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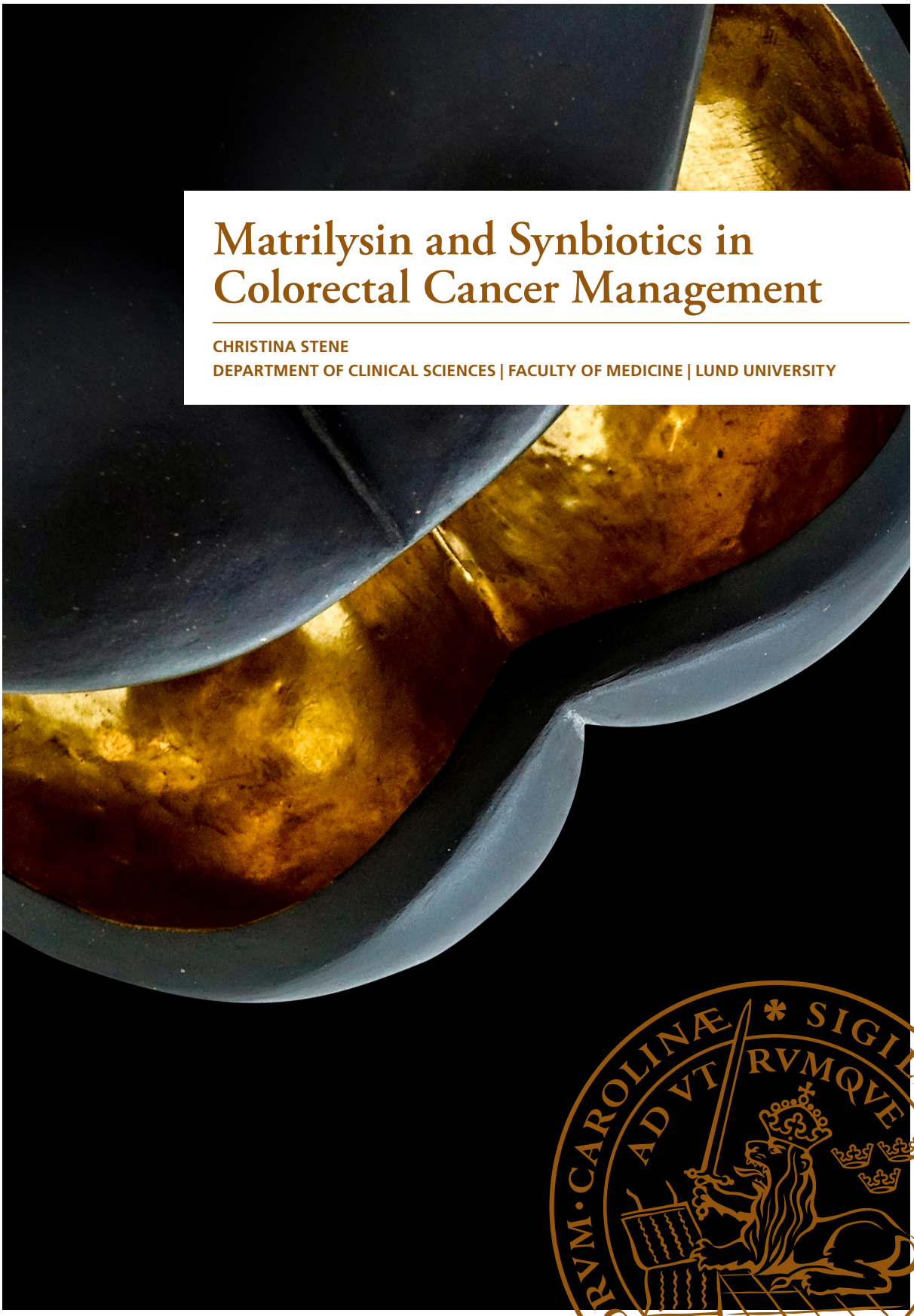
Stene, Christina

2021

Document Version:
Publisher's PDF, also known as Version of record



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Printed by Media-Tryck, Lund 2021 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

FACULTY OF MEDICINE

Department of Clinical Sciences, Malmö

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2021:98
ISBN 978-91-8021-105-5
ISSN 1652-8220

MATRILYSIN AND SYNBIOTICS IN COLORECTAL CANCER MANAGEMENT

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Christina Stene

LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at Agardh-salen, Clinical Research Centre,
Jan Waldenströms gata 35, Skåne University Hospital, Malmö,
on the 1st of October 2021 9.00 AM.

Faculty opponent
Professor Wilhelm Graf
Department of Surgical Sciences, Uppsala University, Sweden

Organization LUND UNIVERSITY	**Document name** DOCTORAL DISSERTATION
Departement of Clinical Sciences, Malmö Faculty of Medicine, Lund University Sweden	**Date of issue** 2021-10-01
Author: Christina Stene	Sponsoring organization

Title and subtitle Matrilysin and Synbiotics in Colorectal Cancer Management

Abstract

Background:

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Aims:

To identify factors of prognostic value in determining disease stage and complications in CRC treatment and to modulate gut microbiota by pre-, pro- & synbiotics to minimise side effects of RT.

Methods:

Studying MMP7 levels in non-irradiated tumours of different grades and in rectal cancer patients undergoing neoadjuvant short- (25 Gy) and long-term (50 Gy) radiotherapy or no irradiation. Examining leukocyte levels and inflammation locally in rectal biopsies of healthy volunteers after probiotic treatment. Investigating inflammatory markers and microbiota diversity in patients undergoing neoadjuvant RT after prebiotic, synbiotic or no pre-treatment.

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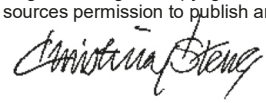
MMP7 increases with dysplasia and disease stage in tumour and regional lymph nodes. MMP7 expression correlates with risk of lymph nodal involvement. Surgery has an overriding effect on the upregulation of MMP7. Neoadjuvant long-term RT induced significantly less MMP7 expression at surgery. Probiotics may reduce inflammatory responses of intestinal mucosa after surgery and/or pelvic irradiation. Pre- & synbiotics enhance bacterial viability and may stabilise intestinal mucosa after radiation injury.

Key words Radiotherapy, Inflammation, Colorectal cancer, Matrilysin, Intestinal microflora, Probiotics, Synbiotics

Classification system and/or index terms (if any)

Supplementary bibliographical information		**Language:** English
ISSN 1652-8220 Lund University, Faculty of Medicine Doctoral Dissertation Series 2021:98		**ISBN** 978-91-8021-105-5
Recipient's notes	**Number of pages** 101	Price
	Security classification	

Signature  Date 2021-08-26

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Cover photo by Katarina Jacobsson, Ystad
Graphical design by Ludvig Jernqvist, Gothenburg
Ceramic sculpture by Sofia Nilsson, Ängelholm

Lund University, Faculty of Medicine
Department of Clinical Sciences, Malmö
Doctoral Dissertation Series 2021:98

ISBN 978-91-8021-105-5
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University, Lund 2021

To Emma & Anna,
lights of my life

Vi får ej välja ramen för vårt öde. Men vi ge den dess innehåll.

...

Insatsen söker oss, inte vi insatsen.

...

Vägen valde dig.

Dag Hammarskjöld, ur ”Vägmärken”

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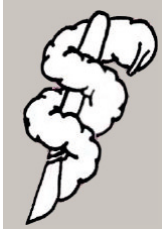
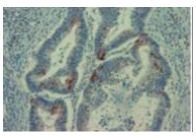
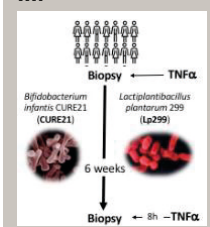
I.  © PROF. I. SYK.	MMP7-expression in different tumour stages	Prospective, descriptive. 28 pat, benign & malignant colorectal tumours. Histology, IHC, ELISA, Western blot. MMP7, tumour	MMP7 ↑ with cancer stage & in reg lymph nodes
II.  © DR. A. POLISTENA	MMP7-expression in different RT-regimens	Prospective, descriptive. 53 pat, ca recti, 3 groups: 25 Gy/50 Gy/control. TMA. MMP7 + different RT/ca recti	Surgery ↑ MMP7, 25 Gy ↑ MMP7
III.  © DR. C. STENE	Local & systemic effects of 2 different probiotics on healthy mucosa and standardized inflammatory injury *in vitro*	Prospective, interventional. 14 healthy volunteers, 2 different probiotics, TNF-α stimulus. Inflam markers incl LPK (blood); IL-6, IL-10, T-RFLP (biopsies); microflora analysis (faeces). Probiotics, healthy mucosa, inflamm injury	Local & systemic ↓ of inflam response, Lp299 ↓ IL-6 in rectal mucosa
IV.  **LACTIPLANTIBACILLUS PLANTARUM HEAL19** © DR. C. STENE	Pre-/synbiotics reduce RT-induced inflammation?	Prospective, interventional. 30 pat, ca recti + RT 25 Gy, 3 groups: pre-/synbiotics/control. T-RFLP, MPO, histology (biopsies); calprotectin (faeces); inflam markers, zonulin (blood). Pre-/synbiotics + RT, ca recti	Progressive inflam, fibrosis in control group. Pre- & synbiotics → ↑ bacterial diversity

List of original papers

This thesis is based upon the following original articles and manuscripts. In the text of the following chapters these papers and manuscripts will be referred to by their Roman numerals in the text.

I. Polistena A, Cucina A, Dinicola S, Stene C, Cavallaro G, Ciardi A, Orlando G, Arena R, D'Ermo G, Cavallaro A, Johnson L B, De Toma G. MMP7 Expression in Colorectal Tumours of Different Stages. *In Vivo.* 2014; 28:105-110.

II. Stene C, Polistena A, Gaber A, Nodin B, Ottochian B, Adawi D, Avenia N, Jirström K, Johnson L B. MMP7 modulation by short-term and long-term radiotherapy in patients with rectal cancer. *In Vivo*. 2018; 32: 133-138.

III. Stene C, Röme A, Palmquist I, Linninge C, Molin G, Ahrné S, Johnson L B, Jeppsson B. Administration of Probiotics to Healthy Volunteers – Effects on Reactivity of Intestinal Mucosa and Systemic Leukocytes. *Submitted.*

IV. Stene C, Håkansson Å, Palmquist I, Molin G, Ahrné S, Thorlacius H, Johnson L B, Jeppsson B. Synbiotics in Radiation Injury and Rectal Cancer Treatment – a Controlled Trial. *Manuscript.*

Published peer-reviewed papers not included in this thesis

Håkansson Å, Stene C, Mihaescu A, Molin G, Ahrné S, Thorlacius H, Jeppsson, B. Rose Hip and Lactobacillus plantarum DSM 9843 Reduce Ischemia/Reperfusion Injury in the Mouse Colon. *Dig Dis Sci.* 2006; 51:2094–2101.

Gaber A, Stene C, Hotakainen K, Nodin B, Palmquist I, Bjartell A, Stenman U-H, Jeppsson B, Johnson L B, Jirström K. Effects of radiation therapy on tissue and serum concentrations of tumour associated trypsin inhibitor and their prognostic significance in rectal cancer patients. *Radiation Oncology.* 2011; I: 6.

Abstract

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Conclusions: MMP7 increases with dysplasia and disease stage in tumour and regional lymph nodes. MMP7 expression correlates with risk of lymph nodal involvement. Surgery has an overriding effect on the upregulation of MMP7. Neoadjuvant long-term RT induced significantly less MMP7 expression at surgery. Probiotics may reduce inflammatory responses of intestinal mucosa after surgery and/or pelvic irradiation. Pre- & synbiotics enhance bacterial viability and may stabilise intestinal mucosa after radiation injury.

Abbreviations

CEA	Carcinoembryonic antigen
CFU	Colony forming unit
CRC	Colorectal cancer
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
FMT	Faecal microbiota transplant
GI	Gastrointestinal
Gy	Gray (SI unit of absorbed radiation)
LR	Local recurrence
MMP	Matrix metalloproteinase
MMP7	Matrilysin, matrix metalloproteinase 7
MPO	Myeloperoxidase
RT	Radiotherapy
SCFA	Short chain fatty acids
SCRCR	Swedish Colorectal Cancer Registry
TGF-β	Transforming growth factor beta
TMA	Tissue Micro Array
TNM	Tumour, Node, Metastasis (UICC [Union Internationale Contre le Cancer; Internationella Cancerunionen]: The TNM Classification of Malignant Tumours)

Non nocere, numquam curae, saepe opem, semper consolare.
Never harm, sometimes cure, often relieve, always comfort.
Aldrig skada, ibland bota, ofta lindra, alltid trösta.

Hippokrates, 460-370 f.Kr.

General background

Colorectal cancer

Epidemiology

Cancer incidence and mortality is increasing worldwide, and cancer is expected to constitute the leading cause of death and the single most important barrier to increasing life expectancy in every country of the world in the 21st century.[1]

Colorectal cancer (CRC) represents, in a global perspective, the third most common diagnosed cancer form in men and the second most common diagnosed cancer form in women, accounting for 1.8 million new cases and almost 900 000 deaths every year, constituting around 10% of cancer incidence and mortality worldwide.[1-3]

Countries in the western world, e g Europe, Japan, North America, Australia, and New Zeeland, show the highest incidences of CRC, with a ten-fold difference between countries of high and low incidence, respectively.[1, 2, 4] It should be noted that high-quality cancer registry data are not available in most of the world's low- and middle-income countries. In Africa and southern Asia, the incidence can be up to six times lower reflecting social and economic development, thus rectal cancer can be regarded as an indicator of socioeconomic development level.[1, 5, 6] In several highly developed countries, decreasing incidence rates as well as mortality rates are seen, in contrast to findings in many low- and middle-income parts of the world.[6]

In Sweden, CRC is the third most common cancer form, with the number of cases steadily rising in the last five decades due to both ageing and growth of population. In 2019, every 9th patient affected with a malignancy in Sweden was diagnosed with CRC.[7] In 2019, a total of 6754 persons were diagnosed with CRC; 69% of the diagnosed tumours were situated in the colon, and 31% in the rectum, resulting in 4645 patients with colon cancer and 2109 patients with rectal cancer that year.[8] Rectal cancer itself constitutes the eighth most common cancer in Sweden.[8] The median age at diagnosis for rectal cancer in 2019 was 72 years for women and 71 years for men.

CRC is more frequent in elderly patients. During 2012-2016, only 4% of all colon cancer and 5% of all rectal cancer was diagnosed in patients under 50 years of age.

During the same period, 29% of colon cancer patients and 21% of rectal cancer patients were 80 years or more at diagnosis.[8]

Colon cancer is evenly distributed between men and women, whereas rectal cancer has a male preponderance with 25 per 100 000 men (62%) and 15 per 100 000 women (38%), with a male mortality rate of 10 per 100 000 and female mortality rate of 5 per 100 000.[8] The incidence of rectal cancer increased in Sweden between 1970 and 2007 (from 15 to 25 new cases per 100 000 inhabitants for men and from 11 to 18 new cases per 100 000 inhabitants for women).[7] The risk of developing CRC in Sweden is 5%.[8]

In Scandinavia, with the exception of Denmark, CRC patients have high survival rates compared to the rest of Europe.[9, 10] A plausible explanation for lower survival may be the selection of patients with more advanced disease and high age for surgical treatment in Denmark.[11]

According to the Swedish Colorectal Cancer Registry, the five-year-survival in Sweden for colon cancer is 54% (56% for women, 53% for men). For rectal cancer, the five-year-survival increased from 48% in 1995 to 58% in 2014 (61% for women, 57% for men).[8]

Carcinogenisis

Carcinogenesis is a multistage process involving modification and mutation to genes that regulate normal cellular function and cell growth control processes.[12] The cells are uncontrollable with regard to cell signalling, and inhibitory signals do not evoke cell apoptosis as scheduled.[13] The conversion from healthy tissue into cells with malignant potential can take between 10 and 35 years and is one explanation of why CRC is more predominant among patients of higher age.[14, 15]

The majority of CRC tumours are derived from adenomatous polyps; in the western world about 25% of people between the ages of 50 and 79 years develop polyps in the colon and rectum, but only a small percentage of the polyps develop into cancer.[16] The adenoma-carcinoma sequence is a process that takes years to decades.[17-20] Of patients with initially curable treated disease, 30-40% finally present with metastatic disease.[21]

Risk factors

Colorectal cancer has a multifaceted and heterogenous aetiology that has been controversial in many respects, although the predominant risk factor, as for many other malignancies, is increasing age.[8] Male gender, earlier colorectal polyps or malignancies, hereditary genetic disorders, and inflammatory bowel diseases as well as environmental factors are linked to high risk for CRC.[22-24]

CRC incidence and mortality rates are linked to socioeconomic development, rising with increasing Human Development Index.[5, 25] It has been shown that people moving from low to high CRC incidence areas have an increased risk for CRC in the new region in less than a generation.

Inflammatory bowel disease has been shown to constitute a strong risk factor for CRC, especially for ulcerative colitis (UC) where 8% of the patients develop CRC within twenty years after their UC diagnosis.[26, 27] However, both UC and Crohn's disease are more associated with cancer in the rectum than with tumours in the colon.[28-30]

An increased risk for CRC has been demonstrated for lifestyle factors such as smoking and obesity, the latter mainly among men and the former more correlated to rectal cancer.[31-36] Dietary factors have been intensely debated, and convincing evidence that processed meat as well as alcohol and body fatness increase CRC risk has recently been presented.[37, 38] The link between the individual's metabolic status comprising adiposity, physical activity, and diseases such as diabetes mellitus, has been deeply investigated and shows a strong association with cancer of the colon and rectum.[33, 39]

Recent studies demonstrate reduced risk for CRC by a high intake of dietary cereal fibre whereas a high intake of fat, red and processed meat as well as alcohol has shown to be associated with an increased risk of CRC.[40-43]

Hereditary genetic alterations constitute nearly 5% of CRC and are most predominantly seen in two forms of CRC, the most frequent being Lynch syndrome (LS), an autosomal dominant non-polyposis hereditary condition. A mutation of the DNA repair systems is a consequence of the genetic aberration. The second form of precancerous hereditary syndrome is familial adenomatous polyposis (FAP), where an autosomal dominant mutation in the adenomatous polyposis coli (APC) gene is the cause.[18] These individuals are diagnosed with numerous polyps in the colon and rectum, predisposing to CRC development.[44, 45] FAP patients are at risk for extracolonic manifestations and although FAP is not as frequent as LS, the patients with FAP have an almost 100% risk of developing CRC.[46]

Diagnosis of rectal cancer and clinical staging

Rectal cancer is, by definition, an adenocarcinoma of mucosal origin with its distant border situated within 15 cm from the anal verge when examined by rigid rectosigmoidoscopy.[4, 47, 48]

A change in bowel habits is usually the initial symptom of rectal cancer whereas rectal bleeding is the most predominant symptom that takes the patient to health care providers. Localized rectal pain and obstruction are usually findings of late disease stage.[49] This may be complicated by fistulas to vagina, urinary bladder or urethra which might be presenting symptoms of the tumour.

Digital examination reveals around 75% of the tumours localized in the lower 2/3 of the rectum. This initial investigation is important to assess the size, fixation and/or mobility of the tumour and should be followed by a rigid rectosigmoidoscopy for biopsy and for determination of tumour height. A colonoscopy, if the tumour is not stricturing, is mandatory to exclude synchronous tumours. The diagnostic procedure is completed with a computerized tomography (CT) of chest and abdomen and magnetic resonance imaging (MRI) of the rectum and adjacent organs, whereas for early tumours, endorectal ultrasound (ERUS) is the examination of choice.[50-55] In a few selected institutions, enhanced MRI and specialist competence is said to be equally as good as ERUS.

Assessment of lymph node involvement remains a challenge for preoperative investigation. Positron emission tomography (PET), where a radioisotope is used to "label" tumour tissue which has a higher metabolism, combined with a CT scan to localize the pathological uptake, has high sensitivity for showing lymph node involvement.[56, 57]

CRC classification systems have been developed throughout the years within various cancer societies, but since 1987 the Tumour-Node-Metastasis (TNM) system has been used by the American Joint Committee on Cancer (AJCC) as well as by the Union International Contre le Cancer (UICC).[58, 59] In the TNM-staging, T represents tumour stage (i e the histological extent of tumour growth and depth of invasion of the bowel wall), graded T1-T4; N represents nodal stage (involvement of regional lymph nodes), graded N0-N2; and M represents distant metastasis (including non-regional lymph nodes), graded M0-M1, whereby M-stage is the most important prognostic factor.[60, 61] To define the type of staging done, different prefixes are used: c for clinical data, p for pathological data, y for RT and/or chemotherapy prior to staging, r for retreatment due to recurrence/disease progression, and a for autopsy data. The TNM system is subject to continuous update, the latest, 8th, edition was issued in 2016.[62]

Prognostic factors have been reported as differentiation grade of the tumour with the majority of CRC tumours being moderately differentiated.[60, 63, 64] Tumour cells found within lymphatic vessels and veins, and at perineural invasion, are coded as follows: lymphatic vessels, L, (X, 0, 1), veins, V, (X, 0, 1, 2) and perineural invasion, PI, (present, absent, not recorded), respectively.[60, 65-67]

Table 1.
5-year survival Sweden 2019 (SCRCR)

Stage	5-year survival
I	81%
II	72%
III	59%
IV	15%

Adjuvant chemotherapy has shown a proven role in the therapy for stage II and in particular stage III colon cancer but is still debated in the multimodal management of rectal cancer where surgery alone is believed to cure 80% of stage II patients.[68, 69]

Age and gender are other established prognostic factors to take into consideration when making clinical decisions. Studies have shown that younger patients tend to have more aggressive tumours; however younger patients are more prone to tolerate a more aggressive chemotherapy and moreover demonstrate better survival rates.[70, 71] Although there is no difference in CRC lifetime risk for men and women, males are diagnosed with CRC earlier in life and have a higher postoperative mortality.[72-74]

Tumour biomarkers e g carcinoembryonic antigen (CEA) have been used in clinical practice for detection of recurrent disease in CRC patients.[75] Elevated CEA has been found to correlate with worse prognosis in CRC.[76, 77]

Multidisciplinary team (MDT) meetings

After investigations are done and tumour staging performed, the ensuing treatment is individually determined preoperatively for each patient by a multidisciplinary board consisting of surgeons, radiologists, pathologist, oncologists, and specialized nurses. The MDT meeting has proven to constitute a prominent means to reach the most favourable treatment strategy for each individual patient and is strongly recommended.[78, 79] Rectal cancer patients discussed in MDT meetings have shown better success rates with lower rates of circumferential resection margin (CRM) involvement (1% vs 26% non-MDT).

After surgery the pathological assessment is evaluated by a new MDT meeting, taking into consideration the clinical outcome for the patient, to decide if any postoperative (adjuvant) treatment would be of value to the individual patient and how the follow-up program is to be undertaken.

Surgical methods

Surgery is the principal treatment for rectal cancer. Of the 2109 patients that were diagnosed with rectal cancer in Sweden in 2019, 68% underwent surgical resection.[8] The location of the tumour in the rectum determines the choice of surgical procedure.

Surgery is always associated with a risk for complications. The Swedish Colorectal Cancer Registry shows a postoperative complication rate of about 40%, with the highest risk being for patients undergoing neoadjuvant treatment.[8]

Local excision may be undertaken in cases of low-risk T1 tumour in rectum,[4, 80, 81] in elderly patients or in patients with multiple concomitant diseases. It may also be an alternative if the malignant diagnosis is not confirmed, as an alternative to surgical resection. The two predominant methods are endoscopic submucosal

dissection (ESD) and transanal endoscopic microsurgery (TEM) resulting in lower morbidity rates, though with an increased risk for local recurrences.

The majority of patients undergo abdominal surgery, either anterior resection, abdomino-perineal resection or Hartmann's procedure.

Amputation of the rectum was introduced by Lisfranc in Paris 1826, resulting in a perineal colostomy. In London in 1908, Miles devised the abdominoperineal excision, influencing rectal surgery for decades, now in use for tumours that endanger the levator muscles and sphincter complex. It is the most extensive procedure, where the distal colon is completely removed as well as the rectum and anal sphincter complex, resulting in a permanent abdominal stoma.[82, 83] Abdominoperineal resection (APR) constituted around 38% of surgeries for rectal cancer in Sweden in 2019.[8]

Since Miles' procedure was afflicted by high mortality rates, a less invasive method with only around 8% mortality was presented in Paris in 1921 by Hartmann.[84, 85] The rectum is resected, and an end colostomy formed, thereby avoiding anastomosis and its connected risks. Hartmann's procedure accounted for approximately 14% of Swedish rectal cancer surgery in 2019.[8]

The most frequently performed surgical procedure for rectal cancer in Sweden in 2019 is the anterior resection (44%), introduced by Dixon at the Mayo Clinic in 1948. It is intended for tumours of the upper and mid rectum,[8] and in special cases for some tumours in the lower rectum. This technique was facilitated by the introduction of the circular stapler technique in 1972.[86-88] Intestinal continuity through an anastomosis resulted in possible avoidance of a colostomy, with improved quality of life for the patient.[89-91] The anastomosis could be created in any of three ways: i) intestinal ends merged end-to-end primarily for tumours in the upper third of the rectum, ii) a side-to-end anastomosis, or iii) a J-pouch (where the proximal bowel end is folded onto itself), used for lower situated tumours aiming to replicate a reservoir function in more distant resections of the rectum.

The introduction of total mesorectal excision (TME) by Sir Bill Heald in Basingstoke in 1982 led to a reduction of local recurrences from around 20% to around 4%.[92, 93] This technique states that the embryological planes which engulf the mesorectum should be followed and respected during dissection. The aim is to take out the bowel with the tumour and its surrounding tissue (the mesorectum) *en bloc*, and thus to reduce the risk of local recurrences.[94]

Any of the three procedures may be performed by open surgery or minimally invasive techniques (laparoscopy with or without robotic assistance) and have shown comparable oncological outcome; however reduced peri- and postoperative implications such as reduced blood loss, decreased postoperative pain and shorter length of hospital stay were seen in the minimally invasive techniques group. [95-98] In Sweden in 2019, 32% of the patients underwent open surgery, 41% underwent

minimally invasive procedures with robotic assistance, and 26% with laparoscopic assistance.

To minimize surgical complications and postoperative morbidity a "watch-and-wait"-concept was initiated in Brazil, where radiotherapy is the mainstay treatment aiming for complete pathological remission with active surveillance of the patient.[99, 100]

Radiotherapy

Radiotherapy (RT) has been instrumental as an adjunct in rectal cancer management for the past thirty years. The use of neoadjuvant short-course RT, 5 x 5 Gray (Gy), a treatment strategy developed in Sweden, is now routine in many countries worldwide and constitutes a core of the multimodal therapy for rectal tumours.[4]

Radiotherapy is used alone or in conjunction with other therapeutic modalities to treat malignant conditions with curative or palliative intention. The maximum applied dosage in clinical use is strictly limited by potential side-effects occurring to normal surrounding organs.[101] The risk of injury to the healthy surrounding tissues limits the radiation dose that can be safely delivered to a tumour.[102]

The goal of the given RT is to achieve as little short-term harm and as much long-term benefit to the largest extent possible. This means to destroy as many tumour cells as possible without harming surrounding normal tissue cells. The mode of action is possible for two reasons: i) the high mitotic activity of tumour cells with a higher proliferation rate giving increased sensitivity to RT; ii) the impaired ability of tumour cells to heal from RT-induced injury. Fractionated RT is thought to be a means of protection of non-tumoral tissue, with the intention of having cells that are able to undergo restorative processes in between radiation doses. RT schedules are continuously being evaluated to optimise tumour destruction and to minimise damage to healthy surrounding tissue, as there is a correlation between volume and dose as well as fraction and intervals of the treatment. Some studies have shown less impact of long-course RT compared to short-course RT in aspects of impairment of the inflammatory response and tumour progression.[103, 104]

Clinical application of radiotherapy in rectal cancer

RT has played an important role in the management of rectal cancer. Symonds reported in 1914 the use of radium bromide in achieving complete tumour remission in a rectal cancer patient.[105-107] The technique was however mainly used until the end of the century for reducing the incidence of pelvic recurrences.

Improved techniques and advanced research have resulted in various procedures for administration of radiation to rectal cancer patients, e g irradiation by external beam or endocavitary means as well as intraoperative therapy (IORT), brachytherapy or combinations.[108] In resectable rectal cancer, this can be given as neoadjuvant (preoperatively) or adjuvant (postoperatively) treatment. Some studies have shown that the effect of RT is optimal when administered preoperatively.[109]

Neoadjuvant treatment with RT and/or chemotherapy for localized rectal cancer can, dependent on the clinical situation, be administered with different intentions: either to shrink the tumour to facilitate radical surgery and enable organ-preserving surgery or to prevent local recurrences through treatment of locoregional micro-metastases.[108] Only trials using a dose greater than 20 Gy for neoadjuvant RT have given lower recurrence rates compared to surgery alone.[110]

Local recurrences (LR) used to be a major clinical problem following surgery for rectal cancer with a recurrence risk in up to one third of the patients treated. The combination of improved or enhanced surgery with neoadjuvant RT has led to a considerable reduction of LR during recent decades. Incidence of LR within 5 years is decreasing and is now below 5% for RT-treated as well as for non-RT-treated patients. LR are decreasing for all stages of disease.

In Sweden, the introduction of the total mesorectal excision technique (TME) in 1982 resulted in the reduction of LR from about 15% to 4%.[8, 92] Studies show that neoadjuvant RT 5 x 5 Gy reduces the risk for LR by 50-70%.

Since the beginning of the 1990s, RT has been increasingly used as an adjunct to surgery in rectal cancer treatment, in order to achieve enhanced local destruction of microscopic tumour growth in the surrounding tissue aiming to bring the rate of local recurrences down and raise cancer specific survival.[111-118] It may be administered as short-course RT 25 Gy (5 x 5 Gy), or as long-course RT 40-50 Gy (1.8-2 x 25 Gy) for advanced tumours, often with concomitant chemotherapy.[49] The number of patients receiving neoadjuvant RT varies considerably between different countries. In Sweden, 60–65% of rectal cancer patients will receive some form of neoadjuvant RT, with only small differences between the different regions.[8, 119] The majority of these will receive 5 x 5 Gy, while around 25% of irradiated patients will receive chemotherapy in addition, for better oncological outcome.

Studies have shown that downstaging and local control are increased when the surgical procedure is not performed until 6 to 8 weeks after completion of long-course RT.[120-123] In recent years, delayed surgery also after short-course RT is well sustained and proved to result in excellent oncological results.[124] In Sweden the approach varies between different regions; some regions still operate within 2-4 days after completed RT whereas other regions practise delayed surgery in the 4-8 weeks after completed 5 x 5 Gy RT for primary resectable tumours.[4]

However, RT is not infrequently complicated with late adverse effects, making careful selection of patients for neoadjuvant treatment very important. The Swedish Colorectal Cancer Registry (SCRCR) has a record of 40% postoperative complications with the highest risk being for patients receiving neoadjuvant RT.[8] In recent years, the combination of chemo- and radiotherapy has been on the increase to achieve better treatment outcomes and one can thus expect further systemic and local gut intestinal effects.

Three different therapeutic choices are in principle at hand for neoadjuvant RT to rectal cancer patients:

The "low risk (good)" group, where the tumour is restricted to the intestinal wall without signs of spread; therefore neoadjuvant RT may be abstained and surgery could be performed without any neoadjuvant RT. This group constitutes around 20-40% of the patients. These tumours are classified as favourable or good as the risk of LR is low and neoadjuvant RT is regarded as overtreatment.

The "intermediate risk (bad)" group, where the tumour growth has penetrated the intestinal wall and/or signs of lymph node metastases are found. In this case around 40–60% of the patients will receive short-course neoadjuvant RT 5 x 5 Gy in order to reduce the risk for future local recurrences. These patients have usually been subject to surgery the week after completed RT. After the promising results of the RAPIDO study, there is now a trend to give short-course RT 25 Gy combined with chemotherapy and delayed surgery.[125]

The "high risk (ugly)" group, with locally advanced tumours, representing almost 40% of the irradiated patients, will receive long-course neoadjuvant RT to achieve tumour shrinkage to enable or facilitate radical surgical procedure, usually undertaken after 6-8 weeks. This treatment is nowadays almost always combined with concomitant chemotherapy. These strategies are subject to continuous development and reassessment, and there are recommendations for applying short-course neoadjuvant RT combined with chemotherapy even in this group.

For fragile patients or patients with massive comorbidity, estimated not to withstand chemoradiotherapy, 5 x 5 Gy with delayed surgery is a standard alternative. Palliative RT is otherwise indicated for a certain group of patients with rectal cancer not amenable to surgery because of comorbidity or for advanced extra-pelvic disease. It may also be effective to use with palliative intention to reduce pain or bleeding.[108, 117]

In selected cases, where no remaining tumour can be detected after neoadjuvant therapy (i e complete clinical and pathological response), a conservative nonsurgical treatment strategy entitled "watch-and-wait" may be used, as mentioned above, though the scientific support for this approach, outside of clinical trials, is still not well established.[99, 100, 126-128] This concept is to avoid surgery and rather monitor patients carefully through clinical investigations including local rectal and endoscopic examination as well as MRI of the rectum. Surgery is undertaken if signs of local

tumour growth are found. There is an ongoing national Swedish study to register and evaluate patients with complete clinical remission after neoadjuvant therapy.[129]

Radiation injury to the gut

Pelvic radiotherapy is an important treatment modality for cancer patients. However, radiotherapy of pelvic organs is associated with severe and often dose-limiting side-effects on the intestines. The pathophysiology of radiation-induced gut injury is not yet fully clarified beyond that it is a multifaceted interaction between the injured epithelium and changes in the intestinal vascular, nervous, and immune systems, preferentially in persons with a genetic predisposition. The ionizing radiation causes an acute inflammatory injury to the gut and a subsequent fibrosis, appearing months to years later. Although modern techniques for RT have decreased the post-irradiation symptoms demonstrated in studies from the 1980s-1990s, a more frequent use of RT in the future for malignant diseases including gynaecological and urological tumours as well as prostate, rectal and anal cancer, will increase the burden of adverse effects with unfavourable gut reaction.

The small intestine is a crucial dose-limiting organ for abdominopelvic RT due to its high radiosensitivity, since gut cells divide rapidly and are thus extremely vulnerable to radiation-induced damage. The turnover rate for the villi of the small intestine is 1.5 days, and for colonic epithelial cells 4.2 days, which put them in the leading position of the body's fastest reproducing fixed tissue cells, thus making the gut one of the most susceptible organs to radiation injury.[130, 131] The result of gut irradiation is determined by the concentration of ionizing radiation leading to diminished integrity of the tight junctions and epithelial cell death in the crypts as well as higher up the villi.[131-134]

The organs most at risk during rectal cancer irradiation besides the small intestine are the anal sphincters as well as the bladder, nerves, and genital organs. Traditionally irradiation damage to the intestinal mucosa has been categorized into two entities: acute radiation injury in which atrophy of crypt and mucosa is characteristic; and subacute or late injuries where inflammatory cell infiltration, vascular destruction and fibrosis are prominent.[135-137] One considerable problem following RT is an acute irradiation-induced intestinal inflammation. Starting within hours after irradiation, an early inflammatory reaction including fibrin and protein deposition and oedema in the gut mucosa is seen.[138, 139] The heights of villi are reduced successively leading to lessened intestinal area, thereby reducing mucosal ability to absorb conjugated bile salts, water, electrolytes, and proteins. This in combination with an altered local gut microbiota that deconjugate bile salts may result in chologenic diarrhoea.[140, 141] Microvascular injury, endothelial and epithelial cell apoptosis, as well as recruitment of platelets and leukocytes, are early

reactions to irradiation. Later, deeper layers of the intestinal wall are the focus for accumulation of connective tissue. As a result of these enduring fibrotic alterations, stricture formation may occur, resulting in intestinal obstruction.[142]

A novel comment on chronic radiation changes on rectal mucosa declares that this state might be divided into two distinct units due to its presentation and underlying pathology: either radiation-associated vascular ectasis, RAVE, or the established denomination Chronic Radiation Proctopathy, CRP.[143, 144] However, in some patients these may overlap.[143]

Irradiated rectal cancer patients are at a 50% increased risk of impaired anal function after an anterior resection.[145] If this anal incontinence is due to direct irradiation towards the anal sphincters or is affected by radiation of the remaining rectal stump or pelvic nerves and vessels is not known. Most studies are performed on sphincter-irradiated patients. However, it is important not to irradiate the sphincter complex if the tumour is not as distant, as an APR is necessary. RT to the pelvic region is reported to induce changes in the bowel habits of 90% of the patients, with half of all patients reporting that quality of life is significantly adversely affected, and that serious complications can persist decades post treatment completion.[146-149]

Radiotherapy is used in an elective setting, which makes knowledge of pathophysiology a basis for prevention. How and in what order the pathological alterations occur is still not fully elucidated but important events include:

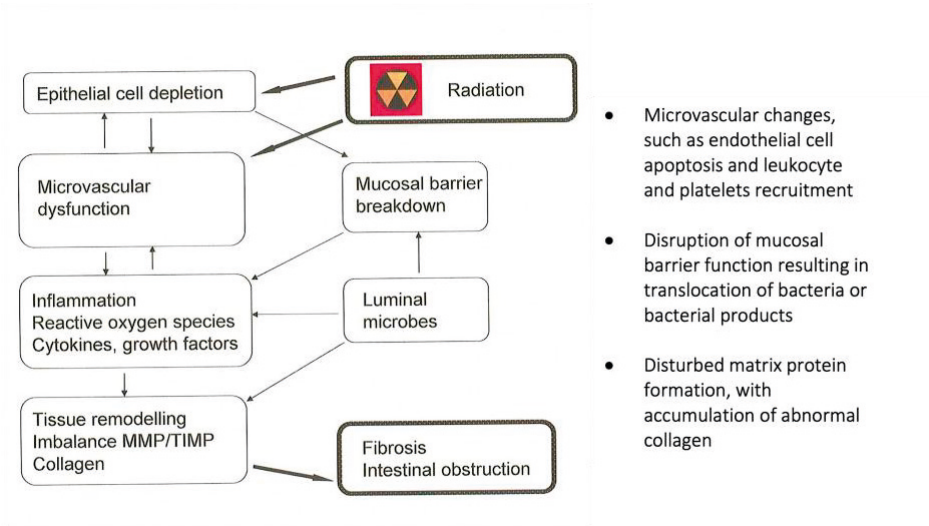

Figure 1.
Plausible important events in pathophysiology of radiotherapy-induced intestinal injury.

In this process there are a number of different procedures regulating the fibrotic response to radiation, including growth factors, metalloproteinases, angiotensin II and protein kinases.

The fibrogenic cytokine transforming growth factor beta (TGF-β), which is situated in the epithelial cells as well as in lamina propria, submucosa, subserosa, and smooth cells, induces collagen synthesis and is associated with radiation-induced fibrosis.[103] A decrease in TGF-β in the first postoperative week and a subsequent increase later on is seen following treatment with radiotherapy and surgery, as well as after chemotherapy and surgery, respectively.[150, 151] One hypothesis is that overwhelming inflammation in response to radiation therapy provokes excessive platelet recruitment and TGF- β deposition in the intestine, which may be a significant mechanism behind radiation-associated fibrosis.

Radiation activates early various cellular signalling pathways leading to activation and expression of pro-inflammatory and pro-fibrotic cytokines, vascular injury, and activation of the coagulation cascade. Mucosal cytokines are activated and the levels of tumour necrosis factor alpha (TNF-α), interleukin 1beta (IL-1β), IL-2, IL-6, and IL-8 are found to be significantly higher.[152]

In the extracellular space matrix degradation occurs predominantly as a consequence of the action of a family of enzymes called the matrix metalloproteinases (MMPs), described in more detail later in this thesis. It has been reported that radiotherapy induces expression of MMP-2, -7, and -9, indicating a potential role in fibrogenesis following radiotherapy.[153] TGF-β increases the expression of MMP-2 and -9. Bacterial exposure of mucosal epithelial cells also induces and activates MMPs.[154] In chronic pouchitis, increased expression of MMP-2 and -9 has been observed, concomitant with proinflammatory cytokines. MMP expression was found to be reduced after therapy of the acute pouchitis with probiotic bacteria that reduced the inflammation.[155] It is not fully elucidated if a similar effect can be observed in radiation enteropathy.

Histopathological changes

Histologically detectable alterations of the intestinal mucosa such as protein and fibrin precipitation, inflammation and oedema of the bowel wall can be found after radiation.[138, 139] Early findings are disruption in vascular architecture leading to microhaemorrhage and cascades of pro-thrombotic and pro-inflammatory events resulting in further inflammation, vascular occlusion and reduced red blood supply leading to ischaemia.[156] Fibrosis sets in with obliterating vasculitis, atrophy, degeneration of muscle fibre, and morphological changes in fibroblasts, endothelial and epithelial cells in a later phase.[157]

Microscopic findings during the early phase of ischaemia include mucosal oedema and pallor, congested vessels, and denuded cells with separation of surface epithelia

cells from the underlying basement membrane. As the ischaemic changes advance, marked ulcerations are seen as the superficial part of the mucosa will be necrotic while the deeper crypts are saved, but atrophied and mucin depleted. Gradually the number and size of the crypts decrease, and fibrosis appears in the lamina propria until necrosis and ulcerations, along with granulation tissue development, extend into the submucosa and muscularis mucosae. In the hyalinised lamina propria, hemosiderin and oedema due to haemorrhage are seen, and abscesses in the crypts may be present, accompanied by pathological changes in vessels e g necrotizing phlebitis or capillary microthrombi.

In chronic gut injuries, mucosal atrophy, submucosal fibrosis, and stricture formations are found.[158, 159] These enteropathic alterations are visualized by chronic inflammation and impaired motility.[131]

Radiation-associated morbidity

Pelvic irradiation results in an inflammatory injury to the intestinal mucosa and thereby following morbidity in the short run as well as in the long run: impaired wound healing, perineal wound infections, postoperative abscesses, fistulas, bowel obstruction, perforation, bleeding, diarrhoea, sexual dysfunction, urinary dysfunction, anal sphincter damage, osteoradionecrosis, cardiovascular and thromboembolic events, and peripheral nerve injuries as well as functional bowel disorders such as urgency, faecal incontinence, abdominal pain, flatulence and faecal disturbances.[145, 160-164] Intestinal obstruction (5-11%), ileus (5%), intestinal perforation as well as genitourinary and anal dysfunction and fractures may appear.[110] Birgisson reported a 7-8% increased risk for late intestinal complications such as ileus or subileus shown to appear first 6-8 years after surgery.[145] After 13-15 years of follow-up, this risk doubled to 12-14%.

Some severe postoperative complications after chemoradiotherapy, or RT alone, include anastomotic leakage or impaired perineal and pelvic healing. Clinical studies have demonstrated around 28% of anastomotic leakage in cases where no defunctioning stoma is constructed along with the primary anastomosis during the surgical procedure for rectal cancer surgery.[165-167] Up to 50% of irradiated patients experience long-term chronic gastrointestinal side-effects to a degree that affects their quality of life.[146]

As radical surgery is a prerequisite for good oncological results in locally advanced rectal cancer, urinary and sexual difficulties are consequences of resected vessels and pelvic nerve bundles.[168] Sexual function may be influenced by radiation as well, in women and in men and an effect on testosterone production could be seen, especially if the tumour is distant located and irradiation of the testicles is difficult to avoid and a high risk for terminated sperm production or ovarian function is

present.[4, 169, 170] There are ongoing studies to investigate postoperative sexual function in both male and female patients following major pelvic surgery.

A risk for secondary malignancies has been seen during long-term follow-up mainly within the irradiated field but also at other locations. Predominant locations were colon, the prostate and urinary bladder, but a trend was seen also in tissue beyond the irradiated field. The risk for radiation-induced secondary cancer was reported in a Swedish study as 7% but nearly twice as high for RT-treated patients compared to non-RT-treated patients.[171] Some recent studies have not been able to confirm this.[172-174]

Radiation and microflora

The gut microbiome denotes the entire genetic material harboured by gut microbiota, a population of microorganisms that inhabit the luminal space as well as mucosal surface of the human GI tract. These terms are however not always distinguished. There are up to 10^{14} microbes in every human being, together accounting for around 2 kg of the body weight, thus more than the weight of the brain and possessing a metabolic capacity equal to that of the human liver.[175, 176]

Each individual person has their own unique configuration of microbiota, which may however be modified as a response to alterations in environment, pharmacological treatment, and state of health. Various bacteria dominate the gastrointestinal microbiota, but other microorganisms like viruses, fungi, archaea, and protists are also represented. The method of child delivery constitutes an important factor for the initiation of instant establishment of microorganisms in the human GI tract.[177, 178] Vaginally born infants are, during the passage through the birth canal, exposed to their mother's vaginal and intestinal microbiota and, as a result, microbes are transmitted from mother to child. A less diverse microbiota, due to deprived connection with the maternal vaginal microbiota, is found in infants born by Caesarean section, who get their first microbes from the surrounding milieu in the operating room, such as nursing and operation staff, equipment, and air. The personally profiled microbiota is accomplished around the age of three; thereafter it remains quite unchanged for the rest of the individual's life.[179, 180]

Besides participating in the control of the immune as well as neurological and endocrine systems, microbiota aid in absorption of nutrients and digestion.[181] Fermentation of degraded polysaccharides is a process resulting in production of short-chain fatty acids (SCFA) e g butyric acid and propionic acid, a fundamental source of energy for colonic epithelial cells as well as for hepatocytes.[37] Producing vitamins (vitamin K and several B-vitamins) is another essential mission for gut microbiota as well as affecting and regulating intestinal motility.[182, 183]

Microbiota challenge pathogenic bacteria for nutrients, thereby preserving the gut barrier function along with improving the competence of mucosal immune system defence mechanisms, demonstrated in studies where germ-free mice who showed decreased gut epithelial cell turnover rate had considerably impaired intestinal immune system reaction.[184] A genetic predisposition has been shown to be associated with the radiosensitivity of an individual.[185] However, recent research advocates that the individual's genetic background is secondary to the impact of environmental factors on the microbiome.[131, 186, 187]

Beyond this, the intestinal microbiota is linked to the brain by the "gut-brain axis", a two-way interaction comprising the central nervous system as well as the peripheral nervous system and gut microbiota.[188, 189]

Intestinal good health emerges from a steady state when a homeostasis exists between microbiota and host, with favourable outcome for both parts. However, when this equilibrium is disturbed, e g by dietary changes, infections and/or antibiotic treatment as well as surgical procedures, the microbiota constitution comes to an imbalance that may cause an adverse course of events, and a dysbiosis develops. Most often this is due to a decreased diversity of microbiota and is commonly seen in gastrointestinal disorders such as inflammatory bowel diseases.[190-194]

Gut microbiota has been found to manage bowel disorder procedures that follow radiation-induced injury.[195] Germ-free mice have a radioresistant small intestine and survive toxic doses of irradiation, thus demonstrating less intestinal radiosensitivity compared to mice with normal microbiota where irradiation produces marked features of radiation enteritis and an increase in mortality.[195, 196] The crucial role of the bowel microbiota in the healthy gut is well known, and growing support for a radioprotective influence of gut bacteria points towards theories that a particular feature of several species of microbiota, in correct composition, may offer protection in the intestinal response to irradiation.[131] A derangement of the barrier function has been verified in many clinical situations, e g shock, radiation treatment, trauma, and intensive care when a multi-organ dysfunction is present, leading to a constant leakage of microbes/microbial products and a prolonged state of inflammation.[197-201]

Intestinal irradiation is associated with a reduced gut microflora opening to possibilities for opportunistic growth of pathogenic germ selection leading to injured intestinal mucosa susceptible to infections.[103, 202-204] Changes in microbiota have been seen after localized irradiation in humans and mouse models.[103, 205-207] The intestinal susceptibility to irradiation and inflammation is enhanced by radiation-induced dysbiosis.[208] The dysbiotic microbiota thus appears to be a promotor of bowel disorders following irradiation.[195, 208] Mice suffering from radiation-induced injury healed better after faecal transplantation, demonstrating a link connecting microbiota composition and radiation injury.[209, 210]

The pathophysiology of radiation-induced intestinal damage is still not clear, although our knowledge of the intricate interactions of the injured epithelial cells

and systemic modifications has increased. With growing technical advances in characterizing gut microbiota, e g 16S rRNA gene amplicon analysis, there will be more ways to find evidence to confirm that the microbiota plays a major role of in the pathogenesis of radiation-induced gut damage.

Substantial and important alterations of intestinal microbiota are found in animal studies as well as in humans following radiation therapy for lower GI tract or gynaecological tumours.[207, 211] An RT-induced reduction in the diversity of microbiota profile was seen in patients who developed post-radiation diarrhoea as well as in patients who did not develop diarrhoea, however to a greater extent in the diarrhoea group.[207] Studies in mice as well as in human epithelial cells have demonstrated that a radiation-induced dysbiosis makes the gut more vulnerable to damage and assumes a pathogenic function in fuelling the gut damage that follows irradiation.[208]

The intestinal bacterial flora contains a wide spectrum of taxa ranging from potentially pathogenic bacteria to lactic acid bacteria and bifidobacteria without any known harmful effects to the body. These probiotic bacteria, described later in this thesis, are defined as providers of specific benefits to host health or physiology upon ingestion. The disruption of the intestinal bacterial balance may as earlier mentioned modify the intestinal barrier function and epithelial turnover. Experimental and clinical studies have demonstrated that a strain of *Lactiplantibacillus* (*Lb*), formerly called *Lactobacillus*, *Lb plantarum* 299v, has an ability to colonize the whole gastrointestinal tract after oral administration, thereby suppressing the growth of potentially pathogenic bacteria and increasing fermentation.[212] Short-chain fatty acids (SCFA) are the main products of fermentation and the main source of energy for colonocytes. One of these SCFAs, butyrate, has been shown to reduce symptoms of acute radiation proctitis when locally administered.[213] Several experimental and clinical studies have shown that altering the luminal milieu by increasing intake of lactobacilli may decrease bacterial translocation.[214-216] It is rational to assume that restoration of barrier function before/during/after radiotherapy may reduce acute as well as late side-effects. Therefore, gut microbiota modification may be useful in a clinical setting to improve the intestinal endurance of irradiation. One way of impacting gut microbiota to reduce intestinal inflammation might be by administration of probiotics.

Evidence showing that microbiota exhibit a different profile in tumour tissue and adjacent mucosa in colorectal cancer patients has been demonstrated in a few small studies.[217] This correlation is seen in both irradiated and non-irradiated tumours, though it is not yet established whether this link is a reason for or a result of the malignant condition. The relation between the personal microbiota and the individual's response to cancer treatment has been demonstrated in recent studies, which indicate that dysbiosis may result in an altered immune system of the host, thereby systemically having an impact on the individual's ability to respond to cancer therapy.[218, 219]

It is postulated that RT-treated rectal cancer patients have an increased mucosal permeability due to atrophy of intestinal mucosa that leads to radiation-induced enteritis.[220] This translocation of pathogenic organisms across the intestinal wall into the bloodstream, the peritoneal cavity and abdominal organs has been extensively demonstrated as a promotor of supervening sepsis entailing potentially life-threatening complications in critically ill patients.[221, 222]

Some qualities of human health may be considered to be determined by the health status of the individual's microbiota since increasing research has identified that human symbiotic bacteria, which possess effective anti-inflammatory properties and may control inflammatory processes that are injurious to the host, have been shown to be lacking during illness. Reassessment of the microbial community in medical as well as social aspects may carry immense implications for the health of generations in the future.[184]

MMPs and colorectal cancer

Matrix metalloproteinases (MMPs) constitute a family of endopeptidases that are Ca^{2+}-dependent and Zn^{2+}-containing.[223-225] They are important in many normal biological processes such as physiological and pathological remodelling of extracellular matrix (ECM), and in tissue repair processes, e g proliferation, angiogenesis, and wound healing. However, MMPs also play an important role in pathological processes such as inflammation, tissue destruction, tumour invasion and metastatic spread. They are all synthesized as zymogens, a latent proactive form. MMPs are divided into different groups named by substrate specificity:

Table 2.
Matrix metalloproteinases (MMPs).

Groups	Member, function
Collagenases	MMP-1, MMP-8, MMP-13 Expressively involved in matrix turnover and remodelling
Gelatinases	MMP-2, MMP-9 Specific for collagen type IV, V, elastin, fibronectin, and denaturated collagens
Stomelysins	MMP-3, MMP-10, MMP-11 Involved in the degradation of proteoglycans, laminin, fibronectin, elastin, and degraded collagens
Matrilysin	MMP-7
Metalloelastase	MMP-12
Membrane-type MMPs	MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, MMP-25 Most of the MMPs are secreted but the MT-MMPs are membrane associated. A number of these have cytoplasmic domains which may be important in cellular signalling and play a role in the tumour invasion process
Other MMPs	MMP-20, MMP-21, MMP-22, MMP-23, MMP-28

MMP7, also known as matrilysin, is the smallest metalloproteinase known, 28 kD, and possesses a broad substrate specificity including casein, some gelatins, fibronectin, proteoglycans and collagen type IV and X, but also MMP9.[226] Furthermore, MMP7 is one of the main regulatory enzymes of apoptosis where it promotes cell survival by resisting apoptosis through cleaving the Fas ligand (FasL).[227-229] Studies have verified a special role for MMP7 amongst other MMPs, established by the fact that it is expressed in normal, uninflamed, and uninjured epithelial cells in various organs while other MMPs most frequently are not detectable in normal tissue.[154, 230] Generally, MMPs are expressed as a consequence of injuries and/or inflammatory processes as a response to immunological signals specialized in e g proliferation and remodelling in physiological and pathological development of tissue growth and differentiation, such as tissue repair and wound healing. However, it is problematic to compare different studies since expression of MMPs is studied at diverse time points and by various methods.

Thus, the activation of MMPs is initiated by different extracellular mechanisms, of which one is irradiation, another is the Ca-binding protein calprotectin.[231-233] However, they are also regulated by specific tissue inhibitors of metalloproteinases, a family of four protease inhibitors (TIMP-1, TIMP-2, TIMP-3, and TIMP-4). A number of synthetic MMP inhibitors have been tried without substantial success.

A critical factor in tumour invasion and metastasis is the balance between MMP expression and their inhibition.[234] An association has been established between overexpression of the MMP7 gene and increased colorectal cancer stage and metastatic potential.[235]

In colorectal cancer human MMP7 mRNA expression is very specific, since its expression is predominantly found in malignant epithelial cells, with an increasing expression associated with proliferating grades of dysplasia from normal tissue to cancer.[235, 236] Gene expression of MMP7 is shown to constitute an early occasion in colorectal tumorigenesis, in contrast to other MMPs that are expressed as a later event.[237] MMP7 and some other members of the MMP-families can however also be expressed in benign colonic mucosa as well as in uninflamed, uninjured epithelial cells in various organs but at much less detectable levels than in tumour tissue.[235] MMP7 and other MMPs have been shown to sustain a distinct function in tumour growth and spread.[234, 238]

MMP7 expression has been shown to be increased in human colorectal carcinomas, in tumour tissue as well as in serum, lymph nodes and in peritoneal liquid, and is found in advanced and even metastatic dicease. These findings confirm that MMP7 may prove useful as a sensitive tumour marker of biologic aggressiveness and serve as a prognostic indicator as well as a potential target for treatment in colorectal cancer patients.[236, 239, 240]

Studies have shown that MMPs are massively upregulated in malignant tissues and possess a unique ability to degrade all components of the ECM and it has been

demonstrated that MMP activity is increased in tumour cell lines after radiation.[241, 242] Adjuvant RT to rectal cancer patients induces an increase of MMP7 gene expression which may influence the abnormal tissue remodelling in the post-radiation phase.[235, 243] The high levels of MMP7 in the RT treated malignant rectal tissue as opposed to low levels in the irradiated normal rectal mucosa points to the intriguing relation to tumour progression and metastatic spreading, as caused by MMP mediated ECM remodelling, in neoadjuvant and adjuvant irradiated colorectal cancer.

MMPs and intestinal microflora

The intestinal microflora has several vital functions beyond conversion of food constituents that otherwise would be indigestible, such as regulation of GI peptide hormone secretion, production of necessary vitamins and cofactors, inflection of intestinal barrier integrity, and stimulation of the innate immune system.[244]

Changes in intestinal microbial diversity have been demonstrated to have an association with activation of certain MMPs and the severity of disease, although this relation is not yet completely elucidated. We know that different MMPs select different substates for their biological action, and they have been demonstrated to be useful as tissue-specific indicators of intestinal damage following chemotherapy and particularly Crohn's disease.[245, 246]

Generally, MMPs are expressed in response to distinct immunological signals after various injuries or inflammation but also after normal physiological procedures e g proliferation, differentiation and tissue remodelling as well as in pathological events such as wound healing and tissue repair. This finding is confirmed in numerous animal studies indicating that MMP7 exerts a crucial function in the mucosal barrier epithelial defence.[247, 248]

MMP7 has been shown to exert an indirect bactericidal effect in an extensively controlled and complex arrangement of the innate immunity system. Animal studies have proved the homeostatic action of MMP7 in murine models *in vitro* as well as *in vivo*. When the mucosal barrier is subject to bacterial exposition, or influence of bacterial antigens, bactericidal proteins are secreted, constituting an essential component of the innate immune system.[249]

In human and murine mucosal epithelial tissues, cell lines and cells, MMP7 is strongly induced by bacterial exposure that seems to produce a physiologically significant and pronounced signal for epithelial cells in controlling expression of MMP7.[154] Under pathological conditions, MMPs are allocated to matrix remodelling after various types of injuries causing a modification of the mucosal barrier which may facilitate infection and bacterial transmigration. However, critical

infection and subsequent inflammation may result in mucosal injury. Therefore, the connection between infection and injury makes MMPs, and especially MMP7, crucial in the regulation of wound healing and moreover in the control of the multifaceted innate immune system.[154]

In tissues with a high bacterial content MMP7 is significantly overexpressed resulting in bactericidal effect indirectly by regulation of the level of antimicrobial peptides (α and β defensins) in the innate immunity. The immunological homeostasis of the gut is to a great extent controlled by α-defensins, whose precursors, cryptidins, are generated in murine Paneth cells and stimulated by intracellular MMP7 and are efficient against Gram-negative as well as Gram-positive bacteria. These peptides function as innate anti-bacterial mediators and exert a controlling effect through adjusting inflammatory cytokines, possessing a "broad-spectrum" anti-microbial capacity.[154, 250] Administration of antibiotics in order to affect microflora after irradiation and/or surgery had no impact on MMP7 expression.[251] Irradiation was shown to have an overriding effect on antibiotics resulting in upregulated MMP7 and TGF-β expression. The combination of antibiotics and preoperative irradiation in the murine gut causes a clear rise in expression of MMP7, to the same extent as irradiation or antibiotics separately.

Human MMP7 has been demonstrated to be increased in connective tissue of the skin by lengthy exposure to sunlight as well as in irradiated rectal tumours and members of various MMP families may take part in tissue damage and remodeling in necrotizing enterocolitis (NEC).[235, 252, 253]

Butyrate, as previously described, is a result of bacterial fermentation of dietary fibre to short- chain fatty acids (SCFAs) in the bowel and has been shown to regulate the molecular mechanisms that influence expression of MMPs.[254] This might constitute one of the means by which dietary or nutritionally alterations could modulate intestinal inflammatory consequences.

Pre-, pro- & synbiotics and gastrointestinal radiation injury

A **prebiotic** is "a substrate that is selectively utilized by host microorganisms conferring a health benefit".[255] It is a selectively fermented element, generally non-digestible carbohydrate, oligosaccharide, or short polysaccharide, that leads to alterations in the action or structure of the gut microbiota and as a result gives benefit(s) to host health.[256] Thus, beneficial microorganisms harboured by the host use these non-viable substrates as nutrients.[255]

Probiotics are live microorganisms that when administered in adequate amounts confer a health benefit to the host.[257, 258] The term has been widely used and not

seldom misused and should be restricted to strains of well-studied species of microbes provided in adequate doses as nutrients or complements in a common population. Examples of these species include strains of the genera *Bifidobacterium* and *Lactobacillus*. Among various interfaces with the host, probiotics exert effects on mucosal barrier function including competitive inhibition of other microbes and interaction with antigen presenting dendritic cells.[259, 260]

Synbiotics are defined as a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit to the host.[261] Synbiotics may be categorized into two entities: complementary synbiotics, consisting of a prebiotic and a probiotic that not necessarily demands a co-dependent function, but which collectively confer health benefit(s); and synergistic synbiotics, where the latter comprises a substrate discriminatory used by the co-administered live microorganism(s).

We are at the beginning of an era when research results advocate a link between the mechanistic role of the microbiota and systemic diseases, as well as a role during acute GI symptoms after RT to abdominopelvic organs. A broad and lively debate concerning the possible therapeutic properties of the microbiota is ongoing.

The intestinal microbiota and its impact on radiation-induced gut damage is exceptionally intricate and as expected thus not dependent on one single factor but a row of dynamic interactions between different parts of the enteric systems and microbial populations that is responsible for these sophisticated alterations.[131]

In a recently published review article, Thomsen and Vitetta demonstrated that probiotics can be used to treat patients and reduce toxic symptoms from the GI tract, caused by RT or chemotherapy, without any considerable adverse effects. Probiotics were found to reduce intestinal mucositis and diarrhoea following these treatments.[149] However, studies exploring the effect of probiotics in radiation-induced GI symptoms are problematic to evaluate, due to divergent categories of patients recruited, to various RT modalities with or without concomitant chemotherapy, as well as the fact that different probiotic bacteria are provided. A meta-analysis comprising six studies stated that ingested probiotics did have a beneficial effect with a significant reduction in the incidence of post-RT diarrhoea.[262] Even if there were variations in the type of bacteria used in the supplied probiotics in the six studies, all of them did contain *Lactobacillus*.

Possible mechanisms of intestinal radioprotection provided by the microbiome could be several, many still not elucidated. Animal studies have shown that radiation-induced injuries could be prevented with a *Lactobacillus*-containing probiotic to achieve reduced endotoxin expression as well as diminished *Pseudomonas* bacteriemia and milder histological damage to the intestinal wall subsequent to RT.[263-266] Moreover, crypt cell apoptosis was less pronounced and a better survival of the cells of the crypts after RT was seen in mice treated with *Lactobacillus rhamnosus*. The mechanism was achieved by a Toll-like receptor 2

(TLR2) related pathway that induced cyclooxygenase-2 (COX-2) influence on mesenchymal stem cells from the lamina propria of villi near the crypt.[267] Probiotics have been used in connection with colorectal surgery for many years with the intention to decrease pathogenic bacteria in the gut and to ameliorate immune response. CRC patients received probiotics pre- and postoperatively (concomitantly administered *Bifidobacterium longum* and *Lactobacillus johnsonii*, 10^9 CFU) resulting in reduced levels of *Enterobacteriacae*, as well as for enterococci, in faeces.[268] In a Chinese study, 100 patients with CRC received a mixture of three probiotic strains (*L. plantarum*, *L. acidophilus* and *B. longum*) in a high dose (2.6 x 10^{14} CFU/day) pre- and postoperatively for 16 days.[269] The transepithelial resistance was found to be increased while the transmucosal permeability was reduced, as was the bacterial translocation. The mucosal tight junction protein expression was improved, and the amount and diversity of faecal bacteria increased. Postoperative outcome in terms of intestinal postoperative gut paralysis, diarrhoea and infectious complications was enhanced as well. Since the late 1980s, probiotics have been shown to reduce diarrhoea in patients with irradiated gynaecological tumours receiving *L. acidophilus* in a Finnish study.[270] In another large study where patients with adjuvant RT for sigmoid, rectal, or cervical cancer were provided with the probiotic VSL#3 (a commercial probiotic mixture of eight bacterial strains: four strains of *Lactobacillus*, three strains of *Bifidobacterium* and one strain of *Streptococcus*) vs placebo, the frequency of diarrhoea following adjuvant RT decreased.[271] However, there was no impact on radiation-induced diarrhoea when patients with gynaecological cancer ingested a probiotic drink containing *L. casei*, expressing the need to be able to identify which probiotic strain is effective for the symptoms to be treated.[272] Even though the link between the microbiome and acute post-RT diarrhoeal symptoms is well established by research in both animals and humans, the effect of probiotics on the relationship between radiation-induced dysbiosis and the chronic type of radiation-induced intestinal injury is yet to be further elucidated. It is difficult to compare studies, due to different strains or combinations of bacteria used, different doses and treatment periods, thus making it hard to issue guidelines or general recommendations. The pathogenesis of the chronic radiation-induced gut damage differs from that of acute injury. In contrast to the acute form that mostly declines with time, the chronic type gradually devastates, not seldom leading to a life-long distress and severely impaired quality of life since the therapeutic alternatives are few.

The importance of the considerable impact irradiation exerts on the modification of the intestinal microbiome has been widely demonstrated. A particular microbiota profile has been identified prior to RT in patients suffering from diarrhoea following RT, but studies on this are heterogenous and the causation is complex, thus it is not easy to conclude if such a profile might be used for prevention or therapy. The pathogenic quality of dysbiosis generated by irradiation, for influence on intestinal damage, was recently demonstrated in an animal study, initiating the understanding of how gut mucosal injuries might be affected by microbiota modulation.[208]

Alternative solutions such as faecal microbiota transplantation (FMT), first reported in 1958 for the treatment of pseudomembranous colitis, have also been demonstrated in mice to be favourable for the recovery of irradiated mucosa damage.[209] Recently a pilot study demonstrated that FMT in patients with chronic radiation enteritis (CRE) gave improvements regarding diarrhoea, rectal haemorrhage, abdominal/rectal pain, and faecal incontinence, and led to a decrease in Karnofsky Performance Status (KPS) score.[273] Even if FMT may seem to offer a safe and effective possibility to enhance intestinal symptoms and mucosal injury in patients with CRE, larger studies are needed to confirm this. FMT has so far appeared to be a safe option with only rare reports of short-term serious events, but potential long-term sequelae are not yet known. Donor selection is another issue that must be carefully considered. One must keep in mind that FMT also includes transmission of not only microbes but also of viruses, funghi, archaea and bacteriophagic particles.

Prebiotics such as dietary fibre from different sources, e g cereals or grains, vegetables, fruits, and legumes, are heterogenous in their composition and properties, and are thus hypothesised to possess varying quantities of anticarcinogenic properties. In a meta-analysis summarising the relationships of different fibre sources with colorectal cancer and adenoma risks, fruit and vegetable fibres were found to act upon the early stages in the process such as adenoma formation whereas cereal and grain fibre are more likely to influence later stages in the process of malignant transformation.[42] Dietary fibre intake shows strong negative correlation with risk for CRC-development and stimulates gut microbiota to butyrogenic production, providing high quantities of butyrate that exhibit extensive anti-neoplastic properties, as previously mentioned.[41] Other prebiotics, such as phenolic compounds, abundant in blueberries and other dark berries such as blackcurrant, crowberries, and chokeberries, as well as cranberries, have been suggested to be capable of reducing colon cancer risk.[274, 275] In a Spanish study of prebiotic supplementation in patients with RT-treated gynaecological malignancies, an improved recovery of microbiota was found.[276]

A recent British-Dutch review of pre-, pro- or synbiotics found that a significant potential for the prevention and treatment of cancer exists but the field is still very much in its early stages. Well-evidenced mechanisms by which probiotics may exert beneficial effects exist, but clinical studies are heterogenous, small and few in number, often suffering from significant biases. The use of pre-, pro- and synbiotics as adjuncts in the treatment of cancer has a better evidence base in particular for use in the prevention of diarrhoea associated with antibiotic or chemo-/radiotherapy treatment as well as infectious complications following surgery.[277]

Numerous publications in recent years have reported on perioperative synbiotic interventions in CRC patients and a recently published meta-analysis supports the clinical use of probiotics/synbiotics to reduce postoperative GI symptoms, adverse side-effects, and complications after colorectal surgery in CRC patients, giving few

side-effects and at low costs compared to alternatives.[278] A Brazilian study of CRC patients undergoing elective surgery found that perioperative administration of synbiotics significantly decreased rates of postoperative infections.[279]

For the future, further research is needed to investigate the potential role of the microbiota and the methods of activity and efficiency of probiotics in clinical praxis of treating RT- and/or chemotherapy-induced gastrointestinal damage and subsequent morbidity, not least due to its documented superior therapeutic index and great safety profile.[131, 149] Intensified research within this field is highly awaited.

Aims of the thesis

Today a broad selection of therapies enables us to present individualised treatments to patients with rectal cancer, based on surgery and radiotherapy. Despite increased knowledge of risk factors regarding patient variables, tumour, and surgical procedures/treatment traits, we are still many steps away from an optimal tailored treatment plan for the individual patient.

The overall aims of this thesis were to shed light on different aspects in the management of rectal cancer patients and to investigate ways of minimising the risks related to radiotherapy in rectal cancer treatment.

The specific aims of this thesis are as follows:

I. To assess and confirm a varied MMP7 expression in different stages of colorectal tumour development.

II. To analyse the effects of short- and long-course radiotherapy on MMP7 modulation during treatment of rectal cancer patients.

III. To study local and systemic effects of probiotics on non-irradiated rectal mucosa after a standardised inflammatory injury *in vitro*.

IV. To investigate the role of pre- and synbiotics in minimising radiation-induced intestinal inflammation injury in rectal cancer patients.

Our findings may hopefully contribute to structuring well studied information in the future, to facilitate maximal advantage and minimal harm in management of colorectal cancer patients.

Det finns mitt i skogen en oväntad glänta
som bara kan hittas av den som gått vilse.

Tomas Tranströmer 1931-2015

Mät aldrig bergets höjd förrän du nått toppen.
Då kommer du att se hur lågt det var.

Dag Hammarskjöld 1905-1961

Study design and methods

Paper I

A prospective descriptive study over three years including 28 patients referred for elective colorectal cancer treatment was implemented. All patients gave informed consent. The study was approved by the Human Ethics Committee at the Sapienza University of Rome and registered with Clinical Trials (ID NCT01570452). Exclusion criteria were neoadjuvant RT or chemoradiotherapy as well as linguistic difficulties and withdrawal of consent. Staging was performed after colonoscopy with biopsies, abdominal computed tomography (CT) and chest radiography. Clinical variables were included in a database. Serum was collected from 10 healthy volunteers after acceptance of informed consent. Patients with severe dysplasia or large symptomatic low-to-moderate dysplastic adenomas and adenocarcinomas underwent surgical resection. Baseline blood parameters, specific oncomarkers (CEA, CA19-9, CA-50) and MMP7 were collected.

Collection of samples

Intraoperative blood samples were collected. Specimens from tumour tissue as well as from normal mucosa at a distance of 2.0-2.5 cm from tumour edge, and mesenteric lymph nodes from the vicinity of the tumour, were collected during surgery. Specimens were fixed in 4% formaldehyde before histopathological examination. Tissue samples and lymph nodes were immediately stored at -80ºC until analysed.

Analyses of samples

The tissue samples and lymph nodes were divided into aliquots of 50 to 100 mg and treated with 300 μl of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% NP-40, 1% CHAPS, 2 mM EDTA dissolved in tetra-distilled water). A mixture of protease inhibitors (Complete-Mini Protease Inhibitor Cocktail Tablets, Roche, Mannheim, Germany) was added just before use. Samples were first homogenated by Ultra-Turrax® (T10 basic, IKA®, Staufen, Germany), thereafter sonicated for 20 seconds and centrifuged at 14000 rpm for 10 minutes whereafter the supernatants were collected.

Venous blood samples were drawn into sterile vacuum tubes and left at room temperature for 30 minutes, then centrifuged at 4000 rpm for 15 minutes. Serum was immediately aliquoted and stored at -80°C until analysed. The protein content of supernatants and serum samples was determined by using the Bradford assay.

Histology

Samples were fixed in 4% phosphate buffered formaldehyde and then embedded in paraffin. Sliced specimens stained with haematoxylin and eosin were analysed under light microscopy. At least 3 slides were studied from each specimen by a blinded observer. Stage definition was stated according to 2002 UICC classification.

Immunohistochemistry

Standard avidin-biotin procedures for human MMP7 were used for immunohistochemistry. After deparaffinisation and washing in phosphate buffered solution (PBS), endogenous peroxidase activity was blocked by incubating the sliced sections in 3% hydrogen peroxide in PBS for 10 minutes. Analysis for matrilysin was performed using anti-MMP-7 (MAB-10756 Immunological Sciences, Rome, Italy) following the manufacturer's instructions. Biotin-conjugated secondary antibody and streptavidin-conjugated horseradish peroxidase (DAKO, USA) were applied to sections for 45 minutes at room temperature and developed using 3,3'-diaminobenzidine (DAB) as substrate. Finally, counterstaining with haematoxylin was performed. Sections were mounted and the grade of staining was determined on randomly selected areas counter-checked for intensity by a blinded observer.

ELISA

In supernatants and serum samples, total human MMP7 levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine®, R&D Systems, Minneapolis, USA). 150 µl of diluted samples were added to a 96-well microtiter plate, precoated with anti-human MMP7 monoclonal antibody, and incubated at room temperature for 2 hours on a microplate shaker. After washing, 200 µl of the secondary antibody solution was added, and the plate was incubated for 2 hours at room temperature on the shaker. After washing, the substrate solution was then added and incubated at room temperature in the dark. A 50 µl stop solution was added after 30 minutes and optical density was measured using a microtiter plate reader (Opsys MR™, Dinex Technologies, Inc.; Chantilly, Virginia, USA) at 450 nm, with the correction wavelength set at 570 nm.

Western blot analysis

For western blot analysis, supernatants obtained from treated lymph nodes were separated on a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with a concentration of acrylamide specific for MMP7 and β-actin. Proteins were blotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA,

USA) and probed with the following antibodies: anti-MMP7 (MAB-10756 Immunological Sciences, Rome, Italy) and anti-β-actin (A 5060, Sigma Chemical Co. St. Louis, MO, USA). Antigens were detected with an enhanced chemoluminescence (ECL) kit from Amersham (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). All western blot images were acquired and analysed through an Imaging Fluor S densitometer (Bio-Rad Laboratories, Hercules, CA, USA). The optical density (O.D.) of each condition was correlated to the signal of the β-actin internal control.

Statistical methods

Data are expressed as mean ± standard deviation (SD). Statistical comparison between groups was performed by using the analysis of variance (ANOVA) followed by the Bonferroni post hoc test. Differences were considered significant at p values less than 0.05. Analyses were performed using GraphPad (GraphPad Software Inc., San Diego, CA, USA).

Paper II

A prospective descriptive case-control study of 77 patients over three years diagnosed with, and treated for, rectal cancer at Skåne University Hospital, Malmö, Sweden was performed. All patients were managed according to the clinical protocol of the Department of Clinical Sciences, Division of Colorectal Surgery, adhering to national guidelines and assessment of the local multidisciplinary treatment board. The study was approved by the Ethics Committee at Lund University (ref 144/2004, amendment 597/2006) and registered with Clinical Trials (ID NCT 03151759). Written consent was obtained after oral and written information. Staging was performed according to the TNM system. Exclusion criteria were previous RT to the pelvic region, inflammatory bowel disease, neoadjuvant therapy as well as ongoing steroid, immunosuppressive or antibiotic therapy. Three treatment groups were outlined: one group receiving short-course neoadjuvant RT 25 Gy (5 x 5 Gy), one group treated with long-course neoadjuvant RT 50 Gy (25 x 1,8 Gy), and a control group that underwent surgery alone.

Collection of samples

A rigid rectosigmoidoscopy was performed before and after irradiation, and at the operating theatre before start of surgery. Two-millimetre punch biopsies were obtained from tumour tissue within the irradiated field. Tissue samples were taken on three occasions: at inclusion before RT; after RT prior to start of surgery to

eliminate possible effects of surgical trauma and ischaemia on MMP expression; and from the excised specimen. Tissue samples were instantly formalin fixed followed by paraffin embedding according to standardised laboratory routines.

Analyses of samples

Tissue microarrays

Two tissue microarray (TMA) series were constructed: one TMA with 1 x 1 mm cores from biopsies with normal tissue (sampled 2 cm from the tumour) and cancer, respectively, and one TMA with normal and cancerous tissue from the surgical specimens, whereby 2 x 1 mm cores were extracted from areas representing viable, non-necrotic tumour, and from adjacent, microscopically benign rectal mucosa, respectively. This was performed using a manual arraying device (MTA-1; Beecher Inc., Sun Prairie, WI, USA) and tissue samples were mounted in a recipient block.

Immunohistochemistry and staining evaluation

The TMAs were cut into 4-µm sections pre-treated in a DAKO PT-link module using a standard protocol and buffer supplied by the manufacturer (DAKO, Glostrup, Denmark). Thereafter slides were stained in a DAKO Autostainer-plus using EnVision™ FLEX including Peroxidase-Blocking Reagent (DAKO) with mouse monoclonal antibody to MMP7 (dilution 1:50; Santa Cruz, Dallas, TX, USA) and rabbit polyclonal antibody to TGF-β (dilution 1:200; Abcam, Cambridge, UK). Immunohistochemistry was performed by an automated staining machine (Ventana Medical Systems, Inc., Tucson, AZ, USA). Three research scientists jointly annotated cytoplasmic expression of MMP7 and TGF-β in tumour tissue for each core. Annotation of the absolute percentage of positively stained cells was multiplied by the annotated intensity (score 0-3) of stained cells, and a mean expression score was subsequently calculated for each patient at each time point. This method was earlier validated.[280, 281] Discrepant cores were discussed until consensus was reached.

Statistical methods

Spearman's Rho and chi-squared tests were used to investigate RT groups and patient characteristics. Mann-Whitney *U*-test/Wilcoxon Z-test were used to investigate differences in MMP7 expression between tissue before and after RT, after surgery, in the RT subgroups as well as for the TGF-β analysis. Fisher's exact test was used in single case analyses. All statistical analyses were performed using SPSS version 21.0 (IBM Corp. Armonk, NY, USA); associations/differences with p values less than 0.05 were considered significant.

Paper III

Fourteen healthy volunteers without symptoms from the gastrointestinal tract were recruited by open invitation and were allocated by a procedure of randomly chosen envelopes to receive 10^{10} colony forming units (CFU)/day orally of either probiotic strains *Lactiplantibacillus plantarum* 299 (Lp299) [DSM 6595], n=7, or *Bifidobacterium infantis* CURE21 (CURE21) [DSM 15159], n=7, for six weeks. During an eight-week period, volunteers were advised to stop consumption of any probiotic products, traditional lactic acid fermented milk products and other lactic acid fermented products (e g brined olives, sauerkraut, and pickled gherkins). The study was approved by the Human Ethics Committee of Lund University, Sweden (LU738-03, Dnr 538/2005) and registered with Clinical Trials (ID NCT01534572).

Collection of samples

Two weeks after abstaining from all fermented products, blood and faecal samples were collected and thereafter the volunteers underwent a rigid recto-sigmoidoscopy where six biopsies were taken at 15 centimetres using a two-millimetre biopsy forceps. All biopsies were weighed. After the initial procedure, volunteers started to ingest probiotics in freeze-dried powder form, dissolved in water, for six weeks. The administration of probiotics was blinded to volunteers and all investigators, except to the research nurse. No gut enema was used before biopsies.

Six weeks after probiotic administration, a new set of samples was taken from blood and faeces as well as from the rectum by rigid recto-sigmoidoscopy, as previously done. Thus, the study subjects served as their own controls. Further, the subjects recorded daily frequency of defecation, stool consistency, flatulence activity, abdominal pain, as well as a subjective estimation of gastrointestinal function on a scale from 1 (best possible) to 10 (worst possible) in a study diary. Any altered habits regarding physical activity, food, tobacco, alcohol, and coffee/tea intake were recorded.

Analyses of samples

Cytokines and composition of the intestinal microbiota were analysed. Faecal specimens were cultured for Lp299 and CURE21. Profiling of the gut microbiota was done by Terminal Restriction Fragment Length Polymorphism (T-RFLP) on rectal mucosal specimens. Separate quantitative PCR (qPCR) assays were used to estimate the presence of bacterial 16S rRNA genes of specified taxa, as described by Karlsson.[178, 282] *In vitro* stimulation of rectal biopsies with TNF- α for detection of the pro-inflammatory cytokine IL-6 and the anti-inflammatory cytokine IL-10 was performed at 0, 4 and 8 h respectively, as well as in different concentrations of

10 ng/ml and 100 ng/ml. Lactate dehydrogenase (LDH) was used as marker for mucosal viability, measured according to clinical routines at the laboratory, at 4 h and 8 h in supernatant and tissue. The ratio of LDH activity in the supernatant over total activity in tissue was calculated and used to estimate tissue viability.

For histology, one biopsy per subject was placed in formalin for 24 h and then embedded in paraffin, according to standard routines. The histological examination was performed by a blinded observer.

Statistical methods

Statistical evaluations were performed with paired t-test and Wilcoxon rank sum test. The results are presented according to the ingested bacteria for each group of volunteers, before and after the administration of probiotics, as median values and ranges. Differences were considered significant at p values less than 0.05. Statistical analyses of qPCR were performed using Mann-Whitney Rank Sum Test (Sigma Stat 3.0, Systat Software, San Jose, CA, USA).

Paper IV

A prospective randomised clinical trial of 30 patients diagnosed with rectal cancer and preoperatively treated with short-course radiotherapy 25 Gy at Skåne University Hospital, Malmö and Helsingborg Hospital was performed. Exclusion criteria were history of inflammatory bowel disease, dysregulated diabetes, steroid- or immunosuppressive treatment, ongoing treatment with antibiotics and previous RT to the pelvic region. Three groups were examined: one prebiotic group receiving 45 g of oat bran only, one synbiotic group with a study product containing 22 g oat bran + 13 g freeze-dried blueberry husks + the probiotic *Lactiplantibacillus plantarum* HEAL19 (HEAL19) [DSM 15313], 10^{10} CFU, and a control group that received no treatment/study product at all. This is equivalent to 8 g of fibre in each of the two treated groups. The study product was ingested during one week before RT and throughout the RT, altogether 2 weeks.

Collection of samples

Inflammatory markers (fibrinogen, white blood cell count [WBC], C-reactive protein [CRP], haptoglobin, orosomucoid and ceruloplasmin), cytokines (IL-1β, IL-2, IL-6, IL-8, IL-10 and TGF-β1) and zonulin were analysed in blood samples collected before start of RT and in the operating room prior to start of surgery. Faecal samples were analysed for calprotectin.

A rigid rectosigmoidoscopy was performed prior to RT, and at the operating room before start of surgery. Two-millimetre punch biopsies were obtained from normal mucosal tissue more than two centimetres from the tumour edge for histological examination, directly formalin fixed before paraffin embedding following standardised routines, as well as for microflora analyses and myeloperoxidase (MPO) analyses. Profiling of the gut microbiota was performed through Terminal Restriction Fragment Length Polymorphism (T-RFLP). To estimate the presence of bacterial 16S rRNA genes of specified taxa, separate quantitative PCR (qPCR) assays were utilised, as earlier defined by Karlsson.[178, 282]

Analyses of samples

Blood samples were collected according to hospital routines and immediately analysed for CRP, WBC, haptoglobin and fibrinogen at the Department of Clinical Chemistry, Skåne University Hospital, Malmö. MPO was analysed in biopsies using a biochemical analysis method previously described by our group.[283] Serum samples were kept in -80°C until analyses of orosomucoid and ceruloplasmin. Levels of zonulin in serum were analysed by ELISA and cytokines were defined in plasma by a V-PLEX immunoassay technology. For histological investigation, one biopsy per patient, at inclusion and before surgery respectively, were placed in formalin for 24 h and examination was performed by a blinded observer.

Statistical methods

Statistical evaluations for MPO, TGF-β, zonulin and WBC were performed using IBM statistics 16.0 (SPSS Inc., Chicago, IL, USA). Mann-Whitney Rank Test was used to analyse changes in expression of MPO, TGF-β, zonulin and WBC between 'before RT' and 'prior to surgery', as well as comparison between the three groups. SigmaPlotVR version 13.0 (SYSTAT Software, Point Richmond, CA) was utilised to perform the statistical analyses of other inflammatory markers as well as for bacterial analyses. Mann-Whitney Rank Sum Test was used to analyse the change within each group from inclusion to surgery. To compare all three groups, changes in data before RT and before surgery were calculated, and ANOVA on ranks was done utilising the differences from the three groups. Bacterial diversity (T-RFLP) was evaluated by calculation of richness (number of T-RFs) and of Shannon–Wiener diversity index (H') and Simpson diversity index (1-D), as described earlier.[282] Calculations of the incidence of T-RFs were done in QuickStat version 2.6 and were evaluated by Fisher's exact test. Multivariate data analysis was operated on T-RFLP by use of UnscramblerVRX software version 10.3 (32-bit) (CAMO Software AS, Oslo, Norway). p values less than 0.05 were considered statistically significant.

Ora et labora (et lege).
S:t Benedict av Nursia 480-543

Ultreia et suseia!
Keep going! Let's go further!
Codex Calixtinus

Results

Paper I

The study included 28 patients, 16 men (57%) and 12 women (43%) with mean age 74 years (55-88 years). Eight patients had benign lesions not suitable for endoscopic procedures and 20 had malignant colorectal tumours. All recruited patients finalised the study. Tumours were located in the right colon (n=8), the left colon (n=12) and the rectum (n=8). Surgical procedures performed were right hemicolectomy (n=8), left hemicolectomy (n=6), sigmoid resection (n=4), anterior resection of the rectum (n=6), abdominoperineal rectal resection (n=2), subtotal colectomy (n=1) and total colectomy (n=1). None of the rectal cancer patients underwent neoadjuvant RT. Serum controls were collected from 10 healthy volunteers, 6 men and 4 women, with a mean age of 70 years (range 50-83 years). No major complication was observed after surgery and no mortality was registered within 30 days after surgery.

Histological examination was performed, and the adenomas and tumours were staged according to three groups: dysplastic adenomas (n=8); no disseminated disease (n=10) of which stage I (n=7) and stage II (n=3) were included; and disseminated disease (n=10) comprising stage III (n=8) and stage IV (n=2).

Immunohistochemistry demonstrated progressively increasing MMP7 expression from benign to malignant tumour among which well differentiated carcinoma had the most evident staining. Normal mucosa had no significant staining for MMP7 (Figure 2).

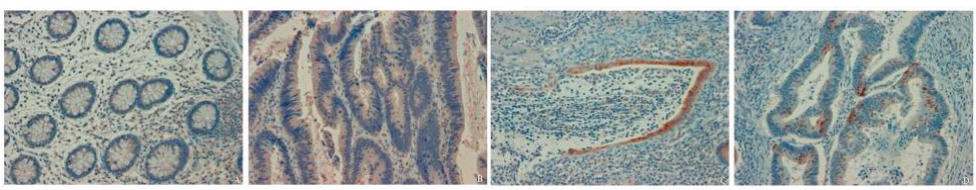

Figure 2.
Immunohistochemistry for matrix metalloproteinase-7 (MMP7). Negative staining in healthy mucosa (A). MMP7 expression in low-grade dysplastic adenoma (B), well-differentiated carcinoma T2N0M0 (C) and in poorly differentiated carcinoma T3N2 M0 (D), original magnification, x20.

ELISA showed that MMP7 levels were significantly higher (p<0.001) in stage I and II cancer tissues, compared to adenomas (low and moderate dysplasia), and significantly higher compared to stage III or IV cancers (Figure 3). MMP7 expression in adenomas was likewise significantly lower (p<0.001) compared to those in stage III and IV disease. No measurable levels of MMP7 were found in normal mucosa. Significant differences were not observed in serum levels of MMP7 when comparing patients with benign adenomas to those with stage I and II disease. In serum, a significant increase (p<0.01) was seen comparing patients with adenomas to those with stage III and IV cancer. However, no significant differences were observed for MMP7 expression, when comparing within the group stage I to II, and when comparing within the group stage III to IV, in both tumour and serum. In serum from healthy controls only low or undetectable levels of MMP7 were found. Lymph nodes demonstrated lower MMP7 expression compared to serum and tumoral tissue. Significant differences in expression in lymph nodes were noticed among groups: adenoma vs stages I and II (p<0.05) and stages III and IV vs both stages I and II and adenomas (p<0.01), (Figure 3).

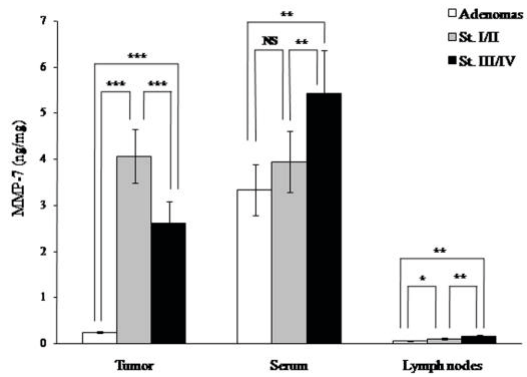

Figure 3.
ELISA analysis showing Matrix metalloproteinase-7 expression in tumoral tissue, serum and lymph nodes. Significantly different at *p<0.05, **p<0.01, ***p<0.001; NS, p>0.05.

Western blot analyses, with a semi-quantitative measurement of MMP7 levels in lymph nodes, verified the ELISA results. A significant increase in MMP7 expression from adenoma to increasing cancer stage was seen. Lymph nodes of patients with stage I and II tumours showed significantly higher MMP7 levels than those in patients with adenomas (p<0.05). Lymph nodes of patients with stage III and IV tumours had significantly higher MMP7 expression compared to those with stage I and II adenocarcinomas and to adenoma-patients (p<0.001), (Figure 4).

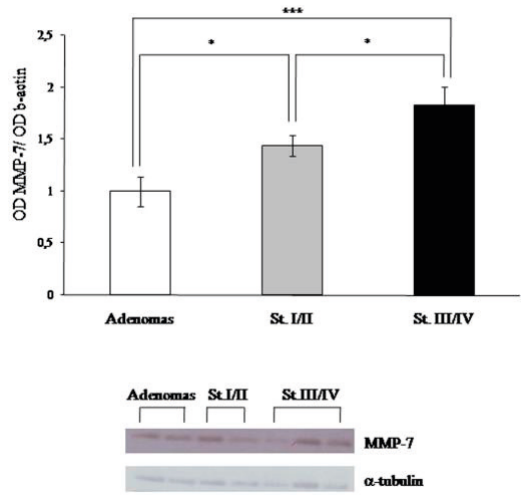

Figure 4.
Western blot analysis showing the expression of MMP7 in lymph nodes. Significantly different at *p<0.05, ***p<0.001.

Paper II

The study cohort consisted of 53 patients of whom 36 were men (68%) and 17 (32%) were women. Twenty-four patients were excluded from the study due to revised pathological diagnosis showing high-grade dysplasia (n=8), impaired general conditions (n=8), declining of participation (n=4), synchronous colonic tumours (n=3) and for logistical reasons (n=1). Three treatment groups were defined: one group of 20 patients (38%) receiving short-course preoperative RT of 25 Gy (5 x 5 Gy), one group of 21 patients (40%) treated with long-course preoperative RT of 50 Gy (25 x 1,8 Gy), and a control group of 12 patients (23%) that underwent surgery alone.

Surgical procedures performed were either anterior resection of the rectum or abdominoperineal rectal resection, by the TME technique. In the control group, 9 patients (75%) went through rectal resection, 2 (17%) underwent abdominoperineal resection, and one patient (8%) Hartmann's procedure. Among short-course irradiated patients, 11 (55%) went through rectal resection, 8 (40%) abdominoperineal resection and one (5%) Hartmann's procedure. In the long-course RT group, 9 patients (43%) had a rectal resection, 8 patients an abdominoperineal resection and 3 (14%) Hartmann's procedure. Surgical procedure was unknown in one patient (5%) in the latter group (Table 3). None of the patients received neoadjuvant chemotherapy but 13 patients received adjuvant chemotherapy after surgery.

Table 3.
Patient characteristics.

	RT before surgery, n (%)			
	None	**Short-term**	**Long-term**	***p*-value**
Characteristic				
Total, n	12 (22.6)	20 (37.8)	21 (39.6)	
Age				
<75 Years	8 (66.7)	15 (75.0)	16 (76.2)	
≥75 Years	4 (33.3)	5 (25.0)	5 (23.8)	0.823
Gender				
Male	9 (75.0)	14 (70.0)	13 (61.9)	
Female	3 (25.0)	6 (30.0)	8 (38.1)	0.717
Clinical staging				
I	2 (16.7)	2 (10.0)	0 (0)	
II	9 (75.0)	8 (40.0)	6 (28.6)	
III	0 (0)	10 (50.0)	15 (71.4)	
IV	1 (8.3)	0 (0)	0 (0)	
Missing	0 (0)	0 (0)	0 (0)	0.005
Pathological staging				
I	3 (25.0)	7 (35.0)	4 (19.0)	
II	4 (33.3)	3 (15.0)	7 (33.3)	
III	4 (33.3)	8 (40.0)	7 (33.3)	
IV	1 (8.3)	2 (10.0)	1 (4.8)	
Missing	0 (0)	0 (0)	2 (9.5)	0.628
Operative procedure				
Anterior resection	9 (75.0)	11 (55.0)	9 (42.9)	
Abdomino-perineal resection	2 (16.7)	8 (40.0)	8 (38.1)	
Hartmann	1 (8.3)	1 (5.0)	3 (14.3)	
Missing	0 (0)	0 (0)	1 (4.8)	0.506

Surgery was found to up-regulate MMP7 in all groups, regardless of RT. Short-course 25 Gy RT induced over-expression of MMP7 before and at the time of surgery but was not observed after 50 Gy RT (Figure 5, Table 4).

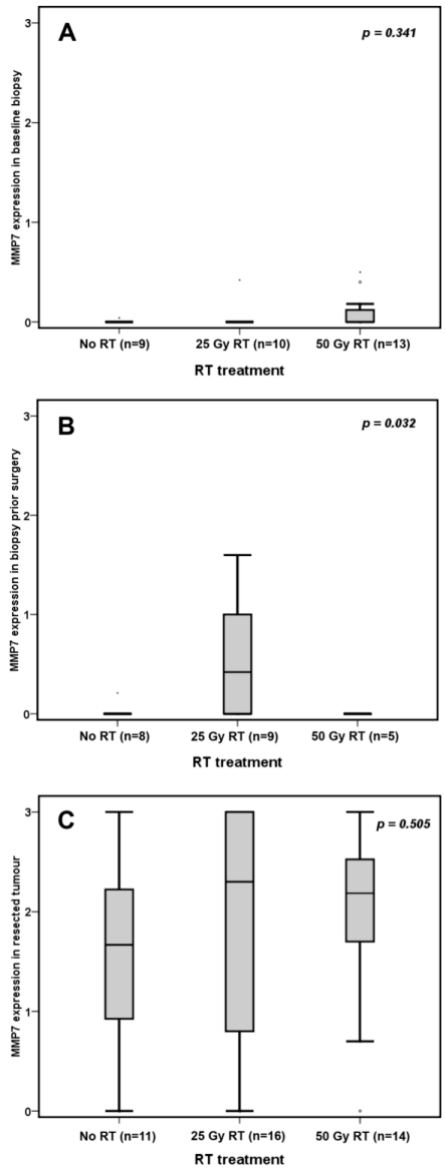

Figure 5.
Matrix metalloproteinase 7 (MMP7) expression in the two radiotherapy (RT) regimen and in controls, in the baseline.

Table 4.
Tumour expression of matrix metalloproteinase 7 (MMP7) before and after treatment. Mean rank is used to approximate relation between the MMP7 in treatment occasions. *p*-value and Z-value are compared with baseline.

Group	Time point	n	MMP7 mean rank	*p*-Value	Z-value
Short-term RT	Baseline	10	8.05		
	Before surgery	9	16.56	0.065	−2.230
	After surgery	16	25.03	<0.00001	−4.147
Long-term RT	Baseline	13	12.92		
	Before surgery	5	9.00	0.336	−1.353
	After surgery	14	22.50	0.005	−2.870
No RT	Baseline	9	10.00		
	Before surgery	8	10.25	0.963	−0.172
	After surgery	11	21.27	0.0016	−3.208

In all three groups, no significant increase of TGF- β was observed before surgery. TGF-β showed significant 2- to 3-fold increase only after surgery (Table 5).

Table 5.
Tumour expression of transforming growth factor beta (TGF-β) before and after treatment compared to baseline.

Group	n	TGF-β mean rank	*p*-value	Z-value
25 Gy RT				
Baseline	9	7.72		
Before surgery	6	9.17	0.529	−0.714
After surgery	18	24.25	<0.0001	−4.010
50 Gy RT				
Baseline	10	6.95		
Before surgery	2	8.00	1.000	0.000
After surgery	14	18.96	<0.0001	−3.862
No RT				
Baseline	8	8.12		
Before surgery	5	5.80	0.435	−0.897
After surgery	11	18.73	0.0002	−3.399

Paper III

All healthy volunteers, nine women (64%) and five men (36%) with seven persons in each group, finalised the study period. Age, gender, and BMI were equally distributed between the two groups (Table 6).

Table 6.
Demographics.

	n	Age median	range	BMI median	range
Total	14	46.0	28-79	22.0	19-27
Women	9	48.0	28-79	21.0	19-27
Men	5	46.0	29-76	23.0	21-24

No side effect of the probiotics was recorded, and the volunteers did not experience any untoward effect of gut function before and after administration of probiotics. The number of defecations was reduced by 45% in the Lp299-group.

Administration of probiotic strains Lp299 and CURE21 to healthy individuals decreased the number of leukocytes although within normal ranges, whereas lymphocytes remained unaltered (Figure 6).

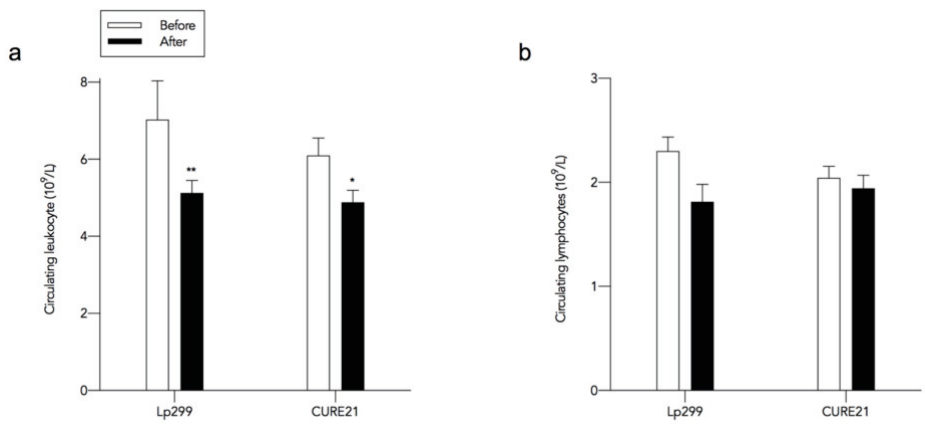

Figure 6.
Leukocytes (a) and lymphocytes (b).

FACS analysis (CD3, CD4, CD8, CD19, CD16+56, HLA-DR, HLA-DR/CD3 and fibrinogen), CRP analysis and IL-6 in blood samples before and after administration of probiotics did not show any significant differences.

No histological changes were seen before or after probiotic administration.

Lp299 could be found in faecal samples from all healthy volunteers ingesting the bacteria, however CURE21 was only found in one out of seven individuals after ingestion. The diversity of intestinal microbiota was not significantly altered in any group after the treatment, nor were any significant changes in the amount of the different taxa found (Table 7).

Table 7.

Concentrations of specific bacterial groups, detected by qPCR, in rectal tissue of healthy adults before and after 6 weeks probiotic consumption. Samples below detection limit were set to the detection limit of its specific qPCR assay. *All samples were below the detection limit of 5.65.

	Median (interquartile range) log 16S rRNA copies/g tissue before probiotic consumption	**Median (interquartile range) log 16S rRNA copies/g tissue after probiotic consumption**	**p-value**
***Lactiplantibacillus plantarum* 299 (n=6)**			
Lactobacillus	5.68 (5.68-5.94)	5.66 (5.65 -5.68)	0.180
Bifidobacterium	6.33 (5.75-7.55)	6.75 (5.73-6.97)	0.589
Akkermansia muciniphilia-like	6.74 (6.20-7.02)	6.55 (6.04-7.49)	1.000
Bacteroides fragilis group	5.82 (5.82-6.77)	5.81 (5.71-5.96)	0.485
Enterobacteriaceae	8.27 (8.06-10.01)	8.51 (7.73-9.46)	0.485
***Bifidobacterium infantis* CURE21 (n=4)**			
Lactobacillus	<5.65*	5.83 (5.65-6.41)	0.114
Bifidobacterium	6.64 (6.12-7.48)	6.56 (5.57-7.14)	0.686
Akkermansia muciniphilia-like	6.56 (5.98-7.28)	7.71 (6.62-8.21)	0.343
Bacteroides fragilis group	5.86 (5.79-5.88)	5.92 (5.80-6.19)	0.343
Enterobacteriaceae	7.73 (7.69-7.76)	8.15 (7.81-8.80)	0.114

Lactate dehydrogenase (LDH) confirmed that all tissue samples were viable.

Intake of *L. plantarum* 299 led to a reduction of the pro-inflammatory cytokine IL-6 in rectal mucosa after in vitro stimulation with a high concentration of TNF-α, however ingestion of *B. infantis* CURE21 did not give the same response. Thus, the release of IL-6 in response to stimulation with TNF-α induced different responses by different bacteria. Lp299 evoked a reduced release of IL-6 upon stimulation with TNF-α10 ng/ml. A higher concentration of TNF-α as well as a longer exposure resulted in a more pronounced reaction, hence only results for the latter period of 8 hours and he higher concentration of 100 ng/ml are given (Figure 7a-b).

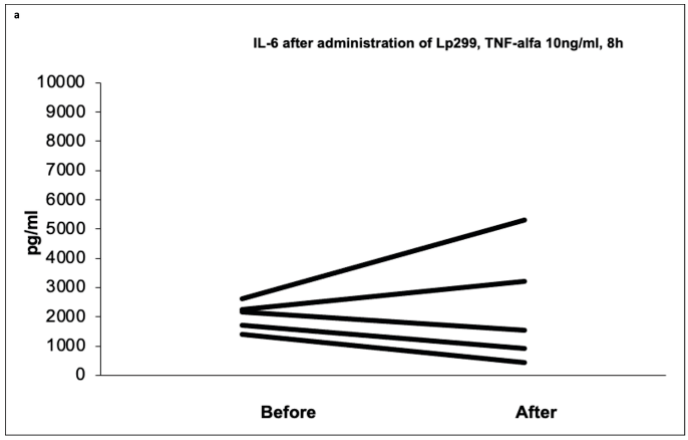

Figure 7a.
IL-6 after administration of Lp299, TNF-α 10 ng/ml, 8h.

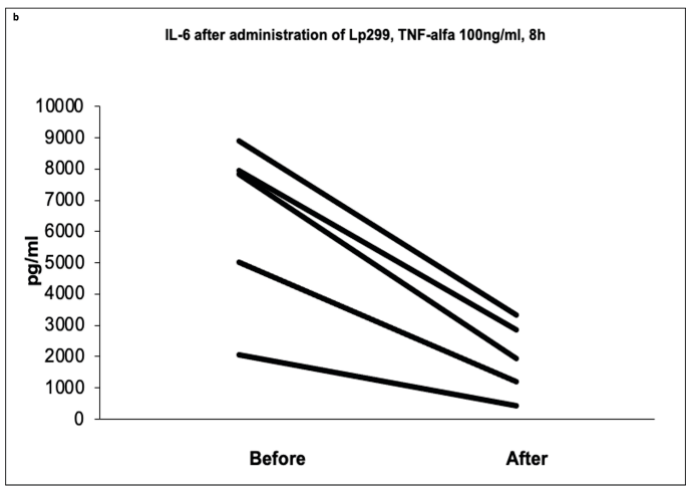

Figure 7b.
IL-6 after administration of Lp299, TNF-α 100 ng/ml, 8h.

Two samples in the Lp299 group did not respond at all before start of ingestion of bacteria (IL-6 <200 pg/ml) and therefore excluded from analysis. In the group with intake of CURE21 the response was more variable and there was no reactivity in three out of seven subjects whose samples were similarly excluded.

Paper IV

The number of patients who completed the study was 30 in total, 6 women (20%) and 24 men (80%), with 10 patients in each group. Two patients from each of the treatment groups were excluded for personal reasons. No side effects were recorded. The median age was 67 years (range 35-80), and the median BMI was 25,15 kg/m^2 (range 18-41,6 kg/m^2).

In the control group, 4 patients went through abdominoperineal resection (APR) and 6 patients underwent resection of the rectum, of which 2 were performed laparoscopically. In the prebiotic group, 2 patients underwent APR, 5 patients a rectal resection and 3 a Hartmann's procedure. In the synbiotic group, 2 patients underwent APR, 7 patients a rectal resection, of which 1 was performed laparoscopically, and one patient underwent Hartmann's procedure.

T-RFLP showed a low number of peaks in the control group compared to the two treated groups, before as well as after RT (Figure 8, Figure 9).

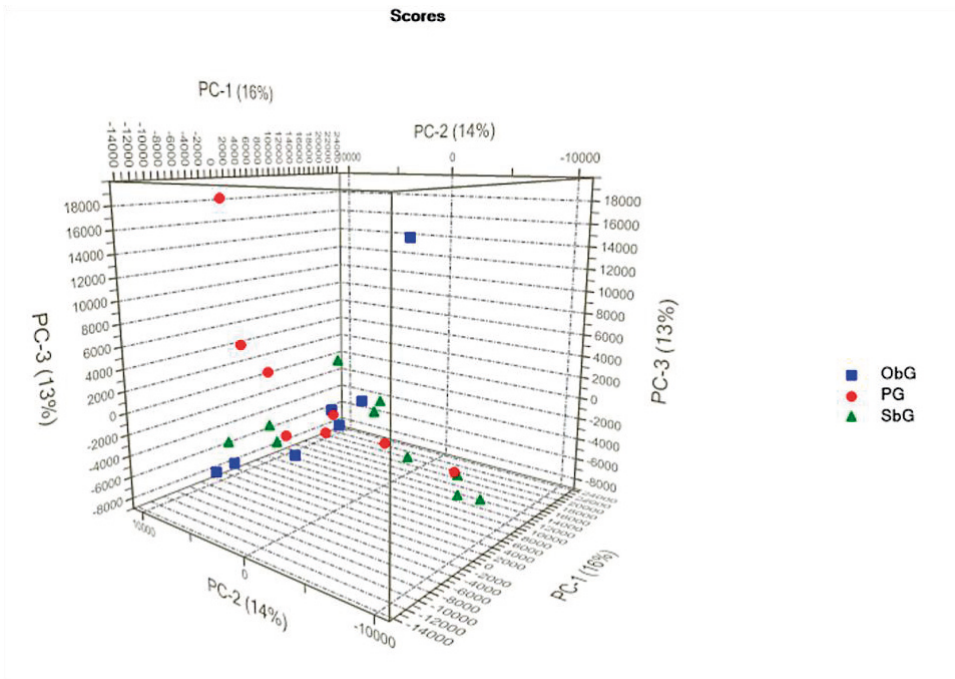

Figure 8.
PCA 3D score scatter plot of T-RFLP data before radiation obtained from *Msp*I digestion.

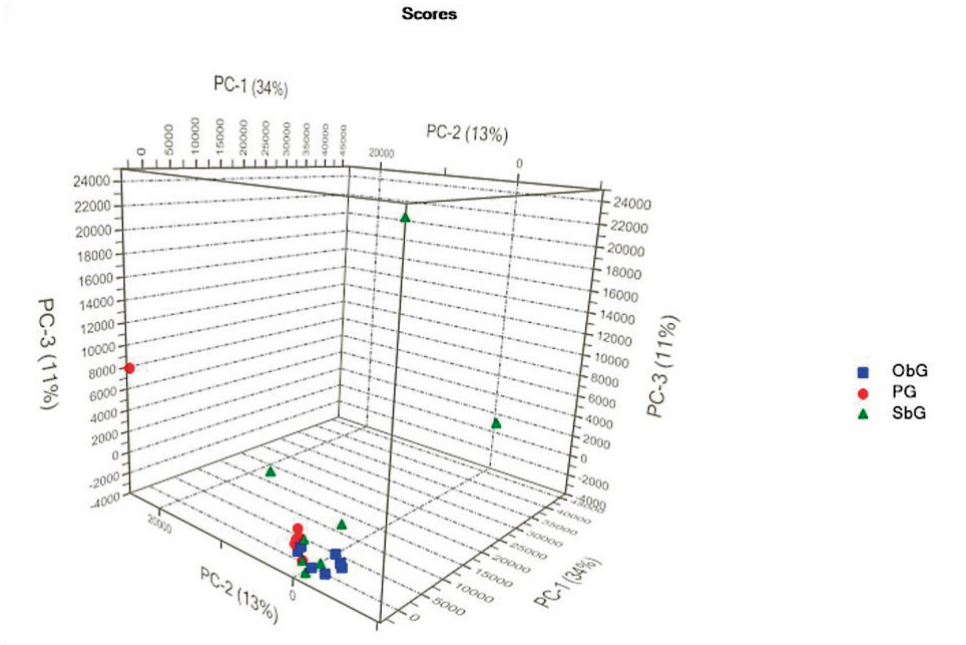

Figure 9.
PCA 3D score scatter plot of T-RFLP data at surgery obtained from *Msp*I digestion.

White blood cell count decreased significantly after radiation in the two treated groups, although not in the control group (Figure 10A). No significant differences in TGF- β were observed (Figure 10B), nor for the cytokines IL-6 or IL-10. MPO expression was higher in all three groups before and after radiotherapy (Figure 10C). No significant changes were seen for other inflammatory markers. No alteration of bowel permeability was noted as zonulin levels did not change significantly after RT (Figure 10D).

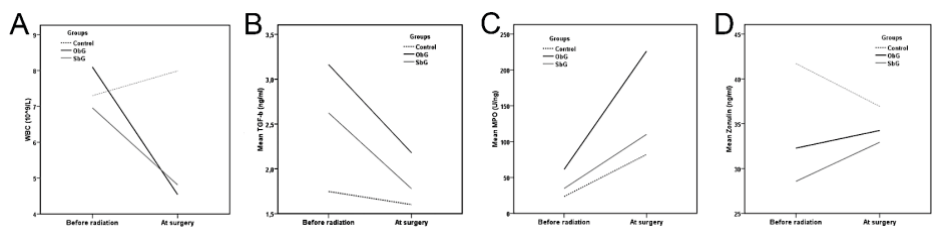

Figure 10 A-D.
Levels of WBC, TGF-β, MPO, and zonulin, before radiation and at surgery.

Histological examination demonstrated milder inflammation following RT in the prebiotic group and more pronounced inflammatory changes in the control group. Signs of marked fibrosis were observed in the latter group.

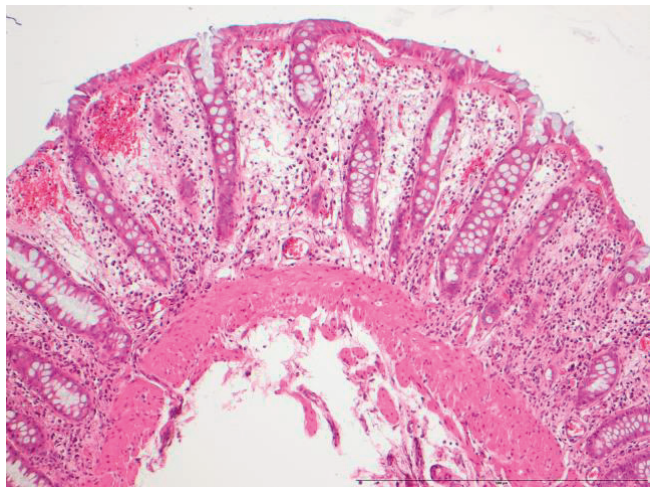

Figure 11.
Prebiotic group (ObG). Mild to moderate inflammation with mild ischaemia.

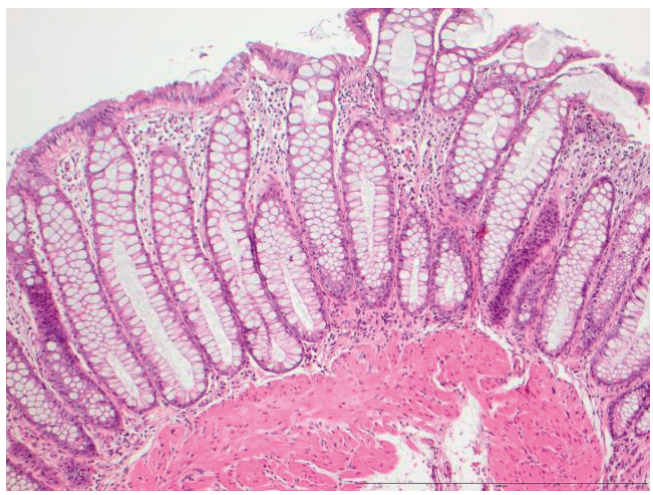

Figure 12.
Synbiotic group (SbG). Mild inflammation with slightly irregular, focally hyperplasic architecture, dilated crypts with some oedema in the lamina propria.

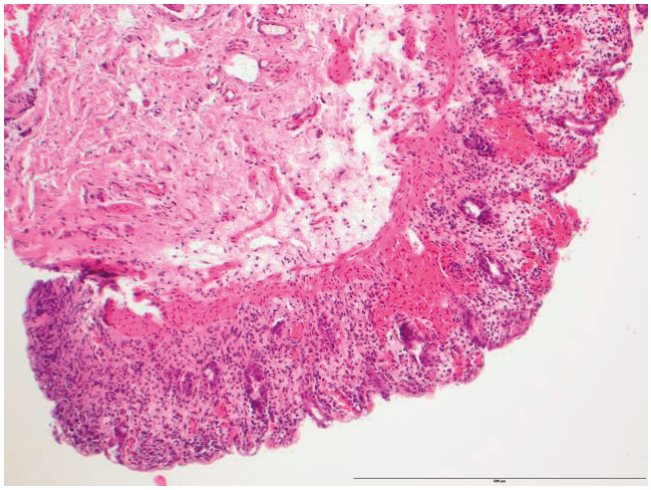

Figure 13.

Control group (CG). More advanced inflammation with highly ischaemic alterations, atrophic microcrypts and reactive alterations of the epithelium. In the lamina propria some focal bleeding and oedema is observed as well as increased amounts of chronically inflamed cells with fibrosis and scattered neutrophil granulocytes. Some active inflammation with cryptites is also observed.

I know we could live tomorrow,
but I don't think we should wait.

Laleh Poukarim

Discussion

The aim of this thesis has emerged from the ambition to find means of reducing risks and drawbacks with radiotherapy for rectal cancer for the two thirds of these patients receiving this treatment. Moreover, a curiosity to explore further possibilities of detecting spread of colorectal disease and to improve patient centred treatment was evoked based on the existing knowledge gap between providing maximal tumoricidal effect and maximal avoidance of adverse effects.

It would be of value for surgeons to improve their knowledge about gut microflora in the context of new methods and treatments based on the microbiota. Knowledge about the microbiota will enable enhanced comprehension of the pathophysiology of trauma as well as of surgical interventions. Emerging technologies may soon make it possible to use knowledge of, and about, the microbiota as a diagnostic tool in clinical settings.

During the past 20 years, awareness has increased of the pivotal significance of the human microbiota for health and disease in cases of impaired homeostasis and dysbiosis. Knowledge of the complex and sophisticated interfaces connecting the human body and the microbiota residing in and on it is evolving and will hopefully soon be fully recognised by the scientific society.

The human body is known to harbour bacteria outnumbering human cells by a ratio of more than 10:1, but this has recently been re-evaluated to a ratio closer to 1:1.[284] One can agree with the Delphic maxim of "know thyself" by examining the contents of the body, and also with Carl Linnaeus when he, in the same spirit, in 1735 described humans with the identical phrase "Nosce te ipsum".

MMPs have been reported as one of the main proteins involved in cancer progression and metastasis formation. It is also known that accurate identification of lymph node involvement is critical for successful treatment of patients with spread colorectal carcinoma. The aim of study I was to investigate a new molecular biological diagnostic and prognostic marker for the malignant potential of tumours. As MMPs are involved in degradation of basal membranes and extracellular matrix, and thereby actively contribute to spread of tumour tissue, we aimed to assess the role of MMP expression in the clinical context. The expression of MMP7 mRNA in humans has high specificity in colorectal cancer, especially in malignant epithelial cells, but weaker expression in normal colorectal mucosa, as well as in different grades of dysplasia to cancer. At the time of the study, to our knowledge no previous

study had investigated MMP7 in tumour and normal mucosa as well as in blood and lymph nodes at the same time, in irradiated rectal cancer patients.

The first study in this thesis is an assessment of MMP7 expression in different stages of colorectal tumours from benign lesions to cancer development. A successive increase of MMP7 was seen from benign polyps to malignant tumour tissue and an association between MMP7 levels in lymph nodes, in serum, and in more advanced tumour stage was observed, indicating that MMP7 might play a role as a biomarker for locally advanced colorectal cancer. If so, it may be an adjunct for evaluating prognosis and best selected therapy. An efficacious therapy for patients with colorectal tumours requires a correct classification of lymph node engagement. We found that increased levels of MMP7 are associated with lymph node involvement, with locally invasive tumour, and with a tendency to metastatic disease. MMP7 used as a marker of malignant potential of tumours might be of value in anti-cancer or anti-metastatic therapy in the future.

The second study examines how neoadjuvant radiotherapy and surgery affect expression of MMP7 in rectal cancer patients. To our knowledge, only one previous study had investigated the association between MMP7 and radiotherapy as the solely neoadjuvant treatment for rectal cancer when this study was carried out.[235] Further, this study examined effects of short-course radiotherapy (25 Gy). At that time, there were two active radiotherapy regimens used for treatment of rectal cancer in Sweden. The short-course (25 Gy) treatment for limited tumours and the long-course (50 Gy) treatment for more advanced tumours. We believed it would be valuable to investigate how these different regimens of radiotherapy influenced MMP7 expression. It is known that elevated MMP levels are related to an increased risk for impaired wound healing and risk for complications such as anastomotic leakage or dehiscence. One hypothesis was that a connection between irradiation, increased MMP7 levels and risk for surgical complications, such as mentioned above, could be found. Since no chemotherapy was routinely administered concomitantly at the time, this study made it possible to investigate the effect of radiation alone. Thus, the material in this study is unique in that it compares the effects of two different regimens of neoadjuvant radiotherapy (short-course/long-course) on MMP7 expression in rectal cancer patients with standardised surgical trauma (TME surgery) where effects of irradiation alone can be evaluated. In addition, a non-irradiated control group was included. We found that short-course radiotherapy induced increased levels of MMP7 before surgery, whereas long-course neoadjuvant radiotherapy resulted in significantly lower MMP7 levels compared to short-course irradiation. The difference could be mainly explained by the period between radiotherapy and surgery, thus allowing the tumour and surrounding tissue to decrease MMP7 levels from acute to lower inflammatory levels. Furthermore, surgery upregulated MMP7 expression in all three groups; thus surgery has been shown to exert an overruling effect on RT on the upregulation of MMP7.

Radiation to the abdominopelvic region inevitably affects healthy gut mucosa, which may lead to acute and chronic toxic injury to the gut, since it is particularly

susceptible to radiation toxicity. It is of value to develop strategies to prevent these side-effects, in order to reduce serious radiation-induced complications and facilitate potentially curable radiotherapy in certain cancer patients. Under these circumstances, when elective surgery is at hand and the RT is scheduled, we are aware of the time point of injury, thus making it possible to prevent.

Radiotherapy to this region affects intestinal bacteria resulting in a change in, and impairment of, gut microbiota. An unfavourable growth of opportunistic microorganisms occurs, leading to a more pronounced damage to the bowel mucosa and an increased inflammation. Gut microbe dysbiosis seems to affect the sensitivity of epithelial cells to irradiation, resulting in apoptosis of epithelial and endothelial cells, as well as promoting leukocyte- and platelet recruitment enhancing the intestinal radiation-induced inflammation.[210] This may lead to increased fibrosis deep in the intestinal wall with risk for stricture formation and eventually bowel obstruction later.

Since pelvic radiotherapy almost invariably is accompanied by acute intestinal inflammation and often followed by a progressive fibrosis, months to years later, it is of interest to find a way to minimise or reduce the injury caused by ionising radiation. Today the evidence of effective preventive action to protect the healthy intestinal mucosa from radiation injury is limited, which often leads to considerable post-radiation morbidity. Since radiotherapy in treatment of cancers affecting gynaecological organs, the urinary bladder, prostate, rectum, and anus is growing, an increase of adverse effects related to the intestines is to be expected in the future. Studies have reported that up to 50% of RT-treated patients have long-term chronic gastrointestinal side-effects affecting quality of life.[146] These drawbacks make it important to develop strategies to reduce some of the negative effects of radiotherapy related to the inflammatory reaction that follows ionising radiation to the bowel mucosa.

Beyond the inflammatory response evoked by irradiation, a direct cell-killing effect is seen, which may lead to a impaired barrier function in the intestines and moreover an injury to the immunological system that to a great extent is located in the gastrointestinal tract.

The epithelial barrier function is found to be substantially disturbed after radiotherapy and a deranged intestinal flora appears to predispose for gut symptoms. A stabilised gut microbiota e g by ingestion of probiotics appears in studies to reduce the bacterial translocation and decrease the inflammatory reaction.[133] Studies in animals have shown that probiotics exert beneficial effects in reducing radiation-induced endotoxin expression, minimising histological changes as well as attenuating bacteriemia.[131] In recent years, human studies also have demonstrated favourable effects of enterally administered non-pathogenic bacteria in conditions such as septicaemia, severe trauma with inflammatory reaction and in gut barrier dysfunction where an overgrowth of potentially pathogenic bacteria is seen.[285-287]

The mechanisms of action are not fully clarified but it can be anticipated that the impaired barrier function is restored, a counterweight to pathogenic bacteria emerges and the immune system is stimulated by the presence of non-pathogenic bacteria. Probiotics stimulate mucosal growth and reinforce the bowel wall. The intestinal mucosa may be "pre-conditioned" to better endure injuries.

Thus, one way of reducing adverse effects on the gut mucosa, evoked by RT, might be by preparing it through administration of probiotics. To investigate how the healthy mucosa reacts, in the third study we created a model for a standardised injury to the intestinal mucosa. Because of difficulties in achieving this *in vivo* we chose to do it *in vitro*, by use of the potent pro-inflammatory cytokine tumour necrosis factor alpha (TNF-α). The inflammatory response to a standardised injury on the healthy gut mucosa *in vitro* was reduced after six weeks' ingestion of *Lactiplantibacillus plantarum* 299 (Lp299) and the systemic leukocyte count decreased after intake of Lp299 as well as after *Bifidobacterium infantis* CURE21.

The risk of damaging the surrounding healthy tissue is a limiting factor when planning target dose to a tumour, thus affecting the possibility to cure the disease. The microbiota emerges as a critical driver of radiation-induced intestinal disease.[208, 210] Prebiotics, e g fibre, may exert potential benefits on acute post-radiation gastrointestinal symptoms such as diarrhoea and bowel symptoms.[288] Probiotics have been reported to be used for prevention and treatment of chemo- and/or radiotherapy-induced gastrointestinal toxic symptoms with the absence of considerable adverse consequences, but the evidence is still not sufficient for standardised clinical recommendations and further research is needed.[149] Synbiotic therapy may be beneficial in bringing additive and/or synergistic effects of combinations of prebiotic and probiotic agents that may be efficient in conditions where an abnormal gut flora is present.

The next step was to investigate if the adverse inflammatory effects of radiation therapy could be equivalently reduced in patients being treated for rectal cancer. Pre- or synbiotics may impact gut microbiota, inflammation, and gut permeability, thereby preventing radiation injury and optimising conditions for surgery in patients with neoadjuvant radiotherapy for rectal cancer. In the fourth study, we found that administered pre- or synbiotics led to higher number of T-RFLP peaks compared to the control group, indicating a higher microbial diversity, which is considered favourable. White blood cell count decreased and mucosal MPO increased after radiotherapy in the pre-treated groups, which might indicate an increased neutrophil activity after irradiation in these groups. A progressive inflammation but not pronounced fibrosis was seen on histologic examination in the pre- and synbiotic treatment groups, whereas fibrosis was more distinct in the control group. Thus, a protective effect of administered pre- and/or synbiotics could be seen in our clinical study.

The preventive effect of probiotics to radiation-induced intestinal injuries has however been predominantly studied for acute gastrointestinal effects. Studies that investigate the influence of probiotics in the prevention of chronic radiation-induced gut damage are lacking. Future studies should focus on the correlation between chronic radiation-induced bowel injury and dysbiosis induced by irradiation and investigate what characteristics the microbiota possess that may offer a protective role in the radiation-induced inflammatory injury to the intestines.[131]

Interestingly, there are at least two opposing aspects of microbiota action: beyond a protective role against radiation-induced gut mucosa injury and morbidity, several studies have shown the importance of microbiota in the development of CRC. Lifestyle factors, including dietary factors mediated by the intestinal microbiota, have shown a strong connection to the risk of CRC development. Patients with CRC exhibit an altered gut microbiota, and methods to recognise potential microbial markers for CRC are indeed required.[289-291] A study by Donohoe *et al* has shown that the composition of the microbiota in samples from patients with colon cancer who received probiotics had a unique profile, with an abundance of butyrate-producing bacteria in tumour, mucosa and faecal samples compared to patients with cancer who did not receive probiotics.[292] Specific probiotic bacteria have been shown to modulate inflammation and reduce tumour proliferation in animal models of carcinogenesis and might offer therapeutic benefits for CRC patients. The colon cancer-associated microbiota can be manipulated by specific probiotic strains, resulting in an altered microbiota enriched with beneficial bacteria. Microbiota modulation by probiotics could thus be considered as part of a therapeutic regimen for CRC patients.[217] The ameliorative effects of synbiotics on acute radiation-induced diarrhoea (ARID) are not simply mediated through the intestinal flora balance, but rather through their influence on the principal pathophysiological components of radiation injury.[293]

It would be of importance to determine if synbiotics might act as radiomitigators or radioprotectors and to study their usefulness in preventing the development of different phenotypes of chronic radiation changes in the rectum.[143]

Today there is no optimal way to prevent radiation-induced damage on surrounding tissue, even if delivery of irradiation is optimised to minimise damage to other than malignant cells. Thus, the results from our studies have strengthened our belief that: MMP7 may be used as an important adjunct in planning of rectal cancer treatment strategies and assessing prognosis; that the limited expression of MMP7 in long-course RT might help reduce tumour progression; and that emphasis in future research should be focused on modulation of the gut and its microbiota as ways of minimising and preventing radiation-induced complications in cancer patients undergoing irradiation therapy.

It always seems impossible until it is done.

Nelson Mandela 1918-2003

It may seem difficult at first,
but all things are difficult at first.

Miyamoto Musashi 1584-1645

Conclusions

- An increased expression of matrilysin/MMP7 correlates to risk for lymph nodal involvement in colorectal cancer disease.
- In advanced stage of disease, significantly increasing levels of matrilysin/MMP7 in serum, tumour tissue and in lymph nodes correlate to increasing grade of dysplasia and adenocarcinoma infiltration.
- Neoadjuvant long-course (50 Gy) irradiation induces a significant reduction in matrilysin/MMP7 expression at surgery compared to short-course radiotherapy (25 Gy). Long-course 50 Gy RT may thus be more favourable in reducing tumour progression.
- Surgery *per se* has an overriding influence on increasing matrilysin/MMP7 expression compared to RT.
- TGF- β showed a significant 2- to 3-fold increase after surgery.
- Administration of the probiotic strains *Lactiplantibacillus plantarum* 299 (Lp299) and *Bifidobacterium infantis* CURE21 to healthy humans leads to systemic and local reduction of inflammatory response to a standardized injury by decreasing leukocyte levels. Lp299 alone reduces levels of the pro-inflammatory cytokine IL-6 in rectal mucosa.
- Probiotic supplementation may be of value in minimising inflammatory injuries to gastrointestinal mucosa in clinical situations such as surgical trauma and/or radiotherapy.
- *L. plantarum* 299 and *B. infantis* CURE21 evoked different reactivity levels locally in mucosa after an inflammatory stimulus. This probably reflects different modes of action with the immune system by different bacteria. The different actions were not reflected systemically.
- Pre- and/or synbiotic therapy influence gut microbiota and enhance bacterial viability, and this may reduce gastrointestinal radiation injury and improve the prerequisites for better outcome after surgery in patients undergoing neoadjuvant radiotherapy for rectal cancer.

Vad som är fördolt och vad som
är uppenbart, allt har jag
lärt känna.

Ty visheten, hon som är mästare
i allt, har undervisat mig
därom.

...

Hon sträcker sig i full kraft från
världens ena ända till den andra,
och hon styr allting väl.

Salomos Vishet 7:21-22, 8:1 (1921)

Future perspectives

To clinically investigate the expression of MMP7 in resected specimens to enable better diagnosis and staging, as well as tailoring and individualising eventual adjuvant therapy in rectal cancer patients.

To further examine the advantages of long-course RT for neoadjuvant rectal cancer treatment due to its restricted effect on MMP7 expression and subsequently on reducing tumour progression.

Further clinical studies on pre-, pro- and/or synbiotics in clinical contexts to investigate their ability to reduce inflammatory injuries on gastrointestinal mucosa caused by surgery, radio- and/or chemotherapy.

Allt kan man ta ifrån människan
Utom en sak – den yttersta friheten
att välja förhållningssätt
till det som livet för med sig.

Viktor Frankl 1905-1997
ur ”Livet måste ha mening”

Allt verkligt liv är möte.

Martin Buber 1878-1965

Populärvetenskaplig sammanfattning

Cancer i tjock- och ändtarm är den tredje vanligaste dödsorsaken i västvärlden. I Sverige diagnostiseras ungefär 6000 personer varje år med tjock- och ändtarmscancer av vilka ca 2000 har sin tumör i ändtarmen. Genomsnittsåldern för insjuknande är 73 år för tjocktarmscancer och 72 år för ändtarmscancer. Endast 4% av tjocktarmscancer- respektive 5% av ändtarmscancerpatienterna är under 50 år vid insjuknandet.

Ändtarmscancer är vanligare bland män (62%) än bland kvinnor (38%). När diagnosen har ställts kan ungefär två tredjedelar av patienterna genomgå operation i syfte att bota sjukdomen men statistiskt sett kommer 30-50% av dessa patienter att få återfall och avlida till följd av sin sjukdom. I syfte att förbättra detta utfall har tilläggsbehandling i form av strålbehandling, med eller utan cellgiftsbehandling, introducerats i behandlingsarsenalen, upptill den kirurgiska operationen. I Sverige får 60-65% av patienterna strålbehandling, antingen för att minska lokalt återfall eller för att krympa tumörer som primärt inte går att operera. Biverkningar av eller komplikationer till dessa tilläggsbehandlingar omfattar strålningsorsakad skada på tarmen och inflammation som kan leda till svåra och besvärande symptom och tillstånd såsom försämrad läkning efter kirurgi, bäckensmärta, diarré, blödningar, avföringsrubbningar och/eller trånghet i tarmen. Idag finns inget enskilt test eller markör som kan förutspå eller prognostisera ett bra behandlingssvar eller ett misslyckande av de olika behandlingsmetoderna, vare sig vid begränsad sjukdom eller vid avancerad/spridd sjukdom.

Matrilysin, även benämnt matrixmetalloproteinas 7 (MMP7), är den minsta medlemmen i en stor familj av proteiner (äggviteämnen) som har förmåga att bl a bryta ner den vävnad som omger celler och därmed påverka t ex tillväxt, kärlnybildning och sårläkning. MMP7 har visats ha ett samband med spridd/avancerad tumörsjukdom och dess utsöndring ökar vid strålning. Tarmbakterier har visat sig ha en nyckelroll för utsöndringen av proteinet MMP7.

Människans bakterieflora i tarmen är viktig för att tarmslemhinnan ska må bra och kunna stå emot inflammation samt upprätthålla tarmväggens skyddsfunktion. Strålbehandling medför en förändrad/försämrad tarmflora med ogynnsam tillväxt av mikroorganismer vilka kan öka skadan i tarmslemhinnan och den inflammation som uppstår i tarmen vid bestrålning. Denna skada förvärras vid strålbehandling av ändtarmscancer eftersom tarmbakterier finns i det bestrålade området. Strålningen

framkallar ett inflammatoriskt svar men har också en direkt celldödande effekt och kan leda till en förlorad barriärfunktion i tarmen och dessutom till en skada på immunsystemet, vilket till stor del finns i magtarmkanalen. Studier har visat att probiotika (”nyttiga/goda” bakterier), liksom dessa bakteriers ”föda/mat” d v s prebiotika (t ex olika typer av fibrer), samt varierande kombinationer av dessa (synbiotika), skulle kunna påverka tarmens bakterieflora och minska den strålningsorsakade tarmslemhinneskadan hos patienter som får strålbehandling vid ändtarmscancer, utan att reducera den eftersträvade effekten av strålbehandlingen på tumören. Tillförd pre-, pro- och/eller synbiotika stimulerar tillväxt av tarmslemhinnan och ”tätar” tarmväggen, som på så sätt kan bli förbehandlad för att bättre kunna stå emot en skada som t ex en strålningsorsakad inflammation.

Syftet med denna avhandling är att undersöka huruvida det inflammatoriska proteinet MMP7 (matrilysin) skulle kunna vara av potentiell klinisk nytta som markör/indikator för komplikationer och prognos vid kirurgi hos patienter med cancer i tjock-/ändtarm samt att studera huruvida pre-, pro- och/eller synbiotika har en inverkan på tarmens slemhinna/vägg så att den bättre kan motstå den inflammatoriska skada som uppstår efter t ex strålbehandling av tumörer.

Avhandlingen består av fyra delarbeten. Arbete I är en studie av MMP7-nivåer vid olika stadier av tumörsjukdom i tjock- och ändtarm, från godartade polyper (slemhinneutväxter) till avancerade cancertumörer, och dess relation till spridd sjukdom hos patienter som genomgår operation. Hos dessa patienter skulle MMP7 kunna ha en prognostisk betydelse som markör vid diagnostik av avancerad sjukdom. I arbete II undersöks hur nivåerna av MMP7 hos patienter med ändtarmscancer påverkas av strålbehandling i tre olika behandlingsgrupper: en grupp med ”kort” strålbehandling (25 Gray, Gy), en grupp med ”lång” strålning (50 Gy) samt en kontrollgrupp utan bestrålning. I arbete III kartläggs hur den friska tarmslemhinnan reagerar när den utsätts för en standardiserad inflammatorisk skada efter att ha blivit förbehandlad med en av två olika probiotiska bakteriestammar för att om möjligt minska den framkallade skadan. Skadan åstadkoms genom att vävnadsprover från tarmslemhinnan hos friska frivilliga försökspersoner utsattes för en inflammatorisk stimulering i stigande doser och det inflammatoriska svaret vid olika tidpunkter värderades. Arbete IV undersöker tre grupper av patienter med ändtarmscancer som alla erhåller ”kort” strålbehandling (25 Gy) under en vecka före operation. En grupp förbehandlas med prebiotika (havre) och den andra gruppen med synbiotika (havre + blåbär + en probiotisk bakterie, *Lactiplantibacillus plantarum* HEAL19) under sammanlagt två veckor före operation, medan den tredje gruppen (en kontrollgrupp) inte får någon förbehandling alls.

Vi fann i studie I att utsöndringen av MMP7 ökade med ökad grad av cancerförändringar såväl i själva tumören som i intilliggande lymfkörtlar. MMP7 skulle därför kunna användas som komplement/markör vid undersökning/utredning av misstänkt lokalt avancerad cancer.

Studie II visade att det kirurgiska ingreppet/operationen ökar MMP7-nivåerna mer än vad strålningen gör i alla tre grupperna, samt att ”lång” strålning framkallade lägre MMP7-utsöndring vid operation jämfört med ”kort” strålbehandling.

I studie III påvisades en minskning av antalet vita blodkroppar efter intag av de probiotiska bakterierna *Lactiplantibacillus plantarum* 299 (Lp299) och *Bifidobacterium infantis* CURE21 (CURE21). Därtill åstadkom Lp299 en minskad lokal inflammation i ändtarmens slemhinna vilket inte var fallet med CURE21. Probiotikatillförsel skulle kunna göra tarmslemhinnan mer motståndskraftig så att den är bättre rustad att klara av en inflammatorisk skada såsom den som uppstår i samband med strålbehandling. Probiotika har en minskande effekt på inflammation och denna egenskap förefaller vara specifik för olika bakteriestammar, vilket är av betydelse då olika probiotiska behandlingsalternativ jämföres.

Studie IV påvisar sänkta nivåer av vita blodkroppar i de båda behandlade grupperna, men inte i kontrollgruppen. Den mikroskopiska undersökningen av vävnadsproverna visade att det förelåg en mild respektive måttlig inflammation i de grupper som fick prebiotika respektive synbiotika, medan kontrollgruppen uppvisade uttalade inflammatoriska förändringar och bindvävsomvandling i tarmvävnaden. Undersökning av bakterierna i tarmslemhinnan påvisade en större variation av bakterier efter synbiotikatillförsel vilket skulle kunna vara av godo för att stärka tarmens förmåga att bättre stå emot en inflammatorisk påverkan, såsom den som orsakas av strålbehandling hos patienter med ändtarmscancer.

Strålbehandling används numera allt mera vid behandling av olika cancerformer. Möjligheterna att kunna påverka strålningens ogynnsamma inverkan på omgivande frisk tarm är idag begränsade, vilket kan leda till svåra negativa konsekvenser för patienterna.

Delarbetena i denna avhandling har kunnat påvisa:

- ett samband mellan proteinet MMP7 (matrilysin) och cancerutveckling och ett möjligt prognostiskt värde av MMP7 för diagnostik av spridd sjukdom
- att MMP7 påverkas mer av den kirurgiska operationen än av strålbehandlingen och att lång strålbehandling (50 Gy) leder till minskad utsöndring av MMP7 vid kirurgi vilket skulle kunna vara fördelaktigt för minskad tumörutveckling
- att intag av pre-, pro- och/eller synbiotika skulle kunna stärka tarmslemhinnan så att den bättre kan stå emot strålningens negativa påverkan på tarmväggen och förbättra återhämtningen efter kirurgisk åtgärd och därmed kunna ge patienter som genomgått strålbehandling av t ex ändtarmscancer en förbättrad livskvalitet.

Skogen skulle
vara mycket tyst
om inga andra
fåglar sjöng än de
som sjunger bäst.

Henry van Dyke 1852-1933

Acknowledgements

This thesis for the degree of Doctor of Philosophy in Medical Science would not have been possible without the contribution, untiring effort, and dedication of many people around me throughout these years (you know who you are). I cannot mention you all here, but I will always be deeply grateful to you.

Still, I would like to express my sincere gratitude in particular to:

All patients and healthy volunteers who have taken part in the studies of this thesis.

Bengt Jeppsson, senior professor of surgery, who initiated this long journey of mutual research many years ago. Thank you for your immense patience from start to finish! Your vast knowledge in the field of this thesis has been my deepest source of inspiration. Heartfelt thanks to you and Christina for your warm hospitality and many good meetings in your homes!

Louis Banka Johnson, associate professor of surgery, who picked up the baton to complete the race. Thank you for your tireless patience and for your ability to 'always look at the bright side of life'! The two of you, my supervisors, share a common exclusive quality in almost always making a PhD student find a ray of hope and confidence even in times of despair.

Henrik Thorlacius, professor of surgery and head of the surgical research group, Department of Clinical Sciences, Malmö, for completing the supervisor team and for support over the years.

Past and present colleagues in the Departments of Surgery in Ängelholm, Lund, Malmö, and Helsingborg, for invaluable and fruitful collaboration, in clinical work and research as well as for your friendship.

Everyone at 'Kirurgmottagningen i Ängelholm' for boundless enthusiastic support, and for taking care of the daily work when I was busy writing this thesis. You are true Angels!

My co-authors, among whom the following deserve my special thankfulness: Dr Andrea Polistena, for kindness and friendship as well as for invaluable input and guidance in the world of matrilysin and statistics. Next year in Lund!

Andrada Röme, dear colleague and my "SuperKompis", for everything you shared with me over the years, in research and clinical work from the first mouse rose-hip study and beyond, in all aspects of, and events in, life. "Faithful friends are a sturdy

shelter: whoever finds one has found a treasure. Faithful friends are beyond price; no amount can balance their worth. Faithful friends are life-saving medicine." *Sirach 6:14-16a*

Ingrid Palmquist, research nurse at the Department of Surgery and Clinical Research Centre, Malmö. You are at the heart of things in the surgical research group, without whom nothing would work. Your ability to keep track of everything, even though a substantial amount of time may have elapsed, is invaluable!

Dear friend Sylvia Idström, @Teamword, for excellent guiding in the art of language, and Sanja Matoničkin, @Sjukhusbiblioteket Helsingborg, for navigating through the world of references.

My greater family, who have always been there for me. Some of you also contributed to this thesis in different ways, for which I am so grateful! Together with my closest family: my late father, Folke, who left this world far too soon; my still-going-strong mother, Kerstin ("Nanja"); and my brother Anders – you all gave me a confident, solid, and creative foundation in life. Thank you for enriching my life and for teaching, throughout the generations, the value of hard work.

This thesis has not been able to finalise until now, maybe because "my crew had to grow… up" – I have been blessed with the most amazing family: my beloved daughters, Emma and Anna, lights of my life, joy of my world. You are always by my side, sharing interest in my work, equipped with never-ending patience and love. Together with dear Nils, you are simply the best team ever, all categories (not to mention when I'm wrestling with the computer and your never-failing encouragement and invaluable support 24/7 is only a phone call away) - thank you from the bottom of my heart, for being who you are! Last but not least, my adorable grandson, Folke – you came with energy, light, and hope. You all are the nucleus of my life – gratitude and endless love!

This thesis was made possible by much-appreciated support from Stig and Ragna Gorthon Foundation, Maggie Stephen Foundation, Eva and Ingemar Nilsson Foundation, the Faculty of Medicine at Lund University, and the Research Department at Skåne Hospital Northwest (FoUU SSNV).

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Kunskapen är stolt över allt den vet.
Visdomen är ödmjuk inför allt den inte vet.

William Cowper 1731-1800