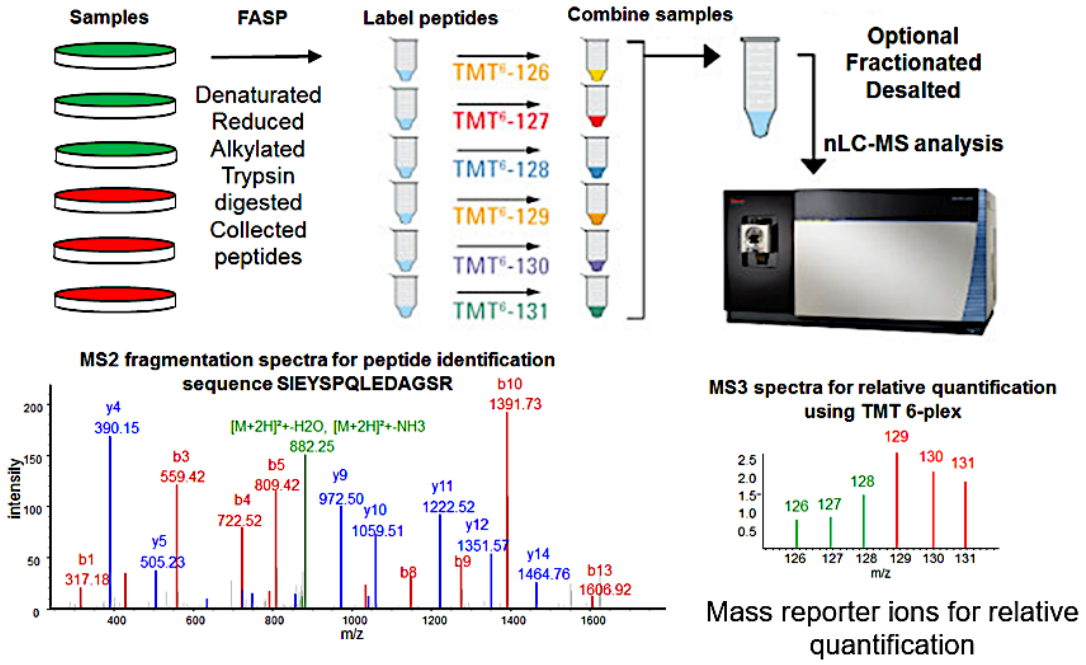

Figure 5. Relative quantitation with isobaric labeling (TMT) is utilized to analyze abundance of peptides and their respective proteins in multiple samples at MS/MS level.
Proteins were extracted from tissue and reduced, alkylated, and enzymatically digested with trypsin into peptides. Samples were labelled with the TMT reagents. Multiplexing of samples was followed by fractionation and clean-up. Labelled samples were analyzed on a high-resolution Orbitrap nanoLC-MS/MS mass spectrometer. The relative quantification was based on the abundance of TMT reporter ions (Modified from Thermo Fisher Scientific).

Mucosal permeability analysis

Mucosal permeability was assessed in order to understand whether the intestinal barrier was compromised after radiotherapy. Using fluorochrome beads applied to the luminal side of the biopsies, we were able to assess the outer and inner mucus layer penetrability with confocal microscopy.

Note: In Gustafsson *et al*., the thick structure of the connected inner mucus layer was studied using size exclusion of µm-sized beads, and it was discovered that beads the size of bacteria were excluded. However, the outer mucus layer allows bacteria-sized beads to get through, showing that the outer mucus layer contains bigger pores than the inner mucus layer[70]. There are other less invasive methods for measuring intestinal permeability, such as measuring lactulose in urine or lipopolysaccharide in serum. With these methods we would not be able to

know the location of a possible barrier breach, and if the disruption of the barrier was subtle, such methods would most likely not be sensitive enough. In addition, sampling urine or serum would have required an additional effort from the study participants.

Isolation of cell populations and flow cytometry

Flow cytometry is a widely used technique to detect immune cell populations from a cell suspension. It relies on fluorochrome-conjugated antibodies to detect the specific cell surface expression or antigens on cells in a suspension. This suspension is injected into flow cytometer (BD LSR Fortessa) instrument and analyzed with FlowJo v.10.8.0 (Tree Star, Ashland, OR, USA). Total populations of the cells were obtained with help of AccuCount beads (Spherotech, Lake Forest, IL, USA).

Cells from the biopsies were extracted using a pre-established protocol and adapted to our study[71]. Markers used to discern the different cell populations were as follows: neutrophils (HLA-DR-CD15+CD11c+), macrophages (HLA-DR+CD11c+CD64+), dendritic cells (HLA-DR+, CD11c+CD64-) and T cells (CD3+). CD15 is expressed by neutrophils but is also expressed on other granulocytes, and also on monocytes. By using gating strategies, we could purify the population of polymononuclear cells from other myeloid cells based on the expression of HLA-DR on dendritic cells and monocytes and all the lymphoid cells based on CD3, CD19 and CD56. The remaining cells that were HLA-DR- were polymononuclear cells. We also used other markers like CD14 and CD64 to test and make sure the population was pure polymononuclear cells. Using CD16, we could separate neutrophils from eosinophils but we found almost no well-defined CD15+ CD16- eosinophil population, thus cells labelled were neutrophils.

Immunohistochemistry and quantification

Biopsies that were fixed in 6% formaldehyde were paraffin-embedded and sectioned to 4 µm thickness. For the staining procedure, slides containing sections from biopsies taken at low-dose areas (25 cm) and high-dose areas (5 cm) were chosen. The slides were dewaxed in an incubator for 1 hour at 60 ºC and then deparaffinized in xylene and alcohol. Sections were then placed in sodium citrate pH 6 for 30 minutes at 80 ºC in an incubator. The slides were then rinsed in TBS and blocked (2 % bovine serum albumin in 0.05 % Triton X-100 in TBS) for an hour followed by incubation overnight in primary antibody (1:250, anti-rabbit

myeloperoxidase, Abcam). The next morning, the sections were rinsed in TBS and incubated in secondary antibody (1:500, Alexa Fluor 555 donkey anti-rabbit, Abcam) for 1 hour. Afterwards, the sections were rinsed in TBS and dehydrated by immersing them in series of alcohol and then xylene. The sections were coverslipped with DAPI mounting media (Prolong Gold antifade with 4,6-diamidine2-phenylindole dihydrochloride, Invitrogen). We quantified the number of neutrophils per area (MPO-positive cells) in 40X using a Leica DM 6000B microscope with a semiautomatic stereology system. The area of the lamina propria was calculated by subtracting the area occupied by crypts from the total area mucosa analyzed per subject. The number of neutrophils per area lamina propria in each subject was then calculated by dividing the total number of neutrophils counted per subject with the total area lamina propria. For Goblet cell quantification, nuclei were stained with Alcian blue followed by Nuclear Fast Red. Alcian Blue has an affinity for acidic tissue components, such as mucin. After incubating with Alcian blue (pH 2.5) for 30 minutes, washing under tap water slides were immersed in Nuclear Fast Red for 10minutes, to stain the nuclei, rinsed and coverslipped. On a slide with six sections, goblet cells were quantified in every second section using a Leica DM 6000B microscope. Three crypts per section were analyzed and the area of each crypt was measured. Only the crypts that reached at least half of the thickness of the mucosa and the crypts that were open to the lumen were included for the quantification.

Note: Myeloperoxidase (MPO) is a neutrophil-derived enzyme that catalyzes the production of many reactive oxygen species[72]. MPO-derived oxidants have been shown to contribute to tissue damage during inflammation, in addition to being an essential component of the innate immune response. Approximately 5% of the neutrophil dry mass is composed of MPO[73], which is mainly found in lysosomal azurophilic granules, It has been claimed that myeloperoxidase (MPO) reflects the level of neutrophil activation[74]. Goblet cells in the colon synthesize and secrete high-molecular-weight glycoproteins called mucins to maintain the protective mucus layer.

Bacterial detection using FISH

Bacteria in the lamina propria and crypts were assessed using the universal probe EUB338 labelled with Alexa-546 and fluorescence *in situ* hybridization. Quantification of the bacteria was performed using stereology software and the Leica DM6000B microscope. The area of the mucosa and crypts were estimated with the help of the Cavalieri point-counting method.

Statistical analysis

Our statistical analyses were carried out using the Graph-Pad Prism 8 software (GraphPad Software LLC, San Diego, CA, USA), version 9.0.2, and R statistics software. Statistical significance was determined by a P-value of ≤ 0.05 (Mann-Whitney or Wilcox signed rank test) and the data expressed as mean + S.E.M. Using cluster analysis in R, we further classified subjects based on their profile with regards to expression of proteins known to be involved in inflammation.

MAIN RESULTS AND DISCUSSION OF PAPER I

Despite the well-known health benefits of a diet rich in fiber, patients who are undergoing pelvic radiotherapy are often recommended to follow a low-fiber diet to avoid the exacerbation of radiation-induced gastrointestinal symptoms[58]. How such advice affects the cancer survivor's bowel health in the long run is unknown. One of the few studies in humans on the subject was carried out by Wedlake and colleagues and published in 2018[75]. Wedlake randomized pelvic cancer patients to eating more fiber than usual, eating less fiber than as usual, or eating their habitual diet during radiotherapy and found that the group that was eating more fiber and the group that was eating less fiber both experienced less gastrointestinal toxicity one year after completed radiotherapy than the group that continued their habitual diet. The results from the study were thus inconclusive, but it is possible that by reducing fiber intake, the participants experienced less discomfort caused by the fermentation of dietary fiber in the colon, which promotes gas formation. The study did not evaluate histological changes, and so it was not possible to draw any conclusions on whether reducing dietary fiber intake was harmful to the bowel health. In Paper I, we evaluated the long-term effects of a fiber-rich diet with bioprocessed oat bran or a fiber-free diet on radiation-induced intestinal injury using a murine model of pelvic radiotherapy. The colorectum was irradiated or sham-irradiated with a linear accelerator (4 fractions of 8 Gy) and the tissue was analyzed at three time points after irradiation: 1, 6, and 18 weeks (Figure 6A). The mice were fed their diets starting 2 weeks before irradiation and maintained throughout the experiment (Figure 6B).

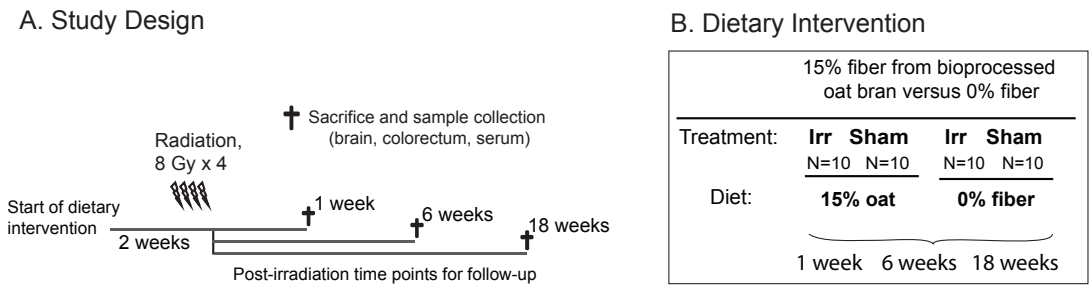

***Figure 6**. A. Study design. B. Dietary groups. Irr=Irradiated, Sham=Sham irradiated*

Body weight and diet

Throughout the experiments, the mice were examined and weighed weekly. There was an effect of diet on body weight. Mice fed with the no-fiber diet had a more pronounced increase in body weight over the course of time (Figure 7). Fiber consumption is often linked to weight loss, and recent studies have found that oatmeal, which is strong in beta-glucan, promotes satiety and reduces calorie intake in subsequent meals[76, 77]. Also of interest, previous research has found a link between a lack of dietary fiber and being overweight[78, 79]. To balance the caloric intake and volume of the porridge in both groups, mice on a no-fiber diet had starch added to the basal diet. In contrast to the dietary fiber being fermented in the colon, the starch is quickly absorbed in the small intestine, producing a weight gain in both the sham-irradiated and irradiated groups. In the irradiated high-oat group, the weight gain over time was slightly (although not statistically significant) smaller than that of their sham-irradiated controls, probably due to damage to the colorectal mucosa in the irradiated group.

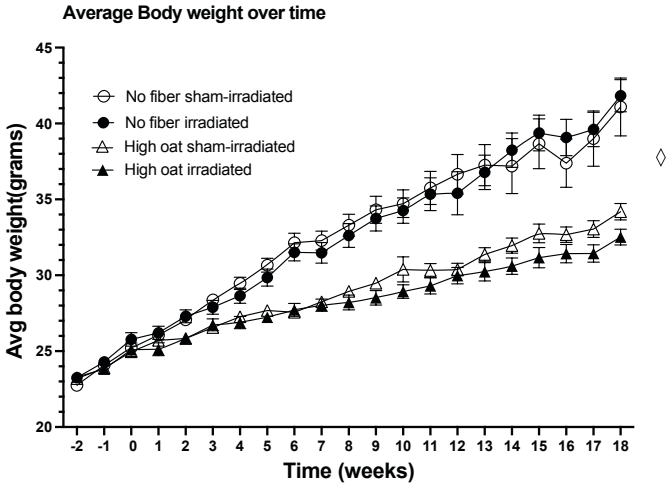

***Figure** 7. Weight curves of mice for 20 weeks showing three-way ANOVA with significance for diet. Modified from Malipatlolla, D.K.,et al., Int. J. Mol. Sci. 2022, 23(1), 439.*

Crypt degeneration

There is no direct evidence showing crypt degeneration in healthy intestines. We did not observe any degenerating crypts in our non-irradiated controls at any time point, irrespective of diet. Degenerated crypts were observed in both diet groups at 1 week and 6 weeks after irradiation. In the fiber-free diet group, we observed severe acute crypt degeneration, whereas crypt degeneration in the fiber-fed group appeared more subtle and consistent over time. Initially at 1 week (Figure 8A) the fiber-fed irradiated group portrayed a slightly protective

effect, but at 18 weeks (Figure 8C) we observed that the fiber- rich diet was not able to rescue the crypts from degeneration due to radiation-induced damage in the long term. This suggests that although repair mechanisms are stimulated by dietary fiber intake, high-dose radiation damage to the stem cell niche will eventually cause depletion of the stem cell pool and result in crypt degeneration.

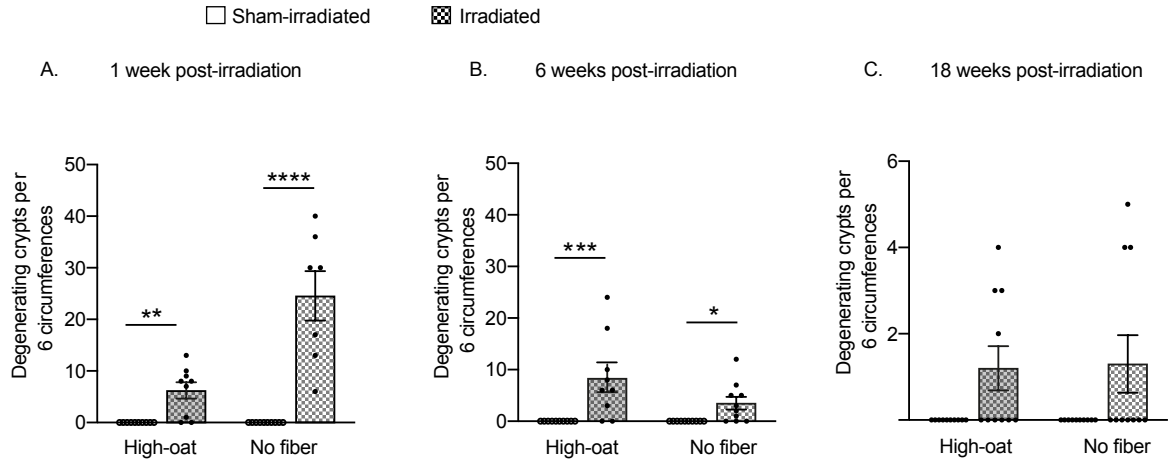

Figure 8. *Number of degenerating crypts at 1 week, 6 weeks and 18 weeks after irradiation.*
**= Post-hoc significance. Modified from Malipatlolla, D.K.,et al., Int. J. Mol. Sci. 2022, 23(1), 439.*

Crypt fission

Other than crypt proliferation, crypt fission, where a crypt will split into two or more new crypts, is another repair mechanism for increasing the number of crypts. Crypt fission plays a major role in maintaining homeostasis of the intestine and increased fission has been observed due to intestinal damage and ulceration[80]. It has been previously discussed that crypt fission maybe a compensatory response to crypt loss/fusion[81]. At one week post-irradiation, we measured the number of crypt fissions per circumference and found more fissions in irradiated mice. The increase appeared slightly larger in mice on a fiber-rich diet. At 6 weeks, there were no significant differences, but we did observe a higher baseline in sham-irradiated mice which could be a result of stimuli due to the diet. At 18 weeks, both irradiated groups still had more crypt fissions than the sham-irradiated, although only the fiber-free group reached a statistical significance. When inspecting the graphs visually, sham-irradiated mice on a fiber-rich diet had a higher baseline of crypt fission at one and 18 weeks compared to sham-irradiated mice (data not shown). Possibly, the fiber-rich diet stimulated crypt fission in the colorectum throughout all time points, and in line with this, the sham-irradiated mice on a fiber-rich diet also had the highest number of crypts per circumference.

Cell proliferation and radiotherapy

Radiation causes single strand breaks or double strand breaks in the genetic material, preventing the cancerous cells from proliferating further. The amount of ionizing radiation necessary to cause cell inactivation and death varies depending on the tumor and surrounding tissues[82]. The potential cell damage is determined not only by the cellular healing mechanisms, but also by the stage of the cell cycle in which the cell is at the time of treatment. Certain phases of the cell cycle enhance the possibility of repairing damage, a function many cancerous cells have lost[83]. Over the years, clinical oncologists have fine-tuned radiation regimens and the development of highly advanced radiation technology has limited the harm to healthy tissue caused by excessively high doses or large field sizes[3]. Nevertheless, intestinal mucosa close to or within the radiation field remains a limiting factor due to the high proliferation rate of stem- and progenitor cells in the crypts. Therefore, approaches to protect or stimulate the stem cell niche in the crypts during or after radiotherapy are of interest. We observed an increase in cell proliferation in both dietary groups at one week after radiotherapy, but the effect lasted longer in the mice fed with a fiber-rich diet and was still present at six weeks, but not 18 weeks post-irradiation. At 6 weeks (Figure 9) after radiotherapy we observed a statistically significant increase in cell proliferation in mice fed with fiber-rich diet compared to fiber free diet. Sureban *et al*. showed that consuming soluble dietary pectin protected the stem cell population and increased the survival of crypts after irradiation, but the mechanism is unknown[61].

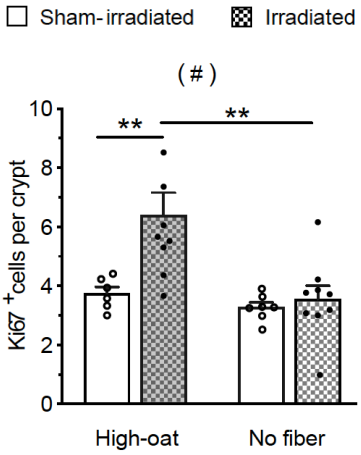

Figure 9. *6 weeks post radiotherapy. Two-way ANOVA, #= diet. *=Post-hoc significance. Modified from Malipatlolla, D.K.,et al., Int. J. Mol. Sci. 2022, 23(1), 439.*

Crypt survival

In mice, the colonic epithelium is renewed every couple of days, whereas in humans renewal takes little less than a week[84]. The total number of crypts is regulated by a homeostatic process whereby the increase in crypts driven by fission is balanced by a process that decreases crypts by fusion. Irradiated mice had fewer crypts than sham-irradiated mice regardless of diet (Figure 10A and B). In comparison to the fiber-deprived animals, the bioprocessed oat bran increased the number of crypts in the sham-irradiated animals over time. Sham-irradiated animals fed a fiber-rich diet increased their crypt numbers by 44 percent between week 1 and week 18, while fiber-deprived animals increased their numbers by only 16 percent.

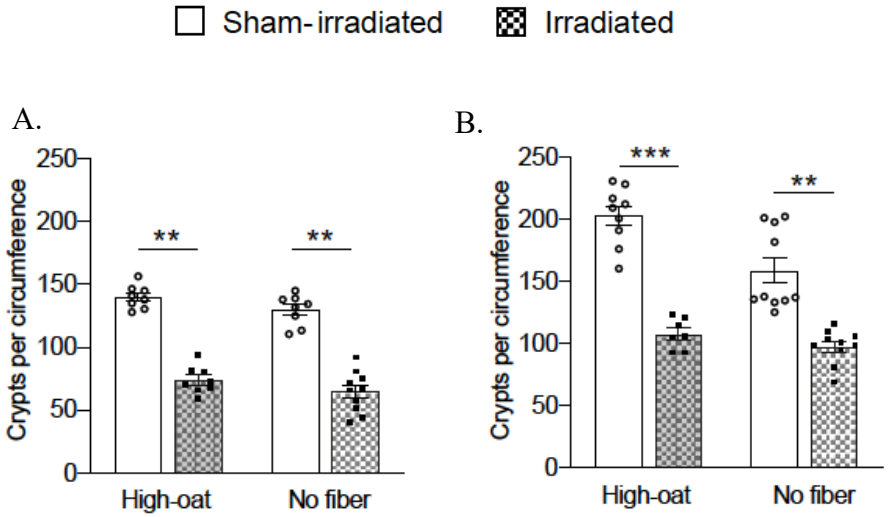

***Figure 10**. A and B Crypts per circumference at 1 and 18 weeks *= Post-hoc significance. Modified from Malipatlolla, D.K.,et al., Int. J. Mol. Sci. 2022, 23(1), 439.*

Correlation analysis- cytokine profile and bacterial infiltration

Serum from the mice was extracted and the presence of 23 cytokines was analyzed for all the three time points in our previously published study[69]. In Paper I, hierarchical clustering of cytokines at the 18-weeks' timepoint in the four groups revealed three different profiles. The most pro-inflammatory profile was expressed by irradiated mice on a fiber-free diet. In a study of DNBS-induced colitis in Sprague–Dawley rats, the intake of oat beta-glucans lowered the level of inflammatory markers and restored the inflammatory signaling pathways and histological alterations[85]. In our study, we observed higher levels of pro-inflammatory

cytokines in our mice with fiber-free diet post radiotherapy compared to fiber-rich diet fed mice.

It has been shown that acutely after ionizing radiation exposure, the intestinal barrier becomes less effective, allowing intestinal bacteria and their components to penetrate the lamina propria where they are detected by antigen-presenting cells. After activation, antigen-presenting release proinflammatory cytokines. We found that a fiber-deprived diet combined with irradiation-induced damage greatly increased intestinal bacterial penetration at 18 weeks post-radiotherapy, which could have promoted the production of pro-inflammatory cytokines (Figure 11A and B). When the cytokine profiles were correlated with the bacterial counts in the 18 weeks groups post radiotherapy, animals with high bacterial counts were found to express cytokine profile 3, the most pro-inflammatory profile (illustrated in Figure 11B).

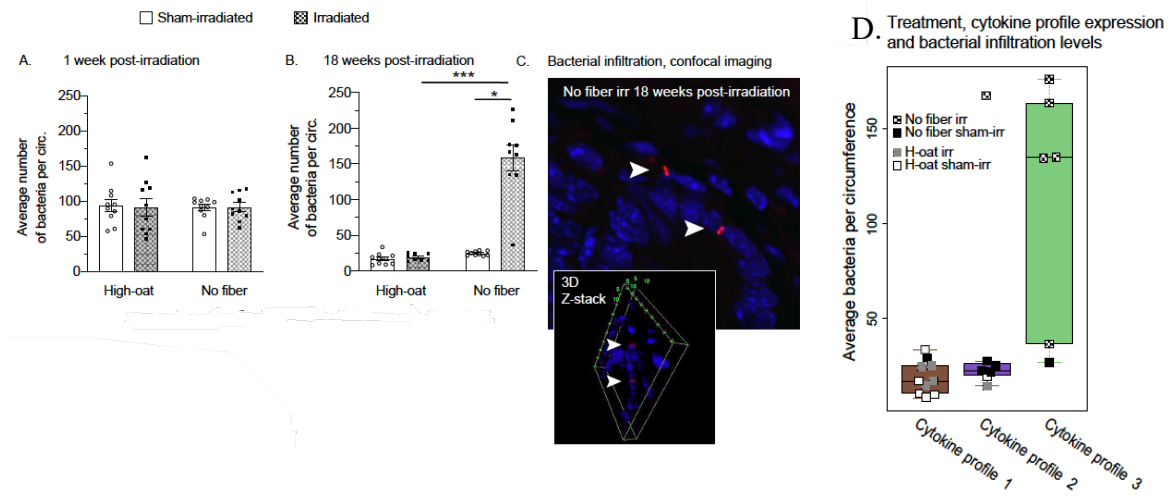

Figure 11. *A and B. Bacterial infiltration 1 and 18 weeks. Circ.= circumference *= Post-hoc significance. C. Confocal image of bacterial infiltration in mice fed with fiber free diet at 18 weeks.* ***D.*** *Box-plots with median and interquartile range showing the number of infiltrating bacteria in the mice within each cytokine profiles. Modified from Malipatlolla, D.K, et al., Int. J. Mol. Sci. 2022, 23(1), 439.*

In contrast to others, we did not see bacterial infiltration acutely after irradiation. Modelling of gastrointestinal injury after radiotherapy is usually done by single-dose radiation. We used fractionated irradiation, which could have changed the dynamics and timing of bacterial infiltration. This is an important notion, since pelvic radiotherapy in the clinic is given in multiple fractions. Changes in the dynamics of bacterial infiltration could be expected to impact

the conclusions that can be drawn from a specific model at a certain time point greatly and influence its validity with regards to the human situation.

Macrophages of the colon

Colonic resident macrophages are highly phagocytic but not overtly inflammatory[86]. The colon is constantly exposed to commensal pathogens and macrophages play important role in differentiating commensals and microbiome pathogens. This process, however, is dysregulated in Crohn's disease and ulcerative colitis, as patients with IBD have enhanced migration of $CD14^{hi}$ monocytes compared to healthy control individuals, resulting in an accumulation of $CD11c^{hi}$ pro-inflammatory monocyte-like cells in the colon of IBD patients[87, 88]. In our mouse model we observed increased macrophage numbers at 6- and 18-weeks' time points in both irradiated groups, irrespective of diet (Figure 12). Since the number of macrophages did not correlate with bacterial presence or peak injury, their role in the tissue was unclear. They could be involved in repair processes after irradiation, as has been shown by Saha et al[89].

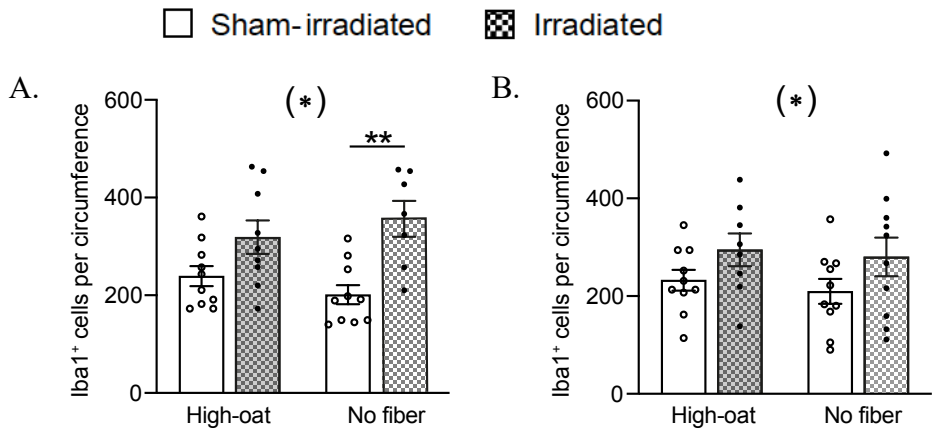

***Figure 12**. Macrophages per circumference. A and B 6 and 18 weeks *=Two-way ANOVA, irradiation, *= Post-hoc significance. Modified from Malipatlolla,D.K., et al., Int. J. Mol. Sci. 2022, 23(1), 439.*

In conclusion, our findings show that dietary fiber had the ability to influence the timing, magnitude and duration of numerous intestinal pathophysiological processes and repair mechanisms following radiation, and could prevent a late, perhaps chronic, radiation-induced bacterial infiltration of the mucosa[90]. Although dietary fiber intake could not protect against crypt loss, a common pathological feature of the irradiated mucosa, our data show that

dietary changes should be considered when developing interventions for gut health protection and restoration during and after pelvic irradiation. Keeping the mucus layer intact is one of the processes through which intestinal homeostasis is maintained. If the mucus barrier is breached, pathogens can move from the lumen into the mucosa, triggering a chain reaction of inflammatory processes in the gut. The fiber source we used in this study, bioprocessed oat bran, is abundant in easily accessible beta-glucan, which produces an especially high amount of butyrate. Butyrate serves as an energy source for colonocytes, and goblet cells use it to build a protective mucus layer that lines the epithelium. A fiber-free diet, on the other hand, has been demonstrated to increase the presence of mucus-degrading bacteria, leading to increased colonic mucus-barrier deterioration and pathogen vulnerability. This could be a reason for the profound increase in bacteria in our mice fed with fiber-free diet 18 weeks post radiotherapy. We also profiled the inflammatory signature in serum following irradiation and reported that it was influenced by diet. Finally, we suggest that with further understanding about the effects of various dietary products on bowel health, we may be able to develop methods other than dose reduction to safeguard intestinal health in pelvic cancer survivors.

MAIN RESULTS AND DISCUSSION OF PAPER II

Adult neurogenesis occurs primarily in two brain regions: the SVZ (subventricular zone) of the lateral ventricles and the SGZ (subgranular zone) of the DG (dentate gyrus). Adult-born neurons appear to contribute to particular brain activities like learning and memory, and mood regulation, according to compelling evidence[91, 92]. Neuroinflammation and cellular alterations caused by systemic inflammation affect cognitive function, particularly learning and memory. The problems associated with CNS functioning during cancer treatment have mainly been linked to the influence of chemotherapy, which, unlike pelvic radiation, is administered systemically. Because radiation is commonly paired with chemotherapy, the degree to which pelvic radiotherapy may contribute to late cognitive damage is uncertain. Both fatigue and cognitive dysfunction after pelvic cancer treatment are vastly underdiagnosed, and few studies exist. Five years or longer after treatment, one-third of cervical cancer survivors who had had pelvic radiation but not chemotherapy experienced persistent fatigue (defined as both physical and mental exhaustion[45]. Studies have shown that chronic inflammation peripherally in the body can disrupt the production, maturation, and functional integration of new neurons in the hippocampus. Crohn's disease patients' hippocampus-dependent cognitive processes have subtle abnormalities correlating with systemic inflammation, as demonstrated by poor performance on SCIT, the Subtle Cognitive Impairment Test[93]. Environmental factors (such as food and stress) and social interactions[94] can have a significant impact on adult neurogenesis on numerous levels including proliferation, fate specification, migration, integration, and survival. Clinical studies have linked microbiota of phylum *Bacteroidetes* to cognition and neurodegenerative diseases. Additionally, a high level of gut *Bacteroides* at 1 year of age correlates with higher cognitive abilities at 2 years of age[95] while *Bacteroides* are less abundant in the gut microbiota of dementia patients at the genus level[96]. β(1,4)/β(1,3)-glucan from oat and barley is fermented mainly by gut microbiota residing in the lower gastrointestinal tract, possibly resulting in a compositional and functional shift in the gut microbiota with abundance in *Bacteroidetes* lower taxa, such as *Bacteroides ovatus* and thereby improving cognition and brain function via the gut-brain axis[97-99].

In our study, in order to determine whether radiation to the colorectum could affect parameters of hippocampal neurogenesis and, if so, to learn if a fiber-rich diet could modify these effects, the hippocampus from the mice in the study for Paper I was analyzed using markers for neurogenesis. Colorectal irradiation reduced cell proliferation in the SGZ transiently.

Irradiation also affected the number of immature neurons, an effect that was modulated by the intake of dietary fiber. The impact was rather small, and the number of mature neurons was unchanged. Likewise, the density of Iba1-positive microglia was unchanged, indicating the absence of overt inflammatory activity.

Cell proliferation

It has been discussed previously about how gut microbiota communicates with the brain through mechanically stimulating intestinal mucosal cells, generating toxins, signaling through receptors, and increasing intestinal permeability[100]. In other studies, it has been shown that the number of newly generated DG neurons is reduced by inflammation and the presence of proinflammatory cytokines[101, 102]. We observed a reduction of proliferating cells caused by irradiation in both dietary groups (Figure 13). The effect was small and would probably have been larger if we had increased the number of fractions or had given a higher total dose of radiotherapy, but had most likely caused weight loss and stress in the mice, affecting the outcome. Increasing the size of the radiation field to irradiate a larger volume of the intestines would have resulted in off-target organ damage, prohibiting the disentangling of the colorectal irradiation effects on the hippocampal stem cell niche per se.

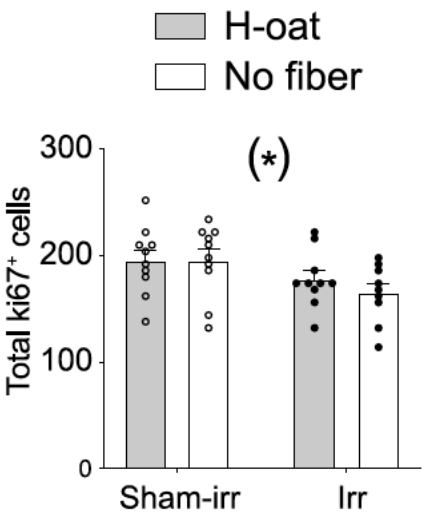

***Figure 13**. At 6 weeks, there was a reduced number of Ki67-positive cells in irradiated mice. *=Two-way ANOVA, irradiation. Modified from S. Devarakonda et. al. Neuroscience 475 (2021) 137–147.*

Immature neurons

Under systemic inflammatory circumstances, induction of hippocampal neurogenesis is increasingly acknowledged as a homeostatic response to the inflammatory stressor[103, 104]. When compared to sham-irradiated, irradiation increased the number of immature neurons per millimeter GCL at 6 weeks in animals fed with fiber-rich diet. At 18 weeks, sham-irradiated fiber-free diet fed mice had fewer immature neurons than sham-irradiated fiber-rich diet fed mice, and irradiation caused a considerable increase in the number of immature neurons in the mice fed with fiber-free diet but not in the mice fed with fiber-rich diet (Figure 14). Exposure to a mild repeated stressor on a regular basis has been demonstrated to promote neurogenesis[105]. We found that if the animals were fed bioprocessed oat bran, there was a shift towards a larger percentage of DCX-positive neurons that had transitioned into the more mature stage following irradiation, although the data regarding the DCX-positive cell population were difficult to interpret. This shift could possibly be due to systemic circulating cytokines and their effect on neurogenesis. An excess of rapidly proliferating progenitor cells produces a large number of immature neurons, which then undergo a strict selection process. We hypothesize that the overproduction "buffers" against a reduction in cell proliferation until a very severe damage in the intestine is generated, in which case the impact on cell production would be so great that it could not be compensated for. Thus, only a fraction of the newly produced cells mature and integrate[106], and as a result the number of DCX-positive cells does not always correspond to the production rate of new cells or new mature neurons.

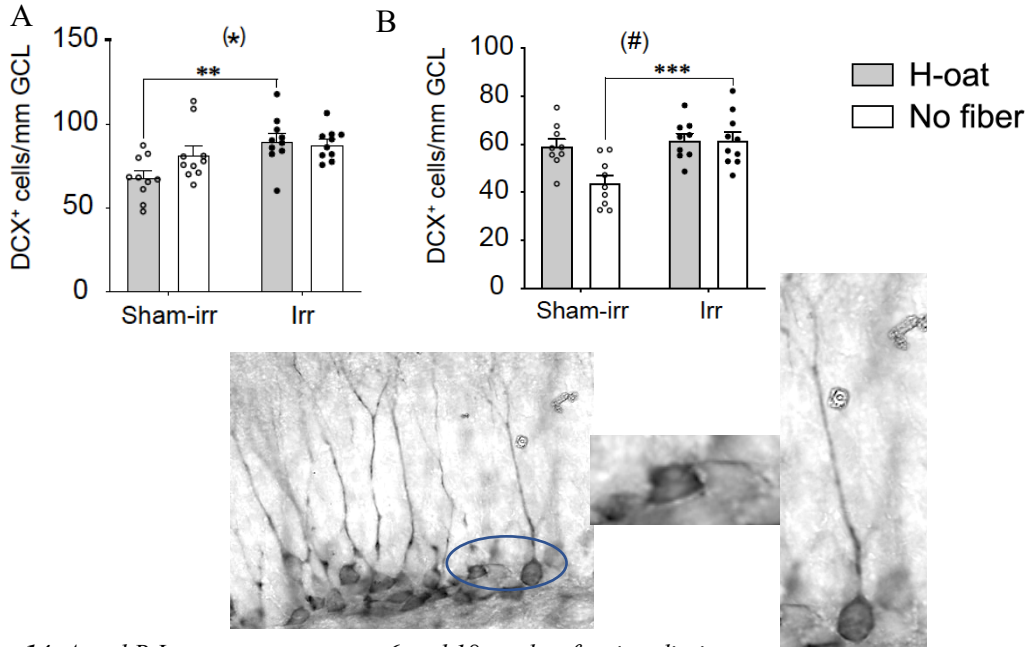

Figure 14. *A and B Immature neurons at 6 and 18 weeks after irradiation, Two-way ANOVA *= irradiation, #= interaction, *=Post-hoc significance. Modified from S. Devarakonda et al. Neuroscience 475 (2021) 137–147.*

When assessing the number of mature neurons in the GCL, we found no changes in the population size of mature granule cells, the major kind of neuron in the hippocampus's dentate gyrus, again likely reflecting the brain's ability to "buffer" for minor alterations in the early phases of neurogenesis. At the 6-week time point, we also quantified microglia in the hippocampus as increases in the population size of microglia would indicate ongoing inflammation. The time point was chosen due to the reduced proliferation at this time point. A separate analysis was conducted on each of the three hippocampal areas, the granule cell layer, the molecular layer, and the hilus and in any of these three locations, we did not find differences in the density of Iba1-positive microglia between the groups. We did not perform any molecular level analysis to further investigate the possibility of inflammation in the hippocampus. However, when we performed a correlation analysis between the serum cytokines levels and immature neuronal counts, we noticed a negative correlation between MIP1a and the number of DCX-positive cells in both irradiated groups at the acute phase (Figure 15 B and C), with a tendency toward higher cytokine levels and fewer DCX-positive cells in the irradiated animals on a fiber-free diet (Figure 15 A). In a study, MIP-1a has been found to inhibit neurogenesis as well as learning and memory activities[107]. When it comes to evaluating correlations, it is important to keep in mind that the cytokines presented in this study are only suggested possible candidates, among many other possible candidates, that could be linking intestinal inflammation to hippocampal health.

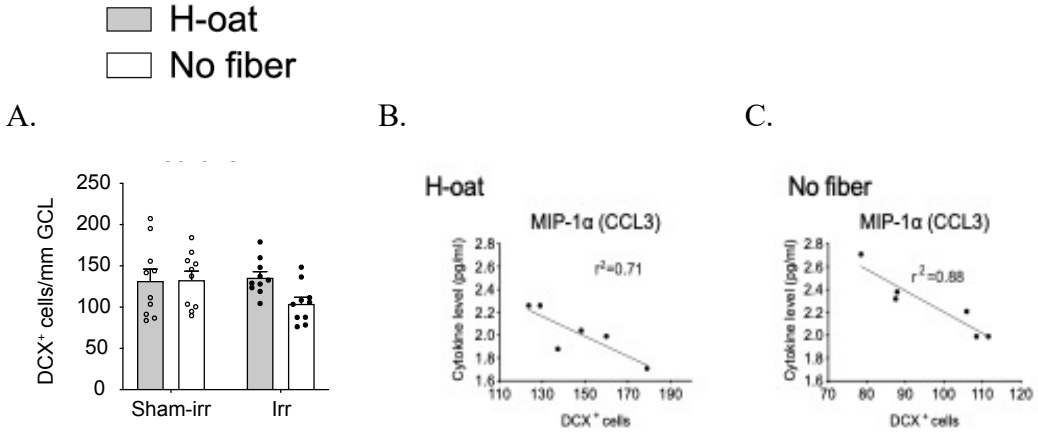

***Figure 15**. A. Immature neurons at 1 week post radiotherapy. B and C. Correlation analysis between serum cytokine versus immature neurons at 1 week post radiotherapy. Modified from S. Devarakonda et al. Neuroscience 475 (2021) 137–147.*

In summary, the effect of colorectal irradiation on neurogenesis parameters was mild, but increasing the radiation field would have resulted in organ damage, prohibiting the disentangling of the intestinal radiation effects on the hippocampal stem cell niche per se. If the mice would had been subjected to a higher dose and/or more fractions, the damage would probably have prevented long-term follow-up. In our model of pelvic radiotherapy, the macroscopic pathophysiological hallmarks in the mucosa were similar to those identified in the mucosa of irradiated pelvic-cancer survivors[90]. Nonetheless, because mice are more radioresistant than humans, we believe that the effects of pelvic irradiation on hippocampal parameters in humans may be more substantial than in mice. For an example, although the tissue damage in the mouse model is very similar to that of irradiated pelvic cancer survivors on a macroscopic level, the mice do not suffer from loose stools or fecal leakage. Thus, there are differences in the manifestations of the disease, which appear milder in mice and could result in an underestimation of the impact of pelvic radiotherapy on the brain. Nevertheless, our findings demonstrate that pelvic radiation may add to the long-term effects of pelvic-organ cancer treatment on cognitive function, and that strategies to minimize intestine damage may potentially help pelvic-cancer survivors maintain brain health.

MAIN RESULTS AND DISCUSSION OF PAPER III

Radiotherapy is a potent cancer-fighting treatment. However, eradicating cancerous cells successfully comes with unavoidable consequences. Clusters of symptoms (syndromes) from the intestines might occur in months, years, or even decades after pelvic radiotherapy. The general belief among care givers is that the initial mucosal inflammation caused by ionizing radiation soon progresses to fibrosis and then subsides. The main pathophysiological processes thereafter are ischemia and irreversible fibrosis, without any further inflammation[3, 108]. Thus, sufferers of chronic PRD are usually only offered symptom-managing care and attempts to prevent disease progression or stimulate repair of the mucosa are considered futile. Findings in animal models of radiotherapy, including our own model of pelvic radiotherapy, do however suggest the presence of a mild chronic inflammation[69, 109-111].

In the study for Paper III, we set out to determine if there is a chronic low-grade inflammation in the intestinal mucosa after pelvic radiotherapy. We biopsied 24 pelvic cancer survivors at two locations; 5 cm (high dose area) and 25cm (low dose area; internal control) from the anal opening in patients who had received pelvic radiotherapy two to twenty years ago. Four non-irradiated subjects were biopsied in the same areas as the cancer survivors and in the same setting. Our findings revealed inflammatory activity up to at least two decades after the cancer treatment. Using tandem mass tag proteomic analysis, we identified over 7000 proteins in the mucosa, of which nearly 1500 were significantly changed between the high-dose area and the low-dose area of the cancer survivors who had received pelvic radiotherapy. Over 60 of these proteins were involved in neutrophil activity. With flow cytometry, we observed increased numbers of neutrophils in the high-dose biopsies compared to the internal control biopsies. In the healthy colon, a two-layer mucus acts a protective barrier and stops infiltration of commensal pathogens into the epithelium. We analyzed the quality of the mucus barrier and quantified bacterial infiltration and found a disrupted mucosal barrier which allowed pathogen invasion.

Under normal circumstances, the single-layered intestinal epithelium acts as a physical and immunological barrier, preventing direct contact between luminal bacteria and intestinal mucosa. In this setting, when the epithelium is damaged, neutrophils play an important role in protecting against invading pathogens. Among circulating leukocytes in humans, neutrophils constitute of 50-70%[112]. Neutrophils responds rapidly to infection and can both phagocytize or release mediators that disarms pathogens[113]. During neutrophil maturation, three kinds of

granules that release these mediators develop sequentially: primary (or azurophilic) granules, secondary specific granules, and tertiary gelatinase granules.

Important enzymes in primary granules are MPO (myeloperoxidase), defensins, BPI (Bactericidal/permeability-increasing protein), and cathepsin G. Some enzymes found in secondary granules are lactoferrin and matrix metalloproteinase-8 (MMP8), and examples of enzymes in tertiary granules are MMP9, cysteine-rich secretory protein 3, lysozyme and heparnase[114, 115]. All three types of granules have overlapping functions.
Enzymes coming from all various subtypes of neutrophil granules, such as neutrophil elastase, defensins, cathepsin G, MPO, lactoferrin, and lysozyme c, were shown to be elevated in biopsies taken from locations that had received a high dose of radiation in our study. Some of the main functions of these enzymes are to inhibit growth and multiplicity of bacteria and neutralizing toxins secreted by pathogens. Different enzymes have different roles to play in pathogen killing. Neutrophil elastase is highly nonspecific, and it focuses mainly on digesting different proteins from pathogens that are essential for their functioning or proteins responsible for invasion[116]. MPO releases oxidant hypochlorous acid and deactivates pathogens and prevents them from invading[117]. Lactotransferrin (or lactoferrin) acts by binding to iron, so the pathogens are deprived of iron for metabolism and growth[118].

A previous proteomic study on biopsies from ulcerative colitis patients found that several proteins associated with neutrophils and NETs (neutrophil extracellular traps) were elevated in UC patients' biopsies compared to controls[119, 120]. Diverse triggers, such as pathogen-associated molecular pattern (PAMPs) and damage-associated molecular pattern (DAMPs) molecules, activate resident sentinel cells, such as macrophages, near the site of inflammation[121]. The cells then produce pro-inflammatory mediators and chemokines, initiating neutrophil mobilization and recruitment. When neutrophils are not effectively removed, they can contribute to considerable tissue damage during acute and chronic diseases[121]. Neutrophils' damaging capacity necessitates strict control over their recruitment and activation in tissues. It is possible that a constant presence of neutrophils in the mucosa of irradiated pelvic cancer survivors promotes the progression of fibrosis and the development of symptoms over time.

Mucins have a crucial role in commensal survival and pathogenic competitor colonization resistance. Recently, a more comprehensive examination of sigmoid colon samples from healthy people and ulcerative colitis patients suggested a core mucus proteome comprising 29

proteins[122]. Several of these proteins, including core structural components, were shown to be decreased in active UC patients. MUC2, chloride channel regulator, calcium-activated-1 (Clca1), and zymogen granule protein 16 (Zg16) are a few mucus proteins that were altered in their study. The list also contained the Fc fragment of IgG binding protein (FCGBP) that was reduced, and is known to have a comparable quantity and distribution as MUC2. We also observed similar regulation of these proteins in our proteomic analysis. Changes in these proteins can alter mucus integrity, leading to pathogen infiltration. In our study, a permeability analysis on biopsies from three cancer survivors revealed poor mucus protection and infiltration of bacteria sized beads into the inner thick mucus layer. When analyzing the density of goblet cells between biopsies collected at high versus low dose areas, we found that the density was similar, but we noticed a reduction in crypt area in the high-dose biopsies which would result in fewer cells capable of producing mucus. We do not know how radiation affects the goblet cells' capacity to produce mucus. In a Cochrane review, five studies were presented that showed that the composition of the gut microbiota before radiation differed between patients with and without postradiotherapy diarrhea[2]. Our mouse studies and others show microbial dysfunction that might result in mucosal degradation[90, 111, 123]. We also saw weak signs of bacterial infiltration in the biopsies from high-dose areas compared to biopsies from unirradiated controls, but since the number analyzed was too low we were unable to draw any firm conclusions. We believe that understanding the exact role of neutrophils in the chronic inflammation after pelvic radiotherapy might open new possibilities to help irradiated pelvic cancer survivors to recover their bowel health. Depending on the role of neutrophils after pelvic irradiation, exploring approaches that prevent their chronic stimulation and strengthen the mucosal barrier might be beneficial.

CONCLUSIONS

I. We found that dietary fiber is able to modulate multiple pathophysiological processes and repair mechanisms after irradiation. Fiber-rich diet is able to protect from the pathogen invasion during the late chronic intestinal inflammation.

II. We found that radiation-induced injury restricted to the colorectum may affect several parameters of neurogenesis and that this injury can be modulated by dietary fiber. Although the effects in our study were relatively small, the findings suggest that radiation, even if restricted to a small volume of the distal bowels, can affect brain health.

III. We found that there is a chronic low-grade inflammatory activity in the irradiated intestinal mucosa even decades after radiotherapy. A poor quality of the protective mucus layers after pelvic radiotherapy could lead to chronic pathogen infiltration across the epithelium.

FUTURE PERSPECTIVES

Data in this thesis suggest that radiotherapy initiates pathophysiological processes in normal intestinal tissue that can last for decades in humans. Our pre-clinical data indicate that a high intake of fiber during radiation therapy can modify these pathophysiological processes (Paper I). The mechanism may be that a dietary fiber intake strengthens the mucus layers that protect the intestinal epithelium and prevent bacterial infiltration. We do not know if and how the fiber composition matters, and if there is a dose-response effect. A future dietary intervention in patients will need careful consideration of all factors that could affect the symptoms. On the basis of our findings and others, our group is currently studying the effects of dietary fiber during and after pelvic radiotherapy in pelvic cancer patients[124]. The pelvic cancer patients are randomized to receive supplementary capsules containing either psyllium husk or placebo, which are ingested daily starting two weeks before radiotherapy and lasting for four weeks after pelvic radiotherapy. Samples of blood and feces are collected before, during, and after pelvic radiotherapy and markers of inflammation are evaluated together with microbial changes. The symptoms of the participants are carefully documented, with the last follow-up one year after pelvic radiotherapy. The purpose of the study is to collect evidence-based information on the possible benefits of dietary fiber intake in humans subjected to pelvic radiotherapy.

The effect of colorectal irradiation on parameters of hippocampal neurogenesis in our study was quite moderate (Paper II). We expect the effects of pelvic radiotherapy on hippocampal parameters and cognitive function to be greater in humans than in mice, since mice are more radioresistant than humans, and our mice did not display symptoms such as loose stools or fecal leakage. A future aim is to evaluate cognitive functioning with highly sensitive tests in the study participants that were enrolled in the fiber study and the biopsy study. Thereby, it would be possible to evaluate whether markers of intestinal inflammation correlate with cognitive health, and also to determine whether the dietary intervention can protect brain heath in the irradiated pelvic cancer survivor.

In the biopsy study (Paper III), the patient group in was limited. We intend to enroll a total of 100 pelvic cancer survivors to understand the radiotherapy effect on pathophysiological mechanisms. The data will be correlated with self-reported symptoms that we are collecting, which can give us information on which mechanisms underlie specific symptoms. We also intend to map the regulation of mRNA in the irradiated mucosa and compare the information

with already existing data on the classical inflammatory bowel diseases, where there is much more knowledge and already existing treatments. We thereby hope to be given entirely new possibilities to develop preventive and curative treatment for pelvic radiation disease.

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