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Modulating the inflammatory response after colorectal cancer surgery: friend or foe?

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To Matilda

My rock-solid foundation and precious partner

I love you for so many reasons

And to my dearest Thelma and Alexis

You are my everything

Register

Abstract

Colorectal cancer was the second most deadly and third most common cancer globally in 2020. In Sweden, more than 5,000 new colonic cancer cases and more than 2,000 rectal cancer cases were reported in 2021, making colorectal cancer the third most common in Sweden (excluding skin malignancies).

Anastomotic leakage after colorectal cancer surgery is a feared complication that confers substantial morbidity, including a higher risk of permanent stoma and cardiovascular morbidity, but can also impart an increased risk of recurrence and mortality; the reason why leakage might cause this is not established. Perioperative inflammation including upregulation of cyclooxygenase-enzymes, which is further increased by anastomotic leakage, can possibly modulate both anastomotic healing as well as impact minimal residual disease. Non-steroidal anti-inflammatory drugs (NSAIDs) act by inhibiting COX-enzymes and can be part of a postoperative multimodal analgesia protocol. However, their postoperative use has been debated, with fears of NSAIDs possibly increasing anastomotic leakage rates.

Study I was a retrospective cohort study on 1,341 patients who had undergone anterior resection for rectal cancer. Exposure was at least two days with NSAIDs during the first postoperative week, and the primary outcome was recurrence-free survival. A Cox regression model could not demonstrate a significant association with a hazard ratio (HR) of 1.02 (95% confidence interval (CI): 0.79–1.33) and neither did a propensity score-matched analysis. An instrumental variable analysis displayed a tentative improvement in recurrence-free survival in the NSAID-exposed (HR 0.61; 95% CI 0.38–0.99), but the core assumptions to perform such an analysis were not fully satisfied.

Study II was a protocol-based retrospective cohort study with a total of 6,945 patients resected for colorectal cancer with a primary anastomosis formed. NSAID-exposure was determined by each individual hospital's postoperative analgesia protocol, while patient data

and outcomes were retrieved from the Swedish colorectal cancer registry. Some 3,996 (58%) patients were treated at a hospital with NSAIDs included in their postoperative analgesia protocol. No significant association with recurrence-free survival was seen (HR 0.97, 95% CI 0.87–1.09). However, a reduction in cancer recurrence was demonstrated (HR 0.83, 95% CI 0.72–0.95), with an increased risk reduction for locoregional (HR 0.68, 95% CI 0.48–0.97) in comparison to distant recurrence (HR 0.85, 95% CI 0.74–0.98). Anastomotic leakage was less frequent as well, mainly because of a reduction in the group with colorectal or ileorectal anastomoses (HR 0.47, 95% CI 0.33–0.68).

In **Study III**, the aim was to explore proteomic and biological pathway alterations in patients with peritoneal infection. This was a 1:1 matched cohort study on patients resected for colorectal cancer with a primary anastomosis formed, including 32 cases who suffered a postoperative peritoneal infection matched with 32 controls with a complication-free postoperative stay. Serum samples were retrieved from their first postoperative visit and at one year postoperatively. Out of a total of 270 proteins tested, 77 were differentially expressed at the first postoperative visit at a median sampling time of 41 days after surgery. Many of the top hub proteins are known actors in colorectal cancer progression, including survival and invasiveness, potentially enhancing minimal residual disease. Over-represented pathways were related to cardiomyopathy, cell-adhesion, extracellular matrix, phosphatidylinositol-3-kinase/Akt (PI3K-Akt) and transforming growth factor beta (TGF-β) signalling.

In **Study IV**, the aim was to evaluate the frequency of a known polymorphism of the COX-2 gene promotor -765G>C in a Swedish cohort of colorectal cancer patients, and whether a previously reported association between this gene variant with an increase in anastomotic leakage could be reproduced. This was a 1:1 matched case-control study on a total of 94 patients who were resected for colorectal cancer with a subsequent primary anastomosis, with cases suffering a peritoneal infection. Preoperative blood and serum samples were genotyped and analysed using pre-defined protein panels. Of the 94 patients in total, one in each group were homozygous for the minor allele C/C, and ten cases and 14 controls were heterozygous with G/C and the rest were homozygous for the major allele. Thus, there were fewer individuals with the minor allele in the case group, with a non-significant odds ratio of 0.71 ($p=0.413$), ultimately not replicating the finding of the previous study. The protein quantitative trait loci analysis rendered no associations of interest.

In conclusion, no statistically significant effects on recurrence-free survival from postoperative NSAIDs in patients resected for colorectal cancer could be demonstrated in **study I**, whereas significant associations between NSAID use and reduction in frequency of anastomotic leaks as well as cancer recurrence could be shown in **study II**. In **study III**, numerous proteins were differentially expressed in patients suffering a postoperative peritoneal infection, even after more than a month's duration, potentially stimulating minimal residual disease. The over-representation analysis found pathways related to cardiomyopathy, which could help explain the increase in cardiovascular morbidity in patients suffering anastomotic leakage. **Study IV** could not reproduce the potentially marked increase in anastomotic leak frequency in carriers of the COX-2 gene promotor -765G>C polymorphism in a Swedish sample.

Whether to include NSAIDs or not in postoperative multimodal analgesia is a question still not answered, and it may depend on the genotype, the patient's preoperative inflammatory state, tumour location, the specific NSAID used, and whether a leak has already occurred. NSAIDs might have effects on both morbidity including cardiovascular and anastomotic leakage as well as minimal residual disease including recurrence and mortality. This thesis suggests potential protective effects regarding both anastomotic leakage as well as cancer recurrence, but it seems to depend on at least some of the aforementioned factors. The proteomic landscape regarding postoperative peritoneal infection has been investigated, and where it has also been demonstrated that the duration of said alterations can be greater than was earlier suspected. Finally, even if a replication attempt was not successful considering the relation between a COX-2 gene promotor polymorphism and anastomotic leakage, it could be worthwhile to attempt further replication studies.

Abbreviations

5-FU – 5 fluoro-uracil

Akt – ak strain transforming

ANOVA – analysis of variance

APC – adenomatous polyposis coli

ASA – The American Society of Anesthesiologists

BMI – body mass index

BRAF - v-raf murine sarcoma viral oncogene homolog B

CAPOX – capecitabine and oxaliplatin

CEA – carcinoembryonic antigen

CI – confidence interval

COX – cyclooxygenase enzyme

DCC – deleted in colorectal cancer

dMMR – defect mismatch repair

DNA – deoxyribonucleic acid

DPD – dihydropyrimidine dehydrogenase

EGFR – epidermal growth factor receptor

EMT – epithelial to mesenchymal transition

GO – gene ontology

HR – hazard ratio

IL - interleukin

ISREC – The International Study Group of Rectal Cancer

KEGG – Kyoto encyclopedia of genes and genomes

KRAS – kirsten rat sarcoma virus

MAPK – mitogen activated protein kinases

MMR – mismatch repair

MSI – microsatellite instability

MSI-H – microsatellite instability high

NSAID – Non-steroidal anti-inflammatory drug

OPLS-EP – orthogonal projections to latent structures effect projections

p53/TP53 – tumour protein 53

PCA – principal component analysis

PGE2 – prostaglandin E2

PI3K – phosphatidylinositol 3-kinase

PME – partial mesorectal excision

pQTL – protein quantitative trait loci

RAS – rat sarcoma

SCRCR – Swedish ColoRectal Cancer Registry

SNP – single-nucleotide polymorphism

STRING – search tool for the retrieval of interacting genes/proteins

TGF-β – transforming growth factor beta

TME – total mesorectal excision

TNM – Tumour Node Metastasis

VEGF – vascular endothelial growth factor

WNT – wingless-related integration site

U-CAN – Uppsala-Umeå Cancer Consortium biobank

Populärvetenskaplig sammanfattning

Bakgrund

Kolorektalcancer, det vill säga den sammanslagna gruppen tjock- och ändtarmscancer, är en av de vanligaste cancerformerna i världen. Varje år drabbas i Sverige över 5000 nya patienter av koloncancer och över 2000 av rektalcancer.

Vid operation av dessa cancerformer avlägsnas tumören med omkringliggande vävnad som tarm och tarmkäx. Därefter kan kirurgen ofta foga samman de två tarmändar som återstår i en tarmskarv, en så kallad anastomos, istället för att sy upp tarmände eller tarmändar så att de sticker ut genom huden, bildande en så kallad stomi. En av de mest fruktade komplikationerna med en anastomos är ett anastomosläckage, vilket uppstår om anastomosen inte läker ihop utan havererar – detta leder till att tarminnehåll sipprar ut i bukhålan. Detta kan bland annat orsaka blodförgiftning och kräva akut reoperation.

Anastomosläckage medför även många andra risker, såsom ökad risk för permanent stomi och komplikationer, exempelvis hjärtinfarkt, men även död och möjligen även canceråterfall. Framförallt för det senare är det oklart varför detta skulle kunna ske. En teori är att den av kirurgin orsakade inflammatoriska reaktionen i kroppen förstärks av ett läckage. Denna inflammation tros kunna i sig självt, men även av biologiska följdeffekter av densamma, förorsaka ökad risk för komplikationer och död och kanske även återfall i cancer.

Vanligt förekommande läkemedel mot smärta efter en operation är så kallade icke-steroida anti-inflammatoriska läkemedel, på engelska förkortat till NSAID. Dessa ger lindring mot smärta men påverkar bland annat även det inflammatoriska svaret som till viss del behövs för sårläkning, vilket skulle kunna påverka riskerna för diverse komplikationer, canceråterfall och död. Det finns en farhåga för att dessa NSAID skulle kunna öka förekomst av anastomosläckage på grund av påverkan på sårläkning, men det finns också en möjlig nytta utifrån att det kan finnas situationer där NSAID skulle kunna förebygga canceråterfall och död.

Det finns även en relativt vanligt förekommande genetisk variant av den så kallad COX-2-genen, där man i en tidigare studie har sett en dramatisk ökning av anastomosläckage hos de som bär på denna variant. Om detta resultat kan återupprepas vore det möjligt att bättre skräddarsy smärtlindring och operationer för de personer som bär på denna genvariant.

Material och metoder

De första två delarbetena är observationsstudier, delvis baserade på det svenska Kolorektalcancerregistret. Detta är ett nationellt register med mycket god täckningsgrad för samtliga kolorektalcancerfall och innehåller data om patienter, deras tumörer, behandlingar, komplikationer och långtidsutfall. För **delarbete I** kompletterades data med journalgranskning för att veta exakt vilka slags NSAID och mängd som patienterna fick efter operationen. För **delarbete II** samlade vi in alla ingående sjukhus smärtlindringsprotokoll, där det anges om och vilka slags NSAID som ingår i den rutinmässiga hanteringen av smärtlindring efter operation.

Delarbete I inkluderade patienter från 15 svenska sjukhus under tidsperioden 2007–2013 vilka genomgått en operation för rektalcancer med anastomos. Här jämfördes gruppen som fått NSAID åtminstone två dagar under sin första postoperativa vecka mot gruppen som inte fått någon NSAID eller bara enstaka dos. Utfallet i studien var återfall i cancer eller död.

Delarbete II omfattade patienter från 21 svenska sjukhus under tidsperioden 2007–2012 vilka genomgått tumörresektion och blivit anastomoserade för kolorektalcancer. I denna studie jämfördes patienter som enligt de inhämtade protokollen ska ha fått NSAID mot övriga patienter. Utfallen i studien var anastomosläckage, canceråterfall och överlevnad.

De sista två delarbetena är också observationsstudier baserade dels på Kolorektalcancerregistret, dels på biobanken Uppsala-Umeå Cancer Consortium. Denna biobank bjuder in samtliga kolorektalcancerpatienter vid Uppsala och Umeå att lämna prover för forskning, inkluderande blodprover före operation, men även blodprover från första återbesöket samt ett år efter kirurgi.

Delarbete III var en matchad kohort av patienter som genomgått planerad kolorektal cancerresektion med anastomos i Uppsala eller Umeå under tidsperioden 2010–2015. Med hjälp av Kolorektalcancerregistret identifierades alla de som drabbats av en bukinfektion, det vill säga anastomosläckage eller en varböld inne i bukhålan (oftast orsakat av ett anastomosläckage). Dessa fall matchades med varsin kontroll som haft ett komplikationsfritt postoperativt vårdtillfälle för att göra grupperna så lika som möjligt. Journalerna i denna kohort granskades för att säkerställa fall- och kontrollstatus. Därefter begärdes blodprover ut från första återbesöket samt vid ett år efter operationen för att kunna jämföra dessa mot preoperativa prover. Alla dessa blodprover analyserades med tre olika proteinpaneler innehållande 92 proteiner var, samt fyra enskilda proteiner ytterligare utöver dessa paneler. Proteinnivåerna jämfördes före och efter operation, och överrepresentations- och nätverksanalyser gjordes för de proteiner som skiljde sig åt.

Delarbete IV var baserad på en utökad version av den matchade kohorten i **delarbete III** med fall som drabbats av bukinfektion och kontroller som haft ett komplikationsfritt postoperativt vårdtillfälle. I denna studie undersöktes blodprover med genetisk analys för att undersöka frekvensen av en känd genvariant av COX-2-genens promotor kallad -765G>C och dess eventuella koppling till bukinfektion.

Resultat

I **delarbete I** inkluderades totalt 1341 patienter, varav 362 (27 %) räknades som NSAID-exponerade. I denna kohort kunde vi inte se någon säker effekt av NSAID på återfallsfri överlevnad, med en hasardkvot (HR) på 1,02 och 95 % konfidensintervall (CI) på 0,79–1,33 vid en så kallad justerad Cox regressionsanalys.

I **delarbete II** inkluderades totalt 6945 patienter, varav 3996 (58 %) patienter hade behandlats enligt smärtlindringsprotokoll som inkluderade NSAID. Ingen association sågs mellan NSAID-protokoll och återfallsfri överlevnad (HR 0,97, 95 % CI 0,87–1,09), men däremot sågs en fördel gällande såväl canceråterfall (HR 0,83, 95 % CI 0,72–0,95) som anastomosläckage (HR 0,69, 95 % CI 0,51–0,94) för gruppen som behandlats med NSAID-protokoll.

I **delarbete III** inkluderades totalt 64 patienter, varav 32 fall med bukinfektion och 32 matchade komplikationsfria kontroller. Nivåer för 77 proteiner skiljde sig åt mellan fall- och kontrollgrupperna vid första återbesöket (i median 41 dagar efter operation för patienterna med bukinfektion). Flertalet av de högst rankade nätverksproteinerna som var påverkade hos de bukinfekterade är kända aktörer när det gäller att stimulera kolorektalcancerceller och deras förmåga till spridning i kroppen. Det fanns en överrepresentation av biologiska signalvägar kopplade dels till hjärtsjuklighet och hur celler fäster i sin omkringliggande miljö, dels i signalvägar som har vedertagen roll i kolorektalcancer och dess progression.

I **delarbete IV** inkluderades totalt 94 patienter, med 47 fall och 47 matchade kontroller. Av dessa var 36 fall och 32 kontroller homozygota för G/G; 10 fall och 14 kontroller var heterozygota för G/C; och en i vardera gruppen var homozygot för C/C. Det var således totalt sett färre med den muterade allelen i fallgruppen, även om detta inte var en statistiskt säkerställd skillnad. I hela kohorten var 2,1 % homozygot muterade (C/C).

Slutsatser

Vi kunde inte se ett samband mellan NSAID-bruk och skillnader i överlevnad. Däremot sågs fördelar hos de grupper som fått NSAID efter operation både gällande återfall i cancer samt minskad frekvens anastomosläckage. Hos de som drabbats av bukinfektion skiljde sig 77 proteinnivåer ännu åt en dryg månad efter operationen, där flertalet av de högst rankade nätverksproteinerna är kända aktörer gällande kolorektala cancercellers invasivitet och metastatiska förmåga; detta skulle kunna vara del i kopplingen mellan anastomosläckage och försämrade cancerutfall. I en överrepresentationsanalys kunde även signalvägar relaterade till bland annat hjärtsjuklighet ses, vilket skulle kunna förklara en del av ökningen av hjärtrelaterad sjuklighet hos de som drabbas av anastomosläckage. Tidigare fynd av ökad frekvens anastomosläckage hos de med genvarianten -765G>C i COX-2 genens promotor kunde inte bekräftas, men det kan vara av värde att undersöka detta ånyo med större studier.

Huruvida NSAID i samband med operation är till nytta eller skada kan sannolikt bero på flertalet faktorer inklusive tumör- och anastomoslokal, grad av inflammatoriskt tillstånd hos patienten, typ av given NSAID, patientens samt tumörens genotyp, och kanske även om ett anastomosläckage redan har inträffat eller ej.

Publications included in this thesis

Study I

Postoperative non-steroidal anti-inflammatory drug use and oncological outcomes of rectal cancer. Oskar Grahn, Mathias Lundin, Marie-Louise Lydrup, Eva Angenete, Martin Rutegård. *BJS Open. 2021 Jan; 5(1).*

Study II

Postoperative nonsteroidal anti-inflammatory drugs in relation to recurrence, survival and anastomotic leakage after surgery for colorectal cancer. Oskar Grahn, Mathias Lundin, Stephen J. Chapman, Jörgen Rutegård, Peter Matthiessen, Martin Rutegård. *Colorectal Dis. 2022 Aug; 24(8): 933–942.*

Study III

Peritoneal infection after colorectal cancer surgery induces substantial alterations in postoperative protein levels: an exploratory study. Oskar Grahn*, Klas Holmgren*, Pär Jonsson, Emmy Borgmästars, Christina Lundin, Malin Sund, Martin Rutegård. * Joint first authors. *Submitted.*

Study IV

Mutation of the COX-2 gene promotor and anastomotic leakage in colorectal cancer patients. Oskar Grahn, Klas Holmgren, Mun-Gwan Hong, Malin Sund, Martin Rutegård. *BJS Open 2024 Jan 3; 8(1).*

Introduction

Epidemiology, risk factors and screening for colorectal cancer

Colorectal cancer was the second most deadly and third most common cancer globally in 2020[1]. In Sweden, more than 5,000 new colonic adenocarcinoma cases[2] and around 2,000 new rectal cancer (these include polyps with high-grade dysplasia and other cancer forms than adenocarcinoma) cases[3] were diagnosed in 2021, making colorectal cancer the third most common cancer (excluding skin malignancies).

The risk of developing colorectal cancer is higher in males, with increasing age, and for those suffering from inflammatory bowel disease. This risk also seems to increase with higher intake of red meat, sodium, alcohol and smoking, a higher body mass index (BMI), and lower grade of physical activity. Low intake of dietary fibre also correlates with an increased colorectal cancer risk[4].

A national colorectal cancer screening has been implemented at the time of writing, following accumulating evidence that screening decreases mortality due to colorectal cancer[5]. Testing is offered every other year to everyone aged 60–74 years in the form of a stool sample which is supposed to detect occult blood; if this test is positive, the patient is investigated further with colonoscopy[6].

Anatomy

The layers in the intestinal wall, starting from the inside, are the mucosa (which contains the epithelium, lamina propria and muscularis mucosae), the submucosa, the muscularis propria (with a circular inner layer and a longitudinal outer layer) and finally the serosa[7]. An adenocarcinoma is a cancer arising from epithelial glandular cells[8], i.e. from the innermost layer of the intestinal wall (Figure 1).

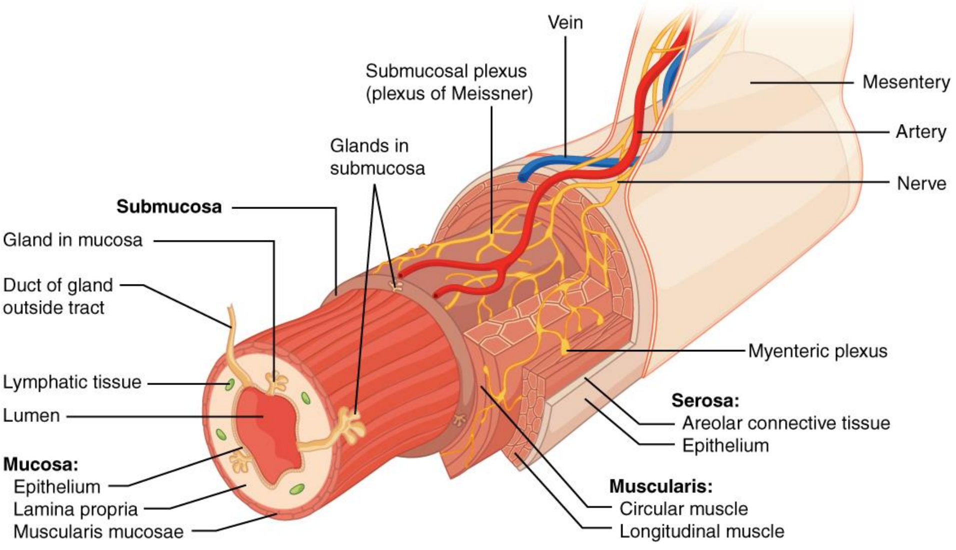


Figure 1. Layers of the gastrointestinal tract. Credit to: "OpenStax College, Rice University – Layers of the Alimentary Canal" at OpenStax.org, by Goran tek-en, license: Creative Commons Attribution 3.0 Unported

The gastrointestinal tract can be divided into three distinct areas, based on the embryological origins: the foregut, midgut and hindgut. The foregut is comprised by the gastrointestinal tract from the oral cavity to the papilla Vateri in the duodenum. The midgut is comprised of the duodenum aboral to the papilla Vateri, jejunum, ileum, caecum, appendix, ascending colon and the oral two thirds of the transverse colon. The hindgut is comprised of the aboral third of transverse colon, descending colon, sigmoid colon and rectum[9]. The midgut and hindgut are chiefly supplied by the superior mesenteric and inferior mesenteric vessels, respectively[10]. The rectum has been given several differing definitions with respect to its length; in this thesis, the rectum was defined as the first 15cm of bowel above the anal verge as measured by a rigid sigmoidoscope. Intraoperatively, it is defined by the disappearance of the colonic taeniae in the pelvis, lack of appendices epiploicae and lack of an intraperitoneal mesentery. The rectum is often divided into three parts from a surgical viewpoint, with the upper third being covered anteriorly and laterally by peritoneum, the middle third being covered by peritoneum anteriorly only and the lower third being completely extraperitoneal. Starting at the anal verge, the rectum can be divided into the lower part being the first 6cm, the middle part being 7–11cm, and the upper part being 12–15cm. Within the perirectal fat, defined as the mesorectum, lie supplying blood vessels and perirectal lymph nodes and this mesorectum is enveloped by its own fascia called the fascia propria of the rectum. This fascia is key to the technique of total mesorectal excision (TME), comprising resection of the rectum and mesorectum with this envelope intact in the avascular 'holy plane', also respecting the presacral/parietal fascia while dissecting close to the mesorectal fascia to preserve autonomic nerves. The anal canal is approximately 4cm long in women and 4.4cm in men, with its starting point at the level of the pelvic diaphragm (the anorectal junction) and terminating at the anal verge[11].

The appendix and caecum are supplied by the ileocolic artery, while the ascending colon and hepatic flexure are supplied by anastomosing vessels between the ileocolic and middle colic artery (or by the right colic artery if present). The transverse colon is supplied by the middle colic artery, while the splenic flexure is supplied by anastomosing vessels between the middle colic and left colic artery. The left colon, sigmoid colon and upper rectum are supplied by the left colic artery, sigmoid arteries and the superior rectal artery (Figure 2). The middle and inferior rectum are supplied by the middle and inferior rectal arteries, both branching from the internal iliac arteries[10].

The colon is visibly distinct from both the small bowel and the rectum, with its three longitudinal taeniae coli which are condensations of the outer muscle coating of the bowel; these cause the radiological sign of haustrations when distended, as the taeniae coli are more rigid than the rest of the intestinal wall which becomes elongated when distended. The colorectal tract varies in length but is often approximately 1.5m in adults[12].

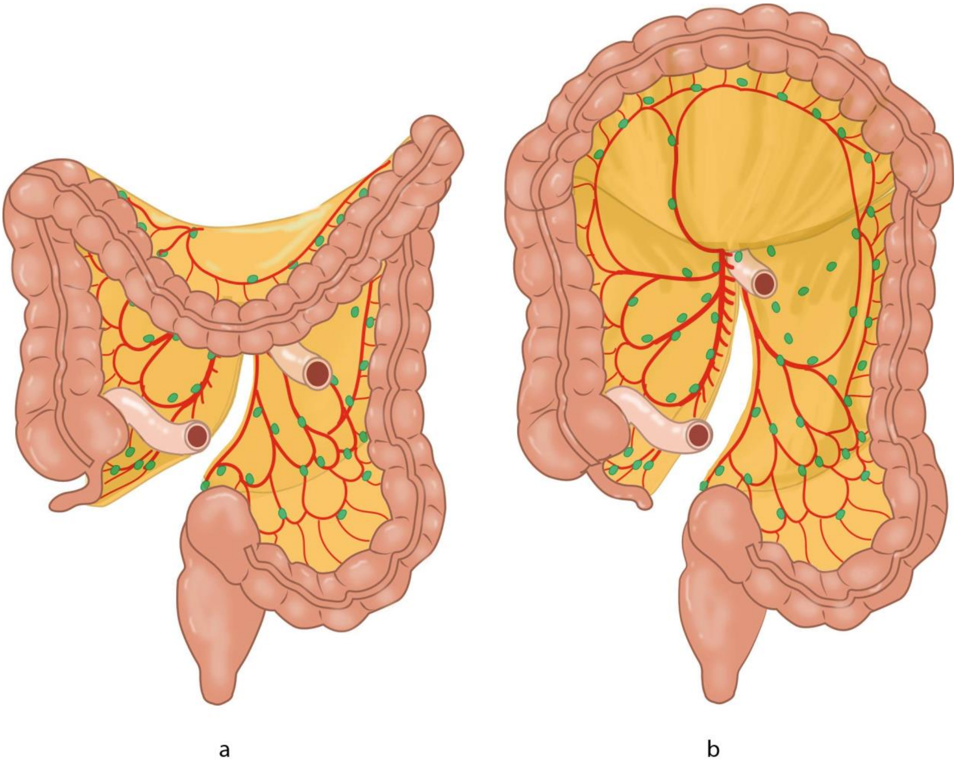


Figure 2. Large intestine with vascularisation and mesocolons. Credit to: "Slagter – Drawing Large intestine with vascularisation and mesocolons – No labels" at AnatomyTOOL.org by Ron Slagter, license: Creative Commons Attribution-NonCommercial-ShareAlike

Colorectal carcinogenesis

There are sporadic, familial and inherited forms of colorectal cancer. The inherited forms comprise about 5% of all colorectal cancers, and the classic example of a hereditary non-polyposis colorectal cancer syndrome is Lynch syndrome, which is caused by germline mutation in a DNA mismatch repair gene, with a lifetime risk of developing colorectal cancer approaching 80%. The chief entity of hereditary polyposis colorectal cancer on the other hand is familial adenomatous polyposis, which is caused by mutation of the tumour suppressor gene APC. APC suppresses WNT signalling, and uninhibited WNT signalling will otherwise inhibit apoptosis and differentiation, and stimulate proliferation, invasion, and tumour progression.

There is also a group of familial colorectal cancers that comprise about 20–30% of all cases, in which there are no single well-defined mutations with a substantially elevated cancer risk; rather, there is a series of polymorphisms, each with low penetrance and, when grouped together, confer an increased risk of colorectal cancer[13].

Thus, the remaining approximately 70% of colorectal cancers are sporadic[13], due to somatic mutations and acquired epigenetic changes. Despite a substantial heterogeneity in sporadic colorectal carcinogenesis, there are common driver mutations. The most frequently occurring molecular pathogenesis begins with a mutation in the aforementioned APC gene, which allows the formation of polyps, with subsequent mutations in KRAS, p53/TP53 and DCC[14] turning it into a colorectal adenocarcinoma (Figure 3).

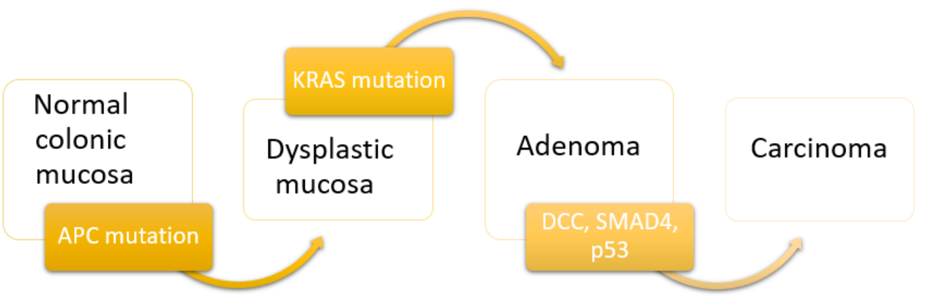


Figure 3. Basic adenoma-carcinoma sequence by Annj716. Credit to: "Basic adenoma-carcinoma sequence" at Wikimedia Commons by Annj716, license: Creative Commons CCO 1.0

APC and KRAS are often seen mutated and are believed to be important for cancer progression including proliferation, migration, transformation, and survival. TP53 has been nicknamed the 'guardian of the genome' as it is responsible for cell cycle arrest and promoting apoptosis when enough cellular stress is detected. The loss of TP53 is sometimes coupled with the transformation of an adenoma to an invasive cancer. The transforming growth factor beta (TGF-β) pathway has a role in both immunological processes and stem cell differentiation and can be a part of the epithelial-to-mesenchymal transition (EMT), and other major effectors in EMT seem to be enhancement of the WNT/β-catenin pathway and loss of E-cadherin. There are possible mutations in the PI3K/AKT pathway, enhancing cell survival and proliferation. BRAF mutation is associated with microsatellite instability (MSI) and is usually not seen in tumours in Lynch patients, thus prompting routine investigation into the possibility of Lynch syndrome in patients with MSI tumours lacking BRAF mutation. Mutations activating the MAPK pathway such as EGFR are not infrequent, and VEGF is of crucial importance for angiogenesis and is in turn often affected in colorectal cancer progression[15].

Diagnostic workup and neoadjuvant treatment

The clinical routine work-up in Sweden of colorectal adenocarcinoma encompasses clinical examination and blood tests including the tumour marker carcinoembryonic antigen (CEA), as well as endoscopy with tumour biopsies. Furthermore, it also includes computed tomography of the chest and abdomen, as well as a magnetic resonance tomography scan of the pelvis in the case of rectal cancer[16]. This radiological evaluation is used to determine a clinical Tumour-Node-Metastasis (cTNM) stage[17], which in turn helps to decide whether the cancer is amenable to curative or palliative therapy; this also guides the multidisciplinary team meeting as to whether to offer any neoadjuvant treatment. Broadly, the T stands for tumour and depicts the invasion depth of the primary tumour, the N stands for regional lymph node involvement and depicts how many are involved (N-positive disease also comprises tumour deposits) and the M stands for distant metastasis and describes the possible extent of metastatic disease (Table 1).

Table 1. TNM status of colorectal cancer

	Primary tumour		Regional lymph nodes
TX	Primary tumour not classifiable	**Nx**	Not classifiable
T0	No primary tumour detected	**N0**	No regional lymph nodes
T1	Invasion into submucosa	**N1**	1–3 lymph nodes
Sm1	Innermost 1/3	**N1a**	1 lymph nodes
Sm2	Middle 1/3	**N1b**	2–3 lymph nodes
Sm3	Outermost 1/3	**N1c**	Tumour deposits
T2	Invasion into muscularis propria	**N2**	4+ invaded regional lymph nodes
T3	Invasion beyond m. propria, into the mesocolon or mesorectum	**N2a**	4–6 invaded regional lymph nodes
		N2b	7+ invaded regional lymph nodes
T4	Locally advanced		
T4a	Invasion through the visceral peritoneum		
T4b	Invasion into adjacent organ(s)		
	Metastatic disease		**Stage**
M0	No distant metastasis	**I**	T1–2 N0 M0
M1	Distant metastasis	**II**	T3–4 N0 M0
M1a	Distant metastasis to one organ	**III**	Any T N+ M0
M1b	Distant metastasis to 2+ organ	**IV**	Any T Any N M+
M1c	Peritoneal spread, with or without other metastatic disease		

Neoadjuvant treatment has historically had little-to-no role in the context of colonic cancer, but this is being challenged particularly in the setting of locally advanced cancer, not least by the recent FOxTROT trial[18]. Briefly, patients with clinically staged T3-4, N0-2, M0 and MSS tumours may benefit from neoadjuvant chemotherapy, but this, as of writing, is not yet part of routine care in Sweden.

In the context of rectal cancer, neoadjuvant therapy has an established role depending on the risk of local and distant recurrence, and possible need for regression of the tumour to make it resectable[16].

Low risk rectal cancers encompass cT1–cT3d if situated above 10cm from the anal verge, and cT1–cT3b below 10cm until the intersphincteric space starts. A maximal of cN1-disease is allowed within this group. This group has a locoregional recurrence of 6–8% at most and is routinely recommended upfront surgery.

Intermediary risk rectal cancers comprise all the previously mentioned tumours above, but with the addition that the mesorectal fascia is invaded by tumour deposits or nodular disease. It also encompasses tumours of cT3c–cT3d situated below 10cm from the anal verge down to where the intersphincteric space starts, as well as cT1–cT3d tumours in the intersphincteric plane. This group has a higher than 6–8% locoregional recurrence risk and a routine recommendation is preoperative short-course radiotherapy of 5x5Gy with a short (2–4 days) or prolonged (4–8 weeks) wait after irradiation, before surgery.

High risk rectal cancers encompass cT4 tumours, all cN2 disease, all cancers not entirely above the peritoneal reflection with extramural vascular invasion, and all cancers with suspected tumour growth in lateral lymph nodes (i.e. within the internal iliac and/or obturator compartments). These cancers are locally advanced with increased risk of distant metastasis, and tumour regression might also be desirable to increase the chance of radical resection and/or to limit the extent of the resection. This group is now recommended sequential neoadjuvant therapy, most often in accordance with the RAPIDO[19] or LARCT-US[20] protocols, each beginning with short-course radiotherapy 5x5Gy and either followed by six or four cycles of capecitabine and oxaliplatin (CAPOX) chemotherapy respectively.

There is an ongoing national study regarding management in the case of complete or near complete clinical response after neoadjuvant treatment, i.e. the tumour has fully regressed, where these patients can be offered to forego surgery and instead be managed according to a so called “Watch and Wait” protocol[21] and remain under close surveillance. Internationally however, this seems to already be a part of routine care in some places[22].

Surgery

Malignant colonic polyps can be endoscopically removed and cured but this may confer a higher risk of recurrence. In contemporary routine care, an endoscopically radically excised Haggitt I–III (i.e. no invasion deeper than the stalk in a pedunculated polyp) or a flat/sessile polyp of no deeper invasion than T1sm1 is considered to be fully treated[16].

Likewise, low risk T1 rectal cancer can be treated and cured with endoscopic submucosal dissection or transanal endoscopic microsurgery, but at the expense of a higher risk of recurrence. A low risk cancer in this context would be a radically excised T1 tumour without tumour budding, and no invasion of vessels or lymphatics and invasion less than 1mm into the submucosa[16].

Most often, however, potentially curable colorectal cancers are surgically resected. In general, this means dissection in the avascular embryological plane all the way to the mesenteric root, including ligating the feeding vessel(s) proximally and keeping the mesocolon (or the mesorectum) intact but also sparing adjacent structures from harm, unless the tumour invades neighbouring organs.

Briefly, a right hemicolectomy can be performed for tumours from the caecum and extended all the way to the distal part of the transverse colon, with ligation of the ileocolic artery and most often the right branch of the middle colic; this sometimes includes the entire middle colic artery if an extended right hemicolectomy is required. A left hemicolectomy encompasses the splenic flexure, descending and sigmoid colon, and can include proximal ligation of the inferior mesenteric artery and vein. A sigmoid resection (Figure 4) entails ligation of the inferior mesenteric artery, either proximally i.e. before the left colic artery take-off (i.e. a high tie) or just distal to the left colic artery (i.e. a low tie), being wary of damaging the hypogastric nerves. For tumours situated at the rectosigmoid junction or high rectal cancers, a partial mesorectal excision (PME) can be performed, which includes dividing the rectum and mesorectum 5cm below the inferior margin of the tumour. For rectal cancers, one can perform both a low tie or a high tie of the inferior mesenteric artery, but in the case of a low anastomosis with a necessary splenic flexure mobilisation, a high tie is typically required[16].

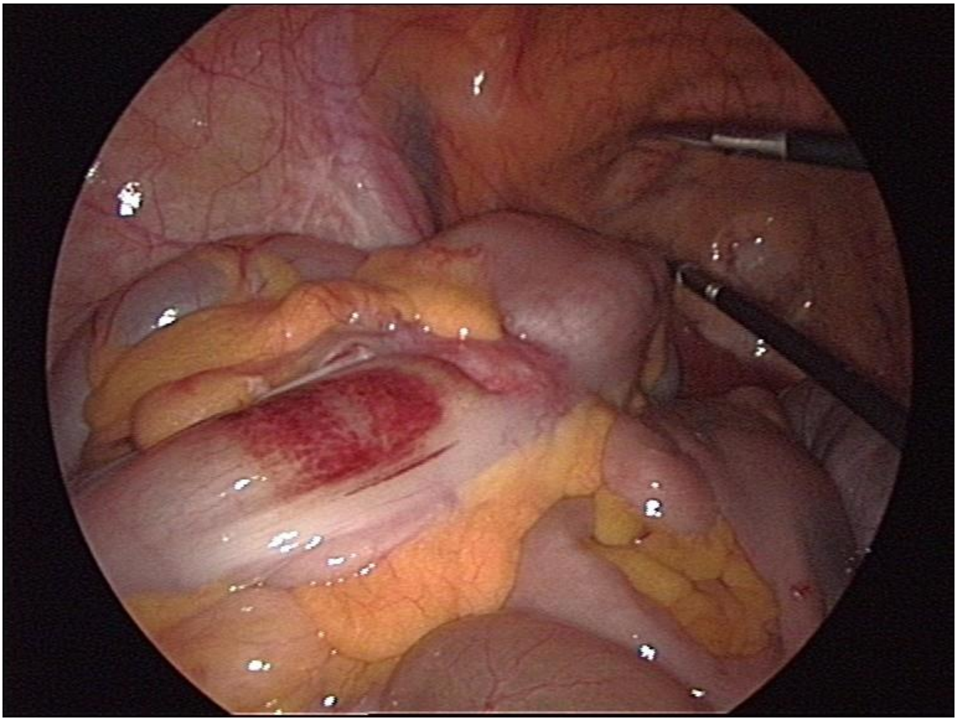


Figure 4. Intraoperative view of a sigmoid colon cancer. "Intraoperative view of the sigmoid colon cancer" at Wikimedia Commons by Anpol42, license: Creative Commons Attribution-Share Alike 4.0 International

The standard procedure for high and mid rectal cancers would be an anterior resection, i.e. either a PME or a TME including an anastomosis (colo-rectal or colo-anal). The standard operation for a low rectal cancer is an abdominoperineal rectal excision, leaving the patient with an end colostomy. Hartmann's procedure is sometimes used instead of either an anterior resection or an inter-sphincteric abdominoperineal rectal excision, also leaving the patient with an end colostomy. For locally invasive colorectal cancers, various types of pelvic exenteration surgery or other tailored operations may be needed[16].

Adjuvant therapy

The commonly used cytotoxic drugs for colorectal cancer are fluoropyrimidines and oxaliplatin. Fluoropyrimidines used are fluorouracil (5-FU) which can be given intravenously or orally administrated as capecitabine (a precursor to 5-FU). Oxaliplatin can be given intravenously[16]. A majority of 5-FU is inactivated in the liver by an enzyme called dihydro-pyrimidine-dehydrogenase (DPD), and the alleles for this gene (also called DPYD) are routinely tested before administration of 5-FU chemotherapy to avoid adverse effects[23]. Adjuvant chemotherapy in the context of colonic cancer has an established role with decreased risk of cancer recurrence and increased overall survival, preferably started within the first 8 postoperative weeks and no later than 12 weeks after surgery. It is usually recommended to stage III, i.e. node-positive disease, but also to stage II disease, which presents with more than one of the following risk factors: pT4 tumour, emergent surgery, tumour perforation, low differentiation, tumour with extramural venous or perineural invasion, few (<12) lymph nodes found in the specimen, and elevated CEA postoperatively[16]. The indication for adjuvant chemotherapy in the context of rectal cancer is not as clear-cut, but patients with stage II disease with risk factors and stage III patients are to be evaluated for adjuvant therapy. Of these patients, those who did not receive neoadjuvant chemotherapy could be offered adjuvant chemotherapy. Those who were down-staged by neoadjuvant radiotherapy to a stage I–II disease without risk factors should not normally be offered adjuvant chemotherapy. Exceptions to this could be pre-therapeutic T3–4 or node-positive disease which was not treated with neoadjuvant chemotherapy. Moreover, patients treated according to RAPIDO[19] or LARCT-US[20] should not be offered additional chemotherapy, nor should patients treated with long-course radiochemotherapy[16].

Considerations for metastatic disease

Even metastatic colorectal cancer disease is potentially resectable and curable, including peritoneal deposits, liver metastasis, lung metastasis, pathological lymph nodes outside of the mesocolic or mesorectal envelopes and perhaps also metastases in other organs. Irinotecan is another cytotoxic drug available for treatment of metastatic disease. Targeted drugs in this context include tyrosine kinase inhibitors, angiogenesis inhibitors and epidermal growth factor-receptor inhibitors (which seem to have the best effect if the tumoral disease is both RAS-wildtype and BRAF-wildtype)[24]. Immunotherapy has also been introduced in the form of checkpoint-inhibitors, which can be used against metastatic disease that exhibits MSI-H/dMMR[16, 25]. Check-point inhibitors have also been tested in a phase II study as single therapy, omitting any surgery, in 12 patients with stage II–III locally advanced rectal cancer, suggesting excellent results at one year follow-up[26].

Overall survival and cancer recurrence

In Sweden, the relative 3-year survival for surgically resected non-metastasised colon cancer patients, i.e. stage I–III, is 94% for planned operations and 73% for emergent operations. The rate of local cancer recurrence for all resected patients within 3 years is 2.9%[27]. For non-metastasised colon cancer patients who were surgically resected in 2019, distant recurrence within 3 years occurred in 12% of cases[3].

The relative 3-year survival for surgically resected non-metastasised rectal cancer patients, i.e. stage I–III, is 95%. The local cancer recurrence rate for all resected patients within 3 years presenting with a T1–3 tumour is 3.2%, and 2.5% vs 3.6% for patients without vs patients with neoadjuvant radiotherapy, respectively[28]. The local recurrence rate for all resected patients within 3 years presenting with a T4-tumour is 17%[28]. For non-metastasised rectal cancer patients who were surgically resected in 2019, distant recurrence within 3 years occurred in 16% of cases[29].

In total in Sweden, reported colorectal cancer recurrence rates at 5 years are 5%, 12% and 33% for stage I, II and III, respectively[30].

Anastomotic leakage

Anastomotic leakage is a dreaded complication as it imparts mortality[31] as well as morbidity, including a higher rate of permanent stoma[32] and cardiovascular events[33]. Anastomotic leakage has also been associated with decreased disease-free and overall survival, more apparently in rectal cancer and less so in colonic cancer, perhaps with a difference between left- and right-sided colonic cancers[34].

The definition of anastomotic leakage varies within the clinical community and, as a result, also within the scientific community. There is no formal definition of anastomotic leakage within the Swedish ColoRectal Cancer Registry (SCRCR), nor a grading of its severity. In this thesis, the definition of anastomotic leakage as proposed by the International Study Group of Rectal Cancer (ISREC) is most often used, where a leak is defined as a communication between intra- and extraluminal compartments due to a defect of the intestinal wall at the anastomotic site, but also the presence of a pelvic abscess when a rectal anastomosis has been constructed[35]. Another problem with anastomotic leakage reporting is that it can present early (within 30 days of resection) or later, where up to a third of anastomotic leakages may be diagnosed after 30 days have passed[36]; this also poses a scientific problem as some registries only account for early leakages or during the index hospital stay (e.g. the SCRCR).

Known risk factors for colorectal anastomotic leakage are male sex, American Society of Anaesthesiologists' (ASA) fitness grade III or higher, co-morbidities (including diabetes mellitus, obstructive pulmonary disease, cardiovascular disease and renal failure), earlier radiotherapy, distal tumour location, emergent surgery, smoking, obesity, excessive alcohol consumption, malnutrition, anaemia and perioperative transfusion[37]. Furthermore, signs of a preoperative inflammatory response seem to be predictive of an increased risk of anastomotic leakage[38, 39].

A defunctioning stoma formed at the time of anterior resection may decrease the severity of the leak, but also postpone its diagnosis, and whether it affects the true incidence of anastomotic leak is still uncertain[37, 40]. However, a recent meta-analysis comprising nine randomised controlled trials demonstrated a beneficial effect on clinical anastomotic leak rates with a defunctioning stoma, possibly only in certain subgroups[41].

The perioperative period, peritoneal infection and possible biological pathways leading to increased morbidity, recurrence and mortality

The reason for why anastomotic leakage might cause increased morbidity[33], decreased disease-free and overall survival[34] is not established. However, it is known that stress, inflammation and modulation of the immune system in the immediate perioperative period affect both the primary tumour until its removal, as well as the ability of any minimal residual disease to survive and establish micro-metastases after surgery[42]. Surgical stress is known to cause an array of changes that can facilitate minimal residual disease[43, 44], and peritoneal infection can further enhance postoperative stress and inflammation. This has been suggested to link e.g. anastomotic leakage and stimulation of minimal residual disease, thus leading to increased recurrence and decreased recurrence-free survival by affecting invasiveness of cancer cells[45], inflammatory and angiogenetic[46], adrenergic[47] and thrombotic pathways[48]. Peritoneal infection has been shown in an experimental murine model to increase metastatic burden, where attenuation of Natural Killer cells cytotoxicity can be implicated as an important factor[49]. Natural Killer cell dysfunction in particular may play an important role in cancer recurrence after curative cancer resection[44].

Non-steroidal anti-inflammatory drugs and cyclooxygenase enzymes

Non-steroidal anti-inflammatory drugs (NSAIDs) can be included as part of postoperative multimodal analgesia, advocated in enhanced recovery after surgery programmes[50]. The main mechanism of NSAIDs consists of inhibition of cyclooxygenase (COX) enzymes -1 and -2, thereby inhibiting thromboxane and prostaglandin production with subsequent effects on pathways related to inflammation, pain, gastrointestinal endothelial homeostasis, angiogenesis, cancer, renal haemodynamics, haemostasis and thrombosis[51].

Though NSAIDs are a diverse group of agents, they are commonly divided into two groups: selective and non-selective. Selective (e.g. diclofenac and coxibs) act mainly on the COX-2 enzyme, while non-selective (e.g ibuprofen and naproxen) act on both COX-1 and COX-2 enzymes[51]. Acetylsalicylic acid, also known as aspirin, is a special case as it is also a non-selective NSAID, irreversibly inhibiting COX-1 in platelets, accounting for its prolific use as prophylaxis against cardiovascular ischemic events[51], but it has also gained an increasing amount of interest regarding its potential as a prophylactic agent in colorectal cancer development[52].

NSAIDs and anastomotic leakage

There is a widespread hesitation in the surgical community to use NSAIDs in the perioperative period for colorectal cancer patients, as there have been reports that they might increase the risk of anastomotic leakage, perhaps due to inhibition of COX-2 enzymes[53]. This potential adverse effect may depend on the type of NSAID used, as the selective agent diclofenac has been associated with increased frequency of anastomotic leakage in several studies as well as meta-analyses[54], whereas certain non-selective agents (e.g. ibuprofen) have been associated with a decreased or at least not an increased risk of anastomotic leakage[55, 56]. A more proximal anastomosis along the colorectum may also be at a higher risk for anastomotic leakage when exposed to NSAIDs[57].

Genetic variation of the COX-2 enzyme

The COX enzymatic expression can also be affected by genetic variation. In a study by Reisinger et al[53], wildtype mice were treated with diclofenac, resulting in a marked increase of anastomotic leakage; similar results were rendered by knocking out the COX-2 gene. In the same study, a consecutive set of 148 patients resected for colorectal cancer with a primary anastomosis were retrospectively studied. Blood samples from these patients were genotyped to tabulate frequencies of the COX-2 gene promotor mutation -765G>C. Seven patients were homozygous for the minor allele with C/C, and three of these seven suffered an anastomotic leakage. Sixteen of the remaining 141 patients, who had either G/C or G/G, suffered anastomotic leakage, amounting to an anastomotic leakage rate of approximately 43% vs 11%.

However, heterozygosity of the minor allele i.e. G/C also seems to infer biological effects, including a decreased postoperative inflammatory state[58], decreased risk of ischaemic stroke and myocardial infarction[59] and a decreased risk of contracting Crohn´s disease[60] as well as gastric cancer[61].

In the context of colorectal cancer and whether this polymorphism associates with alterations of disease incidence, the results have been inconsistent[62-64] and the risk may differ between ethnicities, including a possible increased incidence in Asian populations[64-66].

NSAIDs and oncology

NSAIDs have been associated with favourable oncological outcomes when used perioperatively. One recent cohort study exhibited an association between postoperative NSAID-exposure for colorectal cancer patients and an improved recurrence-free survival (although their outcome should be re-classified as recurrence)[67]. Another recent cohort study on rectal cancer patients exhibited an association between perioperative NSAID-exposure and a decreased recurrence rate, as well as an improved disease-free survival in the subgroup with a more pronounced preoperative inflammatory state[68].

Many adenocarcinomas have upregulated COX-2 enzymes, and an increased inflammatory state seems to have an adverse effect on cancer; several studies have exhibited associations between the use of NSAIDs and an improved recurrence-free survival after tumour resection for different types of adenocarcinomas. Acetylsalicylic acid has been linked to improved oncological outcomes[69] and the Swedish randomised controlled trial ALASCCA has completed its enrolment, testing whether patients with colorectal cancer stage II–III and with a PIK3-mutation may benefit from daily aspirin for 3 years postoperatively[70].

Aims of the thesis

In **study I**, the aim was to evaluate whether postoperative NSAID-exposure influenced recurrence-free survival for patients operated with anterior resection for rectal cancer.

In **study II**, the aim was to investigate whether protocolised postoperative NSAIDs for patients resected for colorectal cancer had an impact on anastomotic leakage or long-term oncological outcomes, while considering different tumour and anastomotic locations.

In **study III**, the aim was to explore potentially important proteins and biological pathways that could help explain the connection between anastomotic leakage and possible detrimental outcomes.

In **study IV**, the aim was to replicate a previous finding of a known COX-2 gene polymorphism that might cause a high rate of anastomotic leakage, as well as to study the frequency of this polymorphism in a Swedish colorectal cancer population.

Data sources, statistical concepts and bioinformatics

Registries and biobanks

The SCRCR was used in all studies in this thesis to identify patients and also for the information it contains. It is a national registry that started recording rectal cancer cases in 1995 and colonic cancer cases since 2007. The registry contains data on demographics including date of death, diagnostic data including histopathological and radiographical evaluations and staging, treatment details including operative and perioperative details, postoperative complications within 30 days, neoadjuvant and/or adjuvant therapy and long-term oncological outcomes. It has been validated for the years 2008–2015, with a reported completeness of 98.5% for colonic cancer and 98.8% for rectal cancer when compared to the Swedish Cancer Registry. Individual registry variables had an average agreement of 90%[71]. In conclusion, it was deemed a high-quality registry although with some weaknesses, including a known underreporting of anastomotic leakage after anterior resection with a false negative rate of 29%[72]. Moreover, it only accounts for anastomotic leakage (and complications in general) within the first 30 postoperative days or within the index hospital stay, whereas later anastomotic leaks (i.e. diagnosed after the first 30 postoperative days) could comprise about a third of the total amount for rectal cancer patients[73]. In a recent review of colonic cancer recurrence reporting into the SCRCR, around 1.6–4.0% recurrences were missing[74].

Material from the Uppsala-Umeå Cancer Consortium biobank (U-CAN) was used in **study III** and **study IV**. This biobank prospectively collects biological material from adult cancer patients in Sweden, and it started as a collaboration between Uppsala and Umeå University Hospitals. The base package consists of blood samples including EDTA plasma, serum, citrate plasma and whole blood. The first sampling is aimed to occur before any therapeutic intervention, while subsequent sampling is done longitudinally at the first postoperative visit and at one year after surgery. This biobank also stores tumour and non-tumoural tissue, as well as patient and cancer data including details on therapy and long-term outcomes. In an evaluation issued by the Swedish government, this biobank was given top ratings for performance, strategy and research output[75].

The Cause of Death registry in Sweden was used in **study III**. In this registry, the presumed cause of death including contributing diseases is required by law to be reported by the physician in charge if the death takes place in-hospital, or otherwise by the general practitioner on-call.

Biological databases

With increasingly large datasets of biologically active molecules such as proteins, it becomes increasingly unfeasible to manually infer biological networks and functions from research findings. Hence, the field of bioinformatics including databases and specialised analytical software is growing.

The aim of the STRING database[76] is to integrate all physical and functional protein-protein interactions, which are fundamental to the biological processes within all humans[77]. The STRING database contains data on more than 14,000 different organisms, including humans. It can be used in many ways, including by inputting lists of differentially expressed proteins to visualise and summarise protein networks and associations, as well as to perform over-representation analyses.

The Reactome database is an archive of human biological processes and serves as a tool to find functional relationships within a dataset, linking proteins to functions[78]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database contains curated pathway maps and also facilitates detection of possible pathways that are affected in a dataset[79], and can be used both for genomics and proteomics. Lastly, the Gene Ontology (GO) database aims to create a unified vocabulary for genes and gene products regarding: i) biological processes; ii) molecular functions; and iii) cellular components for all eucaryotes[80].

Epidemiological concepts and principles

Epidemiology, often defined as the study of distributions and control of disease, also refers to data science in general when applied to the field of medicine. Both experimental and non-experimental studies are considered epidemiological study designs when applied to human populations. Experimental studies are often preferred when determination of causality is the study aim, but may be unethical (e.g. does smoking affect children's' cardiovascular health?), or impractical (e.g. investigating an outcome so rare that it would take tens of thousands of patients in each experimental arm), or too early in the scientific process (no theoretical basis justifying the proposed intervention) for an experimental study to be performed.

Non-experimental studies can be descriptive, hypothesis-generating, or analytical with the potential of inferring causality under the right circumstances[81]. This thesis comprises cohort and case-control studies – both designs are observational and thus non-experimental.

A cohort should be a well-defined group of patients, always with inclusion but often also exclusion criteria. The aim is to create a representative sample of the population one intends to study. It may be retrospective (i.e. the exposure and outcomes have already happened at the start of the study), or prospective (i.e. where exposure and outcomes take place after study start). Retrospective studies can be both affordable and quick, and sometimes with access to large patient materials derived from e.g. national registries. On the other hand, prospective studies have the potential of gathering better data including information on exposure, covariates and outcomes, and are thus generally considered of a higher scientific value. With a cohort study, a single exposure is often evaluated against multiple outcomes. Cohort studies may be appropriate for unusual exposures, but also for exposures that are likely to cause harm, e.g. smoking tobacco.

Case-control studies may be well suited when the outcome is rare, or when the time-to-event is long. Due to its inherent design, only one outcome can be investigated but multiple exposures may be evaluated. At first, the cases are identified, after which the controls are selected from the same population as the cases, often according to a set of matching criteria to construct two groups as similar as possible.

As these study designs are non-experimental and observational in nature, one may be left with various types of bias, in particular confounding[82]. Bias is a systematic error which results in a faulty estimate, i.e. an incorrect evaluation of the association between exposure and outcome. An example of this would be information/misclassification bias, which is when study variables and/or measurements are incorrect, e.g. if the outcome anastomotic leakage is underreported into the registry that serves as the source of data.

Selection bias arises from the application of inclusion and exclusion criteria, and results from constructing a cohort of patients that are not representative of the intended population one set out to study. An example consists of only including hospitalised patients with a certain disease, but where the intention was to infer the results to the entire diseased population.

Confounding refers to the common scenario when there are other factors at play, possibly affecting both exposure and outcome. Experimental trials are designed to avoid this problem, with potential confounding variables randomly distributed between the experimental arms, hopefully leaving the randomised exposure as the main discernible difference between the groups. The major advantage of such a design consists of the random allocation not only of known and measured confounders, but also of unknown and unmeasured ones. An example of confounding would be if frail and co-morbid patients would be less likely to receive medications such as NSAIDs, and this co-morbidity and frailty was the explanation as to why the group not receiving NSAIDs experienced decreased survival, rather than the NSAID exposure itself influencing survival. Confounders need to be considered if possible and this can be done in the inclusion phase (e.g. excluding patients not likely to be eligible for the exposure) and/or in the analytical phase (adjusting for confounding factors). When selecting possible confounders to adjust for, the temporal relationships are important to keep in mind as a true confounder has to be present before the exposure – otherwise it might well be a mediator, affected by the exposure and in turn affecting the outcome; a mediator is situated along the causal pathway, and should not be adjusted for when evaluating the total effect of the exposure on the outcome. A pertinent example could be to not adjust for anastomotic leakage when assessing the effect of NSAIDs on oncological outcomes, as leakage may be influenced by NSAID use at the same time as leakage could affect survival.

Confounding by indication is when the indication for exposure/treatment is related to the outcome, thus creating a bias. An example would be if a group of patients were treated with NSAIDs due to another underlying condition that renders a decreased survival time, and even though NSAIDs could be beneficial regarding the outcome studied, the other underlying condition which may have prompted the NSAID-treatment could be so detrimental that the result could turn into a false negative or even exhibit a detrimental association instead.

Internal validity refers to whether the result of the study reflects the true effect in the study, and can thus be compromised by biases, e.g. information bias or confounding.

External validity refers to whether the study results can be generalised to the intended population, as in that the findings are not only true for the sample from which the results were derived, but also inferable to the population from which the sample was created. Selection bias may decrease this, e.g. only evaluating hospitalised patients with a certain disease and thus not being able to infer the results to the entire diseased population.

Statistical concepts

When adjusting for possible confounders in **studies I and II**, the aim was to establish causal inference by judicious selection of covariates. For this, directed acyclic graphs[83] were used to establish causal frameworks to aid the selection of appropriate sets of covariates (Figure 5). Regarding the matching criteria for the cohort in **studies III and IV**, a select few important confounders, based on the available literature, were chosen to not only ensure comparability between groups but also to allow successful matching of controls to as many cases as possible.

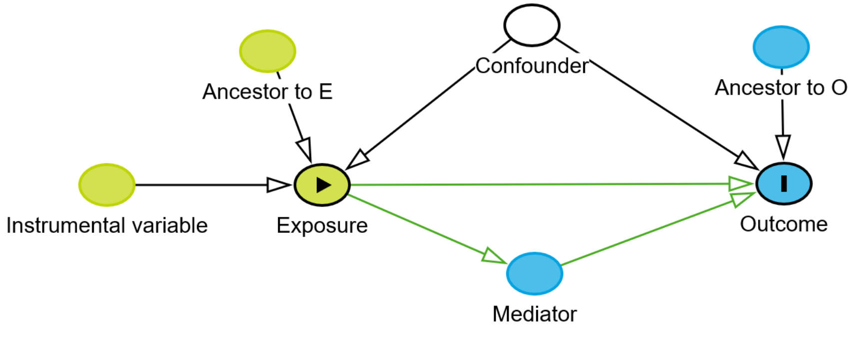


Figure 5. Example of a directed acyclic graph, created using the https://www.dagitty.net/ website. In this example, when e.g. performing a multivariable Cox regression analysis to evaluate the effect of "Exposure" on "Outcome", the only variable to adjust for would be the aptly named "Confounder"

Cox proportional hazards modelling was used in **studies I and II**, both as uni- and multivariable models. This is a survival analysis, which also considers the time to event/outcome. All included independent variables receive a coefficient and a p-value, but these models were constructed with the intent of causal inference, meaning that the only variable to be interpreted in that context is the selected exposure variable (i.e. NSAID-

treatment in this case)[84]. One core assumption for this model is the proportional hazards assumption, i.e. the survival/event curves should never cross each other.
Propensity score matching was also performed for **study I**. Each patient had a propensity score calculated, which is the probability of receiving the exposure. This probability was evaluated by logistic regression, using the same set of covariates selected as in the previously mentioned Cox regression analysis. Subsequently, each patient who received NSAID treatment was matched to a patient who did not receive such treatment, according to their propensity score in a one-to-one nearest-neighbour procedure without replacement.
Briefly, a logistic regression model is a uni- or multivariable method which is constructed to predict a binary outcome, e.g. exposed or non-exposed. As mentioned, logistic regression was used in **study I** to create the propensity score matched sample, in **study II** to evaluate the binary outcome of anastomotic leakage, as well as when evaluating potential associations of different allelic combinations with anastomotic leakage in **study IV**.
A two-stage instrumental variable residual inclusion analysis was also performed for **study I**. The purpose of an instrumental variable analysis is to avoid residual (i.e. unmeasured) confounding. Briefly, the instrumental variable is chosen, with the core assumption being that it should be associated with the outcome only via treatment status. Then, treatment status is regressed on the instrument and the other variables using least squares regression, which results in a predicted treatment status for all patients. The residuals from this regression would then be an estimate of the unmeasured confounding. The original Cox regression analysis would then be performed, with the outcome regressed on the independent variables but also including the residuals from the previous stage.
Kaplan-Meier curves and log-rank tests were performed to display survival time/time-to-event and evaluate possible differences in distributions for **studies I–III**.

For **studies I and II**, patients were included from several different hospitals, and as these patients are not considered independent observations, analyses were adjusted for clustering, i.e. patients belonging to the same hospital belong to one cluster.

To handle missing data for **studies I and II**, multiple imputation was used. Multiple imputation generates values multiple times, based on the other covariates with existing data, and averages the results to replace the missing data. This is to be able to perform statistical analyses on patients with missing values, hopefully leading to less bias than complete case analysis and producing less risk of misleading values when compared to single imputation[85].

The dataset in **study III** entailed 270 unique proteins in total, i.e. a highly multidimensional dataset. Due to the expected multicollinearity and the infeasibility of including more predictors than observations, we chose to evaluate protein levels using orthogonal projections to latent structures effect projections (OPLS-EP). OPLS methods use principal components. Briefly, the first principal component is the fitted line that explains the variation of the data in the best way, i.e. the single line with the best fit. Each subsequent principal component is an orthogonal line to the other(s), each new line drawn with the best possible fit. Thus, there is no multicollinearity between these principal components, and the first few principal components usually explain the absolute majority of the variation in the data[86]. The first two are often used to visualise the data to identify clusters, as in **study IV**, to find ethnic groups that may share genetic disparities when compared to other ethnic groups, and for which subsequent analyses would need adjustment.

OPLS-EP is a method designed for dependent 1:1 samples[87]. For **study III**, one OPLS-EP model was constructed per group per panel per postoperative visit, i.e. two groups (cases and controls) and three panels and two different postoperative visits. These models were then tested with a CV-ANOVA. The models that were tested as significantly different had each protein in their effect matrices tested with a paired t-test.

To control for multiple testing (the multiple t-tests in **study III** and the protein quantitative trait loci analysis in **study IV**), the Benjamini-Hochberg[88] procedure was used, with a false discovery rate set to 0.10. The false discovery rate is the allowed proportion of false positives within the significant results.

Bioinformatics

A functional enrichment analysis was performed in **study III**, which is also known as an over-representation analysis. The list of differentially expressed proteins (up- and downregulated separately) was used, the entire list of all tested proteins was used as background, and then the over-representation analysis was performed using the KEGG, Reactome and GO databases. Briefly, this kind of analysis counts the number of terms or pathways in the list of differentially expressed list of proteins and compares the ratios of the same terms or pathways in the background dataset to determine which, if any, are over-represented.

Network analysis was also performed in **study III**, with up- and downregulated proteins analysed all together. The top ten differentially expressed hub proteins in the dataset were found, i.e. the proteins that were estimated to have the highest degree of protein-protein interactions.

For the genome-wide association analysis in **study IV**, routine quality control was performed for the genetic sampling to omit genetic data that could be wrong or misleading.

For **study IV**, a protein quantitative trait loci analysis was performed. This analysis modelled pairs of one protein with one SNP at a time, using linear regression to test whether the pairs of protein levels and SNPs were significantly correlated.

Methods

Study I: Postoperative non-steroidal anti-inflammatory drug use and oncological outcomes of rectal cancer

Study design

This was a retrospective cohort study on rectal cancer patients who underwent anterior resection during 2007–2013 from 15 Swedish hospitals. Most of the information was retrieved from the SCRCR, but the exposure data were collected via chart review. Exposure was defined as use of any NSAID for at least two days during the first postoperative week. The primary outcome was recurrence-free survival, and secondary outcomes were recurrence and overall survival. Follow-up ended on 11 July 2019.

Statistical analyses

Cox proportional hazards regression modelling was performed, evaluating NSAID exposure versus recurrence-free survival, recurrence and overall survival, with adjustment for age, sex, ASA classification, BMI, diverting stoma, cTNM stage, neoadjuvant therapy, PME or TME surgery, tumour level above the anal verge, intraoperative bleeding, year of surgery and individual hospital. Subsequently, propensity score matching was performed, using the same covariates. This matched sample was then evaluated by a Kaplan-Meier curve, testing for differences with a log rank test and univariable Cox regression.

An instrumental variable analysis was also performed to evaluate the impact of NSAID exposure on recurrence-free survival, using individual hospital as the instrumental variable.

Study II: Postoperative non-steroidal anti-inflammatory drugs in relation to recurrence, survival and anastomotic leakage after surgery for colorectal cancer

Study design

This was a retrospective cohort study on colorectal cancer patients resected with a primary anastomosis, including patients from 21 Swedish hospitals during 2007–2012. Follow-up ended on 30 June 2020. The main data source was the SCRCR, but the individual hospitals' postoperative analgesic protocols were the basis for the exposure: if NSAIDs were included, the patients treated at that hospital were assumed to have received postoperative NSAIDs, and patients treated at the other hospitals were assumed not to have been exposed to NSAIDs. This approach has been validated on a subset of this cohort by chart review, where 79% of patients treated at so-called NSAID hospitals were exposed to NSAIDs, and no patients at the no-NSAID hospitals received NSAIDs[56].

The primary outcome was recurrence-free survival. Secondary outcomes were overall survival, recurrence and anastomotic leakage.

Cancers were divided into right-sided colon cancers, left-sided colon cancers or rectal cancers. Anastomotic locations were defined as ileocolic, colo-colic or colo-/ileo-rectal (i.e. following anterior resection or total colectomy) anastomoses.

Statistical analyses

Several Cox regression models were applied, all with NSAIDs as exposure and with recurrence-free survival, cancer recurrence or overall survival as outcomes; adjustment for confounding was performed in all models, with different sets of covariates depending on the outcome and according to directed acyclic graphs. Tumour location was included as an interaction term, to consider that NSAID exposure may have differing effects depending on tumour locations, as left-sided colon cancers more frequently overexpress COX-2 when compared to right-sided colon cancers[89].

Furthermore, multivariable logistic regression was performed to evaluate the influence of NSAID-exposure on the risk of anastomotic leakage. An interaction term for anastomotic location was included here as well, as the selective NSAID agent diclofenac seems to have an increasing propensity of inducing anastomotic leakage in the proximal direction of colorectum[57].

Study III: Peritoneal infection after colorectal cancer surgery induces substantial alterations in postoperative protein levels: an exploratory study

Study design

This was a retrospective matched case-control study, including patients who were resected for colorectal cancer and with a primary anastomosis formed, at either Uppsala or Umeå University hospitals during 2010–2015. Follow-up ended on 17 June 2021. Case status, i.e. peritoneal infection, comprised either an anastomotic leak and/or an intraabdominal abscess. Control status was defined as having had a complication-free postoperative stay. Cases were matched to controls according to age, sex, tumour stage and operating hospital.

Data was gathered from the SCRCR, and case or control status were verified by chart review. Serum samples from the preoperative visit, from the first postoperative visit and from the visit at one year after surgery were all requested from U-CAN. Samples were sent to the analytics company Olink (Uppsala, Sweden) for analysis using three predefined protein panels called "Inflammation", "Oncology II" and "Immune Response", each consisting of 92 proteins. Four selected proteins were also analysed locally.

Statistical analyses

Overall survival and recurrence were displayed with Kaplan-Meier curves and evaluated with log-rank tests. OPLS-EP models were constructed and then tested with CV-ANOVA, and the significant models had their proteins tested with paired t-tests. These tests were controlled for multiple testing.

Functional enrichment analysis and network analysis

Over-representation analysis was performed, with significantly up- or downregulated proteins analysed separately. Over-represented KEGG, Reactome pathways and GO terms were identified, and a q-value of 0.10 was considered statistically significant.

For network analyses, up- and downregulated proteins were analysed together. The top ten hub proteins were identified using the maximal clique centrality (MCC) method in the CytoScape (version 3.8.2) plugin cytoHubba[90].

Study IV: Mutation of the COX-2 gene promotor and anastomotic leakage in colorectal cancer patients

Study design

This was another retrospective matched case-control study based on the same population as in **study III**, by identifying patients who underwent colorectal cancer resection at Uppsala or Umeå University hospitals during 2010–2015. Case status was peritoneal infection within the first 30 postoperative days, and controls had a complication-free postoperative stay. Data were retrieved from the SCRCR, matching was done as in **study III** and case and control status were likewise checked by chart review. Preoperative whole-blood samples were retrieved for genotyping, and preoperative serum samples were retrieved for analyses with the three predefined protein panels from Olink called "Inflammation", "Oncology II" and "Immune Response". All samples were requested from U-CAN. Quality control of the genetic testing was performed, as well as a principal component analysis to check for clustering. The different versions of the COX-2 gene promotor -765G>C variant were evaluated with logistic regression with anastomotic leakage as the outcome. This analysis was also extended to the neighbouring region in the genome, and this was adjusted for multiple testing with the Westfall's max T permutation based method (n=10000)[91].

Protein quantitative trait loci analysis

An exploratory protein quantitative trait loci (pQTL) analysis was then performed using linear regression modelling, controlling for multiple testing with a false discovery rate set to 0.10.

Results

Study I: Results

In total, 1,341 patients who underwent anterior resection were included. Some 362 (27.0%) patients were NSAID-exposed, of which 298 (82.3%) were administered non-selective NSAIDs and the other 64 (17.7%) were administered selective NSAIDs.

No discernible effect on recurrence-free survival (Table 2) was seen with the Cox regression analysis (HR 1.02, 95% CI 0.79–1.33), in the propensity score matched sample (HR 0.89, 95% CI 0.67–1.20) or on the Kaplan-Meier curve (Figure 6) performed with the matched sample (p=0.69). On the contrary, the instrumental variable analysis indicated a potential protective effect (HR 0.61, 95% CI 0.38–0.99).

Table 2. Long-term oncological outcomes for 1,341 rectal cancer patients who underwent anterior resection, with vs without NSAID exposure

Long-term outcome	Events	Unadjusted HR (95% CI)	Adjusted* HR (95% CI)
Recurrence-free survival	**439**	**0.77** (0.62–0.97)	**1.02** (0.79–1.33)
Locoregional recurrence	**29**	**0.30** (0.09–0.99)	**0.36** (0.10–1.25)
Distant recurrence	**201**	**0.98** (0.72–1.33)	**1.05** (0.74–1.49)
Overall survival	**365**	**0.67** (0.52–0.87)	**0.94** (0.70–1.25)

* With adjustment for American Society of Anesthesiologists' class, age, body mass index, intraoperative bleeding, diverting stoma, hospital, neoadjuvant therapy, sex, extent of mesorectal excision, tumour height, year of surgery and clinical tumour stage.

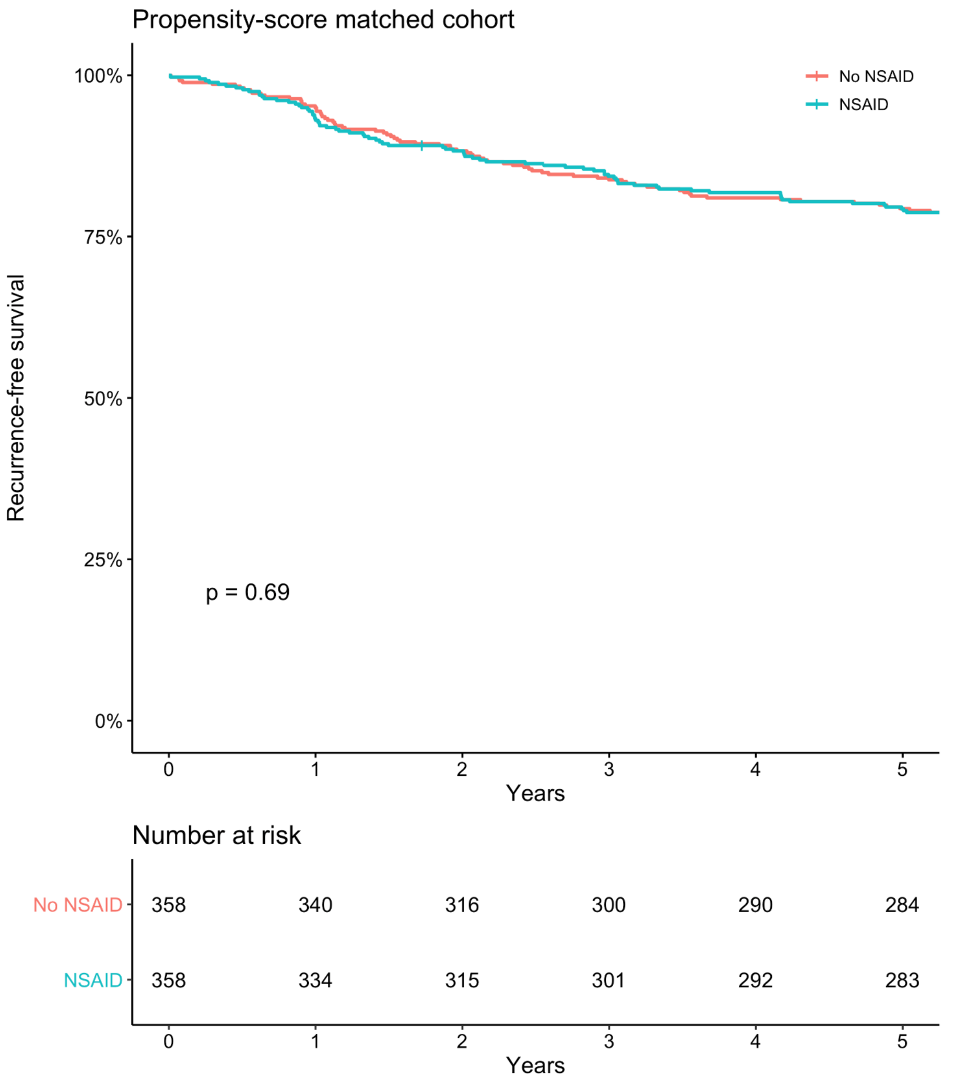


Figure 6. Kaplan-Meier curve on recurrence-free survival using the propensity-score matched cohort

Study II: Results

In this study, 6,945 colorectal cancer patients who underwent tumour resection with a primary anastomosis formed were included. Some 3,996 (58%) of these were in the NSAID-protocol group, with non-selective NSAIDs being the most common agent.

No discernible differences on recurrence-free survival (Figure 7) were seen in the NSAID-exposed group (HR 0.97, 95% CI 0.87–1.09). However, recurrences were significantly less common (Table 3) for the NSAID-exposed (HR 0.83, 95% CI 0.72–0.95), mainly due to a reduced distant recurrence rate in left-sided colon cancers (HR 0.80, 95% CI 0.63–1.00) and perhaps also due to reduced local recurrence rate in rectal cancers (HR 0.56, 95% CI 0.31–1.03). Also, the anastomotic leak rate was significantly reduced (Table 4) in the NSAID-exposed (OR 0.69, 95% CI 0.51–0.94), driven by a reduced leak rate in colo-/ileo-rectal anastomoses (OR 0.47, 95% CI 0.33–0.68); the vast majority of these had undergone an anterior resection for rectal cancer.

Table 3. Total, locoregional and distant recurrences in a cohort of 6,945 colorectal cancer patients resected, with vs without NSAID exposure, only including adjusted* estimates

Primary cancer	**All locations**	**Right-sided colon**	**Left-sided colon**	**Rectal**
Recurrence	**HR (95% CI)**	**HR (95% CI)**	**HR (95% CI)**	**HR (95% CI)**
All	**0.83** (0.72–0.95)	**0.89** (0.72–1.10)	**0.79** (0.63–0.98)	**0.81** (0.66–1.01)
Local	**0.68** (0.48–0.97)	**0.71** (0.43–1.17)	**0.77** (0.45–1.34)	**0.56** (0.31–1.03)
Distant	**0.85** (0.74–0.98)	**0.91** (0.73–1.13)	**0.80** (0.63–1.00)	**0.84** (0.67–1.05)

* With adjustment for American Society of Anesthesiologists' class, age, body mass index, intraoperative bleeding, diverting stoma, hospital volume, neoadjuvant therapy, emergency procedure, sex, tumour location, year of surgery and clinical tumour stage.

Table 4. Anastomotic leakage in a cohort of 6,945 colorectal cancer patients resected, with a primary anastomosis formed, with vs without NSAID exposure, only adjusted* estimates

Anastomosis	Exposed, nr of leaks (%)	Non-exposed, nr of leaks (%)	OR (95% CI)
All locations	**188 (4.7%)**	**182 (6.2%)**	**0.69** (0.51–0.94)
Ileo-colic	**42 (3.0%)**	**27 (2.6%)**	**1.08** (0.63–1.85)
Colo-colic	**61 (5.5%)**	**36 (4.7%)**	**1.20** (0.74–1.93)
Colo/ileo-rectal	**85 (5.7%)**	**119 (10.3%)**	**0.47** (0.33–0.68)

* With adjustment for American Society of Anesthesiologists' class, age, body mass index, intraoperative bleeding, diverting stoma, hospital volume, neoadjuvant therapy, emergency procedure, sex, anastomotic location and year of surgery.

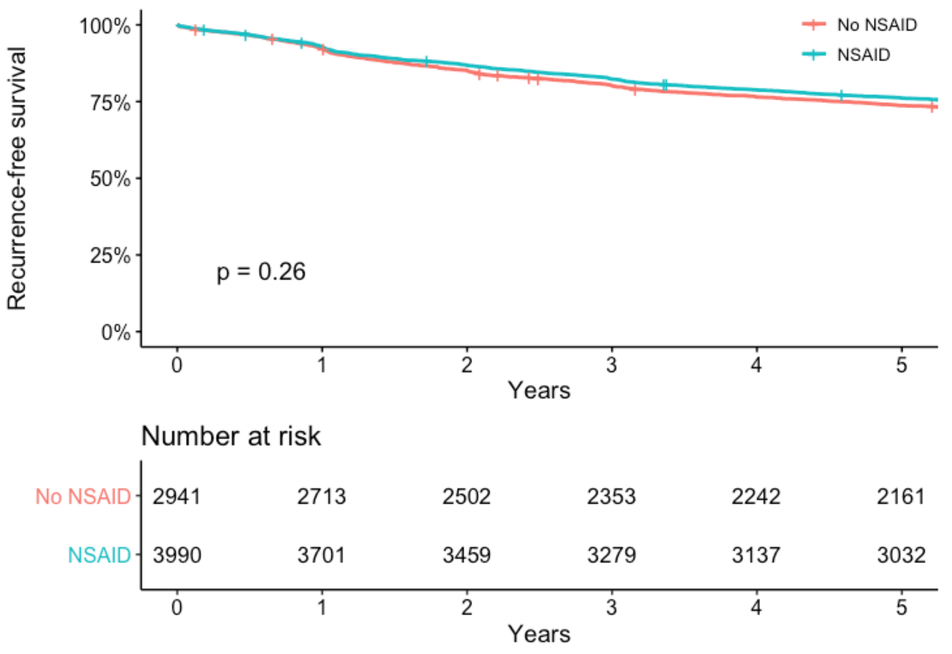


Figure 7. Kaplan-Meier curve for recurrence-free survival in a cohort of 6,945 colorectal cancer patients resected, with vs without NSAID exposure

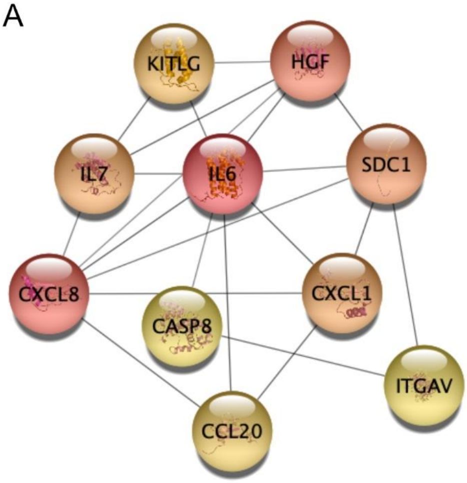
Study III: Results

A total of 64 patients, 32 cases with peritoneal infection matched with 32 controls, were included. For the peritoneally infected, 72 proteins were upregulated and five downregulated at the first postoperative visit at a median of 41 days after surgery. No discernible difference in survival or cause of death were seen between groups.

The top ten ranked hub proteins (Figure 8) were: IL-6, IL-8/CXCL8, HGF, SYND1/Syndecan-1, IL-7, CXCL1, KITLG/SCF, CCL20, CASP8 and ITGAV.

Downregulated proteins were associated with pathways of cardiomyopathy, extracellular matrix (ECM), cell adhesion, PI3K-Akt and TGF-β in the functional enrichment analysis, using the KEGG, Reactome and GO databases (Figure 9). No pathways were over-represented amongst the upregulated proteins.

A



B

Rank	Protein
1	IL6
2	CXCL8(IL8)
3	HGF
4	SDC1 (SYND1)
5	IL7
5	CXCL1
7	KITLG (SCF)
8	CCL20
9	CASP8
9	ITGAV

Figure 8. Top ten differentially expressed hub proteins in patients with peritoneal infection vs patients with a complication-free postoperative stay. A stronger shade of red represents a higher degree of interconnection. Hub proteins are ranked in the table.

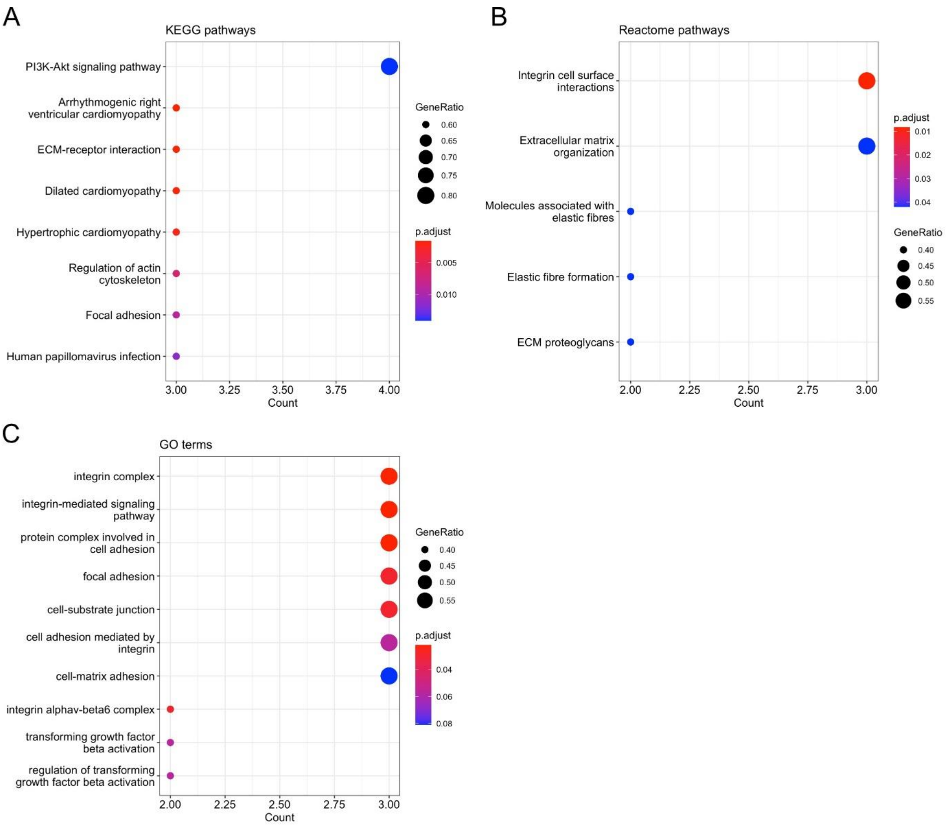


Figure 9. Functional enrichment analysis of downregulated proteins in patients with peritoneal infection vs patients with a complication-free postoperative stay. Significantly over-represented pathways or terms according to KEGG (A), Reactome (B), GO databases. P-values adjusted for false detection rate <0.1. Count (x-axis) displays the number of differentially expressed proteins in each pathway/term. GeneRatio (circle-size) is the ratio of differentially expressed down-regulated proteins in the pathway, divided by the total amount of differentially expressed proteins that were down-regulated.

Study IV: Results

This study comprised 94 patients, where 47 cases who suffered peritoneal infection were matched with 47 complication-free controls. In total, two patients were homozygous for the minor allele -765G>C, one case and one control. There were ten cases and 14 controls who were heterozygous (i.e. G/C), and 36 cases and 32 controls who were homozygous for the major allele (i.e. G/G). Contrary to our expectations, there were more patients with the minor allele in the control group rather than the case group (Table 5), although rendering a non-significant difference with an OR of 0.71 (p=0.413).

In the pQTL analysis, 339 associated pairs of SNPs and protein levels were identified, but all of them were deemed of no further interest when scrutinised.

Table 5. Frequency table of the COX-2 gene promotor -765G>C variations in patients with peritoneal infection vs patients with a complication-free postoperative stay

	G/G	**G/C**	**C/C**
Peritoneally infected	36	10	1
Complication-free	32	14	1

Discussion

Findings

In **studies I and II**, no impact of postoperative NSAIDs on recurrence-free survival could be seen. However, a reduction in cancer recurrence could be demonstrated for left-sided colonic cancers, including a similar trend in patients with rectal cancer. Anastomotic leak rate was also reduced in the NSAID-exposed group, in part due to a reduction in leak rate after restorative rectal cancer surgery.

In **study III**, numerous altered protein levels and biological pathways were demonstrated in patients suffering a postoperative peritoneal infection. These alterations were seen in proteins and pathways that are known actors regarding potentiation of cancer including minimal residual disease, cardiomyopathy and cancer cell invasiveness and metastatic potential.

In **study IV**, frequencies of the COX-2 gene promotor polymorphism of -765G>C in a Swedish colorectal cancer cohort were determined, but a previously noted association between the minor allele and an increased anastomotic leak rate could not be replicated.

Limitations

The studies included in this thesis are retrospective observational studies, leading to potential residual confounding as well as possible correlations without verifiable causation. In attempts to mitigate such confounding, tools such as directed acyclic graphs (for appropriate selection of confounders), matching, propensity scores, and instrumental variable analysis have been employed.

Regarding **study I** and the included instrumental variable analysis, the core assumption for it was most likely not met, i.e. that hospital identity's only effect on the outcome of recurrence-free survival would be through its effect on NSAID-exposure; therefore, this finding must be interpreted with caution.

The protocol-based approach in **study II** enabled a larger sample size, but it may have diluted the effect of the exposure, as around 21% of the patients who were assumed to have received postoperative NSAIDs might ultimately not have been treated with NSAIDs[55]. Moreover, the secondary outcome anastomotic leakage was derived from the SCRCR, which has a known underreporting, at least considering anastomotic leaks after anterior resection[55] and around a third of such leaks may be diagnosed later than 30 days[36].

The protocol-based approach in **study II** presents further analytical difficulties, as one could interpret that the observed differences may not be due to the protocolised NSAID-exposure but rather the individual hospitals' impact on the outcomes via other mechanisms. Adjustment was made for hospital volume, which could be a surrogate marker for each hospital's proficiency in treating and caring for patients. Moreover, several of the hospitals also switched between analgesia protocols, at times including NSAIDs and sometimes not, during the study period itself.

Another limitation to **studies I and II** is the fact that several different NSAIDs were used as a combined exposure, whereas it is known that their profiles differ regarding inhibitory effect on COX-1 and COX-2[51]. In both **studies I and II**, sensitivity analyses were performed to explore this, where the exposure was divided into selective or non-selective NSAIDs.

Studies III and IV have limited sample sizes, with accompanying risks of chance findings but also not detecting actual differences, i.e. both type I and type II errors, even though the risk of a false negative is generally considered higher in smaller cohorts. In addition, a smaller sample size does not allow as many covariates in multivariable analyses or when constructing a matched cohort, thus risking residual confounding to a higher degree when compared to using larger cohorts.

Regarding the functional enrichment analysis in **study III**, a shortcoming of this method was encountered, as the numerous upregulated proteins may have made up such a high proportion of the background set used, so that no pathways were deemed significantly over-represented when compared to it. It may be that this type of analysis is better suited when the differentially expressed proteins are of a smaller proportion when compared to the background data set.

The literature and the thesis

NSAIDs, recurrence and survival

Systematic reviews on the subject of perioperative NSAIDs including aspirin have discerned beneficial long-term effects on oncological outcomes for certain cancer forms, including observational data regarding rectal cancer[69].

An association between NSAIDs after resection for colorectal cancer and improved recurrence-free survival has been reported[67]; of note, this association was no longer significant when emergent procedures were excluded, and it is likely that the outcome itself was recurrence rather than recurrence-free survival in the aforementioned study. Another study reported a link between postoperative NSAIDs and an improved recurrence-free survival in rectal cancer patients with a pronounced preoperative inflammatory state[68].

Only one small pilot trial regarding the combined perioperative use of a NSAID and a beta-adrenergic inhibitor for colorectal cancer patients has been published: an improved five-year disease-free survival could be demonstrated in the intervention group[92].

In **studies I and II**, no such improvement in recurrence-free survival could be reproduced, whereas a decrease in distant recurrence after left-sided colonic cancer resection and perhaps also a reduction in local recurrence after rectal cancer resection could be detected.

Speculatively, the potential beneficial effects may in part depend on the perioperative inflammatory state[68], tumour location[69, 93] and whether the resection was done as a planned or emergent procedure[67]. Furthermore, any difference in survival might further be diluted by the fact that colorectal cancer patients tend to be elderly, with a median of 75 years and 72 years of age at diagnosis for colon and rectal cancer patients, respectively[27, 28], thus, fewer years of life might be gained and a larger sample may be needed to detect a significant difference in overall survival. And many, but not all, colorectal cancers may exhibit overexpression of COX-2 mRNA[94], where left-sided cancers seem more prone to express COX-2 than right-sided colonic cancers[89], which would be in line with the findings of a reduced recurrence in patients resected for left-sided colon cancers who received postoperative NSAIDs.

NSAIDs and anastomotic leakage

A meta-analysis on patients with colorectal cancer did not exhibit any effect of NSAID exposure on the frequency of anastomotic leakage[95]. Nor were any significant associations discernible when evaluating only rectal cancer patients, or when evaluating only non-selective NSAIDs, only selective NSAIDs or only diclofenac[95]. However, two earlier systematic reviews, one of which also included a meta-analysis, both found that diclofenac seemed detrimental[54, 69], and another systematic review indicated that early administration may be particularly negative, perhaps interfering with early wound healing[96].

The above is further corroborated by the findings of another study, that the COX-2 enzyme is essential for colonic anastomotic healing[53].

In **study II**, where the majority were administered non-selective NSAIDs, an association between postoperative NSAIDs and a reduced frequency in anastomotic leakage was exhibited, driven by a risk reduction in patients resected for rectal cancer. No discernible risk decrease or increase was seen in any other anastomotic location. The differing results could be due to the fact that a majority of patients in **study II** received non-selective NSAIDs, and that there is a beneficial interaction between non-selective NSAIDs and anastomotic healing after anterior resection.

Biological perturbations due to intra-abdominal infection

The literature regarding proteomics in the context of intra-abdominal infections has mainly focused on prediction of anastomotic leakage, and often on a single or only a select few biomarkers meant to help detect anastomotic leaks within the first postoperative week. The aim of **study III,** on the other hand, was to explore which proteins and signalling pathways might link anastomotic leakage with worsened oncological outcomes, by mapping out possible biological perturbations.

A review focusing on biomarkers for diagnostic and predictive use listed C-reactive protein (CRP), PCT, IL-6, MMP-9 and calprotectin as affected postoperatively in serum in patients suffering from anastomotic leakage within the first postoperative week, and that I-FABP preoperatively was increased in the group who later suffered anastomotic leakage[97].

IL-6 and VEGF have been measured both in serum and from peritoneal fluid in patients suffering from intra-abdominal infection, and both were increased at postoperative day 4 when compared to patients with a complication-free postoperative course; the patients who suffered from an intra-abdominal infection also suffered from increased cancer recurrence at 2 years[46].

In **study III**, 77 proteins were shown to be differentially expressed at a median of 41 days postoperatively. When examining preoperative samples in an extended version of this cohort[39], only three biomarkers were differentially expressed and out of these only one (i.e. CXCL6) was also differentially expressed in the postoperative sampling, further corroborating that the absolute majority of the results should not be due to preoperative differences. Most of these were novel findings in the context of intra-abdominal infections and the top ten hub proteins found were IL-6, IL-8/CXCL8, HGF, SYND1/Syndecan-1, IL-7, CXCL1, KITLG/SCF, CCL20, CASP8 and ITGAV. Over-represented pathways included cardiomyopathy, extracellular matrix (ECM) organisation, cell adhesion, PI3K-Akt and TGF-β. IL-6 is an important pro-inflammatory protein, also involved in the initiation, proliferation, EMT, invasiveness and metastatic potential of colorectal cancer cells[98], perhaps still potentiating minimal residual disease beyond a month after surgery. Notably, IL-6 was elevated despite a normalised CRP, i.e. even though a clinician may look at a normalised CRP value and conclude that possible detrimental influence from the anastomotic leakage has passed, the pro-inflammatory and pro-cancerogenic IL-6 may still be elevated.

The -765G>C variant of the COX-2 promotor gene

In the seminal experimental study by Reisinger et al[53], by knocking out the COX-2 gene and by administering the selective NSAID diclofenac, it was demonstrated that COX-2 in mice may have an essential function regarding the healing of colonic anastomoses. In the same study, a consecutive cohort of 148 colorectal cancer patients was genotyped for a known polymorphism of the COX-2 gene promotor -765G>C; here, a significant and strong association between C/C status and anastomotic leakage was shown, with a leak rate of approximately 43% vs 11% of the rest (i.e. G/C or G/G). The case-control in this thesis (**study IV**) could not reproduce this finding and, if anything, the estimate even pointed in the opposite direction.

Study IV may have been an underpowered study with 47 cases and 47 controls in total, despite seeming feasible according to a power calculation: assuming anastomotic leak rates of 43% vs 11% [53] and 80% power and significance level set to 0.05, a sample size of 29 cases and 29 controls could suffice, but ultimately there was a lower homozygote rate than anticipated in this cohort, thus reducing the number available for analysis. Heterozygosity was also included in **study IV**, i.e. G/C, as this state can also present with altered phenotypes in other contexts[58, 60].

The previous study [53] did not present their cohort or adjust their analysis; consequently, it is difficult to assess possible influence of confounding in their study. **Study IV** had a matched case-control study design as an attempt to control for such confounding.

In the study by Reisinger, the authors also discussed their findings in view of the reasonable assumption that the -765G>C polymorphism will render a decreased COX-2 gene promotor expression[58]. This notion has been challenged however, with conflicting evidence[99].

Study IV also used the composite outcome of intra-abdominal infection, i.e. anastomotic leakage and/or intra-abdominal abscess, and the study by Reisinger only had anastomotic leakage as outcome. Chart review was used to verify case or control status in **study IV**, whereas in the study by Reisinger et al no presentation was made on how case or control status was determined.

The frequency of C/C state in the study by Reisinger et al was 4.7%, and in **study IV** it was 2.1%, a frequency not previously investigated in a Swedish colorectal cancer cohort.

Pieces of a puzzle

The perioperative period can influence minimal residual disease by several different pathways, including inflammatory and catecholamine pathways, affecting both recurrence risk and mortality[48]. Anastomotic leakage might confer increased risk of recurrence and mortality, at least for some locations[34], but also render increased morbidity including cardiovascular events[33]. Serum as well as peritoneal fluid from peritoneally infected have been shown experimentally to stimulate colorectal cancer cell lines[45] in migration and invasiveness.

In **study III**, the differentially expressed top hub protein was IL-6, which is involved in the inflammatory response as well as stimulating minimal residual disease[98]. IL-6 can be activated by prostaglandin E2 (PGE2), i.e. a product of the COX-2 gene[100-103]. However, none of the COX-2 enzymatic products were analysed with the pre-defined protein panels, so it is not known whether the COX-2 gene expression also was still upregulated at the first postoperative visit. Nevertheless, it has been shown that inhibition of PGE2 in turn can decrease IL-6 in the context of peritonitis [100], and seeing that COX-2 often is overexpressed in colorectal cancer[104], NSAIDs could potentially counteract stimuli on minimal residual disease by inhibiting COX enzymes and their subsequent effects on IL-6.

Perioperative NSAIDs can inhibit postoperative inflammation, neuroendocrine stress and immune response[105], and these factors may otherwise potentiate minimal residual disease including subsequent decreased effect of natural killer cells' activity up to 1–2 months postoperatively[42], being in line with the finding of reduced recurrence rates for the NSAID exposed in **study II**.

Implications and speculations

The preoperative inflammatory state of the patient, whether the procedure is emergent or planned, the tumour and subsequent anastomotic location, both the germline and somatic COX-2 genetic makeup, the specific NSAID used and the timing and duration perioperatively, and whether the patients already have suffered an anastomotic leakage or not may all influence whether the patients may benefit or not from NSAID-exposure.

Non-selective NSAIDs may be neutral or even beneficial to anastomotic healing, and they may be of increased benefit if the patient has an increased preoperative inflammatory state or if emergent surgery is performed, and if the tumour is located in the left colon or the rectum. Speculatively, NSAIDs may also be of additional benefit if the colorectal cancer overexpresses COX-2 mRNA, or perhaps if the patient has already suffered an anastomotic leakage.

It is also possible that any medication meant to counteract the potentially aggravating effect on minimal residual disease in the face of an intra-abdominal infection should be continued for at least 41 days postoperatively or longer.

Conclusions

There was no apparent beneficial survival effect of at least 2 days duration of NSAID exposure during the first postoperative week after anterior resection for rectal cancer. However, a beneficial influence of NSAID exposure on cancer recurrence in a more numerous and mixed colorectal cancer cohort could be demonstrated, including a lower rate of anastomotic leakage after anterior resection. The greatest benefit from NSAID exposure regarding recurrence was seen for left-sided colonic cancers, perhaps due to their tendency of overexpression of the COX-2 enzyme.

The proteomic landscape in patients suffering postoperative peritoneal infection was explored, rendering 77 differentially expressed proteins, where several of the top hub proteins are known actors in colorectal cancer progression including invasiveness and metastatic potential. A previously reported association between a COX-2 gene promotor variant -765G>C and an increased rate of anastomotic leakage could not be replicated. The homozygote minor allele and the heterozygote minor allele frequencies were determined to be 2.1% and 25.5% respectively, in a representative Swedish colorectal cancer cohort.

Future research

Randomised controlled trials testing perioperative NSAID-treatment to evaluate possible effect on short-term outcomes including anastomotic leakage, as well as long-term oncological outcomes, would certainly be of interest. This kind of intervention could be further stratified according to preoperative inflammatory status, tumour location, emergent or planned procedure, and perhaps somatic COX-2 expressional status.

Observational studies with NSAID exposure in a cohort already suffering from anastomotic leakage to evaluate oncological outcomes would be valuable. Such studies could prove to be challenging to design, but a protocol- and register-based approach might work provided ample time for validation if patients presumed to have been administered NSAIDs actually did receive NSAIDs even after anastomotic leakage occurred.

Observational genomic studies regarding anastomotic leakage would be of interest as well, perhaps also including further replication attempts with larger cohorts regarding the COX-2 gene promotor SNP of -765G>C but also performing a genome-wide association study, which most certainly would require a sizable sample.

Observational studies regarding overexpression of COX-2 and whether patients benefit from NSAIDs would certainly be of interest as well, but perhaps difficult to design. One possible option would be to construct a cohort of patients with left-sided colonic cancers and retrieve biological samples to test somatic COX-2 expression and perform chart review to determine perioperative NSAID exposure.

Further research into the proteomic landscape of anastomotic leakage, perhaps with a focus on hub proteins with a high degree of interconnectedness, could continue to map out possible connections between anastomotic leakage and its deleterious impact on morbidity and mortality. This research could branch out in a multitude of ways and could one day result in clinical trials of targeted biological treatment in the event of anastomotic leakage.

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