# Understanding normal-tissue late effects in the intestines after pelvic radiotherapy

**Dilipkumar Malipatlolla**

Department of Oncology

Institute of Clinical Sciences

Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2020

Cover illustration: Radiation-induced crypt degeneration in a mucosal biopsy from a pelvic-organ cancer survivor - Cecilia Bull

Understanding normal-tissue late effects in the intestines after pelvic radiotherapy.


dilip.kumar.malipatlolla@gu.se

ISBN 978-91-7833-828-3 (PRINT)
ISBN 978-91-7833-829-0 (PDF)

Printed in Gothenburg, Sweden 2020
Printed by BrandFactory

Fill the brain with high thoughts, high ideals, place them day and night before you, and out of that will come great work.

- Swami Vivekananda

# Understanding normal-tissue late effects in the intestines after pelvic radiotherapy

**Dilipkumar Malipatlolla**

Department of Oncology, Institute of Clinical Sciences
Sahlgrenska Academy, University of Gothenburg
Gothenburg, Sweden

## ABSTRACT

Radiotherapy cures patients from deadly cancer, alone or in combination with other treatments. The photons must pass through normal tissue to converge on the tumor, and it is unavoidable that radiotherapy causes acute side effects as well as late adverse effects. Roughly one million cancer survivors in Europe are suffering from radiation-induced intestinal symptoms, such as urgency to defecate, leakage of feces and mucus, bleeding, and excessive odorous gas discharge. The symptoms can appear in the pelvic-organ cancer survivor weeks to years after the treatment and severely reduce the quality of life. Very little is known about how radiation-induced pathological processes progress over time and how various factors such as diet influence the disease course. One reason is the lack of preclinical models that allow for a long-term follow-up after irradiation. To better understand the dynamics of intestinal injury after radiotherapy, we have developed a novel model of pelvic radiotherapy and determined the injury and repair mechanisms over time. In the study for **Paper I**, we used the clinic's linear accelerator to irradiate a small field limited to the murine colorectum. The use of the clinic’s linear accelerator protected overall animal health and ensured their

long-term survival. We found that the pathophysiology after 4 fractions of 8 Gy was similar to what is seen in biopsies of pelvic-organ cancer survivors, and that crypt degeneration was fraction-dependent and still ongoing at six weeks post-irradiation. Moreover, there was an increased number of macrophages in the mucosa at six weeks, possibly reflecting a lasting inflammatory activity. In the study for **Paper II**, we characterized the mouse model over a period of 30 weeks. We observed that crypt degeneration was still present at 30 weeks post-irradiation, as well as an increased presence of macrophages, possibly reflecting a chronic, low-grade inflammation. We also found that crypt fission, not cell proliferation, was the main repair mechanism after one week post-irradiation and onwards. In **Papers III & IV**, we studied the effect of bioprocessed oat bran, rich in dietary fiber, on radiation-induced damage to the intestine. In **Paper III**, we observed that the intake of dietary fiber modified the onset, timing, and intensity of radiation-induced pathophysiological processes when compared to a fiber-free diet. In the study for **Paper IV**, we observed that irradiation resulted in a long-lasting increase of serum cytokines indicating a chronic low-grade inflammation and that a fiber-free diet worsened this pro-inflammatory serum profile. In convergence, pelvic irradiation results in long-lasting, possibly chronic, pathophysiological changes in the intestines that may be driven by underlying low-grade inflammation. Nevertheless, even long after irradiation, the intestine attempts to repair itself via crypt fission. This mechanism as well as dietary interventions has the potential to modify the progression of the disease and may be explored further.

Keywords: pelvic radiotherapy, intestinal inflammation, crypt fission, dietary fiber.

ISBN 978-91-7833-828-3 (PRINT)
ISBN 978-91-7833-829-0 (PDF)

# SAMMANFATTNING PÅ SVENSKA

Strålbehandling används för att behandla patienter med malignitet i bäckenområdet, men åtföljs av både akuta biverkningar och seneffekter. Uppskattningsvis en miljon canceröverlevare i Europa lider av strålningsinducerade tarmbesvär, såsom trängningssyndrom, läckage av avföring och slem, blödningar och illaluktande gaser. Symtomen kan uppträda år till decennier efter avslutad behandling, och minskar ofta canceröverlevarens livskvalité drastiskt. Ändå är mycket lite känt om hur strålningsinducerade patologiska processer utvecklas över tid och hur olika faktorer såsom kost påverkar sjukdomsförloppet. En anledning är bristen på prekliniska modeller som möjliggör långtidsuppföljning efter bestrålning. För att bättre förstå de underliggande sjukdomsprocesserna har vi utvecklat en ny modell för bäckencancerstrålning och fastställt skade- och reparationsprocesser över tid. I Artikel I använde vi klinikens linjäraccelerator för att bestråla ett litet fält begränsat till kolorektum hos mus. Genom att använda oss av linjära acceleratorns lilla strålfält bibehölls mössens generella hälsa och de kunde studeras över lång tid. Vi fann att patofysiologin efter 4 fraktioner av 8 Gy liknade den som ses i biopsier från bäckencanceröverlevare. Den strålningsinducerade kryptdegenerationen var beroende av antalet fraktioner och pågick fortfarande sex veckor efter bestrålning. Dessutom fanns ett ökat antal makrofager i slemhinnan vid sex veckor, vilket indikerade en varaktig inflammatorisk aktivitet. I Artikel II följde vi skade- och reparationsprocesser under en period av 30 veckor. Vi observerade att kryptdegenerationen fortfarande pågick 30 veckor efter bestrålning, liksom den ökade förekomsten av makrofager, vilket möjligen återspeglade en kronisk, låggradig inflammation. Vi fann också att kryptfission, inte cellproliferation, var den viktigaste reparationsmekanismen en vecka efter bestrålning och framåt. I Artikel III & IV studerade vi effekten av fiberrikt bioprocessat havrekli på strålningsinducerad skada på tarmen. I artikel III observerade vi att intaget av kostfiber modifierade debuten, varaktigheten och

intensiteten hos strålningsinducerade patofysiologiska processer jämfört med en fiberfri diet. I artikel IV observerade vi att bestrålning resulterade i en långvarig ökning av serumcytokiner som indikerade en kronisk låggradig inflammation, och att en fiberfri diet förvärrade denna pro-inflammatoriska serumprofil. Sammantaget fann vi att bäckenstrålningen resulterade i mycket långvariga patofysiologiska förändringar vilka möjligen drivs av en underliggande låggradig inflammation. Trots det försöker tarmen, även långt efter bestrålning, reparera sig själv via främst kryptfission. Denna mekanism, liksom kostinterventioner, har potential att påverka sjukdomsförloppet.

# LIST OF PAPERS

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

I. Bull C, Malipatlolla D, Kalm M, Sjöberg F, Alevronta E, Grandér R, Sultanian P, Persson L, Boström M, Eriksson Y, Swanpalmer J, Wold AE, Blomgren K, Björk-Eriksson T, Steineck G. **A novel mouse model of radiation-induced cancer survivorship diseases of the gut**. Am J Physiol Gastrointest liver physiol. 2017 Nov 1; 313(5): G456-G466.

II. Dilip K. Malipatlolla, Piyush Patel, Fei Sjöberg, Sravani Devarakonda, Marie Kalm, Eva Angenete, Elinor Bexe Lindskog, Rita Grandér, Linda Persson, Andrea Stringer, Ulrica Wilderäng, John Swanpalmer, Georg Kuhn, Gunnar Steineck, Cecilia Bull. **Long-term mucosal injury and repair in a murine model of pelvic radiotherapy**. Sci Rep. 2019; 9:13803.

III. Malipatlolla DK., Piyush Patel, Sravani Devarakonda, Jolie Danial, Eva Mehdin, Henrietta Norling, Malin Warholm, Rita Grandér, Margareta Nyman, Ana Rascon, Andrea Stringer, Fei Sjöberg, Marie Kalm, Gunnar Steineck, Cecilia Bull. **A fiber-rich diet and persistent pathophysiological processes in the irradiated intestines**.(Manuscript).

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

# CONTENT

# ABBREVIATIONS

| | |
|---|---|
| BrdU | Bromodeoxyuridine |
| CBC | Crypt base columnar cells |
| CD31 | Cluster of differentiation 31 |
| DAB | 3,3′-Diaminobenzidine |
| DNA | Deoxyribonucleic acid |
| ECM | Extracellular matrix |
| Gy | Gray |
| Iba1 | Ionized calcium-binding adapter molecule 1 |
| Krt19 | Keratin, Type I Cytoskeletal 19 |
| Lgr5 | Leucine-rich repeat-containing-G-protein coupled receptor 5 |
| NF-kB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| PBS | Phosphate-buffered saline |
| PFA | Paraformaldehyde |
| SCFA | Short-chain fatty acids |
| TBS | Tris-buffered saline |
| TGF-β | Transforming growth factor beta |
| Tris-EDTA | Tris-Ethylenediamineteraacetic acid |

# INTRODUCTION

*Pelvic radiation – a widespread cause of reduced quality of life among cancer survivors*

Cancer has been part and parcel of the health problems faced by modern society. Patients suffering from cancer can be treated by many different methods such as surgery, radiotherapy, chemotherapy, hormonal therapy, or multimodal treatment (a combination of two or more treatments). Radiotherapy is the most common method of treatment and approximately 60-65 percent of cancer patients receive radiotherapy as a sole treatment modality or in combination with surgery or chemotherapy [1].

Radiotherapy is the use of ionizing radiation to treat cancerous tissue. Radiation not only kills the cancer tissue but also damages the surrounding healthy tissue. Exposing the pelvic area to high-energy particles during pelvic-organ cancer treatment leads to acute (early) and chronic (late) problems, where the various symptoms are manifestations of what is commonly referred to as Pelvic Radiation Disease [2, 3]. It is estimated that approximately 90 percent of the pelvic cancer survivors experience a permanent change in bowel habits. Of them, 50 percent have difficulties in performing daily activities, and 30 percent report moderate to severe symptoms after pelvic radiotherapy, reducing their quality of life [4]. The development and severity of radiation-induced symptoms during and after pelvic radiotherapy not only depend on the total dose given to the patient but also on other factors like radiotherapy techniques, positioning of devices, and life-style related factors [2, 5].

An acute manifestation includes looseness of the bowels, abdominal pain, bloating, loss of appetite, nausea, and fecal urgency. Patients notice acute effects normally during the second week of treatment with a maximum intensity at four to five weeks. Chronic effects may begin to develop six months to three years after pelvic radiotherapy. They

can even appear up to two decades after receiving the treatment [6]. A study by Steineck *et al*. identified nearly 30 symptoms in gynecological survivors, 2-15 years after radiotherapy and they were categorized into five main syndromes [7]. Signs of late effects include fecal urgency, uncontrolled defecation, excessive mucus discharge, excessive bleeding, and odorous flatulence. Moreover, bile malabsorption and bacterial dysbiosis may contribute to the late effects [8].

## Pathophysiological processes involved in normal-tissue injury after irradiation

The pathophysiological processes involved in radiation-induced normal tissue injury start immediately following the exposure, although not all of the histological changes may become apparent until weeks or months after treatment. Some of the problems related to normal tissue injury after radiation include oxidative stress, vascular damage, fibrosis, inflammation that alters the intestinal microenvironment, and disruption of the stem cell niche [9].

The intestinal epithelium is a single-layered and rapidly self-renewing tissue [10]. Regeneration of the epithelium after an injury is dependent on the stem cells present at the base of the crypt [11, 12]. When the dividing stem cells are exposed to ionizing radiation, it directly hits DNA and breaks the bond between the base pairs of the DNA in the cell nuclei and also indirectly through the generation of free radicals that react with the DNA, which leads to structural damage and cell apoptosis. The loss of stem cells is believed to cause the crypts to degenerate and eventually being replaced by fibrotic tissue [13]. Several radiation-induced mechanisms such as hypoxia, vascular injury, loss of tight junctions, and loss of gut wall integrity may further enhance crypt degeneration [14].

The microbiota appears to play a role in the development of the acute and chronic effects following the radiation. In normal conditions, the epithelial barrier is impermeable for the bacteria. Radiation to the

epithelium disrupts the tight junctions of the gut wall, thereby increasing the permeability and allowing a bacterial inflow from the lumen. Radiation-induced apoptosis also leads to disruption of the barrier. This may cause inflammation, which in turn affects the regeneration of the epithelium [14]. A few human studies have shown that patients who received radiotherapy for different cancers had mucosal barrier dysfunction [15, 16]. Rodent studies showed an increased passage of tracers in the extracellular spaces in ileum after irradiation [17, 18]. Furthermore, a study in mice showed that epithelial barrier dysfunction causes an increase in permeability, leading to the activation of immune cells [19].

If the pathophysiological processes involved in acute damage are not resolved, then it could lead to chronic or late damage. Studies in rodents have shown that acute mucosal damage leads to delayed intestinal complications [20, 21]. These observations suggest that acute mucosal injury can contribute to late intestinal toxicity [22]. Several studies have confirmed the protective role of trophic growth factors, such as keratinocyte growth factor (KGF) [23, 24] and glucagon-like peptide-2 (GLP-2) [25] against irradiation in murine models. The clinical use of trophic factors faced a crucial problem in cancer patients related to stimulatory action on tumor growth [26, 27].

## Radiation-induced vascular injury

Despite the improvements in radiotherapy, damage to the intestinal endothelial cells remains a clinical problem. Radiation-induced vascular damage was initially described more than 50 years ago [28]. Several clinical studies demonstrated that cancer patients who received radiotherapy were at increased risk of developing vascular diseases [29, 30]. The radiation effects on vascular tissue occur in 2 waves. The acute effects occur immediately after the irradiation; they include endothelial apoptosis. Preclinical studies have also shown that stem cell apoptosis and depletion of microvascular endothelial cells are associated with acute gastrointestinal manifestations [31, 32].

Radiation causes acute up-regulation of pro-inflammatory cytokines and adhesion molecules that recruit inflammatory cells to the site of vascular injury [33, 34]. In addition to the direct damage, free radicals produced by radiation causes oxidative stress. Oxidative stress up-regulates the pathways related to vascular disease, including matrix metalloproteinases, adhesion molecules, pro-inflammatory cytokines, and smooth muscle cell proliferation and apoptosis, while inactivating vascular-protective nitric oxide [35]. A study in a transgenic mouse model showed that NF-kB serves as a molecular link between oxidative stress and chronic inflammation [36].

Chronic vascular effects occur months after irradiation; they include thickening of the basement membrane, capillary loss, loss of clonogenic capacity and telangiectasia [37]. These late effects of the vascular injury may thus contribute to the progression of the intestinal pathophysiology after irradiation.

## Radiation-induced mucosal inflammation

Radiation induces inflammatory responses by apoptosis, generation of free radicals, mucosal breakdown, and the activation of several pro-inflammatory cytokines and chemokines [38]. The excessive generation of free radicals after irradiation can be considered as a pro-inflammatory signal, subsequently affecting the innate and adaptive immune responses [39]. The early inflammatory response appears only a few hours after radiation. It is a well-regulated process that recruits circulating monocytes as well as the activation of resident macrophages. Depending on the inflammatory conditions, monocytes can be differentiated into M1/ pro-inflammatory and M2/ anti-inflammatory macrophages [40, 41]. Several studies have reported that the infiltration of macrophages to the site of injury occurs after irradiation [42-44]. Macrophages are known to play a role in the resolution of inflammation by cleaning the debris and secreting signals that switch off the inflammatory response. Moreover, radiation-induced epithelial barrier breakdown facilitates the entry of pathogens,

leading to activation of immune cells that could secrete pro-inflammatory cytokines in mucosa such as IL-1, IL-8, and IL-6 [42, 43].

Fibrosis is an important end result of irradiation-induced injury to the intestine and it is described as an irreversible process that occurs under chronic injury conditions caused by a bacterial infection, ischemia, and chronic inflammation [45, 46]. Fibrosis is characterized by excessive accumulation of extracellular matrix (ECM), mainly deposition of collagen and fibronectin in and around the damaged tissue, causing loss of function and permanent scarring [47]. In animal experiments, an increased level of TGF-β has been observed in intestines after irradiation. TGF-β is known as a potent fibrogenic and promotes fibrosis by stimulating the expression of collagen and fibronectin.

## Repair mechanisms after radiation-induced intestinal injury

The epithelium of the intestines displays an impressive regenerative capacity after injury. There are two major mechanisms involved in the regeneration of mucosa after injury; crypt stem cell proliferation and crypt fission.

It is well known that $Lgr5^+$ intestinal stem cells, also called crypt base columnar (CBC) cells, are the major contributors to the epithelial renewal in the small intestine and colon [48]. These CBC cells are very sensitive and are depleted upon exposure to radiotherapy or chemotherapy. When the CBC cells are depleted, radio-resistant $Krt19^+$ reserve stem cells are activated [49]. Several studies have reported the role of reserve stem cells in regeneration after injury, although most have focused on the small intestine (reviewed in [50]).

The effect of radiation on crypt stem cell proliferation has been extensively studied, at least in the early phase after irradiation, but very little attention has been given to the role of crypt fission. Crypt fission is defined as the formation of two daughter crypts from a parent crypt,

and it is more frequently observed during the developmental phase. In the human colon, a crypt will undergo crypt fission every 30 to 40 years [51]. A few studies have proposed that crypt fission is a regenerative process in response to intestinal injuries, such as after irradiation or chemotherapy [52] and in inflammatory bowel disease [53]. A study by Berlanga *et al.* showed that crypt cell proliferation and crypt fission are two independent mechanisms in rodents [54]. However, little is known about how crypt fission is regulated.

## Role of dietary fiber

The term dietary fiber is defined as an edible part of the plant consisting of polysaccharides, resistant to digestion and absorption in the small intestines, with partial or complete fermentation in the colon [55]. Dietary fiber can be categorized in many ways; most commonly based on solubility in water. Pectin, inulin, β-glucan, gums and other polysaccharides are considered water-soluble dietary fibers and mostly act as prebiotics, whereas cellulose, lignin, and hemicellulose are considered water-insoluble dietary fibers, and are responsible for an increase in fecal bulk that affects intestinal transit [56].

The beneficial effect of dietary fiber on intestinal health has been known for a long time, and many studies have evaluated its health benefits [57, 58]. Today's increased scientific interest concerning the role of the microbiota in health and disease has further promoted dietary fiber as a beneficial nutrient to include within the diet. The fermentation of polysaccharides, oligosaccharides and other dietary fiber by the colonic bacteria produces short-chain fatty acids (SCFAs) namely, acetate, propionate, and butyrate [59, 60]. Numerous factors are involved in the production rate, amount, and type of SCFAs produced in the colon, including substrate source, the colonic pH, the abundance and composition gut microbiota, and the gut transit time [60]. Depending on the above-mentioned factors, SCFAs can contribute up to 10% of the total human caloric requirement [61]. Out of the three most common SCFAs formed during fermentation,

butyrate is considered most important for the maintenance of colonic health [62-64].

Butyrate is used as an energy source by colonocytes in the colon and also plays a major role in the regulation of cell proliferation and differentiation [59, 65, 66], while acetate and propionate reach the liver via the portal vein. SCFAs in the colon influences each other's production and function [67], especially converting from acetate into butyrate [68]. In-vitro studies showed that the butyrate and mixture of acetate and propionate protect colonocytes from DNA damage induced by reactive oxygen species [69, 70].

SCFAs maintain colonic homeostasis mainly by protecting colonocytes and maintaining the intestinal barrier. Several studies have confirmed that the supplementation of SCFA improved colonic barrier function [71, 72]. SCFAs also protect the mucus layer by regulating the levels of immune modulators such as prostaglandins [73].

# AIMS

The overall aim of the research leading to this PhD thesis was to increase the understanding of the pathophysiological processes involved in radiation-induced intestinal damage and to acquire knowledge that can be used to prevent such damage. To provide the means to achieve this, my research had the following four specific aims:

**Paper I:** To establish a novel mouse model of radiation-induced late effects in the intestines.

**Paper II:** To define the long-term trajectory of mucosal injury and repair mechanisms in the mouse model.

**Paper III:** To address the hypothesis that a high-fiber diet increases the resiliency of the gut wall and protects from radiation-induced intestinal damage.

**Paper IV:** To investigate whether consuming a high-fiber diet could reduce ongoing systemic inflammation after irradiation.

# MATERIAL AND METHODS

## Animals

All the experimental procedures were approved by the Gothenburg Ethical Committee of the Swedish Animal Welfare Agency (application number for the paper I, II, 22-2015 and paper III, IV 1458-2018). All studies were performed on male young adult C57BL/6J mice. 8 to 10 weeks old mice were purchased from Charles River Laboratories International and were maintained at a constant temperature of 20°C, 42 % relative humidity with a regular 12-hour light/ dark cycle. Animals had free access to food and water. The acclimatization period for the conditions was 1-2 weeks before all the experiments.

*Comment:* Mice intestinal physiology is well characterized and similar to human intestinal physiology. The mouse genome has been entirely mapped, and mice can be genetically modified for the study of mechanisms on a cellular or molecular level. In our experiments, only male mice were used. Female mice were not used in any of the experiments since their reproductive organs would fall within the target area of the radiation.

## Experimental model

### Irradiation Procedure

The irradiation procedure was carried out at Jubileumskliniken at the Sahlgrenska University Hospital in Gothenburg. To ensure that each mouse was in an identical position under the linear accelerator, the mice were kept anesthetized in a silicone mold, using a portable anesthesia unit connected to nose cone delivering 2.5% - 3% isoflurane with airflow of 300mL/min. In paper I, a linear accelerator (Varian Clinac 600 CD; Radiation Oncology System, San Diego, CA) with 4 MV photon energy producing a dose-rate of 3.2 Gy/min was used. In paper II, III, and IV, a linear accelerator (Varian TrueBeam; Varian Medical Systems Inc., Charlottesville, VA, USA), with 6 MV photon

energy producing a dose-rate of 5.9 Gy/min was used to deliver the radiation dose. Other parts of the mice, especially bone marrow and testicles, were avoided by restricting the radiation field to 3x3 $cm^2$ with only the lower quadrant placed over the caudal-dorsal part of the mouse. A 5 mm thick tissue-equivalent bolus was used. The source-to-skin distance was 100 cm, and approximately a total length of 1.5 cm of the distal colon was irradiated (Figure 1). After irradiation, mice were returned to the cages. Sham-irradiated mice were anesthetized but not subjected to radiation, and otherwise treated identically. For a pilot experiment, we developed a protocol based on small-field radiation to the juvenile mouse brain [74]. In the pilot experiment, we analyzed any changes in the histology of the colorectum after 2, 3 or 4 fractions of 6 or 8 Gy with 12 hours interval. We found that the administration of four fractions of 8 Gy (32 Gy total) caused sustained morphological changes similar to that seen in biopsies of irradiated pelvic-organ cancer survivors [75]. This protocol was used in all later experiments.

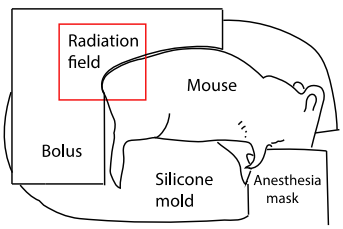
*Comment:* Our ability to study and design new therapies for the late (chronic) effects after irradiation are limited by a lack of proper animal models. In a typical model, the animals die one to two weeks after irradiation due to gastrointestinal or immune-system failure. This occurs when the ionizing irradiation cannot be restricted to a small field. In contrast, our mice had a normal life span and maintained a normal weight, despite receiving high doses of irradiation. This long survival is achieved by limiting target volume and avoiding damage to the immune or gastrointestinal system.

We used a linear accelerator to produce our model. The linear accelerator is used as an external beam radiation treatment to treat cancer patients [1]. It delivers high-energy x-rays with a high dose rate to the cancer cells and limits the damage to surrounding tissue. By using a linear accelerator, we made our mouse model mimic as closely as possible pelvic radiotherapy, delivering a high dose of irradiation (clinically relevant), with a dose rate identical to that given to patients, and in several fractions. In our pilot studies, we found that crypt

degeneration was dependent on the number of fractions rather than the dose given [76], thus fractionation is an important factor when trying to produce intestinal pathophysiology similar to that seen after pelvic radiotherapy.

A. Linear accelerator


B. 3x3 cm radiation field




C. Mouse covered with bolus

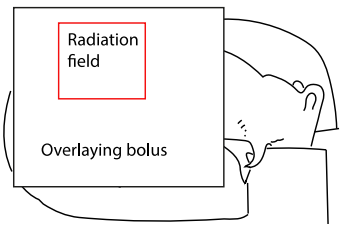



**Figure 1.** A**.** Varian linear accelerator. Courtesy of Varian Medical Systems *Inc*. B and C. The mouse was anesthetized with an anesthesia mask and placed in silicone mold and the body was covered with a 5 mm thick bolus.

## Sacrifice and sample collection

At the chosen time points after irradiation (indicated in each paper as Figure 1A), animals were deeply anesthetized with isoflurane. The abdomen was opened and blood was drawn by cardiac puncture of the left ventricle with the syringe. The intestine was flushed with ice-cold PBS and a thin, soft silicone tubing was inserted. 7 mm of the distal colon was carefully excised and placed immediately in 4% PFA (I & II paper) or methacarn (III & IV paper) overnight before dehydration and embedding into paraffin blocks.

## Serial sectioning procedure

Paraffin blocks were cut into 4-µm thin sections on a microtome (Leica RM2235; Leica Biosystems) to study histological changes. The sections were mounted serially so that each sixth section was positioned on the same slide (1:6 series). Therefore each section was separated from the other sections on the same slide by at least 20 µm, which prevented the person from analyzing the sections from analyzing the same crypt twice.

*Comment:* We used thin silicone tubing while harvesting the tissue. That preserved the shape of the colon and minimized variables such as stretching, and increased the chance of getting transversally sectioned crypts.

Transverse sectioning made it possible to count crypts per circumference instead of mm mucosa, which can be affected by many factors, such as stretching or shrinking. That is also what made it possible to use stereology-based methods to quantify macrophages.

## Assay of radiation-induced damage-acute apoptosis

Colorectal tissue was harvested 4.5 h after the last radiation dose, frozen on dry ice and stored at -80°C. After being thawed, tissue was cut into small pieces and placed in 0.7 ml of ice-cold homogenization buffer. Then the homogenate samples were sonicated on ice and

centrifuged for 10 mins at 10, 000 g. The resultant supernatant was used to measure protein content by Bradford assay.

The DEVDase assay was performed using 20 µl of the supernatant obtained as described above and mixed with 80 µl of extraction buffer. This solution was pre-incubated for 15 min at room temperature before the addition of 100 µl of peptide substrate and 25 m caspase substrate (Ac-Asp-Glu-Val-Asp-aminomethyl coumarin) in an assay buffer. The cleavage of the substrate was measured at 37°C using a SpectraMax Gemini microplate fluorometer with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The degradation was followed at 2-min intervals for two hours, and maximal velocity was calculated from the entire linear part of the curve. Standard curves with 7-amino-4-methyl coumarin in the appropriate buffer was used to express the data in picomoles of AMC formed per minute per milligram of protein.

## Human biopsies

Human biopsies were collected from patients who had received radiotherapy and also had undergone surgery. The biopsies were taken during surgery, approximately 10 cm from the tumor. Nineteen biopsies were acquired 3-5 days after completion of radiotherapy from patients who had received 25 Gy in five fractions (5 Gy x 5). Another thirteen biopsies were obtained 6-11 weeks after completion of radiotherapy from patients who had received 45-50 Gy in 25 fractions (1.8-2 Gy x 25). Biopsies from 13 rectal cancer patients who had not received radiotherapy were also retrieved. Collected biopsies were fixed in paraformaldehyde before embedding in paraffin, and these paraffin blocks were cut on a microtome in 6 µm-thin sections. Informed written consent was obtained from the patients. The ethical committee of the University of Gothenburg approved this study (EPN 118-15).

## Immunohistochemistry (IHC)

The tissues in the studies for this thesis were fixed in different ways prior to IHC: In those for papers I & II, colorectal tissue was fixed in paraformaldehyde and for paper III, colorectal tissue was fixed in methacarn, before embedding in paraffin. After deparaffinization at 60°C, all the slides were rehydrated through xylene and graded washes of ethanol. For Ki-67, CD-31, and Iba1 staining different antigen retrieval was performed. Endogenous peroxidase activity was blocked for 10 minutes using a 0.6% peroxidase blocking solution. For BrdU staining, the tissue was pre-heated with 2 N HCl followed by borate buffer. Slides were washed in TBS and incubated with a blocking solution containing 3% normal donkey serum and Triton in TBS for 30 minutes at room temperature to reduce nonspecific immunostaining. After the blocking, slides were incubated with primary antibodies at 4°C overnight. Then slides were washed and incubated with secondary antibodies for one hour at room temperature followed by washes in TBS and 2-5 minutes incubation in DAB solution. Slides were dehydrated with graded ethanol and xylene washes, mounted with Xtrakitt and coverslipped.

## Primary antibodies

| Antibody | Dilutions & Company | Detecting |
|---|---|---|
| anti-rabbit Ki-67 | 1:150 MerckMillipore | Cell proliferation |
| anti-mouse BrdU | 1:500 DAKO | Cell survival |
| goat anti-CD31 | 1:150 R&D systems | Blood vessels |
| anti-rabbit Iba1 | 1:2000 Wako chemicals | Macrophages |

## Secondary antibodies

| Antibody | Dilutions & Company |
|---|---|
| Biotinylated | 1:250, Vector laboratories |
| Donkey-anti-rabbit | 1:250, Jackson laboratories |

*Comment:* Immunohistochemistry (IHC) is a method in which a target protein (antigen) is detected visually by binding with antibody (immunolabelling). The antigen retrieval process exposes the epitope to an antibody. This process is dependent on the antigen, the type of fixation used for the tissue and also the primary antibody used. In paper I and II, citrate buffer and in the study for paper III Tris-EDTA was used for antigen retrieval method.

## Quantification of cell proliferation and cell survival

Crypt cell proliferation and cell survival were quantified by using a Leica DMi6000 microscope equipped with a semi-automated stereology system. In each section, well-oriented crypts, where the maximum of the crypt axis was visible, were included to quantify cell proliferation and survival. In total per animal, 24 crypts in two sections that were separated by 72 μm were analyzed.

*Comment:* Ki-67 is used as a marker for cell proliferation and expressed throughout the cell cycle except for the G0 phase. Bromodeoxyuridine (BrdU) is a nucleoside analog, which incorporates into DNA during the process of DNA replication by replacing thymidine. BrdU has a short half-life, which means that cells that were dividing at the time of injection will incorporate it into their DNA, and the cell can be followed until it is shed into the lumen. Mice were injected intraperitoneally with BrdU 4 days before the sacrifice because the mucosa is being replaced within a week.

## Quantification of macrophages

To quantify the number of macrophages in the colonic mucosa, sections were incubated with an antibody against ionized calcium-binding adapter molecule 1 (Iba1). In the studies for papers I and II, with a Leica DM6000B microscope equipped with a semi-automated stereology system; we systematically placed a virtual frame at four different places 0°, 90°, 180° and 270°. Then we traced the mucosal area in between the crypts. All the $Iba1^+$ cells within the traced area were counted. In the study for paper III, we used a stereology-based approach: the entire mucosal area per circumference was traced with 5x magnification and $Iba1^+$ cells were counted with 40x magnification. A grid was placed over the traced area and counting frames were placed at each intersection. Around 50 counting frames/section were analyzed for the number of $Iba1^+$ cells. Based on the number of cells counted, the Stereo Investigator software estimated the total number of cells present in the traced area.

## Assessment of blood vessels

Sections incubated with an antibody against CD31 were used to assess the number of blood vessels in the mucosa and submucosa of the colon. Two sections per animal, which were separated by 72 μm, were analyzed.

## Histochemistry

For visualizing crypts, degenerating crypts and crypt fission either Alcian Blue combined with Nuclear Fast Red or Verhoeff's elastic stain was used. Briefly, slides were deparaffinized and rehydrated with descending grades of alcohol. After rehydration, slides were treated with acetic acid for 3 min and followed by Alcian Blue (pH 2.5) for 30 min. Then slides were rinsed with water and counterstained with Nuclear Fast Red for 5 min. In the case of Verhoeff's elastic stain, the same protocol followed until the rehydration step, after rehydration slides were stained according to a standard protocol.

## Assessment of degenerating crypts, crypt fission, and surviving crypts

Alcian blue/Neutral Fast Red or Verhoeff's elastic stain stained sections were used to quantify the number of degenerating crypts and crypt fission. A total of six sections per animal, separated by 24 μm, were analyzed at 40X magnification.

For the quantification of surviving crypts in the colon, three sections per animal, separated by 48 μm, were counted, and the average number was calculated.

## Serum cytokine and chemokines analysis by Luminex Bead-Based Multiple Assay

A Bio-plex Mouse Cytokine 23-plex (Bio-Rad Laboratories AB, Solna, Sweden) was used to measure the concentration of cytokine and chemokines in the serum. The assay plate was pre-wet with a wash buffer initially and then primed with a bead solution. After that, samples and standards were added to the plate containing bead solution and incubated. After washing with a wash buffer, the plate was incubated with the detection antibody followed by streptavidin-PE incubation. Then the plate was re-washed, and the beads were re-suspended with assay buffer. The fluorescence intensity was measured using the Bio-Plex 200 system. The data were processed using the Bio-Plex Manager Software. The concentration of serum cytokines and chemokines was expressed in pg/ml.

## Statistical analysis

Statistical analysis was performed using the GraphPad Prism software. Data in the papers were presented as the mean $\pm$ standard error of the mean (S.E.M). A P-value equal to or below 0.05 when comparing groups was considered statistically significant.

In Paper 1, we used a one-way analysis of variance (ANOVA) followed by Dunnett's test for normally distributed data. When data

was not normally distributed, a nonparametric Kruskal-Wallis test followed by Dunn's multiple-comparison was performed. In Paper II, an analysis of the comparison was done by a Student's t-test. In Papers III and IV, Student's t-test was used for normally distributed data. Mann-Whitney test was performed for non-normally distributed data.

# MAIN RESULTS AND DISCUSSION

## Paper I

To increase our chances of preventing radiation-induced late effects in the intestines, some important questions need to be answered. Some examples of such questions are: When do late symptoms arise after pelvic irradiation, and for how long? How long does inflammation persist? What role does inflammation play in the progression of the radiation-induced disease? What are the repair mechanisms that are activated after radiotherapy? What factors, life-style related or other, influence the repair or progression of radiation-induced intestinal injury?

These questions are, in part, difficult to answer because of a lack of suitable animal models that allow for long-term follow up after high-dose irradiation. Most of the current mouse models rely upon whole-body irradiation, which results in reduced survival rate due to gastrointestinal failure or the collapse of the immune system. To be able to understand radiation-induced late effects of the intestines, and to develop successful strategies to combat them, better preclinical models are needed. A model where it is possible to give relevant doses, with minimal off-target effects and preserved survival that allows for long-term follow-up, is desirable. In paper I, we used the clinic's linear accelerators to make a model that would fulfill these criteria.

### Radiation-induced acute apoptosis in the colorectum

As described in the method part, only the colorectum was irradiated using small-field irradiation. To verify the correct placement of the irradiation field and the restriction of the radiation field to the distal bowel, we measured the acute apoptosis in the non-irradiated proximal colon and the irradiated colorectum of sham-irradiated mice, and mice irradiated with 2 and 4 fractions of 6 Gy. We observed a statistically significant acute cell death in the colorectum of mice irradiated with 2 fractions of 6 Gy but not in mice irradiated with 4 fractions of 6 Gy.

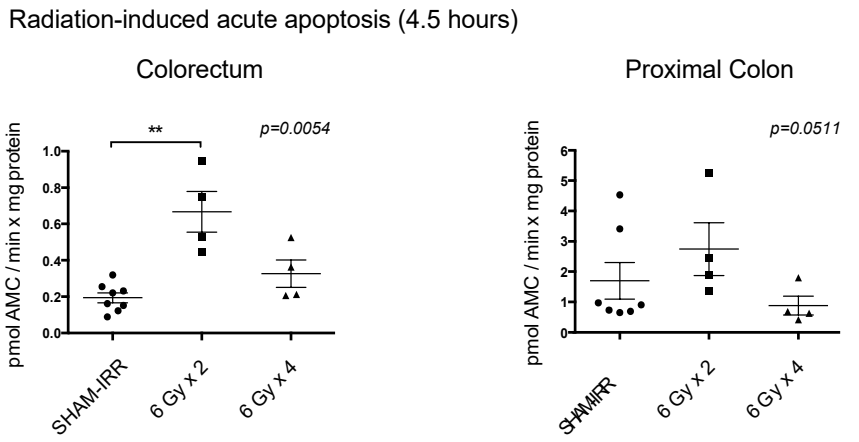



**Figure 2.** Irradiation-induced acute apoptosis in the colorectum and proximal colon. A statistically significant difference was seen in colorectum compared to sham-irradiated. This figure is taken from Bull *et al*. Am J Physiol Gastrointest liver physiol. 2017 Nov 1; 313(5): G456-G466.

There was no statistically significant acute apoptosis in the proximal colon between sham–irradiated and irradiated animals, confirming a minimal spread of radiation outside the boundaries of the radiation field. That we did not see acute apoptosis in the colon after four fractions of 6 Gy, could be explained by the findings by Potten and colleagues, showing that maximum apoptosis of the actively dividing stem cells in the crypt at the moment of irradiation is achieved already at one fraction of 6 Gy. By the time the fourth fraction was given, the pool of stem cells sensitive to apoptosis might have been depleted.

## Survival of the mice over time after pelvic irradiation

There was no overt weight loss observed in mice irradiated with 2, 3, and 4 fractions of 6 Gy or 2 fractions of 8 Gy compared to sham-irradiated mice. However, mice irradiated with 3 or 4 fractions of 8 Gy fractions had a slightly decreased body weight compared to sham-irradiated at 2, 3, and 4 weeks after irradiation. By 6 weeks after irradiation, the differences in body weight were no longer significant.

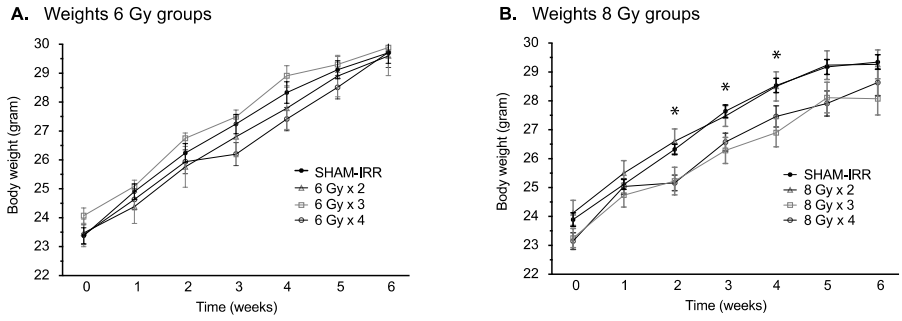



**Figure 3.** A: the 6-Gy groups maintained a normal weight curve throughout the study. B: at 2, 3, and 4 weeks after irradiation, there was a small but significant decrease in weight in the 8 Gy x 3 and 8 Gy x 4 groups. Bull *et al*. Am J Physiol Gastrointest liver physiol. 2017 Nov 1; 313(5): G456-G466.

Our mice received a total of either 24 Gy or 32 Gy of radiation and were followed for six weeks. Studies performed by others have shown that mice exposed to 8-20 Gy of total body irradiation, succumb due to bone marrow damage [32, 77, 78]. In one study, the survival time of mice after total-body irradiation was drastically reduced when the radiation dose increased above 14 Gy [31]. The main reason for the survival of the mice in our studies was the small irradiation field that prevented damage to the other parts of the body.

## Radiation-induced crypt loss

Mice irradiated with 2 or 3 fractions of 6 Gy did not show crypt loss. A statistically significant loss was only seen in mice irradiated with 4 fractions of 6 Gy compared to sham-irradiated mice. However, when using 6 Gy fractions, we did not induce enough tissue damage to produce a profound effect. Therefore, we increased the dose to 8 Gy per fraction, where we found similar morphological damage to what is seen in biopsies from cancer patients having undergone pelvic radiotherapy (Paper II, Figure 1g).

In a similar manner, we found extensive crypt degeneration only at the fourth fraction, regardless of whether 6 Gy or 8 Gy was given [76]. Thus, although 3 x 8 Gy is the same total dose as 4 x 6 Gy (24 Gy), and 3 x 8 Gy is a higher biologically effective dose, the fourth fraction was required to induce the desired effect.

A. Crypt loss at 6 weeks post-irradiation

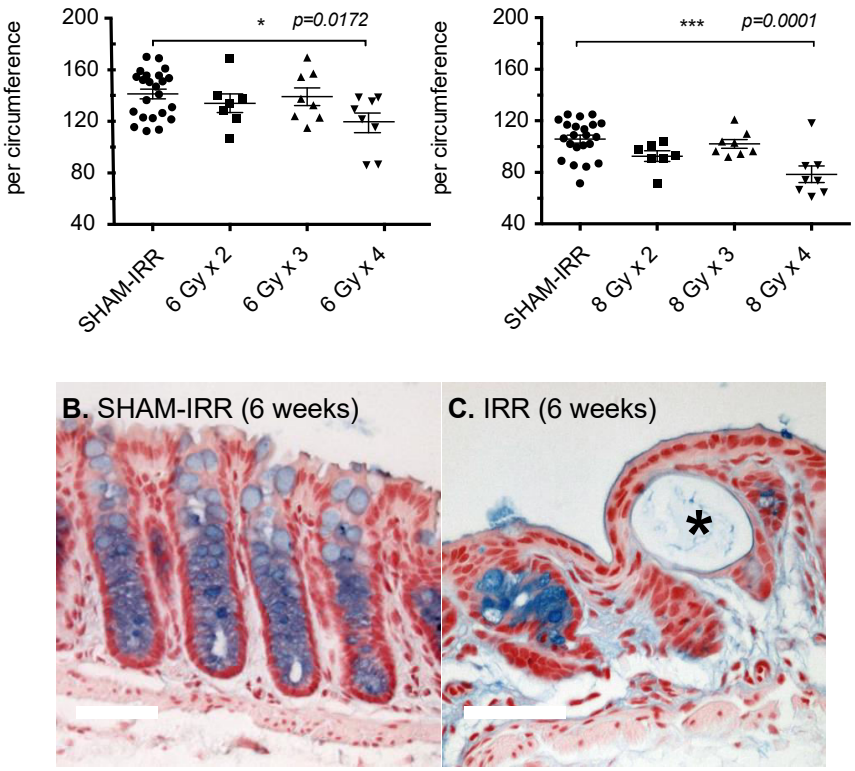



**Figure 4.** A. A decrease in crypt numbers (reflecting crypt loss) was evident first at the fourth fraction in mice irradiated with 4 fractions of 6 Gy or 8 Gy. B and C. Representative microscopic images of sham-irradiated and irradiated mice colorectal mucosa stained with Alcian Blue/Nuclear Fast Red. Bull *et al.* Am J Physiol Gastrointest liver physiol. 2017 Nov 1; 313(5): G456-G466.

Our results suggest a threshold for crypt degeneration and crypt loss that is dependent on the number of fractions delivered during

irradiation. Potten reported that one surviving stem cell is capable of repopulating and rescuing the crypt [79]. One explanation for our results may be that the fourth fraction eliminated the last reserve stem cell in many of the crypts, leading to crypt degeneration. Another explanation may be that irradiation causes damage to the blood vessels resulting in reduced blood flow leading to hypoxia. Hypoxia, in turn, triggers ischemia, ultimately leading to crypt death. Studies have suggested that vascular injury is a primary source of damage from unwanted irradiation and thereby included in the pathways leading to radiation-induced crypt stem cell death. We also observed an increase in the number of macrophages in the gut mucosa at the fourth fraction indicating the inflammatory activity. Inflammatory activity is a strong modulator of stem cell activity and health [80].

Although a fourth fraction was required to induce crypt loss at 6 weeks post-irradiation, we also observed that 8 Gy induced a more profound crypt loss than 6 Gy. Our data and other data support the notion that crypt degeneration is dependent on the total dose delivered to the animals [81, 82].

In conclusion, our mouse model is well suited for studying underlying pathophysiological processes in the intestines after radiation, especially concerning long-term effects and the importance of fractionation. The placement of the radiation field over the colorectum mimics treatment regiments for rectal, anal, urinary and gynecological cancers, where the sigmoid colon and rectum receive the highest radiation dose. Furthermore, the distal placement also makes the model well suited for trying especially topical interventions.

# Paper II

A comprehensive understanding of the gross injury and repair dynamics after pelvic radiotherapy would aid in the deciphering of their underlying mechanisms. We performed successive short and long-term experiments where the progression of crypt degeneration, inflammatory activity, crypt-cell proliferation, and cell survival was determined at different time points after irradiation. The time points evaluated were 24 hours (acute), one week (early), 6 weeks (intermediate), 18 weeks (late) and 30 weeks (chronic).

## Long-term survival of the mice after pelvic radiation

All the mice that received four fractions of 8 Gy survived and displayed a normal weight curve. At 30 weeks, irradiated mice had a non-significant increase in body weight compared to sham-irradiated mice.

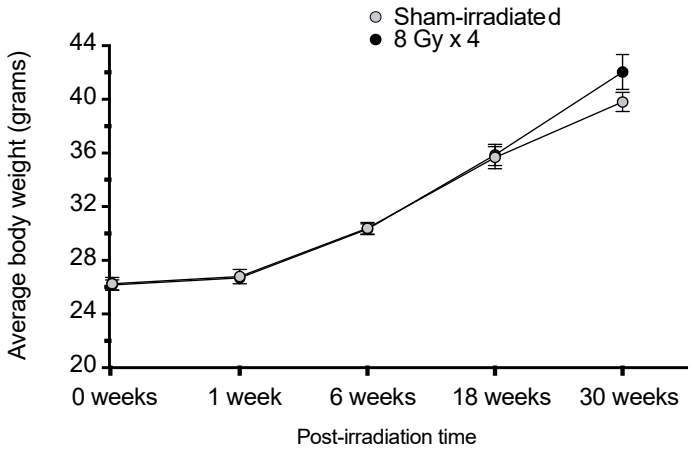



**Figure 5.** Weight graph (in grams) for the 30-week group. Sham-irradiated and irradiated mice gained weight in similar manner. Malipatlolla *et al.* Sci Rep. 2019; 9: 13803.

## Irradiation-induced crypt-loss mucosal damage

Degenerating crypts were never observed in the sham-irradiated mice. However, the irradiated mice displayed crypt degeneration at all the time points, peaking at one week (Figure 6). This was reflected in a statistically significant crypt loss at all time points except at 24 hours and 1 week post-irradiation. Thus, crypt loss lagged somewhat behind the peak of crypt degeneration. This is possibly a reason why we were unable to find a correlation between the number of degenerating crypts and surviving crypts at the given time points (data not shown).

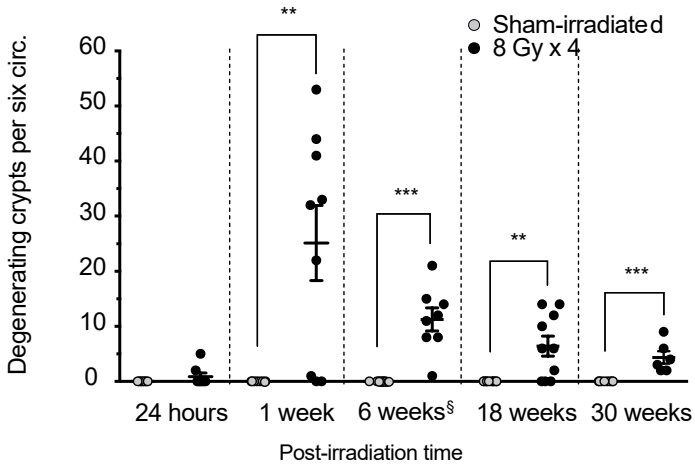



**Figure 6.** Number of degenerating crypts over time. Degenerating crypts were found at all time points studied, peaking at 1 week. Crypt degeneration did not occur in sham-irradiated mice. Malipatlolla *et al*. Sci Rep. 2019; 9: 13803.

Following exposure to ionizing radiation, crypt stem cells undergo apoptosis due to their rapid proliferation activity, which makes them more sensitive to radiation [82]. This may lead to crypt degeneration. A study by Potten showed that the crypt cell apoptosis peaked around 4 hours after post-irradiation [13]. In contrast, our results showed very few degenerating crypts at 24 hours after the last fraction, suggesting that crypt degeneration is a slower process. Our quantification of

surviving crypts also revealed that the crypt loss most likely was permanent after 4x8 Gy, since the crypt density in the irradiated mucosa never recovered.

## Radiation-induced angiogenesis

We quantified the number of CD31 positive blood vessels at various time points after irradiation. No difference in number was observed between irradiated and sham-irradiated at 24h, 6w, and 18 weeks post-irradiation.

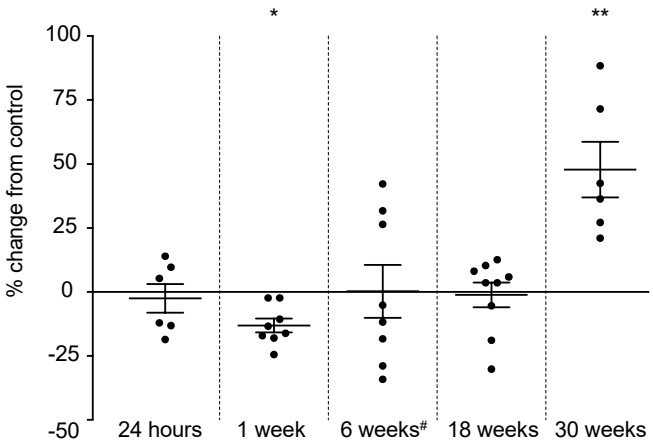



**Figure 7.** Percentage change in number of blood vessels compared to sham-irradiated over time after irradiation. This figure was taken from Malipatlolla *et al.* Sci Rep. 2019; 9: 13803.

At 1 week, there was a decrease in the number of blood vessels between irradiated and sham-irradiated mice. In contrast, an increase in the number of mucosal blood vessels was observed in irradiated mice compared to sham-irradiated mice at 30 weeks (Figure 7).

Along with crypt stem cell death, vascular damage plays a vital role in the development of acute and chronic effects in the intestines after radiotherapy [83]. Several studies propose that damage to the blood vessels is the primary injury after irradiation [31, 84]. Similar to the trajectory of crypt degeneration, loss of blood vessels was seen first at

one week post-irradiation. One study of rectal biopsies taken from cancer patients, four months after pelvic radiotherapy showed severe vascular changes associated with crypt distortion and severe fibrosis of lamina propria [85]. A study by Okunieff showed that mice treated with angiogenic growth factors had more surviving crypts in the intestines after irradiation [86]. This indicates an important role of blood vessels in crypt survival.

## Inflammatory activity

In this study, we followed up our previous results of infiltrating macrophages indicating a low-grade inflammation at six weeks, by quantifying the number of macrophages at 24h, 1w, 6w, 18w, and 30 weeks post-irradiation. Irradiated mice did not show signs of macrophage infiltration in the mucosa at 24 hours and only a slight increase in infiltration of macrophages was seen at 1 week but this was not statistically significant. We found that the higher abundance of infiltrating macrophages at 6 weeks lasted throughout 18 weeks and 30 weeks post-irradiation (Figure 8).

This late-occurring and long-lasting increase in the density of mucosal macrophages could reflect a chronic low-grade inflammation after irradiation. Recent studies have confirmed the infiltration of macrophages within the site of injury after irradiation [87, 88]. A study by Ibuki showed that high doses of irradiation stimulate the activation of macrophages, which are crucial mediators in the inflammation process [89]. We observed inflation of the macrophages in areas where “gut leakiness” might be suspected, such as right above the degenerating crypts (Figure 8E) suggesting that a leakiness of the epithelial barrier attracts the macrophages to the site of injury [90, 91]. Similar to the increase in the number of mucosal macrophages, a significant decrease in the number of the surviving crypt was seen at 6w, 18w and 30 weeks post-irradiation. Previous studies have confirmed that loss of crypts can lead to the breakdown of the intestinal epithelial barrier and also mucosal damage [79, 92]

A. Radiation-induced infiltration of macrophages in the mucosa

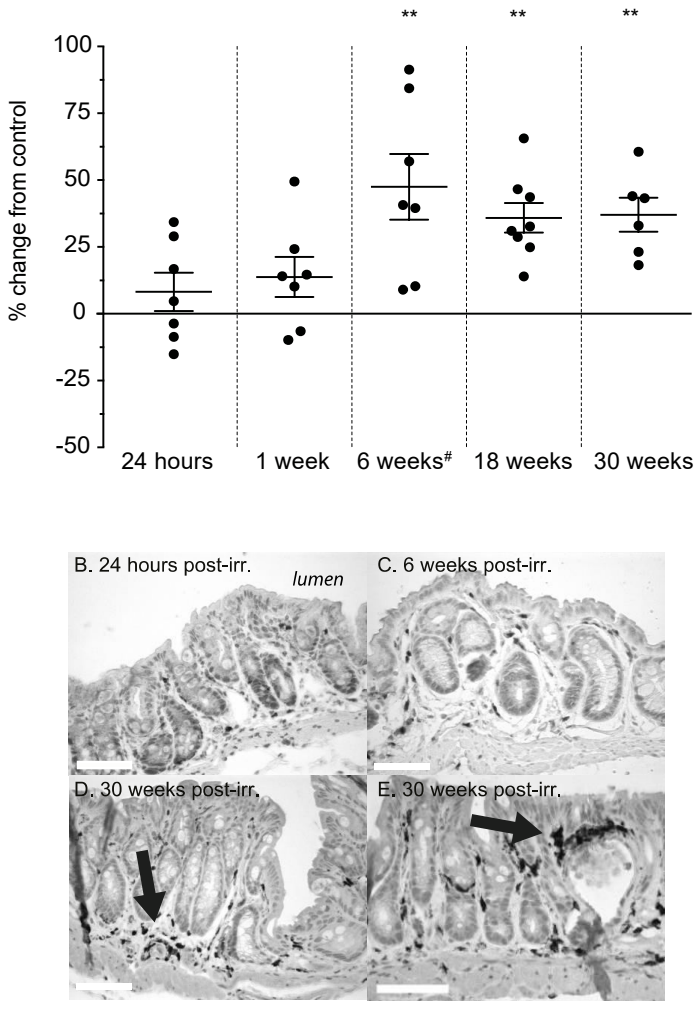



**Figure 8.** Radiation induced mucosal macrophage infiltration. A statistically significant increase was seen at 6, 18 and 30 weeks post-irradiation. Malipatlolla *et al.* Sci Rep. 2019; 9: 13803.

## Crypt fission- a repair mechanism

Crypt fission, which is a natural phenomenon of colonic growth and a repair mechanism after injury, was observed more frequently in irradiated animals than in sham-irradiated. It was dependent on dose and/or fraction since mice irradiated with 4 fractions of 8 Gy displayed more crypt fissions than mice irradiated with 2 or 3 fractions of 8 Gy (Figure 9 A).

At 24 hours post-irradiation, there was a slight but non-significant increase in crypt fission that became more obvious in one week. By six weeks, all irradiated mice displayed crypt fission in the analyzed tissue. At 18 weeks, the peak of fissions subsided and was back to control levels at 30 weeks post-irradiation (Figure 9 B).

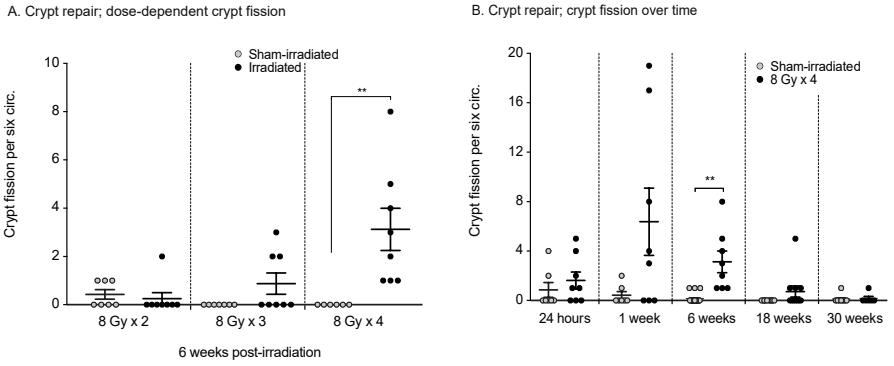



**Figure 9.** A. Crypt fission showing damage-dependent response. B. Crypt fission over time. Irradiated mice had a greater number of crypt fissions than did sham-irradiated at 6 weeks. Malipatlolla *et al*. Sci Rep. 2019; 9: 13803.

We observed what appeared to be a very small increase in the number of proliferating cells at 6, 18 and 30 weeks post-irradiation, but this was not statistically significant (Paper II, Figure 4a).

After the initial injury, a mucosal repair can occur through two different mechanisms; crypt cell proliferation and crypt fission. Previous studies demonstrated the role of crypt fission in colonic growth and repair [54, 93]. Our data indicate an important finding; that

it is crypt fission, not cell proliferation that repairs the damaged tissue in the long-term. Most studies have focused on cell proliferation, thus possibly missing an important repair mechanism. Crypt fission is known to be able to occur without signs of increased cell proliferation [94]. Our results are supported by a study where irradiated mice showed an increase in crypt fission compared to control mice [95].

In conclusion, our results suggest that the irradiation of the colorectum causes permanent loss of crypts, eventually replaced by fibrotic tissue, and also increases in the infiltration of mucosal macrophages over time indicating persistent intestinal inflammation. If this can be confirmed in survivors who underwent pelvic radiotherapy, a successful approach to restoring intestinal health in these patients could take into account the ongoing low-grade intestinal inflammation. Additionally, we suggest that crypt fission is a more important long-term repair mechanism after irradiation than crypt cell proliferation. Since crypt fission was seen at all the time points after the initial injury, the therapeutic window of opportunity for the mucosal healing after radiotherapy may be much wider than what has been previously estimated. Further studies are needed to explain how crypt fission is regulated and why it fails to repair mucosal damage completely.

# Paper III

A recent dietary intervention study performed by Wedlake and colleagues revealed that pelvic cancer survivors that consumed a diet rich in fiber had reduced gastrointestinal toxicity compared to cancer survivors with a habitual-fiber intake [96]. Despite the increasing number of reports on the beneficial effects of fiber in conditions such as irritable bowel syndrome and inflammatory bowel disease [97, 98], pelvic cancer patients are still commonly advised to consume a low or no-fiber diet during pelvic radiotherapy [99]. In the study leading to Paper III, we extended our knowledge reported in Paper II to investigate the hypothesis that a diet high in fiber increases the resiliency of the gut wall and protects against radiation-induced intestinal damage.

Mice were fed either with a diet rich in fiber from bioprocessed oat bran ("high-oat") or a fiber-free ("no-fiber") diet. The dietary intervention started two weeks before irradiation and was maintained for 1, 6, and 18 weeks after irradiation. The colorectal tissue was collected at the different time points and analyzed for the occurrence of degenerated crypts, the number of surviving crypts, crypt fission, crypt cell proliferation, and mucosal infiltration of macrophages.

We found that mice on the no-fiber diet had more degenerating crypts at one week after irradiation than mice on the high-oat diet. We also observed that the fiber-deprived irradiated mice had fewer surviving crypts at the short (1 week) and intermediate (6 weeks) time points (Paper III Figure 4). There are other studies pointing in the same direction; rodents fed with a fiber-free diet had fewer surviving crypts compared to rodents on a fiber-rich diet after irradiation [100, 101]. However, our follow-up at 18 weeks post-irradiation revealed that the high-oat diet was not able to rescue the crypts in the long run. This highlighted the progressive and long-lasting effects of radiation on intestinal health, and that conclusions based on short-term follow-up might be misleading. It is plausible that the initial DNA damage

caused by the ionizing irradiation will eventually deplete the stem cells in the crypts, regardless of measures taken to improve the microenvironment. Nevertheless, the course of the disease can be delayed, which may have important clinical implications.

As mentioned before, radiation to the intestine is believed to increase the permeability of the intestinal wall to pathogens. One mechanism causing a delay of crypt loss could be that fiber-rich diets prevent the entry of pathogens into the colonic tract by enhancing the formation of a thicker protective inner mucus layer [102]. The dietary fibers undergo degradation in the colon by fiber-degrading bacteria, producing energy that is used to form the mucus, which prevents the entry of a pathogen [103]. This could reduce inflammation, which is known to be deleterious to especially stem cells. Another aspect is that a diet deprived of fiber starves the colonocytes, which are dependent on fiber-derived butyrate as their main source of energy [72, 104, 105]. It is reasonable to believe that the starved colon becomes more susceptible to irradiation-induced pathophysiological processes since repair processes such as crypt fission or cell proliferation would require extra energy.

A statistically significant increase in crypt cell proliferation was seen in high-oat irradiated mice compared to no-fiber irradiated mice (Paper III Figures 3 and 5). As explained earlier, crypt cell proliferation and crypt fission are the two main repair mechanisms that play a vital role in the regeneration of crypts after damage. Sureban *et al*. found that soluble dietary pectin increased cell proliferation in the crypts after irradiation [101]. The mechanism by which diet rich in fiber increases crypt cell proliferation is suggested to be the breakdown of fiber into short-chain fatty acids in the colon to produce butyrate, acetate, and propionate, out of which butyrate serves as the main energy source for the colonocytes [60]. In our previous studies (Paper I and II), we did not see any changes in cell proliferation after irradiation, leading us to believe that repair was executed via crypt fission, not cell proliferation. The mice in that study consumed standard chow, consisting of a nearly

the same amount of dietary fiber as the high-oat diet (13% vs 15%, respectively), but having a high fraction of insoluble fiber (85%) with low levels of β-glucan (0.6% vs 28%, respectively). The very high amounts of β-glucan in the current study could explain the discrepancies in our findings concerning cell proliferation after irradiation. β-glucan efficiently produce butyrate, induce proliferation, and stimulate the immune system, and possibly, the butyrate production allowed the proliferative response to last longer in our high-oat mice [106, 107]. However, it cannot explain why the no-fiber mice also responded to irradiation with cell proliferation at one week, while animals on standard chow only had a slight but insignificant increase at 6 weeks and onwards. An explanation for this can be that every fiber-composition causes a different time peak of proliferation and that in the study for Paper II, we missed the proliferation response of animals fed standard chow at 1, 6, and 18 weeks. Alternatively, the composition of the standard chow does not allow for a proliferative response, for an unknown reason. After all, animals fed standard chow lost as many crypts as the no fiber at the one week after irradiation, which supports the notion that it was the composition of the bioprocessed oat bran that was especially important in determining what took place.

Crypt fission, a growth and repair mechanism that has largely been overlooked could be responsible for the increase in the number of crypts both in the sham-irradiated and irradiated high-oat mice. We observed that mice fed with high-oat diet appeared to have more intense crypt fission early on compared to the no-fiber mice but the underlying mechanism is unknown.

In conclusion, a diet rich in fiber modified the onset, timing, and intensity of intestinal pathophysiological processes and repair mechanisms after radiation. This does not mean that we now know what dietary advice to give to pelvic cancer patients. For example, we do not know how various types of fiber modulate the outcome, and diets rich in fiber normally also contain other compounds such as

polyphenols that could influence bowel health. In addition, despite being able to stimulate cell proliferation and crypt fission we were not able to rescue the crypts; this may have unknown implications for bowel health. Nevertheless, further studies on the importance of the dietary fiber given before, during and after pelvic radiotherapy might give us a means other than reducing the radiation dose to protect the normal tissue in the cancer survivor.

# Paper IV

Radiation alters the gut homeostasis balance by activating both pro- and anti-inflammatory pathways [108, 109]. As mentioned before, radiation causes acute intestinal inflammation, where increased permeability of the intestinal epithelial barrier is believed to allow the bacteria to translocate into the lumen [16, 110]. The general perception is, however, that the inflammation quickly subsides when the intestine either repairs itself or becomes fibrotic [2]. Our data from the mouse model did not support this and in the study for paper IV, we set out to investigate whether the increased macrophage density is seen in Paper I-III reflected a persisting low-grade inflammation that could be modified by a diet rich in fiber.

Cytokine and chemokines are important mediators involved in both acute and chronic inflammatory processes acting locally at the site of injury or systemically. We aimed to determine the systemic cytokine levels, reflecting possible inflammatory processes in the tissue, at the various time points after irradiation in the mice from the dietary fiber study presented in Paper III. Thus, at the time of sacrifice, the blood was harvested and serum samples were prepared. The samples were then analyzed with a Luminex Bead-Based Multiplex Assay. In this assay, the color-coded beads were used to measure the analytes in the sample.

Our results indicate an increase in pro-inflammatory cytokine levels in both the irradiated groups fed with the high-oat or no-fiber diets compared to sham-irradiated mice fed with the high-oat or no-fiber diets at all the time points after irradiation. This supports our hypothesis that there is a long-lasting low-grade inflammation after irradiation (Paper IV, Figures 2, 4 and 6). At one week post-irradiation, a significant increase in levels of IL-1$\alpha$ was seen in the irradiated mice compared to sham-irradiated mice regardless of diet. A few studies in rodents have shown that the irradiation increased the levels of IL-1$\alpha$ in irradiated animals compared to controls 2 weeks after irradiation [111,

112]. IL-1α is a pro-inflammatory cytokine known to be released by activated macrophages, neutrophils, epithelial cells, and endothelial cells. Similarly, our unpublished data showed a significant increase in mucosal macrophages in high-oat diet irradiated mice compared to high-oat diet sham-irradiated at one week post-irradiation (Paper III Figure 6).

At six weeks post-treatment, irradiated mice fed the no fiber diet had a statistically significant increase in G-CSF cytokine levels compared to the no fiber sham-irradiated mice. A study by Tanji *et al.* found increased levels of G-CSF in the serum during radiotherapy in prostate cancer patients [113]. G-CSF plays a role in both innate and adaptive immune pathways, modulating the activation of both macrophages and dendritic cells. Similarly, at six weeks post-irradiation the no-fiber diet irradiated mice had a significant increase in macrophages compared to the no-fiber diet sham-irradiated (Paper III Figure 6).

At all the time points, the high-oat diet mice had reduced levels of the circulating pro-inflammatory cytokines in the serum compared to mice fed a no-fiber diet, indicating a protective role of a high-oat diet. Studies have shown that oat β-glucan decreases the levels of pro-inflammatory cytokines in the inflamed intestine [114, 115].

We also observed the up-regulation of the canonical pathways and biological functions in irradiated mice compared to sham-irradiated. Irradiated mice fed a high-oat diet down-regulated the canonical pathways, biological functions, and upstream regulators compared to irradiated mice fed a no-fiber diet (Paper IV figure S6, S7, S8).

In conclusion, our results indicate that irradiation increased both acute and chronic inflammatory cytokines levels in the serum and consuming a high-oat diet reduced both acute and chronic systemic inflammation in these mice compared to mice on a fiber-free diet.

# CONCLUSIONS

Here I highlight the most important conclusions and new insights from my research for this thesis.

1. The employment of linear accelerators and a very small irradiation field resulted in preserved animal survival and overall health, despite the high dose given. The gross pathophysiological changes found in the irradiated colorectum were very similar to those found in the human intestine after pelvic radiotherapy.

2. Infiltration of macrophages in the mucosa was persistent over time, indicating a subtle inflammatory activity that might be life-long. Regeneration of damaged tissue occurred through crypt fission at one week after irradiation and persisted for many weeks.

3. A diet rich in fiber profoundly changed the onset, timing, and intensity of pathophysiological processes in the intestines after irradiation.

4. A diet rich in fiber reduced the inflammatory-related serum-cytokine levels, whereas a fiber-free diet worsened the inflammatory-related serum-cytokine levels, both acutely and chronically.

# FUTURE PERSPECTIVES

## Evaluation of mucosal biopsies from pelvic cancer survivors

We are collecting biopsies from survivors who underwent pelvic radiotherapy two to 20 years earlier. The biopsies will be investigated for the occurrence of chronic, low-grade inflammation and other pathophysiological processes.

## Mucosal injury - repair via crypt fission

Our studies showed that after irradiation, crypt fission as a long-term repair mechanism was favored over crypt cell proliferation. Two important goals to be pursued in the future are to try to learn how crypt fission is regulated and to determine if promoting crypt fission would promote healing after irradiation.

## Dietary interventions in pelvic cancer survivors

We will be evaluating a possible beneficial effect of eating dietary fiber during and after pelvic radiotherapy. Patients will receive around 21 grams of fiber/day two weeks before, during, and 6 weeks after pelvic radiotherapy. Blood and fecal samples will be collected before the start of pelvic radiotherapy, during, 5 weeks and 12 months after the pelvic radiotherapy. Blood samples will be analyzed for acute or chronic inflammatory cytokine levels, and fecal samples will be analyzed for any changes in microbial composition and inflammatory markers.

# ACKNOWLEDGEMENT

I have been very fortunate to receive kind support and help from many wonderful people during my Ph.D. study. Here are the people that I need to thank for all the help during the past four years working on this project.

First and foremost, I would like to express my sincere gratitude to Professor. Gunnar Steineck for his continuous support, patience, and motivation throughout the four years of my study. I could not have imagined having a better advisor and mentor for my Ph.D. and I want you to know that you have been a true inspiration.

I want to thank Cecilia Bull, for accepting me as a Ph.D. student and keeping me under your supervision from start to finish. I am thankful for your inputs, questions and scientific discussions regarding each study. I consider myself fortunate and understand how important your support has been for me.

It is a pleasure to thank Marie Kalm for continuous support, inputs and critical comments regarding the project and manuscripts.

My sincere thanks go to Fei Sjöberg, for your help and constant support during my Ph.D.

I would also like to thank Professor. Hans-Georg Kuhn for sharing his knowledge with me and for always having the door open for me when I had questions.

I thank collaborators Margarita Nyman and Ana Rascon for their help in designing the projects and for their inputs concerning various manuscripts.

Special thanks to Piyush Patel and Sravani Devarakonda for their continuous support throughout my Ph.D. No matter what the situation

was that I faced, you guys were always there for me. I will miss your company.

Special thanks also to a very special person, Rita Grander, for teaching me practical laboratory techniques and for all the assistance you have been giving me since day one. I will definitely miss you.

I would like to thank Ahmed Alrifaiy, Alexandra Hansson, Ali Komai, Amelie Toft, Ann Egardt, Annika Wettergren, Eva Mehdin, Gabriella Wendt, Henrietta Norling, Ida Sigvardsson, Jill Christiansen, Julia Eliasson, Lawrence Lundgren, Lars Karlsson, Linda Åkeflo, Linda Persson, Malin Warholm, Mohammedreza Abdolalian, Pedram Sultanian, Rebecca Ahlin, Reza Motalleb, Saliha Musovic, Toshima Parris and Ulrica Wilderäng.

Finally, I would like to express my thanks to my parents, Yeshwanth Rao and Rukmini Bai and my wife Divya for their support and love.

# REFERENCES


1. Mehta, S.R., et al., *Radiotherapy: Basic Concepts and Recent Advances.* Med J Armed Forces India, 2010. **66**(2): p. 158-62.
2. Morris, K.A. and N.Y. Haboubi, *Pelvic radiation therapy: Between delight and disaster.* World J Gastrointest Surg, 2015. **7**(11): p. 279-88.
3. Andreyev, H.J., et al., *"Pelvic radiation disease": new understanding and new solutions for a new disease in the era of cancer survivorship.* Scand J Gastroenterol, 2011. **46**(4): p. 389-97.
4. Andreyev, H.J., *Gastrointestinal problems after pelvic radiotherapy: the past, the present and the future.* Clin Oncol (R Coll Radiol), 2007. **19**(10): p. 790-9.
5. Alsadius, D., et al., *Tobacco smoking and long-lasting symptoms from the bowel and the anal-sphincter region after radiotherapy for prostate cancer.* Radiother Oncol, 2011. **101**(3): p. 495-501.
6. Lind, H., et al., *Late symptoms in long-term gynaecological cancer survivors after radiation therapy: a population-based cohort study.* Br J Cancer, 2011. **105**(6): p. 737-45.
7. Steineck, G., et al., *Identifying radiation-induced survivorship syndromes affecting bowel health in a cohort of gynecological cancer survivors.* PLoS One, 2017. **12**(2): p. e0171461.
8. Harris, V., et al., *Bile acid malabsorption after pelvic and prostate intensity modulated radiation therapy: an uncommon but treatable condition.* Int J Radiat Oncol Biol Phys, 2012. **84**(5): p. e601-6.
9. Citrin, D.E. and J.B. Mitchell, *Mechanisms of Normal Tissue Injury From Irradiation.* Semin Radiat Oncol, 2017. **27**(4): p. 316-324.
10. Heath, J.P., *Epithelial cell migration in the intestine.* Cell Biol Int, 1996. **20**(2): p. 139-46.
11. Potten, C.S., *Stem cells in gastrointestinal epithelium: numbers, characteristics and death.* Philos Trans R Soc Lond B Biol Sci, 1998. **353**(1370): p. 821-30.
12. Potten, C.S., C. Booth, and D.M. Pritchard, *The intestinal epithelial stem cell: the mucosal governor.* Int J Exp Pathol, 1997. **78**(4): p. 219-43.
13. Potten, C.S. and H.K. Grant, *The relationship between ionizing radiation-induced apoptosis and stem cells in the small and large intestine.* Br J Cancer, 1998. **78**(8): p. 993-1003.
14. Ferreira, M.R., et al., *Microbiota and radiation-induced bowel toxicity: lessons from inflammatory bowel disease for the radiation oncologist.* Lancet Oncol, 2014. **15**(3): p. e139-47.
15. Dvorak, J., et al., *Intestinal permeability, vitamin A absorption, alpha-tocopherol, and neopterin in patients with rectal carcinoma treated with chemoradiation.* Med Oncol, 2010. **27**(3): p. 690-6.

16. Nejdfors, P., et al., *Intestinal permeability in humans is increased after radiation therapy.* Dis Colon Rectum, 2000. **43**(11): p. 1582-1587; discussion 1587-8.
17. Porvaznik, M., *Tight junction disruption and recovery after sublethal gamma irradiation.* Radiat Res, 1979. **78**(2): p. 233-50.
18. Somosy, Z., et al., *Morphological and histochemical changes in intercellular junctional complexes in epithelial cells of mouse small intestine upon X-irradiation: changes of ruthenium red permeability and calcium content.* Scanning Microsc, 1993. **7**(3): p. 961-71.
19. Su, L., et al., *Targeted epithelial tight junction dysfunction causes immune activation and contributes to development of experimental colitis.* Gastroenterology, 2009. **136**(2): p. 551-63.
20. Denham, J.W., et al., *Treatment-time-dependence models of early and delayed radiation injury in rat small intestine.* Int J Radiat Oncol Biol Phys, 2000. **48**(3): p. 871-87.
21. Osborne, J.W., K.N. Prasad, and G.R. Zimmerman, *Changes in the rat intestine after x-irradiation of exteriorized short segments of ileum.* Radiat Res, 1970. **43**(1): p. 131-42.
22. Dorr, W. and J.H. Hendry, *Consequential late effects in normal tissues.* Radiother Oncol, 2001. **61**(3): p. 223-31.
23. Goodman, K.A., et al., *Whole abdominopelvic radiotherapy for desmoplastic small round-cell tumor.* Int J Radiat Oncol Biol Phys, 2002. **54**(1): p. 170-6.
24. Terry, N.H., et al., *Cellular kinetics of murine lung: model system to determine basis for radioprotection with keratinocyte growth factor.* Int J Radiat Oncol Biol Phys, 2004. **58**(2): p. 435-44.
25. Booth, C., et al., *Teduglutide ([Gly2]GLP-2) protects small intestinal stem cells from radiation damage.* Cell Prolif, 2004. **37**(6): p. 385-400.
26. Thulesen, J., et al., *Glucagon-like peptide 2 (GLP-2) accelerates the growth of colonic neoplasms in mice.* Gut, 2004. **53**(8): p. 1145-50.
27. Finch, P.W. and J.S. Rubin, *Keratinocyte growth factor expression and activity in cancer: implications for use in patients with solid tumors.* J Natl Cancer Inst, 2006. **98**(12): p. 812-24.
28. Rubin, P. and G.W. Casarett, *Clinical radiation pathology as applied to curative radiotherapy.* Cancer, 1968. **22**(4): p. 767-78.
29. Russell, N.S., et al., *Novel insights into pathological changes in muscular arteries of radiotherapy patients.* Radiother Oncol, 2009. **92**(3): p. 477-83.
30. Fajardo, L.F. and M. Berthrong, *Vascular lesions following radiation.* Pathol Annu, 1988. **23 Pt 1**: p. 297-330.
31. Paris, F., et al., *Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice.* Science, 2001. **293**(5528): p. 293-7.

32. Potten, C.S., *Radiation, the ideal cytotoxic agent for studying the cell biology of tissues such as the small intestine.* Radiat Res, 2004. **161**(2): p. 123-36.
33. Johnson, L.B., et al., *Radiation enteropathy and leucocyte-endothelial cell reactions in a refined small bowel model.* BMC Surg, 2004. **4**: p. 10.
34. Mihaescu, A., et al., *Rho kinase signalling mediates radiation-induced inflammation and intestinal barrier dysfunction.* Br J Surg, 2011. **98**(1): p. 124-31.
35. Gloire, G., S. Legrand-Poels, and J. Piette, *NF-kappaB activation by reactive oxygen species: fifteen years later.* Biochem Pharmacol, 2006. **72**(11): p. 1493-505.
36. Brown, M., et al., *Cardiac-specific blockade of NF-kappaB in cardiac pathophysiology: differences between acute and chronic stimuli in vivo.* Am J Physiol Heart Circ Physiol, 2005. **289**(1): p. H466-76.
37. Baker, D.G. and R.J. Krochak, *The response of the microvascular system to radiation: a review.* Cancer Invest, 1989. **7**(3): p. 287-94.
38. Ong, Z.Y., et al., *Pro-inflammatory cytokines play a key role in the development of radiotherapy-induced gastrointestinal mucositis.* Radiat Oncol, 2010. **5**: p. 22.
39. Francois, A., et al., *Inflammation and immunity in radiation damage to the gut mucosa.* Biomed Res Int, 2013. **2013**: p. 123241.
40. Gordon, S., *The macrophage: past, present and future.* Eur J Immunol, 2007. **37 Suppl 1**: p. S9-17.
41. Mills, C.D., *Anatomy of a discovery: m1 and m2 macrophages.* Front Immunol, 2015. **6**: p. 212.
42. Bessout, R., et al., *Mesenchymal stem cell therapy induces glucocorticoid synthesis in colonic mucosa and suppresses radiation-activated T cells: new insights into MSC immunomodulation.* Mucosal Immunol, 2014. **7**(3): p. 656-69.
43. Blirando, K., et al., *Mast cells are an essential component of human radiation proctitis and contribute to experimental colorectal damage in mice.* Am J Pathol, 2011. **178**(2): p. 640-51.
44. Hovdenak, N., et al., *Clinical significance of increased gelatinolytic activity in the rectal mucosa during external beam radiation therapy of prostate cancer.* Int J Radiat Oncol Biol Phys, 2002. **53**(4): p. 919-27.
45. Wynn, T.A. and T.R. Ramalingam, *Mechanisms of fibrosis: therapeutic translation for fibrotic disease.* Nat Med, 2012. **18**(7): p. 1028-40.
46. Yarnold, J. and M.C. Brotons, *Pathogenetic mechanisms in radiation fibrosis.* Radiother Oncol, 2010. **97**(1): p. 149-61.

47. Citrin, D.E., et al., *Radiation-Induced Fibrosis: Mechanisms and Opportunities to Mitigate. Report of an NCI Workshop, September 19, 2016.* Radiat Res, 2017. **188**(1): p. 1-20.
48. Barker, N., et al., *Identification of stem cells in small intestine and colon by marker gene Lgr5.* Nature, 2007. **449**(7165): p. 1003-7.
49. Asfaha, S., et al., *Krt19(+)/Lgr5(-) Cells Are Radioresistant Cancer-Initiating Stem Cells in the Colon and Intestine.* Cell Stem Cell, 2015. **16**(6): p. 627-38.
50. Karmakar, S., et al., *Intestinal epithelial regeneration: active versus reserve stem cells and plasticity mechanisms.* Am J Physiol Gastrointest Liver Physiol, 2020.
51. Baker, A.M., et al., *Quantification of crypt and stem cell evolution in the normal and neoplastic human colon.* Cell Rep, 2014. **8**(4): p. 940-7.
52. Zeki, S.S., T.A. Graham, and N.A. Wright, *Stem cells and their implications for colorectal cancer.* Nat Rev Gastroenterol Hepatol, 2011. **8**(2): p. 90-100.
53. Miyoshi, H., et al., *Wnt5a potentiates TGF-beta signaling to promote colonic crypt regeneration after tissue injury.* Science, 2012. **338**(6103): p. 108-13.
54. Berlanga-Acosta, J., et al., *Gastrointestinal cell proliferation and crypt fission are separate but complementary means of increasing tissue mass following infusion of epidermal growth factor in rats.* Gut, 2001. **48**(6): p. 803-7.
55. Trowell, H., *Crude fibre, dietary fibre and atherosclerosis.* Atherosclerosis, 1972. **16**(1): p. 138-40.
56. Dai, F.J. and C.F. Chau, *Classification and regulatory perspectives of dietary fiber.* J Food Drug Anal, 2017. **25**(1): p. 37-42.
57. Burkitt, D.P., A.R. Walker, and N.S. Painter, *Effect of dietary fibre on stools and the transit-times, and its role in the causation of disease.* Lancet, 1972. **2**(7792): p. 1408-12.
58. Trowell, H.C., *Western diseases, Western diets and fibre.* East Afr Med J, 1978. **55**(6): p. 283-9.
59. Topping, D.L. and P.M. Clifton, *Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides.* Physiol Rev, 2001. **81**(3): p. 1031-64.
60. Wong, J.M., et al., *Colonic health: fermentation and short chain fatty acids.* J Clin Gastroenterol, 2006. **40**(3): p. 235-43.
61. Bergman, E.N., *Energy contributions of volatile fatty acids from the gastrointestinal tract in various species.* Physiol Rev, 1990. **70**(2): p. 567-90.
62. Canani, R.B., et al., *Potential beneficial effects of butyrate in intestinal and extraintestinal diseases.* World J Gastroenterol, 2011. **17**(12): p. 1519-28.

63. Hamer, H.M., et al., *Review article: the role of butyrate on colonic function.* Aliment Pharmacol Ther, 2008. **27**(2): p. 104-19.
64. Koh, A., et al., *From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites.* Cell, 2016. **165**(6): p. 1332-1345.
65. Roediger, W.E., *Utilization of nutrients by isolated epithelial cells of the rat colon.* Gastroenterology, 1982. **83**(2): p. 424-9.
66. Roediger, W.E., *Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man.* Gut, 1980. **21**(9): p. 793-8.
67. Wolever, T.M., P. Spadafora, and H. Eshuis, *Interaction between colonic acetate and propionate in humans.* Am J Clin Nutr, 1991. **53**(3): p. 681-7.
68. Boets, E., et al., *Systemic availability and metabolism of colonic-derived short-chain fatty acids in healthy subjects: a stable isotope study.* J Physiol, 2017. **595**(2): p. 541-555.
69. Abrahamse, S.L., B.L. Pool-Zobel, and G. Rechkemmer, *Potential of short chain fatty acids to modulate the induction of DNA damage and changes in the intracellular calcium concentration by oxidative stress in isolated rat distal colon cells.* Carcinogenesis, 1999. **20**(4): p. 629-34.
70. Rosignoli, P., et al., *Protective activity of butyrate on hydrogen peroxide-induced DNA damage in isolated human colonocytes and HT29 tumour cells.* Carcinogenesis, 2001. **22**(10): p. 1675-80.
71. Suzuki, T., S. Yoshida, and H. Hara, *Physiological concentrations of short-chain fatty acids immediately suppress colonic epithelial permeability.* Br J Nutr, 2008. **100**(2): p. 297-305.
72. Kelly, C.J., et al., *Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function.* Cell Host Microbe, 2015. **17**(5): p. 662-71.
73. Willemsen, L.E., et al., *Short chain fatty acids stimulate epithelial mucin 2 expression through differential effects on prostaglandin E(1) and E(2) production by intestinal myofibroblasts.* Gut, 2003. **52**(10): p. 1442-7.
74. Fukuda, H., et al., *Irradiation-induced progenitor cell death in the developing brain is resistant to erythropoietin treatment and caspase inhibition.* Cell Death Differ, 2004. **11**(11): p. 1166-78.
75. Malipatlolla, D.K., et al., *Long-term mucosal injury and repair in a murine model of pelvic radiotherapy.* Sci Rep, 2019. **9**(1): p. 13803.
76. Steineck, G., et al., *Radiation physiology - evidence for a higher biological effect of 24 Gy in four fractions as compared to three.* Acta Oncol, 2017. **56**(9): p. 1240-1243.
77. Rotolo, J.A., R. Kolesnick, and Z. Fuks, *Timing of lethality from gastrointestinal syndrome in mice revisited.* Int J Radiat Oncol Biol Phys, 2009. **73**(1): p. 6-8.

78. van Bekkum, D.W., *Radiation sensitivity of the hemopoietic stem cell.* Radiat Res, 1991. **128**(1 Suppl): p. S4-8.
79. Potten, C.S., *A comprehensive study of the radiobiological response of the murine (BDF1) small intestine.* Int J Radiat Biol, 1990. **58**(6): p. 925-73.
80. Asfaha, S., *Intestinal stem cells and inflammation.* Curr Opin Pharmacol, 2015. **25**: p. 62-6.
81. Roberts, S.A. and C.S. Potten, *Clonogen content of intestinal crypts: its deduction using a microcolony assay on whole mount preparations and its dependence on radiation dose.* Int J Radiat Biol, 1994. **65**(4): p. 477-81.
82. Booth, C., et al., *Acute gastrointestinal syndrome in high-dose irradiated mice.* Health Phys, 2012. **103**(4): p. 383-99.
83. Wang, J., et al., *Significance of endothelial dysfunction in the pathogenesis of early and delayed radiation enteropathy.* World J Gastroenterol, 2007. **13**(22): p. 3047-55.
84. Hasleton, P.S., N. Carr, and P.F. Schofield, *Vascular changes in radiation bowel disease.* Histopathology, 1985. **9**(5): p. 517-34.
85. Haboubi, N.Y., P.F. Schofield, and P.L. Rowland, *The light and electron microscopic features of early and late phase radiation-induced proctitis.* Am J Gastroenterol, 1988. **83**(10): p. 1140-4.
86. Okunieff, P., et al., *In vivo radioprotective effects of angiogenic growth factors on the small bowel of C3H mice.* Radiat Res, 1998. **150**(2): p. 204-11.
87. Monceau, V., et al., *Enhanced sensitivity to low dose irradiation of ApoE-/- mice mediated by early pro-inflammatory profile and delayed activation of the TGFbeta1 cascade involved in fibrogenesis.* PLoS One, 2013. **8**(2): p. e57052.
88. Bickelhaupt, S., et al., *Effects of CTGF Blockade on Attenuation and Reversal of Radiation-Induced Pulmonary Fibrosis.* J Natl Cancer Inst, 2017. **109**(8).
89. Ibuki, Y. and R. Goto, *Ionizing radiation-induced macrophage activation: augmentation of nitric oxide production and its significance.* Cell Mol Biol (Noisy-le-grand), 2004. **50 Online Pub**: p. OL617-26.
90. Hume, D.A., V.H. Perry, and S. Gordon, *The mononuclear phagocyte system of the mouse defined by immunohistochemical localisation of antigen F4/80: macrophages associated with epithelia.* Anat Rec, 1984. **210**(3): p. 503-12.
91. Moyes, S.M., et al., *Changes produced by external radiation in parameters influencing intestinal permeability and microparticle uptake in vitro.* Int J Radiat Biol, 2008. **84**(6): p. 467-86.
92. Harfouche, G. and M.T. Martin, *Response of normal stem cells to ionizing radiation: a balance between homeostasis and genomic stability.* Mutat Res, 2010. **704**(1-3): p. 167-74.

93. Booth, C. and C.S. Potten, *Gut instincts: thoughts on intestinal epithelial stem cells.* J Clin Invest, 2000. **105**(11): p. 1493-9.
94. Park, H.S., et al., *Effects of epidermal growth factor and dimethylhydrazine on crypt size, cell proliferation, and crypt fission in the rat colon. Cell proliferation and crypt fission are controlled independently.* Am J Pathol, 1997. **151**(3): p. 843-52.
95. Cairnie, A.B. and B.H. Millen, *Fission of crypts in the small intestine of the irradiated mouse.* Cell Tissue Kinet, 1975. **8**(2): p. 189-96.
96. Wedlake, L., et al., *Randomized controlled trial of dietary fiber for the prevention of radiation-induced gastrointestinal toxicity during pelvic radiotherapy.* Am J Clin Nutr, 2017. **106**(3): p. 849-857.
97. Birchenough, G., et al., *Dietary destabilisation of the balance between the microbiota and the colonic mucus barrier.* Gut Microbes, 2019. **10**(2): p. 246-250.
98. Castro, F. and H.S.P. de Souza, *Dietary Composition and Effects in Inflammatory Bowel Disease.* Nutrients, 2019. **11**(6).
99. Ahlin, R., et al., *[Differing dietary advice are given to gynaecological and prostate cancer patients receiving radiotherapy in Sweden].* Lakartidningen, 2018. **115**.
100. Nagai, T., et al., *Dietary sugar beet fiber prevents the increase in aberrant crypt foci induced by gamma-irradiation in the colorectum of rats treated with an immunosuppressant.* J Nutr, 2000. **130**(7): p. 1682-7.
101. Sureban, S.M., et al., *Dietary Pectin Increases Intestinal Crypt Stem Cell Survival following Radiation Injury.* PLoS One, 2015. **10**(8): p. e0135561.
102. Schroeder, B.O., et al., *Bifidobacteria or Fiber Protects against Diet-Induced Microbiota-Mediated Colonic Mucus Deterioration.* Cell Host Microbe, 2018. **23**(1): p. 27-40 e7.
103. Desai, M.S., et al., *A Dietary Fiber-Deprived Gut Microbiota Degrades the Colonic Mucus Barrier and Enhances Pathogen Susceptibility.* Cell, 2016. **167**(5): p. 1339-1353 e21.
104. Byndloss, M.X., et al., *Microbiota-activated PPAR-gamma signaling inhibits dysbiotic Enterobacteriaceae expansion.* Science, 2017. **357**(6351): p. 570-575.
105. den Besten, G., et al., *The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism.* J Lipid Res, 2013. **54**(9): p. 2325-40.
106. Jacobasch, G., et al., *Dietary resistant starch and chronic inflammatory bowel diseases.* Int J Colorectal Dis, 1999. **14**(4-5): p. 201-11.
107. Alrawi, S.J., et al., *Aberrant crypt foci.* Anticancer Res, 2006. **26**(1A): p. 107-19.

108. Schaue, D., E.L. Kachikwu, and W.H. McBride, *Cytokines in radiobiological responses: a review.* Radiat Res, 2012. **178**(6): p. 505-23.
109. Hong, J.H., et al., *Rapid induction of cytokine gene expression in the lung after single and fractionated doses of radiation.* Int J Radiat Biol, 1999. **75**(11): p. 1421-7.
110. Shukla, P.K., et al., *Rapid disruption of intestinal epithelial tight junction and barrier dysfunction by ionizing radiation in mouse colon in vivo: protection by N-acetyl-l-cysteine.* Am J Physiol Gastrointest Liver Physiol, 2016. **310**(9): p. G705-15.
111. Richter, K.K., et al., *Association of granulocyte transmigration with structural and cellular parameters of injury in experimental radiation enteropathy.* Radiat Oncol Investig, 1997. **5**(6): p. 275-82.
112. Rubin, P., et al., *A perpetual cascade of cytokines postirradiation leads to pulmonary fibrosis.* Int J Radiat Oncol Biol Phys, 1995. **33**(1): p. 99-109.
113. Tanji, N., et al., *Circulating Cytokine Levels in Patients with Prostate Cancer: Effects of Neoadjuvant Hormonal Therapy and External-beam Radiotherapy.* Anticancer Res, 2015. **35**(6): p. 3379-83.
114. Liu, B., et al., *Oat beta-glucan ameliorates dextran sulfate sodium (DSS)-induced ulcerative colitis in mice.* Food Funct, 2015. **6**(11): p. 3454-63.
115. Wilczak, J., et al., *The effect of low or high molecular weight oat beta-glucans on the inflammatory and oxidative stress status in the colon of rats with LPS-induced enteritis.* Food Funct, 2015. **6**(2): p. 590-603.