ANASTOMOTIC LEAKAGE AFTER COLON CANCER RESECTION:

Incidence, management, outcome, risk factors and experimental modeling

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The *Danaides* by John William Waterhouse (1903)

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This doctoral thesis was based on the following papers, referred to in the text by Study and their roman numerals

I. Krarup PM, Jørgensen LN, Andreasen AH, Harling H.
A nationwide study on anastomotic leakage after colonic cancer surgery.
Colorectal Disease 2012;14:e661-e667

II. Krarup PM, Nordholm-Carstensen A, Jørgensen LN, Harling H.
Association of comorbidity with anastomotic leak, 30-day mortality, and length of stay in elective surgery for colonic cancer: A nationwide cohort study.
Diseases of the Colon & Rectum 2015;58:668-676

III. Krarup PM, Jørgensen LN, Harling H.
Management of anastomotic leakage in a nationwide cohort of colonic cancer patients.
Journal of the American College of Surgeons 2014;218:940-949

IV. Krarup PM, Nordholm-Carstensen A, Jørgensen LN, Harling H.
Anastomotic leak increases distant recurrence and long-term mortality after curative resection for colonic cancer: a nationwide cohort study
Annals of Surgery 2014;259:930-938

V. Krarup PM, Eld M, Heinemeier K, Jørgensen LN, Hansen MB, Ågren MS.
Expression and inhibition of matrix metalloproteinase (MMP)-8, MMP-9 and MMP-12 in early colonic anastomotic repair.
International Journal of Colorectal Disease 2013;28:1151-1159

VI. Krarup PM, Rehn M, Sand-Dejmek J, Ehrnström R, Ågren MS, Syk I.
Rapid morphological changes and loss of collagen following experimental acute colonic obstruction.
International Journal of Colorectal Disease 2013;28:341-347

VII. Krarup PM, Eld M, Jørgensen LN, Hansen MB, Ågren MS.
Selective matrix metalloproteinase inhibition increases breaking strength and reduces anastomotic leakage in experimentally obstructed colon.
International Journal of Colorectal Disease 2017;32:1277-1284

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1 PREFACE

"Any fool can know. The point is to understand." The quote by Albert Einstein, embodies the years I spend in medical school at the University of Aarhus. In the first couple of semesters I struggled with the huge curriculum, where the end goal was to *know* it all at the biannually exams.

My farther, who was a urologist said to me at some point, that I had to do research. During the third semester I volunteered to assist a research scholar with an experimental study on hydronephrosis in piglets at the Institute of Clinical Medicine. The institute, led by Professor Jens Christian Djurhuus, was a place of inspiration. There was a firm belief in the value of experimental research and in the value of engaging junior doctors and medical students. This led to a sabbatical year, where I was trusted with my own study, which ultimately led to my first publication, *Regional changes in renal cortical glucose, lactate and urea during acute unilateral ureteral obstruction: A microdialysis study.* During that year I gained a new perspective; knowing is stationary while understanding is dynamic, and research can make you understand – or at least make you *know* what you don't *understand*.

In 2010, during my surgical training at Bispebjerg Hospital in Copenhagen, I began another sabbatical year of research. Anastomotic leakage after resection for colonic cancer was just adopted as a quality indicator in the database of the Danish Colorectal Cancer Group.

Anastomotic leakage was regarded as inevitable following colorectal cancer surgery and while all surgeons *know* about anastomotic leakage, the *understanding* of the incidence, the management algorithms, the impact on short- and long-term outcomes, the risk factors and the processes of anastomotic healing seem inadequate.

Anastomotic leakage has the ability to ruin a perfectly planned and executed curative resection for colonic cancer. *Understanding* of this problem was my primary motivator for the presented thesis.

The series of studies, compressed here, was planned and executed in collaboration with Henrik Harling, Lars Nannestad Jørgensen and Magnus Sven Ågren.

2 ABBREVIATIONS

ACS NSQIP	AMERICAN COLLEGE OF SURGEONS NATIONAL SURGICAL QUALITY IMPROVEMENT PROGRAM
ASA	AMERICAN SOCIETY OF ANAESTHESIOLOGISTS
AUC	AREA UNDER THE CURVE
CCI	CHARLSON COMORBIDITY INDEX
CI	CONFIDENCE INTERVAL
DCCG	DANISH COLORECTAL CANCER GROUP
ERAS	ENHANCED RECOVERY AFTER SURGERY
ESCP	EUROPEAN SOCIETY OF COLOPROCTOLOGY
HR	HAZARD RATION
IL	INTERLEUKIN
IQR	INTERQUARTILE RANGE
MMP	MATRIX METALLOPROTEINASES
MRNA	MESSENGER RIBOSOMAL NUCLEAR ACID
N	NEWTON
NRP	NATIONAL PATIENT REGISTRY
NSAID	NON STEROID ANTI-INFLAMMATORY DRUG
OR	ODDS RATIO
ROC	RECEIVER OPERATING CHARACTERISTICS
RPLP0	RIBOSOMAL PROTEIN LATERAL STALK SUBUNIT P0
SEM	STANDARD ERROR OF THE MEAN
SD	STANDARD DEVIATION
TGF-β	TRANSFORMING GROWTH FACTOR BETA
TNF-α	TUMOUR NECROTIC FACTOR ALPHA
UICC	UNION FOR INTERNATIONAL CANCER CONTROL
VEGF	VASCULAR ENDOTHELIAL GROWTH FACTOR

3 HISTORICAL INTRODUCTION

The roman poet Publius Ovidius Naso (43 B.C. – 17 A.C.), best known for the *Metamorphoses*, a 15-volume book including the Heroides XIV, told a story about the fifty daughters of Danaus. One version of the myth reads, that the daughters of Danaus were to marry the sons of Danaus' twin brother. On their wedding nights, all but one daughter, killed their newly wed husbands on orders from Danaus himself. Because of their wrongdoings, the daughters were condemned to carry jars with water, filling a bathtub, to wash off their sins. However, the bathtub had a *leak* making fulfillment of the task impossible. This particular situation has later been described as futile and it may also be the first reference to the consequences of a *leak* – a *leak* with life lasting significance.

At the time of the great poets, repair of intestinal lesions was considered futile, a notion lasting till the end of the 19th century. By then, development of surgical techniques and material encouraged surgeons to attempt surgical relief of patients with obstructive tumors in the colon.

Prior to 1882 only about 90 cases of intestinal anastomoses were reported in English literature, and in these cases the fistula and mortality rates were high.[1] In 1833 the French surgeon *Jean- Francois Reybard* (1795-1863) performed the first one-stage resection of the sigmoid colon because of cancer. The intestinal ends were joined with a simple continuous (furrier´s) suture of silk applying one layer in the back row and two layers in the front row.[2,3] In 1880, *Vincenz Czerny* (1842 – 1916), who was Professor at Heidelberg in Germany, resected a large tumor involving the sigmoid, descending and transverse colon and performed an immediate anastomosis.[4] Even though both patients survived the operations, the approach of resection and immediate bowel reconstruction was not accepted by the Royal Academy.[3] Most surgeons at that time recommended a staged procedure for colonic malignancies. In 1891 the Danish surgeon *Oscar Thorvald Bloch* (1847 – 1926) held a lecture on *extra-abdominal treatment of intestinal cancer* at the Danish Medical Society.[5] He described a staged procedure where exteriorization of the tumor-bearing colonic segment was followed by excision of the growth a few days later creating a double-barreled colostomy for possible subsequent closure. Similar approaches were advocated by *Frank T Paul* (1851 – 1941),[6] *Herbert William Allingham* (1862 – 1904)[7] and *Johann Freiherr von Mikulicz-Radecki* (1850 - 1905).[8] Later, the procedure was acknowledged as the Mikulicz procedure. Only few surgeons performed a one-stage procedure with immediate anastomosis, and in 1903 *Von Mikulicz* gave a reading at the American Surgical Association reporting that surgery for colonic cancer with resection and immediate bowel reconstruction carried a mortality of 30 to 50 per cent as compared with approximately 16 per cent after the staged procedure.[8] The higher mortality was attributed peritonitis because of fecal content leaking through yielding sutures of the anastomosis, essentially anastomotic leakage.[8]

During the next 5 decades several surgeons attempted resection with immediate anastomosis[9-11] but the controversy of the one-stage versus the two-stage procedures continued. In 1941, *Richard Barley Cattell* (1900 – 1964), director of the Lahey Clinic in Massachusetts, reported: "*...no resections with primary anastomosis are now being, or have for many years, been undertaken here....*".[12-14]

The discussions primarily involved tumors from the hepatic flexure to the distal part of the sigmoid colon. Subsequently, the disputes also concerned the approach for right sided colectomy. In 1945, *Rodney Maingot* (1893 – 1982) wrote, that he had abandoned primary resection and anastomosis in favor of the modified Mikulitz approach,[14] adopted for cecal and right colonic tumors by *Frank Howard Lahey* (1880 – 1953) in 1932. The main argument for adaptation of the Mikulitz approach was a significant reduction in operative mortality to 10 per cent or even less. *Lahey* further reported, that not only did they reduce mortality, they also increase the operability from 69 to 88 per cent by applying the Mikulitz plan for all procedures.[12]

From the early 1900s up till the 1950s, the principals of asepsis were the only preventive measure during surgery. *Sir Joseph Lister* (1827 – 1912) formulated the aseptic surgical principals combining the germ theory of *Louis Pasteur* (1822 – 1895) with the findings by *Ignác Semmelweis* (1818 – 1865), that hand wash with carbolic acid significantly reduced mortality in women giving birth. The discovery of penicillin by *Alexander Fleming* (1881 – 1955) in 1929 revolutionized surgery with a shift from asepsis to antisepsis. The development of new and more potent antibiotics has continued throughout the years, dramatically reducing mortality and infections rates associated with surgery and thus allowing longer and more complicated surgical procedures.

As early as 1923, *John Percy Lockhart-Mummery* (1875 – 1957) pointed out; "*The fact that the surgeon is now able to operate upon patients with tumours in the colon before serious obstruction has occurred has entirely altered the type of operation that is performed, and greatly improved the results."* He was a firm believer in the one stage procedure and advocated an end-to-end anastomosis as opposed to a side-to-side anastomosis due to the risk of fecal contents being forced by peristalsis through the closed ends causing leakages.[11] He continued; "...to attempt to resect or anastomose the large bowel in a patient suffering from any degree of obstruction is wrong...".[15] This is a principal that is still practiced.

Today, elective colonic resection with primary anastomosis is the cornerstone of colonic cancer treatment. However, anastomotic leakage continues to challenge the concept of primary bowel reconstruction due to life threatening consequences.

4 BACKGROUND

In more recent times, numerous studies on anastomotic leakage after colorectal surgery have been published. However, the main focus has been on patients undergoing low anterior resection for rectal cancer. This procedure is considered more difficult and carries a higher risk of anastomotic leakage compared with colonic resection.16-21

In 1994 the Danish Colorectal Cancer Group (DCCG) assembled for the first time and the National Rectal Cancer Registry was created. In May 2001, this registry was transformed to the DCCG database, also including patients with colonic cancer. From the beginning, anastomotic leakage after low anterior resection was selected as a clinical quality indicator with an acceptable upper limit of 15 per cent decreasing to 10 per cent in 2002. It was not until 2010 that anastomotic leakage after resection for colonic cancer received the status as a DCCG indicator variable. The indicator upper limit was set at 7 per cent not taking into account, that the rate of anastomotic leakage depends on the anastomotic location along the colon.

4.1 Incidence of anastomotic leakage

The reported incidence of anastomotic leakage varies from 1 per cent in small bowel anastomoses to more than 10 per cent in colo-rectal and colo-anal anastomoses.[17,18,20-23]

In 1970, *John Cedric Goligher* (1922 – 1998) published a study on anastomotic leakage rates after left sided colectomy. He reported an overall rate of 40 per cent, but only 9 per cent were clinically important leaks.[24] The strength of the study was the rigid and routinely examination of patients using contrast enema enhanced x-ray. A few years later, *Schrock et al.* reported leakage rates of 3.3 per cent after ileocolic anastomosis, 3.2 per cent in colo-colic anastomosis and 9.2 per cent in colorectal anastomoses.25 In the 1990's the incidence of anastomotic leakage was reported even lower, 1.8 per cent after ileocolic anastomosis, 4.1 per cent in colo-colic anastomosis and 4.5 per cent in colorectal anastomosis.[17] These low rates of anastomotic leakage have in general been supported in the available literature.[18] However, studies presenting very low leakage rates are often results of personal series, small cohorts or single center designs,[26,27] increasing the risk of selection bias and limiting the external validity. Case mix of patients with colonic- or rectal cancer may also cloud the location specific rate of anastomotic leakage. It is essential to distinguish between colonic- and rectal resection because the anatomy, surgical techniques and complication rates differ. A *realistic* level of anastomotic leakage after colonic resection by experienced colorectal surgeons was reported between 3 to 6 per cent.[28] This was confirmed by the first large-scale study on anastomotic leakage after colonic resection for cancer published by Kube et al. In 28271 patients, who underwent colonic cancer resection in 346 German centers, the overall leakage rate was 3.0 per cent.[16]

Anastomotic leakage is important because of its consequences.

4.2 Early mortality after anastomotic leakage

Robust data on short- and long-term outcome following anastomotic leakage after colonic resection were sparse before 2010. In 1973, the overall mortality rate in patients with anastomotic leakage after colorectal surgery was reported between 26.5 per cent and 36.8 per cent.[25] In another study of 151 gastrointestinal leaks, the 71 per cent involving the colon or rectum, carried a low mortality rate of 3.3 per cent.[29] More recently, mortality rates between 5.0 per cent and 12.9 per cent in patients with anastomotic leakage have been published.[18,30,31] In all of the above mentioned studies there was no differentiation between leakages after colonic and rectal resection. In general, the mortality rate following anastomotic leakage is higher after colonic[18,25,29-31] compared with rectal resection[21,32], as further demonstrated by two single-center studies reporting 30-day mortality rates between 29 – 33 per cent following anastomotic leakage in colonic cancer patients[21,32] as compared with 11 per cent after low anterior resection.[33-35] In the large German study from 2009, the in-hospital mortality was 18.6 per cent in patients with anastomotic leakage and 2.6 per cent in patients without anastomotic leakage, confirming the devastating high mortality rates seen in patients with anastomotic leakage.[16] Mortality rates may depend on the grade of the leak,[36] which is closely related to its management.

4.3 Management of anastomotic leakage

It has been speculated, that timely and proper management of patients with anastomotic leakage could reduce the associated morbidity and mortality. However, the literature on management of anastomotic leakage is scarce and diverse.[30,37-44]

In general, anastomotic leakage is associated with increased morbidity and mortality rates regardless of treatment plan.[45] The strategy is often surgical[38] and mainly by *anastomotic takedown* with resection of the anastomosis and creation of an end-ostomy.[38] The procedure carries an inherent risk of a permanent fecal diversion in surviving patients[30,46] with associated stomal complications.[47,48] Recently, another strategy termed *anastomotic salvage* has emerged. Using a proximal loop-ostomy, either alone or in combination with repair or redo of the anastomosis, the bowel continuity through the anastomosis is preserved. This approach seems feasible and safe in selected patients,[30,49-51] and it is probably associated with favorable short- and long-term outcomes, including a decreased risk of a permanent ostomy.[49,52] However, selection bias and lack of control for confounding may limit the external validity of these studies. Despite the lack of robust data, 350 members of the Dutch Society of Gastrointestinal Surgery have expressed a preference for preservation of left-sided anastomosis in physically fit patients.[53]

Timing of re-intervention for anastomotic leakage is difficult to investigate. Because of the retrospective nature of the available literature, the risk of selection and recall bias is immense. In one study of 655 patients, the median time to diagnosis of anastomotic leakage in 39 (6 per cent) patients was 8 days, ranging from 4 to 25 days.[37] The authors demonstrated that re-operation for anastomotic leakage within 5 days from the index operation reduced mortality significantly compared with re-operation beyond day 5.[37] These data suggest that the diagnostic delay was associated with a *failure-to-rescue*.

Serial measurements of procalcitonin and C-reactive protein have demonstrated elevated levels, beyond the regular surgical stress response, from postoperative day 1 and 2, respectively, in patients with anastomotic leakage.[54,55] This is in discrepancy with the time of the leak diagnosis on postoperative day [6.54,55] The diagnosis of anastomotic leakage is difficult. Abnormal vital signs are common in patients subjected to bowel resection[56,57] and comorbidity may cloud the postoperative evaluation of the patients.

4.4 Long-term outcomes following anastomotic leakage

In addition to the immense risk of short-term mortality, a decrease in overall 5-year survival following anastomotic leakage has been observed.[16,21] In the study by *Kube et al.* the survival curves separated early in the postoperative period suggesting that the high in-hospital mortality accounted for the overall reduction in long-term survival.[16] In another study, exclusion of patients dying within 30 days of the index operation did not change the inferior long-term survival in patients with anastomotic leakage.[21] The influence of anastomotic leakage on overall survival is still controversial and published data are sparse. It has been speculated that one reason for the impaired long-term survival in patients with anastomotic leakage could be an increased risk of cancer recurrence. However, in contrast to patients operated on for rectal cancer, increased risk of recurrence has not been demonstrated for patients with anastomotic leakage following colonic cancer resection.[21,32,58,59]

Robust follow-up data on patients surviving anastomotic leakage is sparse. Reversal rates of temporary ostomies as low as 46% following management of anastomotic leakage have been reported,3[0,37,38,46,49,51] which is a little unsettling, considering that patients with permanent ostomies have a significant reduction in quality of life.[47]

4.5 Risk factors for anastomotic leakage

Studies on risk factors for anastomotic leakage are common in colorectal research. Previously, intrinsic variables such as age > 60 years,[25,60] male gender,[61,62] low serum albumin levels[17,31] and transverse colectomy63 have been associated with an increased risk of anastomotic leakage, whereas assessment of comorbidity using the American Society of Anaesthesiologists (ASA) score has failed to demonstrate a clear association with anastomotic leakage64. Potentially adjustable extrinsic risk factors include prolonged duration of surgery,[18,31,65] intraoperative blood loss[31] and blood transfusion.[17,25] However, these factors could indeed reflect difficult or poorly conducted surgery. Lifestyle factors such as smoking and alcohol abuse seem to increase the risk of anastomotic leakage.[66] Both are potentially adjustable, however, smoking cessation for 4 weeks prior surgery did not reduce the rate of anastomotic leakage.[67] Postponement of a resection for cancer beyond this timeframe may not be ethically sound due to the potential risk of stage migration.

Presently, there is no valid explanation to how these risk factors may cause anastomotic leakage. The reason for the increased risk of anastomotic leakage in male patients was recently investigated in an experimental study on anastomotic collagen and breaking strength. The findings suggested an inferior collagen metabolism in male rats compared with female rats.[68] In another explorative clinical study, the gene expression of collagen type I and III were lower in rectal tissue compared with colonic tissue, which was hypo-

thesized as a potential cause of the higher leakage rate after low anterior resection as compared with colonic resection .[69]

4.6 Anastomotic healing

An understanding of the complex processes of anastomotic wound healing is imperative to better apprehend the dynamics of anastomotic leakage. Studying anastomotic healing may also lead to identification of potential target for enhancement of anastomotic integrity.

Healing of an intestinal anastomosis follows the basic principal of wound healing. Four overlapping phases; hemostasis, inflammation, proliferation and remodeling characterize the process of uncomplicated healing.

Briefly, intestinal transection activates platelet aggregation and the intrinsic coagulation cascade, forming a fibrin clot at the cut intestinal ends. Subsequently, neutrophils and later macrophages and lymphocytes migrate into the intestinal wound, especially around the suture channels of the anastomosis,[70] removing debris and phagocytizing pathogenic organisms. This inflammatory phase lasts from hours to days. Following the inflammatory response, fibroblasts and endothelial cells migrate into the wound and proliferate to form a granulation tissue. New type III collagen is synthesized, and reorganization of the extracellular matrix commences. After about a week, remodeling of the extracellular matrix, deposition of type I and III collagen and finally re-epithelization will ultimately strengthen the anastomosis. This last phase may take months.

Early on, *William Stewart Halsted* (1852 – 1922) payed attention to the differences between the layers of the intestinal wall. Through his remarkable experimental studies at Johns Hopkins University in Baltimore, he discovered that the submucosal coat was important to ensure anastomotic strength.[71] It is within this layer the strength-bearing collagen fibers are located and accordingly he argued, that the submucosa should be included in the suture line when constructing an intestinal anastomosis.[71]

Following anastomotic construction, the concentration of collagen and the anastomotic breaking strength decrease to a minimum around postoperative day 3. During this part of the inflammatory phase, collagen degradation predominates over collagen synthesis.[72-74] Neutrophils and macrophages express matrix metalloproteinases (MMP), which are a family of zinc-dependent endopeptidases with the ability to collectively degrade collagen, elastin and other extracellular proteins. First described in 1962 by *Jerome Gross* and *Charles Lapiere*,[75] MMPs are now acknowledged as an important factor of wound healing, but because of the *dualistic nature* of MMPs they can also be detrimental to anastomotic healing.[76] MMPs may play a significant role in the decrease of anastomotic strength during this critical phase of healing.[30,70,77-79]

In human studies, excessive levels of MMP-2 and MMP-9 has been measured in the normal bowel wall of patients that later on developed anastomotic leakage,[80] and MMP-8 and MMP-9 have been identified in the peritoneal fluid from patients with anastomotic leakage suggesting an association between excessive levels of MMPs and anastomotic leakage.[81]

4.7 Compromised anastomotic healing

Anastomotic healing may become compromised in patients with an unbalance in the intestinal wall such as colonic obstruction. About 10 to 15 per cent of patients with colorectal cancer present with an obstructive mass.[82] In rodent studies, dilatation of the colon has been associated with loss of collagen in the intestinal wall.[83-86] The healing properties of the obstructed intestine may per se be compromised. Several studies have demonstrated that colonic obstruction serves as an intrinsic risk factor for the development of anastomotic leakage.[17,87]

4.8 Improvement of anastomotic healing

Inhibition of MMP activity is a tempting concept for preservation of collagen in the intestinal wall and thus improvement of anastomotic healing.

In a recent systematic review, MMP inhibition was the most compelling concept for pharmacological enhancement of anastomotic strength.[88] Administration of seven different MMP-inhibitors in six studies demonstrated significant increases in anastomotic breaking strength or bursting pressure during the inflammatory phase on post-anastomotic day three or four.[72,89-93] In another review on pharmacological enhancement of anastomotic strength during complicated conditions such as colonic obstruction or perforation, *Nerstrøm et al.* confirmed that MMP inhibition may currently be the most interesting concept for optimizing anastomotic healing and thus prevention of leakage.[94]

In all of the experimental studies, the MMP-inhibitors had a non-selective inhibitory profile. In one study, the non-selective MMP-inhibitor GM6001 surprisingly increased the risk intraabdominal abscess formation in relation with anastomotic leakage following colonic obstruction in rats.[95] It was speculated, that inhibition of epithelialization may have caused this finding. The clinical use of non-selective MMP-inhibitors may also be limited due to adverse effects such as significant bone destruction and Dupuytren like musculoskeletal disease.[96-98] Finally, only one clinical study has investigated the impact of a broad proteinase inhibitor (aprotinin) on anastomotic leakage and the results were discouraging.[99]

Anastomotic breaking strength or bursting pressure were used to assess the efficacy of the tested pharmacological compounds in all experimental studies. These biomechanical measures are surrogates for assessment of anastomotic healing in experimental animal studies.[70,72,100-103] Currently, there is no causative association between MMP inhibition, collagen levels or metabolism and increased anastomotic strength. Total anastomotic collagen seems unaffected by MMP-inhibitory treatment,[72] but *Kiyama et al.* reported, that non-selective MMP inhibition increased soluble but not insoluble collagen in experimental anastomoses.[90] Soluble collagen represents the newly formed collagen, whereas insoluble collagen is mature and cross-linked.

In conclusion, anastomotic leakage is a major problem after resection for colonic cancer. The literature has primarily been based on small studies with insufficient statistical power, single centre designs with inherent risks of selection bias and confounding, case mix with rectal cancer patients and collectively poor external validity. There is a knowledge gap in the understanding of the incidence, management, outcome, risk factors and anastomotic healing and there is an unfulfilled potential for enhancement of anastomotic strength. The present doctoral thesis was therefore undertaken to elucidate elements hereof.

5 OBJECTIVES

The main objectives of this thesis were to investigate the

1. incidence of anastomotic leakage in a nationwide, unselected cohort of patients with colonic cancer (*Study I*),

2. impact of anastomotic leakage and management on short-term mortality *(Studies I, II, III*),

3. impact of anastomotic leakage management on long-term mortality and rate of permanent ostomies (*Study III*),

4. influence of anastomotic leakage on local and distant cancer recurrence and long-term survival (*Study* IV),

5. risk factors associated with anastomotic leakage (*Study I, II*),

6. expression and inhibition of matrix metalloproteinases on early experimental anastomotic wound healing (*Study V*),

7. time-dependant biochemical and morphological changes in the colon following acute experimental colonic obstruction (*Study VI*),

8. effect of a selective matrix metalloproteinase inhibitor on anastomotic leakage and strength of experimentally obstructed colon (*Study VII*)

6 METHODS

A brief introduction to methodology and limitations for the studies is presented below.

6.1 Epidemiological studies

The studies were designed as retrospective observational cohort studies using nationwide and unselected patient data. The unique 10-digit personal identification number given to all Danish citizens was used to collect and merge data from the DCCG database, the National Patient Registry (NPR) and the Danish Pathology Registry. In order to ensure proper data quality, the cohort was cross referenced against the NPR with respect to surgical procedure and date of surgery.

Registration in the DCCG database is mandatory in Denmark and the overall coverage is thus more than 95% of all Danish patients with colorectal cancer,[104] which may minimize the risk of selection bias in the retrospective design.

6.1.1 The cohort

All Danish patients, 18 years or older, with a first-time diagnosis of colonic adenocarcinoma, subjected to a curatively intended segmental colonic resection with primary anastomosis and without a protecting stoma, between the 1st of May 2001 and 31st of December 2008, were included. Patients, who presented with distant metastasis at the time of diagnosis or had residual disease as stated in the pathology report, were excluded. These inclusion and exclusion criteria ensured a homogenous cohort, although the external validity may be limited.

Data for the index cohort were extracted on the 16th of December 2009. The Charlson comorbidity index (CCI) scores was included on the 25th of November 2010. The medical file review was finished on the 29th of November 2010.

6.1.2 Short-term outcomes

Data on length of hospital stay and 30-day mortality were extracted from the NPR. Length of stay was defined as the duration of the primary admission. This could underestimate the "true" impact of anastomotic leakage on the entire length of hospital stay, if patients were transferred between departments and/or hospitals. Likewise, the 30-day mortality rate used here may underestimate the "true" short-term mortality.[105]

6.1.3 Long-term outcomes

Disease recurrence was categorised as local or distant. In case of both local and distant recurrence, recurrence status was categorised as distant. This may limit the impact of anastomotic leakage on local recurrence but mitigate the risk of over-interpretation of the influence of local recurrence. Recurrence was confirmed by data from the NPR on histology, radiology or surgery.

Long-term mortality was calculated for patients surviving at least 120 days from the index operation. This was chosen in order to estimate the "true" long-term effect of anastomotic leakage on mortality. However, this could introduce an immortal time bias, where patients in the cohort cannot die within the excluded period.

Administration of adjuvant chemotherapy was examined in patients with Union for International Cancer Control (UICC) III disease, thus excluding UICC II patients with high risk features from the analyses. Time to adjuvant chemotherapy was defined as the time gap between the index operation and the first administration. Information on treatment completion or number of treatment series was not available. This could potentially confound the effect of adjuvant chemotherapy.

The rate of permanent ostomies was calculated as the number of patients subjected to an ostomy minus the number of patients undergoing reversal. The latter was based on operations codes for ostomy reversal extracted from the NPR.

6.1.4 Dependant variable, anastomotic leakage

The dependant variable *anastomotic leakage* was defined as *clinical symptoms suggesting anastomotic leakage and confirmed by contrast enema, CT scan or re-operation*. These data were primarily extracted from the DCCG database. The retrospective nature of the data, regardless of the prospectively collection in the DCCG database, makes it impossible to verify the anastomotic leakage further and misclassification cannot be ruled out. To minimize the risk of underestimation of anastomotic leakage, a supplemental survey was performed in the NPR using the codes for diagnosis and reoperation associated with anastomotic leakage (DT813A, KJWF00). The incidence of anastomotic leakage was thus defined as the cumulative rate of anastomotic leakage.

For the benefit of *Study III*, records describing the treatment of patients with anastomotic leakage were later on collected and graded according to the classification of *Rahbari et al*.[106]:

A: Anastomotic leakage requiring no active therapeutic intervention

B: Anastomotic leakage requiring active therapeutic intervention but manageable without re-laparotomy

C: Anastomotic leakage requiring re-laparotomy

Patients with grade C anastomotic leakage were then grouped as I) *takedown of the anastomosis*, defined as interruption of the bowel continuity with resection or transection of the anastomosis in combination with formation of an end-ileostomy, end-colostomy or both, or as II) *salvage of the anastomosis* with preservation of the bowel continuity by anastomotic repair or redo-anastomosis with or without a protective proximal loop-ostomy.

6.1.5 Independent variables

Independent variables included patient demography, cancer stage and surgical variables. The selection of variables was limited by the nature of data in the DCCG database and the integration with the NPR and the Danish Pathology registry. Data on smoking and alcohol consumption were initially assessed but the completion rate of these parameters in the DCCG database was less than 50%, and the variables were thus excluded.

Comorbidity was defined according to the ASA score[107] and the CCI score.[108,109] The CCI was retrieved after completion of the original dataset and thus not included in study I. Because the CCI score was right-skewed, the variable was categorised as normal (0), moderate (1), severe (2), or very severe (≥ 3)[110] (*Study II*), or dichotomized (*Studies II and IV*). The CCI depends on the registration of individual disease conditions in the NPR. The positive predictive value of CCI in the NPR is very high,[111] whereas the negative predictive value is unknown. This may impose a risk of underestimation of the burden of comorbidity.

Cancer stage was defined according to the UICC. Surgical procedures were defined as right hemicolectomy, transverse colectomy, left hemicolectomy or sigmoid colectomy. Surgical approach was classified as open or completed laparoscopically. An emergency procedure was defined as surgery within 24 hours after hospital admittance. Blood transfusion applied to the whole intra-, per and postoperative period, while blood loss was defined as intraoperative. The association between blood transfusion and anastomotic leakage may thus not describe the "true" association between the two.

A post-hoc collection of additional data associated with the findings at re-operation and management of anastomotic leakage was carried out. These data included the size of the anastomotic defect and the degree of peritonitis using the Hinchey classification.112 These variables are at risk of information bias.

6.1.6 Statistics

Duration of follow-up was calculated from the day of the index operation till end of follow-up or death using the reverse Kaplan-Meier approach.[113] Missing values were considered missing at random and were thus not replaced.

Univariable comparisons were performed by chi-square test, Mann-Whitney test or logistic regression analysis for non-time dependant outcomes and by the log-rank test for time dependant outcomes. Multivariable analysis of time independent outcomes was achieved by adjusted logistic regression analysis. Cox proportional hazard analysis was used for time dependant analyses. A limitation of the disease recurrence analysis is the competing risk of mortality in patients dying before the potential event of recurrence can occur.

In some regression analyses a goodness of fit estimate was included based on the Hosmer-Lemeshow test.[114] Different prediction models for anastomotic leakage were built and evaluated using receiver operating characteristics (ROC). The area under the curve was used to evaluate the level of prediction.[115]

Data were presented as number and percentages, median and interquartile range (IQR) or range, adjusted odds ratios (OR) or adjusted hazard ratios (HR) with 95% confidence intervals (CI). All analyses were two-sided, and a p-value below 0.05 was considered statistically significant.

6.2 Experimental studies

Different experimental designs were employed to investigate aspects of anastomotic healing and leakage in the non-obstructed- and the obstructed colon.

In *Study V* (non-obstructed colon) and *Study VII* (obstructed colon) the animals were randomized to selective MMP inhibition or vehicle by subcutaneous injections daily for three consecutive days starting two hours prior construction of the anastomoses. On postoperative day three, the anastomotic strength was tested, and histopathology and biochemical properties of the colonic wall assessed (Figure 1).

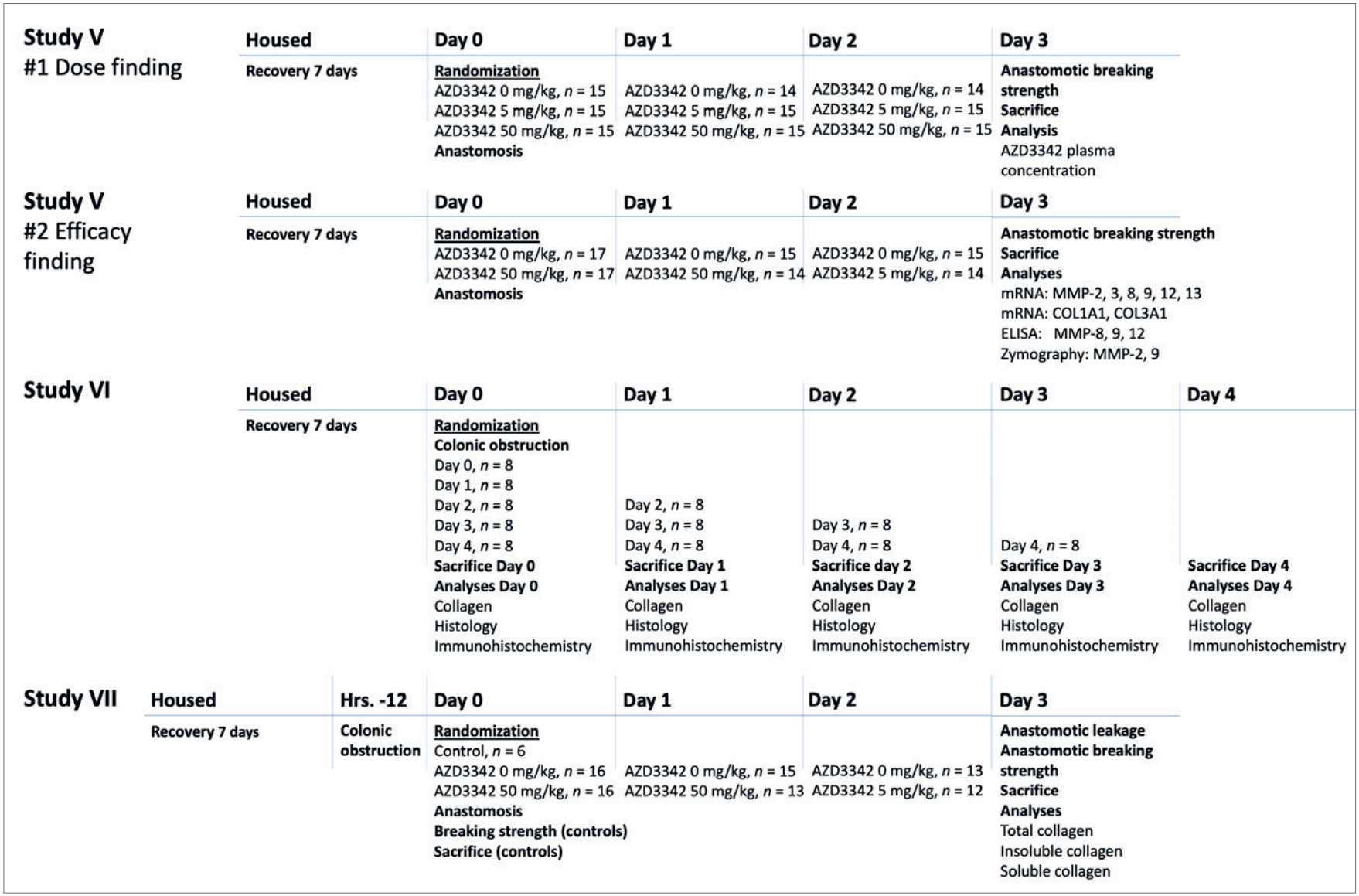
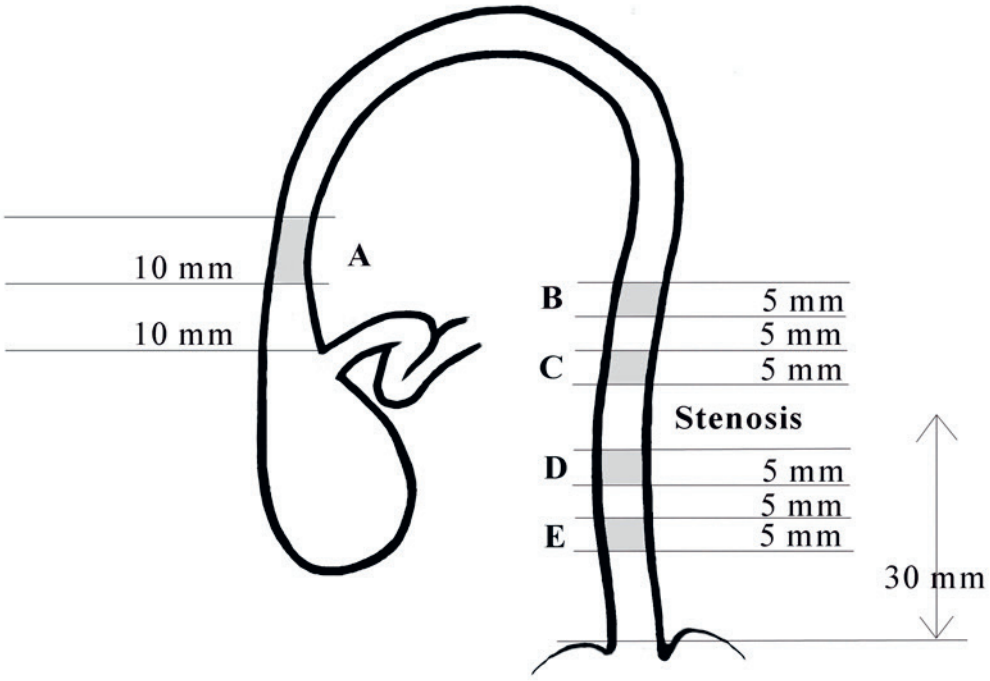


Figure 1 Overview of the experimental designs for studies V, VI and VII

In *Study VI*, time dependent morphological and biochemical changes in in the colonic wall were investigated in different locations along the colon, after 24, 48, 72 and 96 hours of obstruction, (Figure 2).

Figure 2 Schematic presentation of the level of colonic obstruction and sample sites used in Study VI and VII



In *Study VII,* 12 hours of colonic obstruction was chosen as exposure. A series of pre-study experiments developing the novel laparoscopic approach of colonic obstruction, demonstrated that 12 hours of obstruction produced the same colonic dilation as 24 hours, but with a less severe impact on the postoperative well-being of the animals.

6.2.1 Animals

All experimental studies were performed on inbred Sprague-Dawley albino rats, acclimatized for 7 days prior to surgery with free access to water and food pellets.

Rats has the advantages, that they only requires limited space, are inexpensive and have an intestinal anatomy similar to humans except for a slightly different cecum.[116] It has been proposed that a mouse model would be more suitable for studying anastomotic leakage, because mice are less resilient to infections compared with rats, which may enable an experimental model with close resemblance to the event of "real" clinical anastomotic leakage.[117] However, in order to produce anastomotic leakage in a mouse model, insufficient anastomoses with fewer sutures were constructed.[117] This does not

necessarily resemble the clinical situation of anastomotic leakage, which in the present context is regarded as a complication of healing rather that the result of insufficient surgery.

6.2.2 Anastomoses

Two different clinical scenarios were addressed: the elective non-obstructive and the emergency obstructive setting. In both scenarios, the anastomoses were constructed 60 mm from the anal verge, corresponding to approximately 30 mm proximal to the peritoneal reflection. Following a 10 mm resection of the colon, the bowel ends were combined in an end-to-end fashion using 8 (non-obstructive) or 9 (obstructive) interrupted, inverted 6/0 polyamid sutures. Each suture was meticulously placed 3 to 5 mm from the cut edge of the colon to ensure the best possible anastomosis.

Three days after construction, the anastomoses were tested.

6.2.3 Anastomotic breaking strength

Anastomotic breaking strength is a quantitative mechanical method to assess the longitudinal tensile strength of the anastomosis. The method may be sensitive to the surgical technique of the anastomosis,[118] and especially the distance from the cut end of the intestine to where the sutures are placed seems critical.[119] Anastomotic breaking strength is thus an estimate of the suture holding capacity of the anastomoses,[70] which may be reflected by the different phases of anastomotic healing (See section 2.4). Postoperative day three after construction of the anastomosis was regarded as the most critical time point of healing[72] and thus chosen as the day of interest (*Studies V and VII*).

A re-laparotomy was performed on day 3, and the anastomoses were macroscopically evaluated. Anastomotic leakage was defined as a visible defect in the anastomotic suture line. The anastomoses were resected with a 20-mm margin on each side of the suture line, freed of adhesions and placed in saline, after which the animals were sacrificed. The colonic segments with the anastomosis in the middle were fastened by clamps positioned 10 mm apart in a material testing device and pulled apart at 10 mm/min until the ultimate strength of the anastomosis was determined from the load-deformation curve.

6.2.4 Selective MMP-8, MMP-9 and MMP-12 inhibitor (AZD3342)

The encouraging results of the two reviews on pharmacological enhancement of anastomotic strength[88,94] led us to focus on selective MMP inhibition.

In *Studies V* and *VII* the impact of selective MMP inhibition on anastomotic breaking strength was investigated. AZD3342 (AstraZeneca Research and Development, Mölndal, Sweden) is a 403 D, synthetic non-hydroxamate MMP-8, MMP-9, and MMP-12 inhibitor. Data supplied by AstraZeneca demonstrated a half-maximal inhibitory concentration at 16 nM with respect to MMP-8, 10 nM for MMP-9 and 6 nM for MMP-12. AZD3342 offers more than a 3-fold selectivity for MMP-8, MMP-9, and MMP-12 compared with MMP-1 and tumour necrosis factor-α converting enzyme.

The dosage used in both studies was chosen based on the results of a dose-finding study (data included in *Study V*).

6.2.5 Colonic obstruction

Establishing colonic obstruction in experimental rat models has traditionally been undertaken through a midline incision, placing a hollow silicone tube around the left colon.[84,85] This approach was adopted to study the time-dependant biochemical and morphological changes in the colonic wall between 24 and 96 hours following onset of obstruction (Study VI). However, as demonstrated by *Syk et al.*, the midline incision itself inflicts a 1.7-fold increase in MMP activity.[84] In addition, blood levels of TNF-α are also higher in rats subjected to laparotomy compared with laparoscopy,120 while pneumoperitoneum does not seem to influence anastomotic strength.[121] In order to minimize the surgical stress of the procedure required to establish the experimental colonic obstruction, a novel laparoscopic approach was developed for *Study VII*.

Briefly, pneumoperitoneum was established using a 21-gauge needle and the abdominal cavity was inflated with CO2 to a maximal pressure of 4 mmHg. A sheet and trocar were introduced through a 1-mm incision in the midline and a 2.7 mm, 30 degree, videoscope was inserted through the sheet. A 3-mm skin incision was made in the right lower quadrant and the laparoscopic instruments were inserted directly into the abdominal cavity. The incision was tightened with a purse string suture to avoid spillage of CO2. A titanium clip was applied around the colon approximately 30 mm proximal from the peritoneal reflection, between two marginal veins (Figure 3). In 10 sham animals, the mesentery was only pierced between two marginal veins. The abdominal cavity was deflated, and the incisions were closed with interrupted sutures in the abdominal wall and titanium clips in the skin.

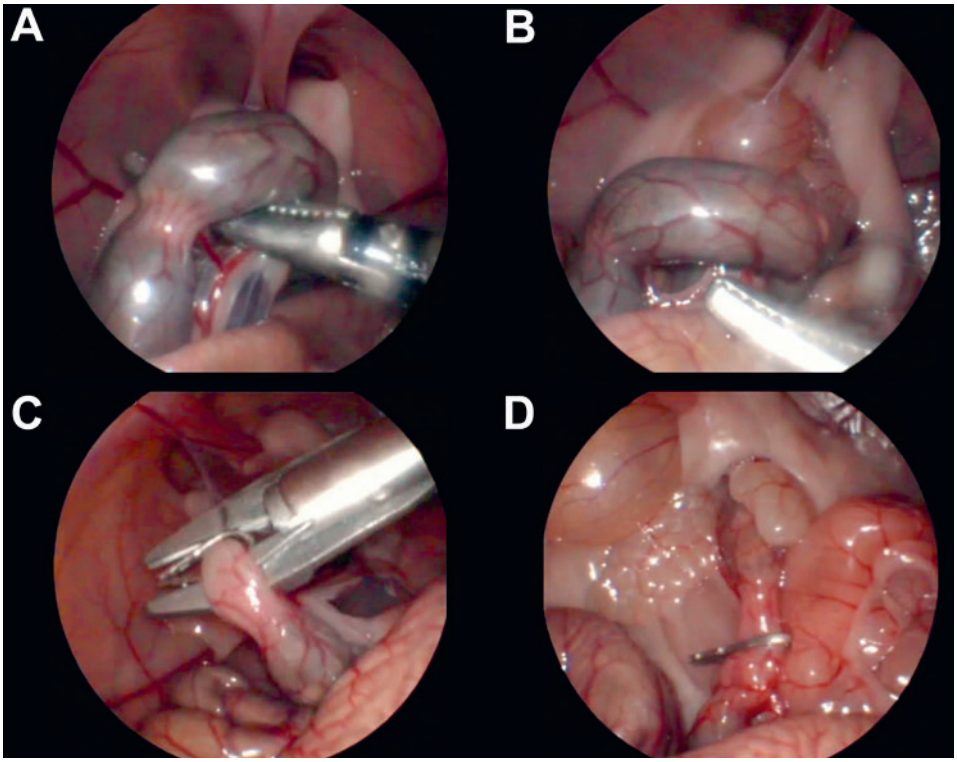
6.2.6 Molecular, immunohistochemical, biochemical and histopathological analyses

Various analytic approaches were undertaken to characterize aspects of anastomotic healing. A special focus was on the role of MMP and MMP-inhibition and the relationship between collagen and anastomotic breaking strength.

The mRNA expressions of MMP-2, MMP-3, MMP-8, MMP-9, MMP-12 and MMP-13 were investigated by quantitative real time PCR in uninjured colon and in the anastomotic suture line three days after construction of the anastomoses. Changes in anastomotic mRNA levels were verified by protein analyses. The gelatinases MMP-2 and MMP-9 were semi-quantified by zymography. Protein levels of MMP-8, MMP-9 and MMP-12, corresponding to the inhibitory profile of AZD3342, were assessed by commercial enzyme-linked immunosorbent assays kits. Anastomotic macrophage counts and MMP-12 expressions were further examined by CD68 and MMP-12 immunohistochemistry (*Study V*). Because the rat lacks expression of MMP-1, MMP-8 and MMP-13 are the dominating collagenases. The protein expression of MMP-13 was not assessed in this thesis.

In the obstructed colon, temporal structural changes were evaluated histologically by haematoxylin–eosin staining and concurrent expression of neutrophils and CD68 posi-

Figure 3 Laparoscopic induction of colonic obstruction in rats (Study VII). After identification of the correct location, the mesentery was pierced between two marginal vessels (A, B), creating room for application of a titanium clip (C, D). The clip was left for 12 hours.



tive macrophages by immunohistochemical double labeling (*Study V*). Collagen levels in the intestinal wall (*Study VI*) and in the anastomoses (*Study VII*) were assessed by concentration of hydroxyproline. The impact of AZD3342 on anastomotic collagen in the obstructed colon was further characterized after pepsin fractionation as insoluble and total soluble collagen by the commercial SirCol collagen assay.

6.2.7 Statistics

Mortality rates were compared using Fisher's exact test, anastomotic breaking strength was analyzed by the unpaired t-test and presented as mean ± SD, while plasma levels of AZD3342 were assessed by the Mann-Whitney test and presented as medians and IQR.

mRNA and protein data were log-transformed to obtain normal distribution. Results of MMP and collagen mRNA were normalized to ribosomal protein lateral stalk subunit P0 (RPLP0), log-transformed and presented as fold change relative to proximal uninjured colon as geometric means ± back-transformed SEM. $P < 0.05$ was considered statistically significant.

7 RESULTS

7.1 Short-term outcomes

A total of 18484 patients were identified in the DCCG database. After exclusion of 9151 patients, 9333 patients were eligible for inclusion. The median age was 72 years (range 23 – 99 years) and 4844 (51.9 per cent) were females. In table 1 an overview of the results of studies I, II, III and IV is presented.

During the study period, the ongoing centralization of colorectal cancer surgery resulted in a reduction of hospitals conducting colon cancer surgery from 48 to 28. Accordingly, the mean annual hospital case load increased from 23 in 2001 to 43 in 2008, ranging from 4 to 98 cases in 2008. Concomitantly, laparoscopic colon cancer surgery was implemented in 25 hospitals during the study period and the rate of laparoscopy increased from 2 per cent in 2001 to 41 per cent in 2008.

Table 1 Overview of the epidemiological studies

Study	Cohort	No. of patients	Outcomes	Main findings
I	Entire Cohort	9333	Incidence of AL	6.4 per cent
			Risk factors for AL	Laparoscopy, left/sigmoid colectomies, blood transfusion, blood loss, male gender
II	Elective Surgery	8597	Prediction of AL	Charlson comorbidity index -OR 1.07 (0.99-1.15) -ROC curve AUC 0.548
			30-day mortality	AL: 20.4 per cent vs. No AL 3.9 per cent
			Length of stay (days)	AL: 23.3 (21.5-25.1) vs. No AL 8.7 (8.4-9.2)
III	Patients With Grade C AI	509	Management of AL	Salvage: 14.6 per cent, takedown 85.4 per cent
			30-day mortality	Salvage vs. takedown: OR 0.65 (0.27-1.53)
			Long-term mortality	Salvage vs. takedown: HR 1.44 (0.91-2.28)
			Permanent ostomy	Salvage vs. takedown: HR 0.41 (0.21-0.68)
VII	Patients Surviving 120 Days	8589	Distant recurrence	AL vs. no AL: HR 0.78 (0.55-1.12)
			Local recurrence	AL vs. no AL: HR 1.42 (1.13-1.78)
			Long-term mortality	AL vs. no AL: HR 1.20 (1.01-1.44)
			Adjuvant chemotherapy	AL vs. no AL: HR 0.58 (0.45-0.74)

OR/HR < 1 indicates decreased likelihood of mortality/ostomy diversion/recurrence/chemotherapy, whereas HR > 1 indicates increased likelihood. All analyses were adjusted for confounding. AL: anastomotic leakage, ROC: receiver operating characteristics, AUC: area under the curve. Length of stay estimated in days.

7.1.1 Incidence of anastomotic leakage

The overall rate of anastomotic leakage was 593 of 9333 (6.4 per cent) with the highest incidence of 10.2 per cent after left hemicolectomy and the lowest incidence of 5.5 per cent after right hemicolectomy. The incidence after transverse- and sigmoid colectomies were 5.6 per cent and 6.5 per cent, respectively.

There was a significant difference in the reported rate of anastomotic leakage between the DCCG database and the NPR. The DCCG database included 544/593 (92 per cent) of the total number of patients with anastomotic leakage, thus 49 patients (8 per cent) were not registered with a diagnosis of anastomotic leakage in the DCCG database (Table 2).

Centralization had no impact on the rate of anastomotic leakage over time, and there was no relationship between hospital case volume and the rate of anastomotic leakage (*Study I*).

Table 2 Incidence of anastomotic leakage

Anastomotic leakage		NPR		
		Yes	No	Total
DCCG	Yes	333	211	544
	No	49	0	49
	Total	382	211	593

DCCG: Danish Colorectal Cancer Group

NPR: National Patient Registry

7.1.2 Length of hospital stay

Four of the 9333 patients were lost to follow up. The overall median length of hospital stay in electively operated patients was 8 days (IQR 5 – 11 days), divided into median 21 days (IQR 9-32 days) for patients with anastomotic leakage as compared with 7 days (IQR 5-10 days) in patients without anastomotic leakage, $P < 0.001$ (*Study II*). In the multiple linear regression model, anastomotic leakage was associated with increased length of stay by an average of 12.9 days, CI 12.1-13.7, $P < 0001$ in patients with limited comorbidity (CCI <2). Mean length of stay increased by additional 2 days in comorbid patients (CCI ≥ 2) developing anastomotic leakage (*Study II*).

7.1.3 Early mortality

A total of 524/9329 (5.6 per cent) patients died within 30 days from the operation. The 30-day mortality was 124/593 (20.9 per cent) in patients with anastomotic leakage compared with 400/8736 (4.6 per cent) in patients without anastomotic leakage, $P < 0.001$, corresponding to a univariable HR = 4.83, 95% CI 3.95-5.90, P < 0.001. After adjustment for confounding factors, anastomotic leakage remained strongly associated with 30-day mortality, HR = 3.90, 95% CI 3.10-4.99, $P < 0.001$. Among the 593 patients with anastomotic leakage, 30-day mortality was 24.1 per cent after right hemicolectomy, 28.6 per cent after transverse colectomy, 19.8 per cent following left hemicolectomy and 17.2 per cent after sigmoid colectomy (*Study I*). During the study period, 30-day mortality in patients with or without anastomotic leakage decreased significantly by 2.31 and 0.26 per cent per year, respectively (Figure 4).

In 8597 electively operated patients, 30-day mortality was 4.9 per cent and the impact of anastomotic leakage on mortality was influenced by the level of comorbidity (Table 3). Severe comorbidity (CCI ≥ 2) further increased the risk of a fatal outcome after anastomotic leakage by HR = 1.58, 95% CI 1.00-2.51, P = 0.033 (*Study II*).

Figure 4 Development in 30-day mortality in patients with (grey) and without (white) anastomotic leakage. The decrease in mortality in patients with anastomotic leakage was 2% annually during the study period as compared with 0.2% for patients without anastomotic leakage.

The linear regression for patients with a leak; slope = -2.31, $R^2 = 0.59$, $P = 0.025$

The linear regression for patients without a leak; slope = -0.26, $R^2 = 0.60$, $P = 0.024$

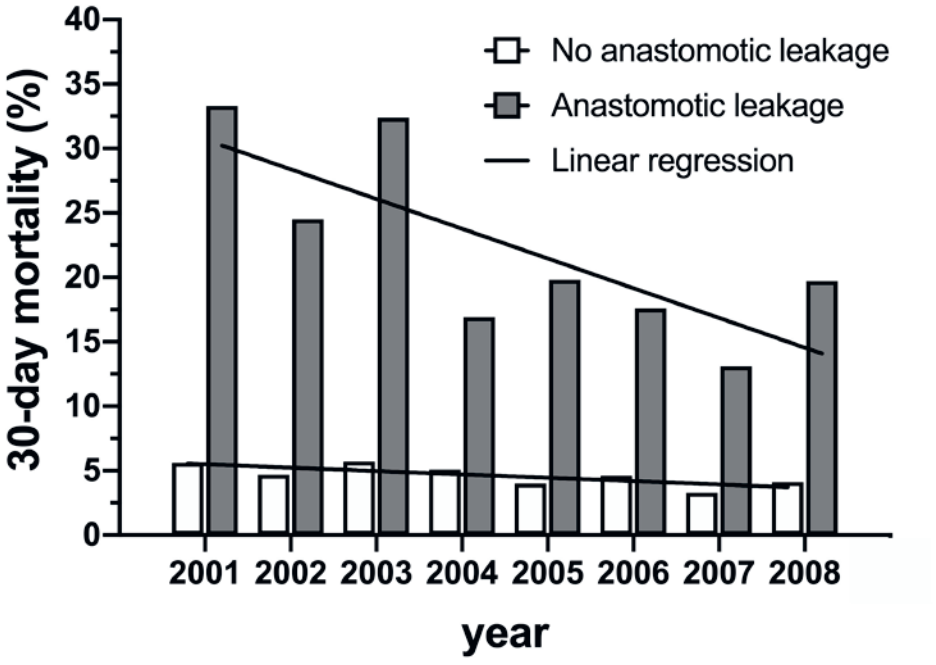


Table 3 30-day mortality following elective resection for colonic cancer

	HR (95% CI)	*P*
No AL and CCI < 2 (reference)	1.00	
No AL and CCI ≥ 2	2.75 (2.14-3.54)	<0.001
AL and CCI < 2	6.24 (4.56-8.53)	<0.001
AL and CCI ≥ 2	7.48 (4.99-11.19)	<0.001

Analysis adjusted for gender, age, tumour stage, surgical procedure and approach, specialization, adjacent organ resection, intraoperative blood loss and transfusion. AL: anastomotic leakage; CCI: Charlson comorbidity index. CCI < 2 indicates no or moderate comorbidity; CCI ≥ 2 indicates severe comorbidity.

7.1.4 Management of anastomotic leakage

Anastomotic leakage was retrospectively graded after the *Rahbari* classification[106] into grade A (n = 33), grade B (n = 33) and grade C (n = 507), while 21 patients were ungradable or died before treatment for anastomotic leakage was initiated. In graded patients, a significant association between grade of anastomotic leakage and mortality was demonstrated (Table 4).[122]

Table 4 Association between grade of anastomotic leakage and 30-day mortality

	30-day mortality
Grade of anastomotic leak	n / N (Per cent)
A	1 / 32 (3.1)
B	4 / 33 (12.1)
C	113 / 507 (22.3)
Total	*118 / 572 (20.6)*

Cochran-Armitage test for trend, P = 0.009

Among the 507 patients who were re-operated for anastomotic leakage, 433 (85.4 per cent) had a takedown procedure with construction of an end-ileostomy or end-colostomy. The remaining 74 patients underwent a salvage procedure with preservation of the intestinal continuity, with- or without a proximal loop-diversion. Factors associated with salvage were a) younger age, b) a low degree of peritonitis corresponding to Hinchey I or II or c) defects in the anastomoses less than one-fourth of the circumference. A total of 185 patients had this favourable presentation of the leak (categories b and c), but only 49 (26 per cent) of these underwent a salvage procedure.

There was no difference in adjusted 30-day mortality between takedown or salvage procedures, OR = 0.65, 95% CI 0.27-1.53, *P* = 0.323. Once again, a CCI score ≥ 2 was associated with increased mortality in patients with grade C anastomotic leakage, OR = 2.13, CI 1.20-3.76, *P* = 0.010.

In the group of patients undergoing a salvage procedure combined with a proximal loop-ostomy, seven of 54 (13 per cent) died within 30 days as compared with six of 20 (30 per cent) without the protection provided by a proximal ostomy, *P* = 0.087. There was no increase in the rate of anastomotic salvage over time (*Study III*).

7.2 Long-term outcomes

The long-term results of anastomotic salvage compared with takedown was further investigated.

7.2.1 Permanent faecal diversion

A total of 487 of 507 patients (96 per cent) with a grade C leak underwent formation of an ostomy (end-ileostomy, loop-ostomy or end-colostomy). After median 5.0 years (IQR 4.7 – 5.2 years) follow-up, the crude overall rate of a permanent ostomy was 54.5 per cent (*Study III*). The adjusted risk of a permanent ostomy was significantly reduced in the anastomotic salvage group compared with the takedown group with an end-ileostomy, HR = 0.47, 95% CI 0.29 - 0.77, *P* = 0.003. Patients, in the takedown group, with an end-colostomy were least likely to undergo stoma-reversal compared with patients with a loop- or an end-ileostomy.

The overall risk of re-anastomotic leakage following stoma reversal in patients with a primary leak was 14/164 (8.5 per cent) with significant differences between stoma types (Table 5) and thus higher than the incidence at the primary resection.

Table 5 Risk of re-anastomotic leakage following stoma reversal

	Re-anastomotic leakage
Stoma type	n / N (Per cent)
End-ileostomy	10 / 64 (15.6)
Loop-ostomy	3 / 36 (8.3)
End-colostomy	1 / 64 (4.9)
Overall	14 / 164 (8.5)

Data from the National Patient Registry and patient files

7.2.2 Recurrence, adjuvant chemotherapy and mortality

Another benchmark of colonic cancer surgery is the rates of recurrence and survival. After exclusion of patients dying within 120 days from the index operation, 8589 patients were eligible for analysis of the relationship between anastomotic leakage, recurrence and long-term overall mortality.

After median 5.3 years, recurrence developed in 2142 (24.9 per cent) patients of whom 861 (10.0 per cent) had only local recurrence and 1281 (14.9 per cent) distant recurrence. Anastomotic leakage was significantly associated with occurrence of distant recurrence, HR=1.42, 95% CI 1.13-1.78, $P = 0.003$, while there was no association between anastomotic leakage and local recurrence.

In a post-hoc subgroup analysis of the association between anastomotic leakage and administration of adjuvant chemotherapy in 2841 patients with UICC stage III disease, the overall rate of administration of adjuvant chemotherapy was 60.7 per cent. Patients with anastomotic leakage were less likely to receive adjuvant chemotherapy compared with patients without anastomotic leakage, HR = 0.58, 95% CI 0.45-0.74, $P < 0.001$. Furthermore, the median time to first administration of adjuvant chemotherapy was significantly increased in patients with anastomotic leakage (59 days, IQR 48-82 days) compared with patients without leakage (43 days, IQR 35-54 days), $P < 0.001$. By multiple linear regression analysis, the average difference was 16 days, CI 12 – 20 days, $P < 0.001$.

Administration of adjuvant chemotherapy did not reduce the rate of distant recurrence in patients with anastomotic leakage. It did reduce the risk of dying, however, this was only true if adjuvant chemotherapy was administered within 55 days from the primary operation.

The impact of anastomotic leakage extended beyond distant recurrence and adjuvant chemotherapy, with an increase in long-term overall mortality, HR = 1.20, 95% CI 1.01-1.44, $P = 0.042$. However, after inclusion of recurrence status as a time-dependant variable in the Cox regression mortality analysis, the association between anastomotic leakage and mortality disappeared, HR = 1.10, 95% CI 0.92-1.32, $P = 0.289$.

7.3 Risk factors

The implementation of laparoscopic surgery during the study period led to a transient non-significant increase in anastomotic leakage. However, multivariable logistic regression analysis identified laparoscopy as a risk factor for anastomotic leakage (OR = 1.34, 95% CI 1.05-1.70, P = 0.003). Additional variables associated with anastomotic leakage were age, male gender, left- and sigmoid colectomies, intraoperative blood loss and transfusion, whereas comorbidity assessed by the ASA classification and emergency procedures did not reach statistical significance (*Study I*).

The surprising finding, that ASA score was not associated with anastomotic leakage, led to additional analyses (study II) assessing comorbidity by the Charlson comorbidity in-

dex in 8597 electively operated patients of the same cohort. In this analysis, a CCI score ≥ 2 was associated with increased risk of anastomotic leakage in the adjusted analysis, OR = 1.33, 95% CI 1.06-1.66, $P < 0.016$. The only disease condition of the CCI, that remained associated with anastomotic leakage after adjustment for confounding was renal disease, OR = 1.68, 95% CI 1.02-2.78, $P = 0.044$. The prediction of anastomotic leakage by ROC curve analysis using the variables associated with anastomotic leakage, was only fair (AUC = 0.745), and the addition of CCI did not improve the predictive power (AUC = 0.740).

The next move was to explore the rationale for enhancement of anastomotic strength by selective inhibition of MMP-8, MMP-9 and MMP-12.

7.4 Experimental outcomes

A total of 157 rats was used of which 15 died prematurely. Six from *Study V*, one from *Study VI* and eight from *Study VII*, leaving 142 rats available for studies on the expression and inhibition of matrix metalloproteinases in elective experimental anastomoses, temporal consequences of colonic obstruction and inhibition of matrix metalloproteinases in anastomoses in the obstructed colon.

7.4.1 Matrix metalloproteinases in experimental anastomoses

Three days after construction of the elective anastomoses, the gene expressions of MMP-8, MMP-9, MMP-12, MMP-13 and to a lesser degree MMP-2 increased significantly in the anastomotic line (Table 6). The largest increases were observed in MMP-8, MMP-9 and MMP-12. The upregulation of mRNAs was confirmed by quantitative protein analyses of MMP-8, MMP-9 and MMP-12, and semiquantitative gelatin zymography of MMP-2 and MMP-9 (Table 6). Interestingly, MMP-9 was only present in its latent form.

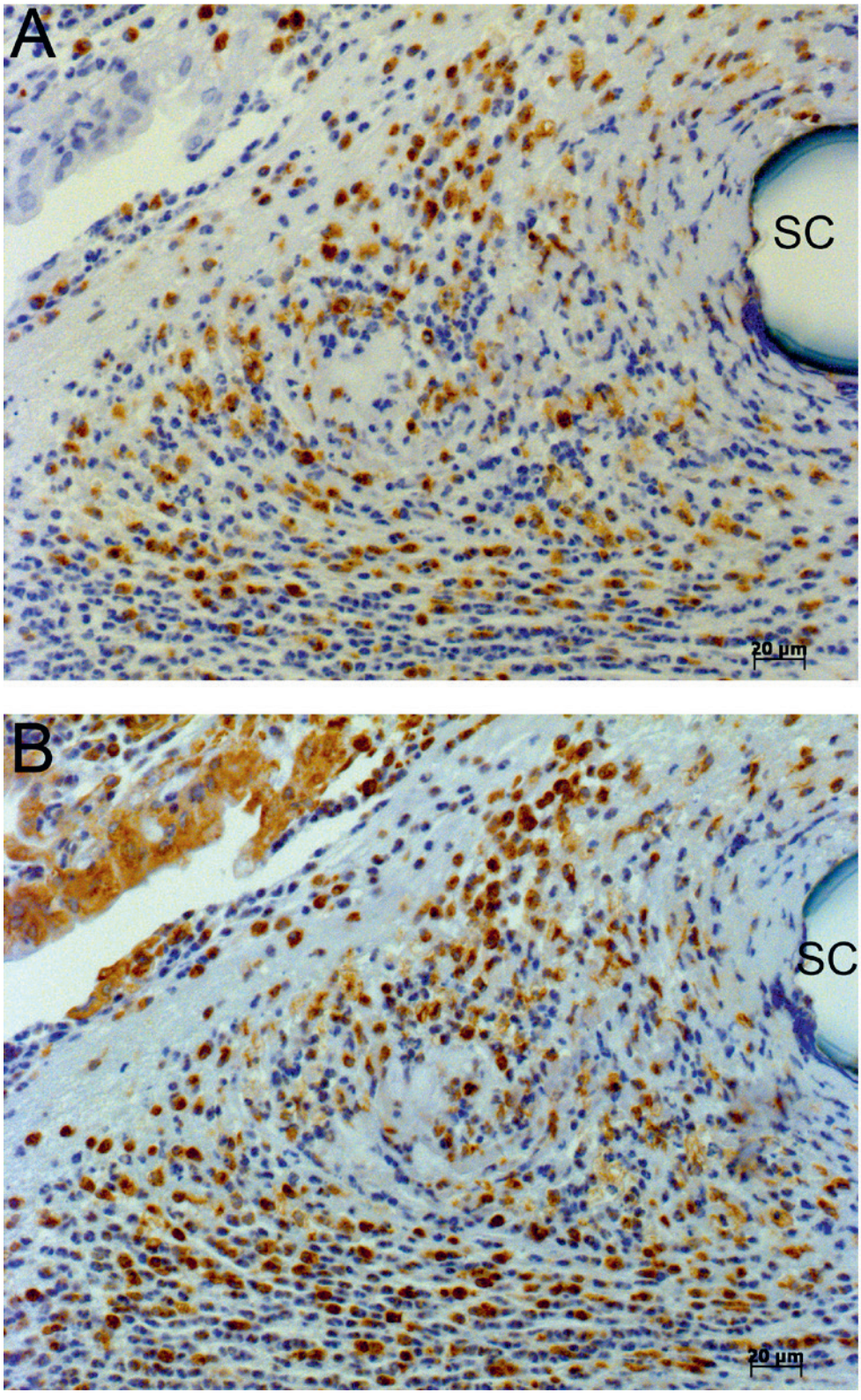
Corresponding to the influx of inflammatory cells around the suture channels, the immunohistochemical analysis demonstrated an upregulation of CD68- and MMP-12 positive macrophages in day three anastomoses (Figure 5).

Table 6 MMP mRNA and protein expressions in day-3 anastomoses (Mean fold changes ± SE)

	Number of cDNA molecules in uninjured colon	mRNA expressions in anastomoses vs. control colon (qRT PCR)	Protein levels in anastomoses vs. control colon (ELISA)	Protein levels in anastomoses vs. control colon (zymography)
MMP-2	33145	1.5 ± 0.2		2.0 ± 0.4
MMP-3	775	NS		
MMP-8	355	81 ± 19	17 ± 5	
MMP-9	48	71 ± 19	14 ± 3	24 ± 8
MMP-12	240	29 ± 6	15 ± 2	
MMP-13	266	16 ± 5		

MMP, matrix metalloproteinases; NS, non-significant

Figure 5 Colonic anastomoses three days after construction (Study V). The brown immunostains, in close vicinity to the suture channel (SC), represent an upregulation of CD68 positive macrophages (A) and MMP-12 (B).

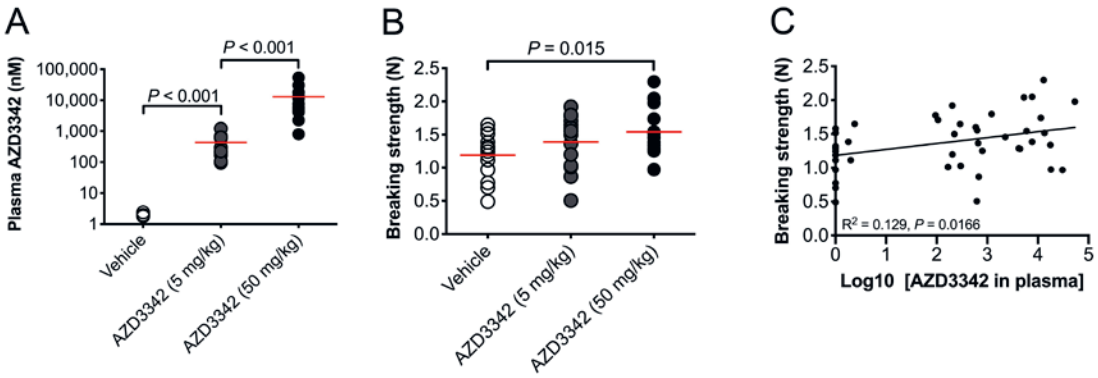


The anastomotic MMP-fingerprint reported here substantiated the rationale for the selective MMP inhibitory profile of AZD3342 as a test drug for optimization of experimental anastomotic healing.

7.4.2 Enhancement of anastomotic breaking strength in the normal colon

The dose-finding study demonstrated, that 50 mg/kg AZD3342 for three days was required to significantly increase the anastomotic breaking strength from mean 1.19 ± 0.34 N in vehicle treated rats to 1.54 ± 0.40 N in AZD3342 treated rats, P = 0.015 (Figure 6). The reproducibility of the result was tested in an independent experimental series. Again, AZD3342 50 mg/kg increased anastomotic breaking strength from 1.52 ± 0.30 N in vehicle treated rats to 1.78 ± 0.28 N, *P* = 0.024. There was no evidence of anastomotic leakage in any of the rats.

Figure 6 Dosages of the selective MMP-8, MMP-9 and MMP-12 inhibitor AZD3342 (AstraZeneca) and corresponding plasma concentrations (A) and anastomotic breaking strength (B). Dose-response relationship was calculated for log-transformed plasma concentrations and anastomotic breaking strength (C).



7.4.3 Changes in the obstructed colon

Contrary to the normal colon, obstruction may diminish the healing potential of a colonic anastomosis.

Partial obstruction increased the proximal colonic circumference by 100 per cent at segment B and 150 per cent at segment C after 24 hours (*Study V*), whereas complete obstruction for 12 hours increased the circumference by 80 per cent at segment C (*Study VI*). The colonic circumference did not increase in segments B and C beyond 24 hours (*Study V*). The following description refers to observations in segments B/C (proximal) compared with segments D/E (distal) following partial colonic obstruction for 24 to 96 hours unless stated otherwise (Figure_2).

7.4.3.1 Water content, collagen and matrix metalloproteinases in the colonic wall

The water content in the colonic wall increased temporarily in the proximal segments from 80 ± 2 per cent to 86 ± 3 per cent. By 72 hours the water content was normalized.

The concentration of collagen proximal to the partial obstruction decreased by 39 to 48 per cent after 24 hours. No further changes in collagen was observed. In the most distal part (Segment E), the concentration of collagen increased by 58 per cent between 0 to 72 hours.

In an unpublished series of 36 rats randomized to control (n = 10), laparoscopic sham (n = 10) or laparoscopic obstruction for 24 hours (n = 16), the gene expression of MMP-8 and MMP-9 increased 30-fold proximal to the obstruction compared with controls. MMP-13 increased 8-fold, while MMP-12 and MMP-3 both increased 4-fold. MMP-2 mRNA did not change (Figure 7). Interestingly, the sham procedure also increased MMP-8 and MMP-9 by 16- and 11-fold, respectively.

7.4.3.2 Histological changes in the colonic wall

After 24 hours of obstruction, a slight mucosal oedema was observed along with influx of neutrophils. Scattered necroses were observed in the muscularis mucosae, while a pronounced oedema and moderate infiltration of neutrophils and CD68-positive macrophages were observed in the submucosa.

After 48 hours, the mucosal oedema was reduced, but inflammation persisted. Fibrinoid necroses and thrombosed arteries were apparent in the submucosa, while inflammation and necroses were seen in the tunica muscularis. In the submucosa, oedema and inflammation were unaltered.

Between 72 and 96 hours, only few neutrophils were seen in the mucosa. The oedema in the submucosa was now normalized leading to increased cell density. While neutrophils persisted, the proportion of CD68-positive cells increased progressively both proximal and distal to the obstruction. By now, neuritis, ganglionitis and peritonitis was present.

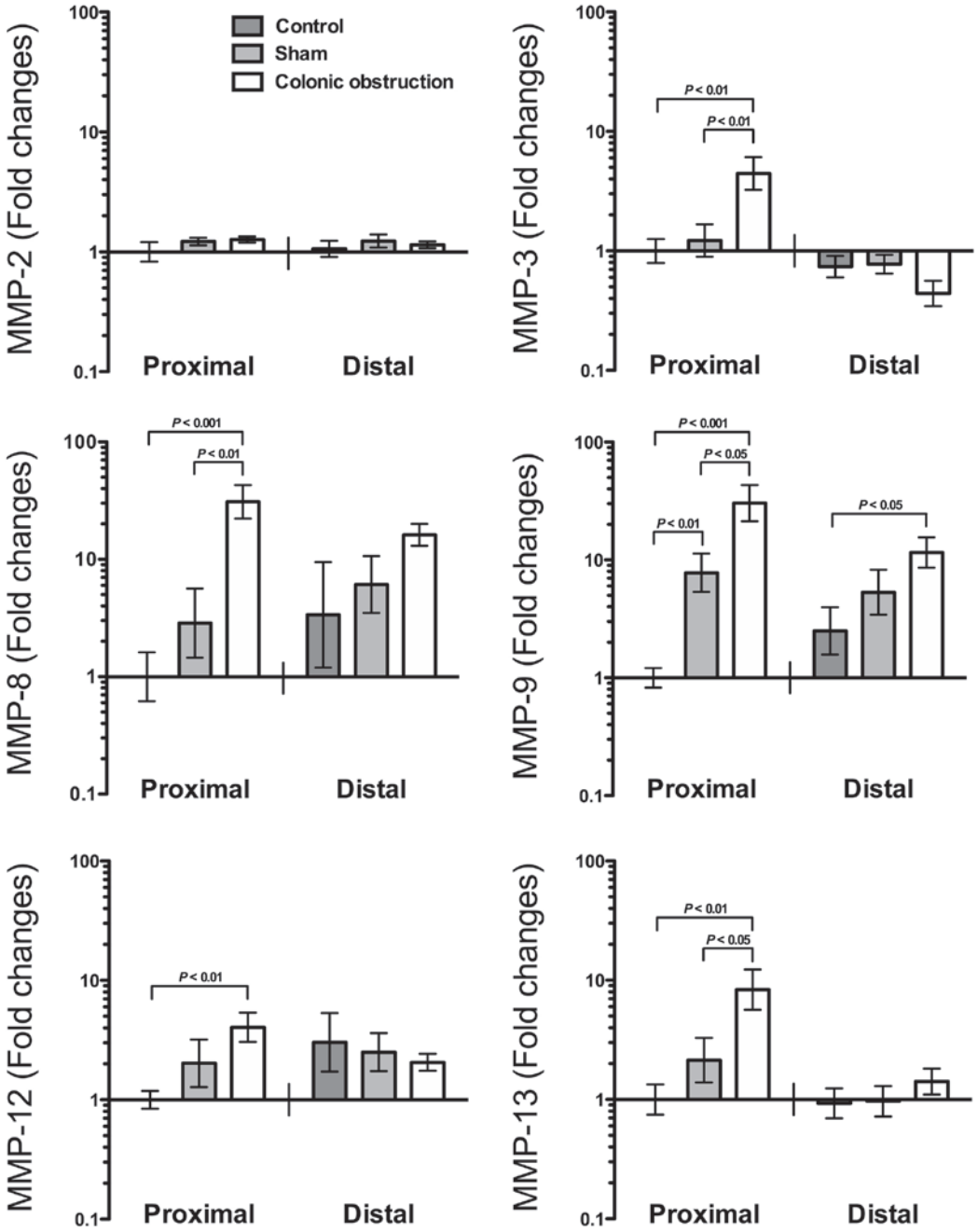
In the model of 12 hours complete colonic obstruction, the colonic dilatation resulted in submucosal oedema and influx of neutrophils and CD68-positive macrophages, corresponding to the model of partial obstruction (Figure 8).

7.4.4 Enhancement of anastomotic breaking strength in the obstructed colon

Following 12 hours of colonic obstruction, 10 mm of colon including the obstructing clip was resected, and a primary anastomosis constructed.

The anastomotic breaking strength decreased from 1.69 ± 0.19 N in control rats on day 0 to 1.26 ± 0.54 N in vehicle treated rats on day 3, $P = 0.023$. Treatment with AZD3342

Figure 7 Expression of indicated matrix metalloproteinases (MMPs) in the dilated colonic wall 24 hours after onset of colonic obstruction measured by quantitative real-time PCR. MMP mRNA were normalized to RPLP0, log-transformed and presented as fold change relative to colon proximal to the stenosis in control rats (=1) as geometric means ± back-transformed SEM.



prevented this natural decrease in anastomotic breaking strength (1.82 ± 0.38 N), corresponding to a significant increase compared with vehicle treated rats, $P = 0.008$.

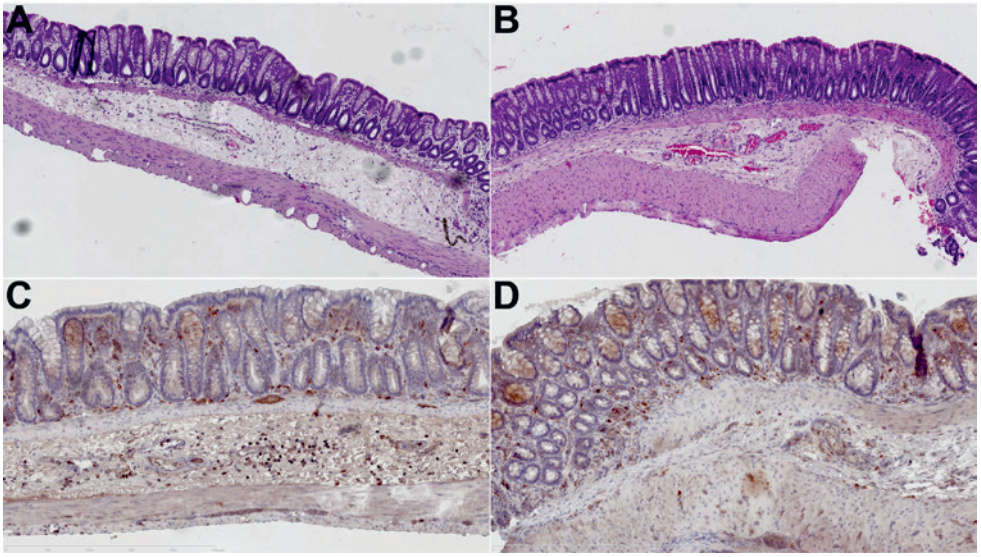
Anastomotic leakage was observed in 1/15 rats in the AZD3342 group compared with 7/16 rats in the vehicle group, $P = 0.037$.

7.4.5 Anastomotic collagen in normal and obstructed colon

Three days after surgery in the normal colon, the gene expressions of type I (COL1A1) and type III (COL3A1) collagen increased 4-fold and 2-fold, respectively, in the anastomoses compared with uninjured adjacent colon. However, there were no differences between AZD3342 and vehicle treated rats (*Study V*).

In anastomoses of obstructed colon, the total collagen concentration decreased from day 0 to day 3 with no difference between AZD3342 and vehicle treated rats on day 3. Subsequent characterization of the anastomotic collagen revealed no differences in pepsin-insoluble or pepsin-soluble collagen or the ratios hereof (*Study VII*).

Figure 8 Section of the left colon after 12 hours of obstruction (A, C) and the non-obstructed left colon (B, D) (Study VII). The specimens are stained with hematoxylin-eosin (A, B) and immunohistochemical double labeled (C, D) for neutrophils (black) and CD68 positive cells (red-brown). Notice the oedema (A) and the expression of neutrophils (C) in the submucosa.



8 DISCUSSION

Modern colorectal surgery was founded by master surgeons with innovative mindsets, technical skills and courage to push the limit beyond contemporary colorectal surgery. They paved the way for surgical treatment of colorectal cancer with the potential of long-term cure. They did struggle with the same problems as we do today. Anastomotic leakage is common, difficult to diagnose and associated with long-term implications in survivors. The importance of anastomotic leakage has not ceased over time, and the fact that this complication was recently chosen as one of the most important outcomes in colorectal cancer research by nurses, surgeons and patients, confirms its significance.[123]

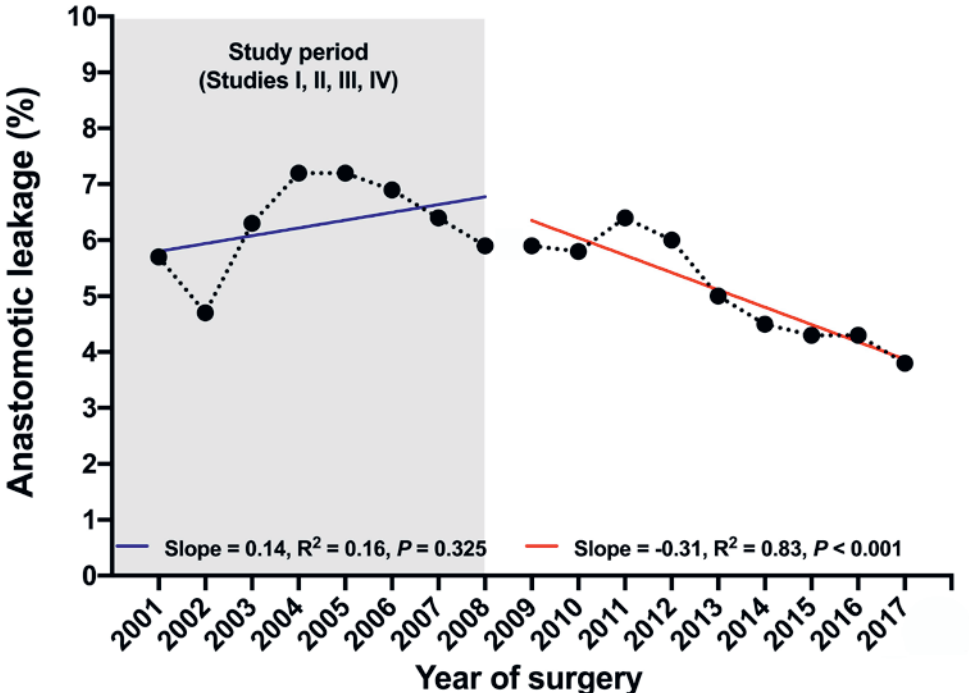
The present thesis is an attempt to elucidate different aspects of anastomotic leakage in four longitudinal retrospective cohort studies, with the inherent advantages and disadvantages. Specific data on anastomotic leakage from randomized controlled trials are seldom and may lack sufficient statistical power to detect different outcomes in important subgroups. Merkow and Ko claimed in their JAMA editorial that "*Well-designed observational research can address a relevant clinical question that could not be answered with an RCT.*"[124] Hopefully, *Studies I, II, III* and *IV* emphasize this statement.

8.1 Incidence of anastomotic leakage

During the last 50 years the rate of anastomotic leakage has been reasonably stable. The incidence of 6.4 per cent after colonic resection in this thesis was higher than expected and may reflect the nationwide data of unselected patients. The rate did not change during the study period between 2001 and 2008. In the slipstream of *Study I*, a comparable nationwide study from the Netherlands demonstrated a 7.5 per cent leakage rate after colonic cancer surgery,[125] whereas selected patient data from the American College of Surgeons National Surgical Quality Improvement Program (ACS NSQIP) revealed a much lower leakage rate of 3.8 per cent.[126] The latter only included grade C leakages,[126] and the differences in definition of anastomotic leakage are a major confounder when comparing results across registries.[127] Most registries consist of prospectively collected data. However, classification- and recall bias cannot be ruled out, and recent evidence of underreporting of anastomotic leakage in the Dutch and Swedish registries has been reported.[128,129] In this thesis, great efforts were made to avoid underestimation of leakage rates by merging data from two nationwide registries. However, patients with stage IV disease were not included here, which is also the case in most other studies. We recently investigated this in a similar nationwide setting and found similar leakage rates in patients with or without distant metastasis.[130]

The data for this thesis was extracted in 2010, the same year as anastomotic leakage after colonic cancer resection was adopted as a quality indicator variable in the DCCG database. I would like to think, that reporting of these studies on anastomotic leakage and its implications has increased the awareness of this severe surgical complication in Denmark, and thus contributed to the decreasing incidence in the following years (Figure 9).

Figure 9 Changes in the incidence of anastomotic leakage after elective colonic cancer surgery in Denmark between 2001 and 2017. During the study period (2001 to 2008) there were no development in the incidence of anastomotic leakage. In the subsequent period from 2009 to 2017 there was a significant decrease in anastomotic leakage rate. Data from the Danish Colorectal Cancer Group.



8.2 Length of hospital stay

It is more difficult to estimate if length of hospital stay in patients with anastomotic leakage has decreased concomitantly. In S*tudy II*, patients with anastomotic leakage were admitted for a mean of 21 days, 13 days longer compared with patients without leakage. Interestingly, a recent paper reported similar results in an optimized ERAS setting, where anastomotic leakage increased length of stay from median two to 15 days,[131] confirming previous results.[132,133]

In the current thesis, length of stay was defined as the duration of the primary admittance. We did not capture patients, who were transferred to another department or for rehabilitation outside the hospital, which may lead to underestimation of the total hospital stay. There is no doubt that the calculated length of stay is a minimum figure - particularly in patients with complications. The increased length of stay will add significantly to the overall costs of the treatment.[134,135]

8.3 Short-term mortality

Postoperative mortality is the caveat of colorectal surgery. During the study period, 30-day mortality decreased significantly and the reduction continued beyond 2008.[136] Today, the national 30-day mortality rate after elective colonic cancer surgery is 1.4 per cent[137] as compared with 4.9 per cent in *Study II*. The mortality after anastomotic leakage was immense even after adjustment for confounding. However, it did decrease between 2001 and 2008. Although the studies here did not capture the cause of death, most patients with a fatal outcome after anastomotic leakage probably died from sepsis and multiorgan failure. This hypothesis is supported by the fact that anastomotic leakage increases the rates of organ dysfunction, including pulmonary, cardiac and renal failure.[16,138] In addition, anastomotic leakage is associated with increased transfer to the intensive care unit,36 re-operation and readmission.[126] Mortality rates in patients with anastomotic leakage are similar in large European studies,[16,125] but much lower in the American study,[126] even though the latter only included patients with grade C leakages. Case mix and registration practice may in part explain the disparities in mortality rates. The American data were extracted from the ASC NSQIP, established to measure and improve surgical care in private sector hospitals. In the European studies, nationwide data were used, thus covering a more diverse cohort including frail and socially vulnerable patient.

Despite the relative decrease in mortality after anastomotic leakage, we recently demonstrated a continuous high 30-day mortality rate of 15.5 per cent in patients with anastomotic leak after right hemicolectomy.[139]

8.4 Impact of comorbidity

In the subgroup analysis of length of stay and 30-day mortality in patients undergoing elective surgery (*Study III*), the explaining variable, anastomotic leakage, was stratified according to the degree of comorbidity, offering a high-resolution analysis of the relationship between anastomotic leakage, comorbidity and length of stay or postoperative death.

Comorbidity in patients without leaks did not impact the length of hospital stay, whereas anastomotic leakage in patients without comorbidity prolonged length of stay by almost two weeks. In patients with significant pre-existing comorbidity and anastomotic leakage, the mean duration of admission was further prolonged by 2 days, suggesting that patients with comorbidity require more time to recover from anastomotic leakage compared with healthy patients.

A previous study demonstrated that anastomotic leakage and comorbidity were independently associated with increased 30-day mortality.[125] This was confirmed in *Study II*, but more interestingly, patients with anastomotic leakage and pre-existing comorbidity had a 1.6-fold increase in mortality compared with patients with anastomotic leakage alone.

These findings are important and underlines that patients with comorbidity cannot cope with the adverse event of an anastomotic leakage. The results offer an objective measure to advice very comorbid patient against a primary anastomosis. However, there is no

data in *Study II* or - to my knowledge - in the available literature to suggest that morbidity and mortality can be reduced in comorbid patients undergoing colonic resection for cancer by avoiding a primary anastomosis. The use of the Charlson comorbidity index herein is a crude measure, where treatment and optimization of the patient's comorbidity are not captured. Disease optimization may be more important for the surgical outcome than the disease itself. In non-optimized or frail patients promising data suggest, that prehabilitation have the potential to improve the postoperative outcome.[140,141]

8.5 Management of anastomotic leakage

The post-hoc stratification of leaks into grade A, B and C enabled a more in-depth analysis of the impact of management. However, the classification merely reflects the severity of the leak at time of diagnosis, exemplified by a trend analysis of the data in *Study III*, published as an Authors reply, demonstrating higher mortality rates with higher grades of leakage.[122]

Anastomotic salvage was the main focus in *Study III*. Salvage was feasible, safe and associated with a reduction in the rate of permanent ostomies as compared with anastomotic takedown. Furthermore, the data suggested that salvage should be performed under the protection of a proximal loop-ostomy. A protecting ostomy may be more relevant for salvage of left sided leaks, but there was not sufficient statistical power to differentiate between the right and the left side. Anastomotic salvage was primarily attempted in younger patients with a low to moderate degree of peritonitis and smaller anastomotic defects as reported by others.[49] This particular presentation may be more frequent with early detection of the leak. However, only a quarter of the patients with this presentation, underwent a salvage procedure, suggesting a need for guidelines on management of anastomotic leakage. Somewhat unexpectedly, participation of a specialized colorectal surgeon at the re-operation did not increase the rate of anastomotic salvage, in contrast to a previous report.[52] Moreover, only 40 per cent of the re-operations were undertaken by colorectal surgeons.

Management of anastomotic leakage was only attempted laparoscopically in four patients, but all were converted to open surgery, probably reflecting the lack of laparoscopic adoption for treatment of anastomotic leakage at the time of the study period. More recently, laparoscopy has increasingly been used as a diagnostic tool as well as the primary approach for management.[142] Laparoscopy has a high degree of diagnostic accuracy,[143] is feasible and effective in the management of anastomotic leakage[142,144,145] and has been associated with reductions in length of hospital stay and permanent ostomy rate.[145]

The data in *Study III* have contributed to the Danish national guideline for management of anastomotic leakage, published in 2016, addressing anastomotic salvage, minimally invasive approach and specialization.[146]

8.6 Long-term outcomes

A significant advantage of the salvage approach was the decreased rate of permanent ostomies, which could significantly improve patients' quality of life.[47] However, the re-anastomotic leakage rate of 8.5% after stoma reversal was higher than the initial rate of

6.4%, confirming the high leakage rates following stoma reversal in a previous study.[147] It was not possible to find an explanation for this in the available data, but it underlines the fact, that a stoma reversal is not just a minor procedure and surgical expertise is mandatory.[148] A perfectly salvaged anastomosis could otherwise be sabotaged by a failed stoma reversal, once again jeopardizing the patients' survival.

A main finding in *Study IV* was the significant association between anastomotic leakage and long-term mortality in patients surviving at least three months after the index resection. The choice to exclude patients dying within the first three months is controversial, because it introduces immortal time bias. In addition, the excluded patients were not accounted for in the analyses, and the impact of anastomotic leakage was not examined between postoperative day 31 and 120. The immortal time bias could lead to an underestimation of the impact of anastomotic leakage on overall mortality, in other words "*... this long run is a misleading guide to current affairs. In the long run we are all dead.*"[149] However, the primary purpose was to investigate if the influence of anastomotic leakage continued beyond the first critical phase. In a study by *Branagan et al.*, where patients dying within thirty days were excluded, the authors were unable to demonstrate a relationship between anastomotic leakage and increased long-term mortality.[32] Three other studies shared the findings of *Study IV*.[21,150,151] In studies calculating overall mortality, without excluding the event of early mortality, all[16,152-158] but two[58,159] have reported a significant association between anastomotic leakage and long-term mortality. So why does anastomotic leakage continue to reduce patient's survival? A possible explanation could be an increased risk of cancer recurrence.

In *Study IV* there was a compelling association between anastomotic leakage and subsequent development of distant recurrence. This finding is rather controversial, whereas it is accepted, that anastomotic leakage after low anterior resection increases the incidence of local recurrence.[59] The analytic approach may be of great importance in the investigation of recurrence. Three studies have examined the impact of anastomotic leakage after colonic resection on distant recurrence using crude frequency analyses of absolute events and found no associations.[21,58,160] Recurrence is a time-dependant outcome and should be investigated accordingly and with control for confounding in case of an observational study design. In *Study IV*, the Cox proportional hazard regression analysis was employed. However, the competing risk analysis might have been more appropriate, as death before recurrence is treated as a competing risk. The impact of anastomotic leakage on cancer recurrence using multivariable Cox regression analysis has only been investigated in one additional study, where there was no significant association.[155] There was however an important difference between *Study IV* and the study by *Goto et al.* regarding the administration of adjuvant chemotherapy.

In this thesis, patients with UICC stage III colon cancer and anastomotic leakage were less likely to receive adjuvant chemotherapy compared with patients without leakage. In addition, the time to first administration was delayed in patients with anastomotic leakage. In a post-hoc analysis published in a *Letter to the Editor*, administration of adjuvant chemotherapy and time to first administration did not differ between patients with grades A, B and C leaks,[122] although this result was challenged by the small numbers of patients with grades A and B leaks.

Chemotherapy as an adjuvant to radical surgery for stage III colon cancer is associated with a 30 per cent relative reduction in 5-year mortality.[161] However, delayed administration reduces this beneficial effect and decrease overall survival by 14 per cent for each 4 weeks of delay.162 In the present cohort, omission or delayed administration of adjuvant chemotherapy could in part explain the association between anastomotic leakage and increased long-term mortality mediated by increased distant recurrence. Interestingly, the impact on long-term mortality disappeared when distant recurrence was included in the multivariable cox regression analysis, confirming this hypothesis. Furthermore, patients with anastomotic leakage and stage III disease, who did receive adjuvant chemotherapy within 8 weeks from the index operation, had a similar survival probability compared with patients without leaks. A limitation is the omission of high-risk patients with stage II disease, that would benefit from adjuvant chemotherapy. In the study by *Goto et al*, adjuvant chemotherapy was equally administrated in patients with- and without anastomotic leakage, which to some degree could explain the negative finding.[155] This *treatment pathway* of omitted or delayed adjuvant chemotherapy is interchangeably connected with a *biological pathway* that may promote recurrence in patients with anastomotic leakage.

The biological pathway is extremely complex and not fully understood. Briefly, the systemic stress response to surgery entails a favourable environment for seeding and progression of tumour cells in the vascular and lymphatic compartments and promotion of micrometastases.[163,164] Activation of catecholamines and prostaglandins with direct tumour affinity[165-167] increases the metastatic potential.[163] Suppression of anti-metastatic immunity by downregulation of natural killer cells, IL-12 and IL-10 and promotion of pro-metastatic immunity by upregulation of IL-8, IL-6, IL-1 and expression of growth- (TNF-α TGF-β) and angiogenic (VEGF) factors promote dissemination.[163,164,168] However, cancer recurrence is dependent on the presence of malignant cells. Spillage of tumour cells during surgery, especially when the mesocolic plane is violated,[164,169,170] and the presence of luminal exfoliated tumour cells provide the building blocks for dissemination. In the event of anastomotic leakage, inflammation is accelerated, which may be a driving force for increased metastatic progression. In the context of *Studies V* and *VII* it is of special interest, that MMPs and in particular MMP-2 and MMP-9, seem to play a role in the epithelial-to-mesenchymal transition and thus tumour metastasis.[168,171] Whether synthetic MMP-inhibition influences the metastatic cascade is unknown.

The course of the *treatment-* and *biological pathways* may be altered by administration of neoadjuvant chemotherapy, clearing the circulating tumour cells. While awaiting the end-results of the FOxTROT (NCT00647530) and NeoCOL (NCT01918527) trials on neoadjuvant chemotherapy in high-risk, non-metastatic patients with colonic cancer, anastomotic leakage is considered a risk factor for recurrence. Patients with stage II disease and anastomotic leakage should thus be eligible for adjuvant chemotherapy.

8.7 Risk factors

The exploration of risk factors for anastomotic leakage may be the most frequent exercise in complication research. Risk factors can be divided in pre-, intra- and postoperative factors,[172] or in host- and treatment related factors.[173] A separation of adjustable and non-adjustable risk factors provide a more operational categorization. In *Study I,* six

risk factors associated with anastomotic leakage were identified. One of the risk factors, laparoscopic surgery, was adjustable, but the increased risk of anastomotic leakage in patients subjected to this surgical approach was a transient phenomenon and probably related with the implementation of the technique.

Other adjustable risk factors not investigated in this thesis include excessive fluid administration,[174] use of NSAIDs,[64,175,176] and use of systemic corticosteroids.[177] Interestingly, data from the DREAMS trial on dexamethasone for postoperative nausea demonstrated a tendency towards a lower leak rate in the dexamethasone group, 1.66% versus 3.1%, *P* = 0.08.178 The combination of oral antibiotics and mechanical bowel preparation has recently been shown to reduce the incidence of anastomotic leakage[179-182] and vascular high-tie with division of the inferior mesenteric artery at the root of the aorta has been proposed as a risk factor.[183] In a more recent Swedish study this only applied to patients with pre-existing cardiovascular disease[184]. Meticulous testing of the perfusion in the arch of Riolan and the marginal of Drummond should minimize anastomotic leakage due to insufficient blood supply. The method for construction of the anastomoses are potentially adjustable. Anastomotic configuration, apposition of the intestinal ends, suture materials and suturing techniques including one-layer *versus* two-layer and stapled *versus* handsewn have all been elegantly reviewed.[185-187] However, the construction of ileocolic anastomoses has recently been the subject of attention. Unfortunately, anastomotic method was not registered in the DCCG database at the time of this thesis. In a 2011 Cochrane review on ileocolic anastomoses there were no differences in the rates of clinical anastomotic leakage between handsewn and stapled anastomoses. However, the results were in favour of the stapled approach when non-clinical leaks were included in the meta-analysis.[188] Recently, several large well-conducted cohort studies including the 2015 European Society of Coloproctology's (ESCP) snapshot study came to the opposite conclusion, that the stapled approach was a risk factor for anastomotic leakage.[133,189-191] In 2015, anastomotic method was included in the DCCG database, and in the first nationwide study, the stapled as compared with the handsewn technique was associated with a two-fold increase in anastomotic leakage.[139] There was no exploration of causality in these studies, but in a spin-off, different stapling devices or closuring techniques for the transverse defect did not explain the increased rate of anastomotic leakage.[192]

The remaining non-adjustable risk factors in this thesis were age, gender, surgical procedure, blood loss and blood transfusion. Interestingly, ASA classification was not associated with anastomotic leakage in the multivariable analysis in *Study I*, contrasting previous findings.[125,172,173,193] We therefore conducted a more thorough analysis of the relationship between comorbidity and anastomotic leakage in patients undergoing elective surgery (*Study II*). This study revealed a vague, but significant association between comorbidity and anastomotic leakage. However, the prediction of anastomotic leakage by comorbidity was only fair and thus not precise enough to advice patients against a primary anastomosis.

Another important and strong risk factor for anastomotic leakage, not captured in *Studies I* and *II*, is smoking.[66,133,194] Smoking is potentially adjustable but short-term smoking cessation does not seem to mitigate the risk of anastomotic leakage.[195] The impact of smoking cessation for longer duration has not been investigated.

Several authors have published significant variations in anastomotic leak rates between surgeons at the same institution.[196-198] However, it is difficult to adjust for case mix between surgeons, and comparisons are thus subjected to significant bias. Differences in approach, anastomotic technique and surgical skills may explain some of these differences.

Further exploration of non-adjustable risk factors may not be worth the effort. Most patients with risk factors do not develop a leak, making prediction difficult. Even the high-resolution investigation of comorbidity and individual disease conditions in this thesis did not improve the prediction of anastomotic leakage. Patient selection for construction of a primary anastomosis or ostomy remains important but difficult, and the current data is not sufficient to guide the surgeon, however, it is clear that patients with significant comorbidity cannot cope with the event of a leak, and some off these frail patients may be better off with an ostomy.

8.8 Experimental outcomes

The use of experimental study designs provides an array of scientific options not applicable in clinical and epidemiological studies. Most importantly, the use of experimental animals should be weighed against the information potentially gained and the capabilities within the study group. Extensive knowledge on experimental wound- and anastomotic healing already existed within our laboratory. In the absence of valid prediction models and patient selection criteria for a primary anastomosis, pharmacological enhancement of anastomotic heling is desirable. The selection of the test compound was supplemented by two systematic reviews.[88,94] Collectively, MMP-inhibition was judged the most promising concept, although this therapy just constitutes one brick in the complex puzzle of anastomotic healing.[199]

8.8.1 Anastomosis in the normal colon

The more than 20 different MMPs are categorized according to substrate specificity and molecular structure. The collagenases (MMP-1, MMP-8 and MMP-13) primarily degrade structural collagen types I and III, whereas the gelatinases (MMP-2 and MMP-9) cleave collagen type IV and partially degraded collagen. Acting together, they degrade collagen synergistically. The macrophage metalloelastase MMP-12 primarily degrades elastin, but it also possesses some collagenolytic activity.[200-202]

The predominant MMP gene transcript in normal colon was MMP-2, while the expression of MMP-8 and MMP-9 was low. Interestingly, the gene expression of MMP-2 only increased 2-fold in the healing anastomosis as compared with MMP-8 (81-fold), MMP-9 (71-fold) and MMP-12 (29-fold), suggesting that MMP-2 is important in maintaining homeostasis in the colon but not during anastomotic healing. The relevance of the increased MMP-8, MMP-9 and MMP-12 gene transcripts was confirmed by protein analyses. The MMP fingerprint of early anastomotic healing, identified in *Study V*, was suggestive of the inflammatory phase with accumulation of neutrophils and macrophages, expressing MMP-8 and MMP-9, around the suture channels.[70] In *Study V*, we also demonstrated MMP-12 positive macrophages around the suture channels, further justifying the rationale of the selective MMP-8, MMP-9 and MMP-12 inhibitor, AZD3342.

Administration of AZD3342 increased the anastomotic breaking strength, here, in two independent experimental series of anastomotic healing in the normal colon. Similar results have recently been obtained using a different selective MMP inhibitor, AG3340 (Prinomastat), with a different inhibitory profile, targeting MMP-2, MMP-3, MMP-9, MMP-13 and MMP-14.[93] Only MMP-9 was inhibited by AZD3343 and AG3340. Ideally, only MMPs with a deleterious impact on anastomotic healing should be inhibited. According to the MMP-fingerprint (*Study V*), MMP-3 did not increase during anastomotic healing, MMP-2 increased only slightly, whereas MMP-13 increased 16-fold. MMP-14 was not assessed. Interestingly, pre-existing excessive levels of MMP-9 in the intestinal wall has been associated with subsequent development of anastomotic leakage.[80] In addition, recent and compelling data have demonstrated important associations between gut bacteria, collagen degradation, MMP-9, and anastomotic leakage in rats.[203]

Several gut bacteria possess collagenolytic capability, but in human samples only *Enterococcus faecalis* and *pseudomonas aeruginosa* displayed this specific phenotype.[203] The abundance of Enterococcus faecalis increases up to 500-fold in the anastomosis during healing. The collagenolytic activity of Enterococcus faecalis was increased in rats with anastomotic leakage.[204] Furthermore, MMP-9 activity was significantly increased in leaking anastomoses. In *Study V*, the major increase in anastomotic MMP-9, may only represent the response of normal wound healing in the intestine, but interestingly, gelatin zymography revealed that MMP-9 protein was only present in its latent form (proMMP-9) rather than its active form (*Study V*). It remains unknown if activation of proMMP-9 is required in the process of anastomotic digestion. The proMMP-9 possesses some enzymatic activity without activation,[205] but *Enterococcus faecalis* has been shown to activate proMMP-9 by direct cleavage.[203]

Until now, the rationale for MMP-inhibition during anastomotic healing has been to reduce the "normal" collagenase activity in the anastomosis, preserving collagen and thus enhance anastomotic strength. However, this concept has been surpassed by the *bacterial theory*. The major increase in anastomotic MMP-9, demonstrated in *Study V*, provides the source of collagen-degradation and by bacterial activation, MMP-9 may cause anastomotic leakage. In the study by Shogan et al., selective MMP-9 inhibition prevented the development of anastomotic dehiscence in rats.[203] MMP-9 is a key mediator of both normal anastomotic healing and anastomotic leakage. The latter is further exemplified by the finding of excessive levels of MMP-9 in the peritoneal fluid of patients with anastomotic leakage.[206,207]

Overexpression of MMP-9 could be regarded as an intrinsic and potentially adjustable risk factor for anastomotic leakage - modifiable by MMP-inhibition.

8.8.2 Anastomosis in the obstructed colon

Anastomotic surgery during complicated conditions such as colonic obstruction is a completely different discipline. Left-sided colonic obstruction is considered a relative contraindication for primary anastomosis during emergency procedures due to an increased inherent risk of leakage.[17,87] In *Study I,* emergency procedure was not associated with anastomotic leakage, which may suggest a safe practice where surgeons favour construction of an ostomy over a primary anastomosis. However, primary restoration of

bowel continuity would be preferable. It is therefore important to understand the structural ground for the increased risk of leakage.

An important finding in *Study VI* was the rapid loss of collagen. Within 24 hours, the level of collagen nearly halved proximal to the stenosis, which correlated with a 2-fold increase in colonic circumference. Neither the circumference nor the collagen level changed further after the 24 hours of obstruction. At the same time, the water content in the submucosa increased and a vast influx of neutrophils, but not macrophages, was observed. This corresponded with a 30-fold increase in MMP-8 and MMP-9 mRNA in the wall of the obstructed colon. MMP-8 and MMP-9 are commonly expressed by neutrophils, and increased MMP activity in the obstructed colon was previously demonstrated as the cause of collagen degradation.[70,84] The expression of MMP-3, MMP-12 and MMP-13 were less pronounced compared with MMP-8 and MMP-9 at this early time point of colonic obstruction, while MMP-2 did not change. Colonic obstruction also leads to a significant shift in gut microbiota and bacteria translocation to the mesenteric lymph nodes, spleen, liver and blood.[208] Whether this intestinal bacterial shift is associated with increased collagenase activity in anastomosis of obstructed colon is unclear.

The methodological approach in *Study VI*, using a partial obstruction of the left colon, allowing passage of flatus but not stool, was a more physiological approach compared with the complete obstruction in *Study VII*[84-86] and may better resemble the clinical state of colonic obstruction. In patients with an obstructing tumour, the colonic dilation is commonly relieved by endoluminal stenting, which restores the loss of collagen in the intestinal wall. This may take up till 10 days.[86] On the other hand, stenting is associated with an increased risk of tumour perforation and thus a potentially inferior prognosis.[209]

The results of the dynamic changes in the obstructed colonic wall suggest, that even a short period of colonic dilation results in a substantial loss of collagen mediated by increased MMP-8 and MMP-9 expressed by neutrophils. We suggest, that this may be the mechanism for the impaired anastomotic healing in the obstructed colon.[71,100]

MMP-inhibition has the potential to reverse this inferior starting point for anastomotic healing. While non-selective MMP-inhibition with GM6001 has been associated with an increased risk of anastomotic leakage by impeding epithelialization in the obstructed colon,[95] the inhibitory profile of AZD3342 may be more appropriate, based on the molecular and structural changes, demonstrated in *Study VI*.

This hypothesis was examined in *Study VII*, where AZD3342 restored anastomotic breaking strength on postoperative day three to baseline levels of control rats, corresponding to a 44% increase in strength compared with vehicle-treated rats. In addition, the rate of anastomotic leakage in this high-risk model was reduced. Interestingly, the relative increase in anastomotic strength induced by AZD3342 was higher in the obstructive model compared with the uncomplicated models in *Study V*.

Other compounds have been investigated including povidone iodine,[210,211] erythropoietin[212,213] and the prostaglandin analogue iloprost,[214] all of which were previously reviewed by our group.[94] Iloprost had the strongest effect on anastomotic bursting pressure, but interestingly, the level of anastomotic MMP-13 decreased.[214]

The increase in anastomotic breaking strength was not accompanied by changes in the total level or composition of collagen. Total collagen was assessed by hydroxyproline, which is also observed in elastin, but in negligible amounts.[215] Contrary to the expected, AZD3342 treatment did not protect the soluble, newly synthesized collagen from degradation, nor did it alter the insoluble cross-linked collagen. This is in contrast with the understanding, that the beneficial effects of MMP-inhibition correlate with collagen quality.[216,217] AZD3342 may have increased anastomotic breaking strength by other important factors, such as alteration of the ratio of type I to type III collagen, level of type V collagen and fiber diameter/orientation, none of which were investigated in this thesis. In addition, the optimal MMP selectivity may not be the same for anastomoses in normal- and obstructed colon.

9 CONCLUSION

The results of this doctoral thesis provide novel and important information of the short- and long-term consequences of anastomotic leakage after colonic cancer surgery. One in five patients with anastomotic leakage died within thirty days from the index operation, and pre-existing comorbidity increased mortality even further. A change in management strategy towards salvage of the intestinal continuity may mitigate the risk of a permanent ostomy without jeopardizing survival. Long-term survival was significantly affected by anastomotic leakage because of an increased risk of distant recurrence. Delayed or omitted adjuvant chemotherapy was the main cause of cancer recurrence in patients with anastomotic leakage. A thorough exploration of risk factors for anastomotic leakage did not identify adjustable factors that can be used in the decision making of a primary anastomosis or an ostomy. Therefore, experimental models of anastomotic healing were developed including employment of selective MMP-inhibition to enhance anastomotic strength. Inflammation associated MMP-8 and MMP-9 were key mediators of anastomotic healing in normal and obstructed colon. Their inhibition increased anastomotic strength and these findings are in agreement with recent novel studies pointing towards MMP-9 as the driving force for development of anastomotic leakage.

10 PERSPECTIVE

It has been said that anastomotic leakage is an inevitable part of colorectal cancer surgery and that surgeons must learn to cope with the defeat of having leaks. Although that might be true to some degree, it is not an acceptable opinion. The goal must be to eliminate anastomotic leaks.

Most studies are experimental- or register based and there are only few randomized clinical trials where anastomotic leakage is the primary endpoint. The best and most recent randomized clinical trials have been terminated prematurely. The *Pillar III* study (Identifier: NCT02205307), on indocyanine green-enhanced fluorescence angiography, was terminated due to slow recruitment, and the *Lifeseal* study (Identifier: NCT02907385) on anastomotic line enforcement by gelatine matrix was terminated by the data and safety monitoring board. These are examples of the difficulties associated with investigating anastomotic leakage in a randomized setting indicating the need for other scientific approaches. Anastomotic leakage is a multifactorial complication without a definitive aetiology. The mystery of anastomotic leakage may not be solved until each factor is included in the scientific modelling.

In this thesis, we confirmed the theoretically foundation for bacterial activated anastomotic leakage by a major upregulation of proMMP-9 in the anastomoses. However, several aspects of the linkage between the microbiome, collagenase and anastomotic leakage remain unknown, including the influence of anastomotic ischemia on the interplay between the microbiome, anastomotic healing and bowel preparation with oral antibiotic. In the most recent randomized controlled trial on oral preparation, the MOBILE trial, the authors were unable to demonstrate superiority of bowel preparation with oral antibiotic, which is in sharp contrast to recent cohort studies.[218] However, more than half of the patients underwent right-sided hemicolectomies, which may obscure the results. The conflicting results may be explained by methodological approach but may also embody the challenges of studying a multifactorial complication. Although translational research may the key to understanding anastomotic leakage, a multitude of influencing factors must be addressed before the biology is investigated.

The introduction of an anastomotic leakage care bundle including pre-, intra- and post-operative factors (Table 7) may have the potential to both minimize anastomotic leakage and give the foundation for a translational study approach. The concept of ERAS, where single factors are less important than the whole, could serve as inspiration and may be studied by robust, large-scale cohort designs.

Table 7 Potential care-bundle for minimizing anastomotic leakage

Preoperative	Intraoperative	Postoperative
Patient selection	Reduce surgical duration[2]	Early mobilisation
Correct anaemia[1]	Reduce blood loss[2]	Early feeding
Halt weight loss[1]	Avoid overhydration	No NSAIDS
High fiber, high protein, low carbohydrate[1]	Assessment of anastomotic blood supply[3]	ERAS
Smoking cessation1	Tension free anastomosis	
Mechanical bowel prep. + oral antibiotics	Prophylactic intravenous antibiotics	

[1] These factors could be assessed in a prehabilitation program either individually or bundled.
[2] These are achievable by proper preparation and critically radiological evaluation of tumour- and vascular anatomy and by operating teams of specialized colorectal surgeons.
[3] Manual testing of the blood flow in the marginals and/or by fluorescence enhances angiography.

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12 SUMMARY

Anastomotic leakage is a severe complication following colonic cancer surgery. The incidence of anastomotic leakage and the impact on short- and long-term outcomes vary significantly between studies. The understanding of anastomotic leakage management and its impact on outcome is limited. And the identification of risk factors has been subjected to bias.

Based on a merged dataset from the database of the Danish Colorectal Cancer Group and the National Patient Registry, 9329 patients with colonic cancer were identified for the investigation of several aspects of anastomotic leakage and its implications.

The incidence of anastomotic leakage was 6.4 per cent. In these patients, the length of stay and the thirty-day mortality were 21 days and 20.9 per cent, respectively, compared with seven days and 4.6 per cent in patients without anastomotic leakage. Mortality rates did not change with management modality but increased with burden of comorbidity. In surviving patients with anastomotic leakage, the rate of permanent ostomies was 55 per cent. Moreover, there was an increased risk of distant recurrence and long-term mortality, primarily due to delayed or omitted adjuvant chemotherapy. No adjustable risk factors for anastomotic leakage were found in the risk analysis.

We therefore looked at the phases of anastomotic healing to identify a potential target for enhancement of anastomotic healing in both normal and obstructed colon. During the inflammatory phase, collagenase activity is upregulated, and several studies have suggested that board-spectrum inhibition of matrix metalloproteinases increased anastomotic breaking strength but with the risk of undesirable side effects.

By experimental modelling in rats, we identified three key MMPs upregulated in the newly constructed anastomosis. Subsequent inhibition of MMP-8, 9 and 12 resulted in a 29 per cent increase in anastomotic breaking strength. Next, we studied the time-dependant biochemical and morphological changes in the colonic wall following onset of obstruction. Colonic dilation, oedema and a rapid loss of submucosal collagen was observed along with influx of neutrophils and macrophages. Again, inhibition of MMP-8, 9 and 12 increased anastomotic breaking strength. However, changes in collagen levels or composition were not correlated with anastomotic healing.

In conclusion, during the study period the incidences of anastomotic leakage was high with an unbearable high mortality rate, length of stay and recurrence rate. The inferior long-term outcome could be explained by omitted or delayed adjuvant chemotherapy. The identification of MMP-8, 9 and 12 as key molecules of anastomotic healing could lead to future translational research combining knowledge of anastomotic heling with novel information on the relationship between MMP-9 and enteral bacteria, anastomotic leakage and outcomes.

13 DANSK LÆGMANDSRESUMÉ

Anastomoselækage, hvor sammensyningen mellem tarmender brister, er en alvorlig komplikation efter operation for tyktarmskræft. Forekomsten af anastomoselækage og påvirkningen af kort- og langtidsresultater varierer markant mellem eksisterende undersøgelserne. Behandling af anastomoselækage og indflydelsen af de forskellige behandlingsmuligheder på overlevelsen er dårligt undersøgt, og identificering af risikofaktorer er ofte fejlbehæftet.

I 4 artikler, baseret på data fra den Danske Kolorektalcancer Gruppes database og Landspatientregisteret, undersøgte vi 9329 patienter, opereret for tyktarmskræft, for bidrage til forståelse af flere aspekter af anastomoselækage og dens implikationer.

Forekomsten af anastomoselækage var 6,4 procent. Hos disse patienter var indlæggelsestiden og 30-dages dødeligheden henholdsvis 21 dage og 20,9 procent sammenlignet med syv dage og 4,6 procent hos patienter uden anastomoselækage. Den valgte behandling af anastomoselækage ændrede ikke risikoen for død. Derimod steg dødeligheden betydeligt med patienternes grad af komorbiditet. Bland de patienter der overlevede anastomoselækage endte 55 procent med permanent stomi. Derudover var der en øget risiko for tilbagefald af kræftsygdommen samt nedsat langtidsoverlevelse. Dette skyldtes primært forsinket eller udeladt efterbehandling med kemoterapi. Vi fandt ikke fundet nogen justerbare risikofaktorer for anastomoselækage.

Herefter undersøgte vi i 3 eksperimentelle artikler anastomoseheling i normal og udspilet tyktarm med det formål at identificeret muligheden for farmakologisk optimering. Udspilet tyktarm ses hos ca. 10-20 procent af patienter med nyopdaget tarmkræft. I den inflammatoriske fase af anastomosehelingen øges aktiviteten af matrix metalloproteinase (MMP), der nedbryder det stærke og bindende collagen. Derved mister anastomosen sin brudstyrke. Flere tidligere undersøgelser har antydet, at bredspektret blokering af disse MMP'er øger brudstyrken af anastomosen, men på bekostning af bivirkninger.

I de eksperimentelle dyremodeller, identificerede vi tre nøgle MMP'er, der blev kraftigt opreguleret i den ny-konstruerede anastomose. Efterfølgende selektiv blokering af MMP-8, 9 og 12 resulterede i en 29 procent stigning i anastomosens brudstyrke. Dernæst undersøgte vi de tidsafhængige biokemiske og morfologiske ændringer i tarmvæggen efter udspiling af tarmen. Vi fandt et hurtigt tab af collagen i tarmvæggen sammen med tilstrømning af inflammatoriske celler (neutrofiler granulocytter og makrofager). Den selektive blokering af MMP-8, 9 og 12 øgede også brudstyrken i anastomoser konstrueret i udspilet tyktarm. Ændringer i collagenniveau eller sammensætning var ikke korreleret med den øgede brudstyrke.

Konklusivt var forekomsten af anastomoselækage i undersøgelsesperioden høj og med en meget høj dødelighed, lang indlæggelsestid og risiko for tilbagefald af kræften. De dårlige langtidsresultater kunne forklares ved udeladt eller forsinket kemoterapi. Identificeringen af MMP-8, 9 og 12 som nøglemolekyler for anastomoseheling kan føre til fremtidig translationel forskning, der kombinerer viden om anastomoseheling med ny information om forholdet mellem MMP-9, tarmbakterier og anastomoselækage.

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15 STUDIES

Original article

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A nationwide study on anastomotic leakage after colonic cancer surgery

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Abstract

Aim Anastomotic leakage (AL) is a major challenge in colorectal cancer surgery due to increased morbidity and mortality. Possible risk factors should be investigated differentially, distinguishing between rectal and colonic surgery in large-scale studies to avoid selection bias and confounding.

Method The incidence and risk factors associated with AL were analysed in an unselected nationwide prospective cohort of patient subjected to curative colonic cancer surgery with primary anastomosis and entered into The Danish Colorectal Cancer Group database between May 2001 and December 2008.

Results AL occurred in 593 (6.4%) of 9333 patients. Laparoscopic surgery [odds ratio (OR) 1.34; 95% confidence interval (CI) 1.05–1.70; $P = 0.03$); left hemicolectomy (OR 2.02; 95% CI 1.50–2.72; $P = 0.01$) or sigmoid colectomy (OR 1.69; 95% CI 1.32–2.17; $P = 0.01$); intra-operative blood loss (OR 1.04; 95% CI 1.01–1.07; $P = 0.03$); blood transfusion (OR 10.27; 95% CI 6.82–15.45; $P < 0.001$) and male gender (OR 1.41; 95% CI 1.12–1.75; $P = 0.02$) were associated with AL in the multivariate analysis.

Conclusion The main finding that a laparoscopic approach was associated with an increased risk of AL should prompt close future monitoring. There was no evidence that centralization of surgery to high-volume hospitals reduced the rate of AL.

Keywords Anastomotic leakage, colon, risk factors, nationwide

What is new in this paper?

We have investigated the risk factors of anastomotic leakage following colonic cancer surgery in an unselected nationwide cohort prospectively entered into The Danish Colorectal Cancer Group database. Risk factors were laparoscopic approach, left and sigmoid colectomy, blood loss, blood transfusion and male gender.

Introduction

Anastomotic leakage (AL) is a major complication after colorectal surgery due to increased rates of morbidity, mortality and permanent stomas in survivors [1,2]. Most studies on AL are prone to selection bias and confounding due to small single-centre series and the inclusion of both colonic and rectal surgery [3,4]. It is essential to distinguish between colonic and rectal surgery, because the anatomy, surgical techniques and complication rates differ as reflected by a leakage rate of 11–12% after rectal surgery [5,6] compared with 3–4% after colonic surgery [1,3,4,7].

Several risk factors including age above 60 years [8], male gender [9,10], low serum albumin levels [3,11], transverse colectomy [12], emergency surgery [3,10], prolonged duration of surgery [4,11,13], increased intra-operative blood loss [11] and blood transfusion [3,8] have been associated with AL. These variables should be investigated prospectively in large-scale studies using multivariate statistics to identify specific risk factors for preoperative identification of high-risk patients.

In the present nationwide study on AL, we focused on patient, disease and treatment factors in a prospective 8-year cohort of patients who underwent curative surgery for colonic cancer.

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Method

Study population

All patients included in this study with a first-time diagnosis of colonic adenocarcinoma were prospectively entered into the Danish Colorectal Cancer Group (DCCG) database between May 2001 and December 2008. The DCCG database is approved by The Danish Data Protection Agency (ref. no. 2000-53-0073) and includes at least 95% of all Danish patients with colorectal cancer [6]. Patients below 18 years of age or without a Danish civil registration number were not registered in the database. All patients included in the study had a curative colonic resection with a primary anastomosis without a protecting stoma. Surgery on the left colon was categorized into left hemicolectomy for resection of tumours in the splenic flexure or descending colon with preservation of the superior haemorrhoidal artery and sigmoid colectomy. Patients with tumours at the recto-sigmoid junction and anastomoses below 15 cm from the anal verge were excluded (Fig. 1). The curative resection criterion required a colonic excisional specimen with at least 2 mm between the tumour and the circumferential resection margin and no distal disease.

The patient cohort was verified against the Danish Patient Register to ensure a perfect match with respect to date and type of surgery. In cases of discrepancy the patients were excluded.

Variables

The dependent variable, AL was defined according to the guidelines of the DCCG: 'Clinical symptoms suggesting AL and confirmed by contrast enema or CT scan'. Patients with AL were identified in the DCCG database or in the Danish Patient Register using the International Classification of Disease (ICD-10) for diagnosis and reoperation codes associated with AL (DT813A, KJWF00).

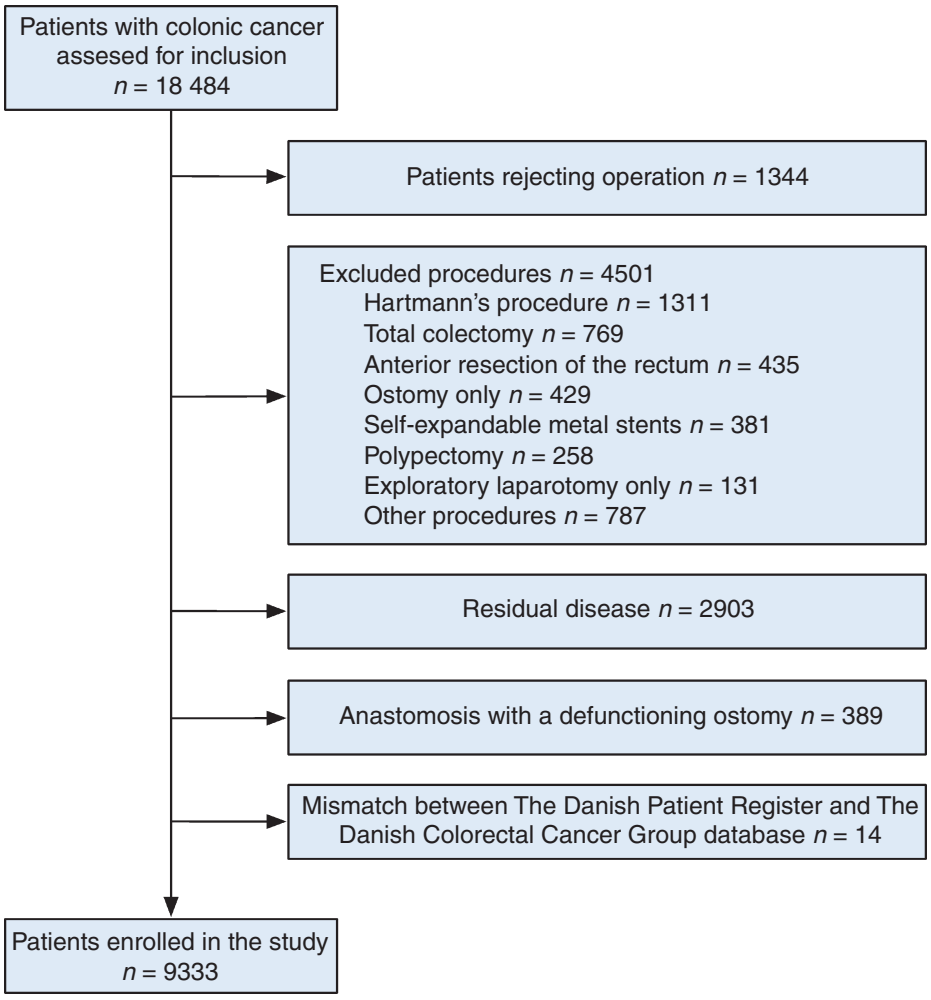

Figure 1 Inclusion chart.

Independent variables including patient demographics, comorbidity, tumour stage and surgical treatment were extracted from the database. During the study period there was an on-going centralization of colonic cancer surgery to fewer hospitals, thus hospital case volume was defined as the mean annual number of surgical procedures for colonic cancer. Laparoscopic colorectal surgery was introduced in Denmark in 2001. It was therefore investigated whether the rate of AL varied significantly throughout the study period to assess the potential impact of a laparoscopic learning curve. The timeframe for blood transfusion was from the day of surgery until discharge.

Statistics

Variables and their association to AL were investigated in univariate analyses using the chi-square test for categorical and the Mann–Whitney–Wilcoxon test for continuous variables, respectively. A logistic regression model with generalized estimating equations was used to examine the influence of several variables associated with the risk of AL. A statistical interaction between blood transfusion and intra-operative blood loss was included in all analyses.

The results of the multivariate analysis are presented as odds ratios (OR) with 95% confidence intervals (CI). All analyses were two-sided and considered statistically significant if the overall test (type III analysis) showed a *P*-value < 0.05.

Results

Data from 18 484 patients with colonic cancer were entered into the database. A total of 9333 (50.5%) patients remained in the study after exclusion of 9151 patients (Fig. 1). AL occurred in 593 patients (6.4%). The rate of AL was 257/4693 (5.5%), 14/250 (5.6%), 101/991 (10.2%) and 221/3399 (6.5%) after right, transverse, left and sigmoid colectomies, respectively.

At the beginning of the study period in 2001 colonic cancer surgery was performed in 48 hospitals compared with 28 hospitals in 2008. Accordingly, the average annual hospital case volume increased from 23 in 2001 to 43 in 2008. Centralization of surgery to fewer hospitals did not reduce the overall rate of AL, and there was no association between hospital case volume and AL (Fig. 2). At the same time, the rate of laparoscopic

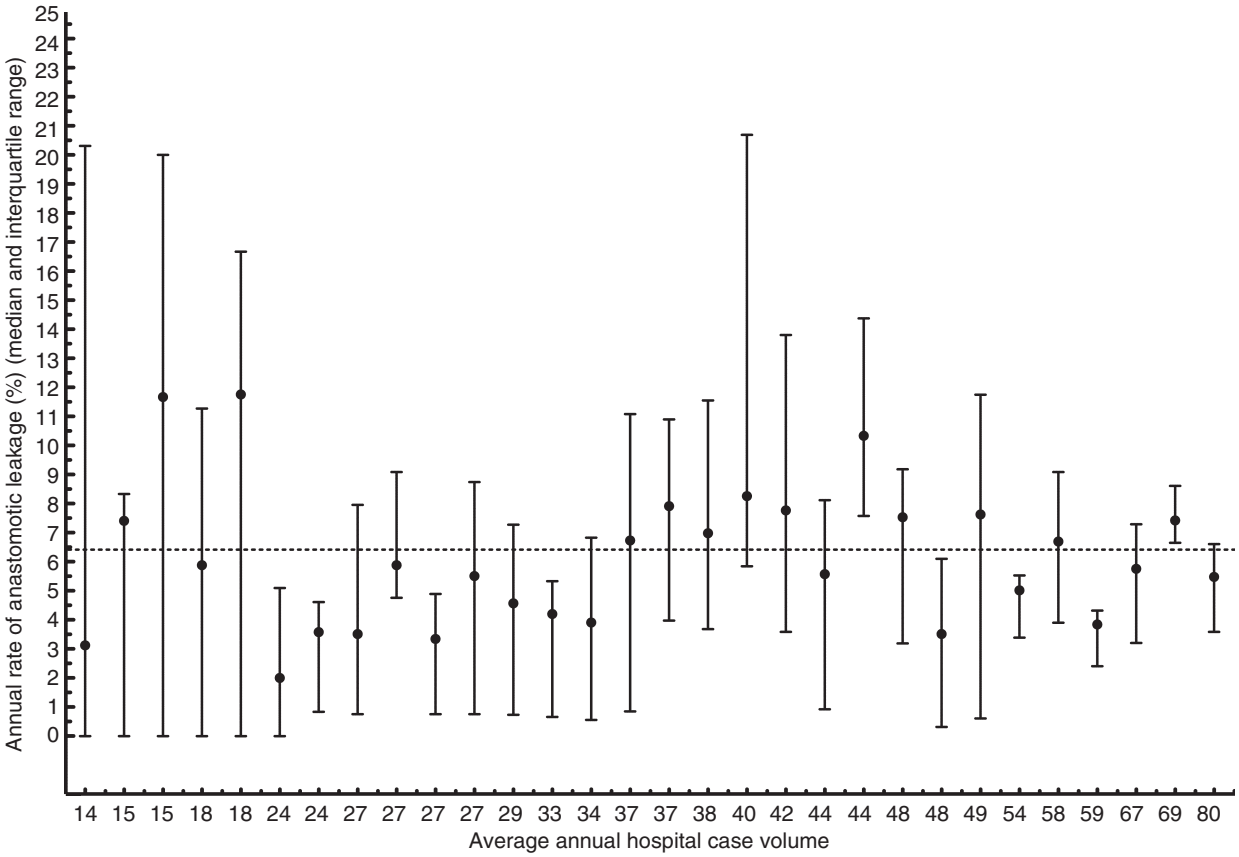

Figure 2 Anastomotic leakage according to hospital case volume, 2001–2008. Hospitals with a case volume of more than one operation per month (n = 8934, 96%). The horizontal line represents the overall anastomotic leakage rate of 6.4%.

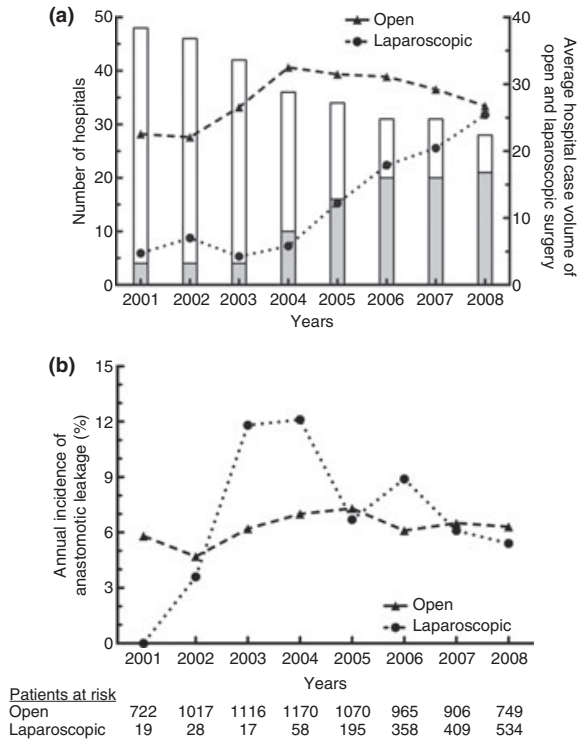

Figure 3 Number of hospitals, average case volume and incidence of anastomotic leakage (AL) for the period 2001–2008. (a) Annual number of hospitals performing open (white bars) and laparoscopic (grey bars) surgery for colonic cancer and the annual average case volume of open and laparoscopic surgery. (b) Annual incidence of AL according to open and laparoscopic surgery. There was no difference between the annual rates of AL in patients undergoing laparoscopic surgery ($P = 0.32$).

surgery increased from 2% in 2001 to 41% in 2008, accordingly the average annual number of laparoscopic procedures increased from five in 2001 to 24 per hospital in 2008 (Fig. 3a). The laparoscopic approach was implemented in 25 different hospitals during the study period, and in 2008 22 of the 28 operating hospitals performed laparoscopic surgery for colonic cancer (Fig. 3a). There was no statistically significant difference between the annual rates of AL in patients undergoing laparoscopic surgery ($P = 0.32$) (Fig. 3b).

Table 1 shows the outcome of the univariate analyses of variables and their association with AL. The variables that reached statistical significance were male gender, high American Society of Anesthesiologists (ASA) score, left hemicolectomy or sigmoid colectomy, splenectomy, intra-operative blood loss and blood transfusion. The multivariate analysis demonstrated that left hemicolectomy or sigmoid colectomy, operative blood loss, blood transfusion and male gender remained independent risk factors for AL (Table 2). In addition, laparoscopic surgery and decreasing age reached statistical significance in the multivariate analysis, associating these factors with an increased risk of AL. There was no significant association between AL and hospital case volume ($P = 0.07$) or tumour stage ($P = 0.14$). Of the 593 patients who developed AL, 124 (20.9%) died within 30 days compared with 400 of 8735 patients (4.6%) without AL ($P < 0.001$). Five patients were lost to follow-up.

Discussion

This is the first nationwide study on AL after colonic cancer surgery yielding the following variables significantly predictive for AL: laparoscopic surgery, intra-operative blood loss, blood transfusion, left hemicolectomy or sigmoid colectomy, male gender and decreasing age. The 6.4% leakage rate after colonic cancer surgery is relatively high compared with the 3–4% rates reported in previous studies [1,3,4,7]. This reflects the complication rate in an unselected national cohort characterized by implementation of laparoscopic surgery and a certain latency in the centralization of surgical procedures.

The striking result, that laparoscopic surgery for colonic cancer was associated with an increased risk of AL, has not previously been demonstrated. A reason for this finding could be the prolonged duration of surgery associated with laparoscopic colectomies compared with open surgery [14]. Previous studies demonstrated that an operating time longer than 200–240 min increased the risk of AL [11,13]; however, these data were not available for the present study. Increased risk of AL in stapled anastomoses using multiple firing has also been reported [15], but data on anastomotic technique were not registered in the present study. Randomized clinical trials have compared the laparoscopic approach with open surgery for colonic cancer and reported equal rates of AL [14,16]. While these studies implied selection of surgeons and hospitals, laparoscopic surgery was also performed in low-volume hospitals in the present national study, and the results are therefore not comparable.

Laparoscopic surgery for colonic cancer was implemented in Denmark without national guidelines. Individual hospitals, including low-volume units, could thus apply this new technique. This could explain the increased risk of AL as low-volume hospitals did not have the case volume needed to complete the learning curve for laparoscopic colectomies [17]. However, the rate of AL did not change significantly with time, probably due to low patient numbers in the laparoscopic group during the beginning of the study period. Selection of low-risk patients could also hide the true impact of the implementation of laparoscopic colonic surgery on the rate of AL. This selection may also account for the

Table I Patient characteristics and univariate analyses of possible factors associated with anastomotic leakage in 9333 patients undergoing colonic cancer surgery with primary anastomosis.

	Patients with anastomotic leakage n = 593 (6.4%)	Patients without anastomotic leakage n = 8740 (93.6%)	P
Age (years), median (range)	72 (36–94)	72 (23–99)	0.99
Gender			
Female	246/593 (41.5%)	4598/8740 (52.6%)	< 0.001
Male	347/593 (58.5%)	4142/8740 (47.4%)	
ASA score			
ASA I	91/569 (16.0%)	1967/8341 (23.6%)	< 0.001
ASA II	282/569 (49.6%)	4 464/8341 (53.5%)	
ASA III–V	196/569 (34.4%)	1 910/8341 (22.9%)	
Tumour stage			
UICC I	93/432 (21.5%)	1095/5949 (18.4%)	0.17
UICC II	238/432 (55.1%)	3286/5949 (55.2%)	
UICC III	101/432 (23.4%)	1568/5949 (26.4%)	
Surgical procedure			
Right hemicolectomy	257/593 (43.3%)	4436/8740 (50.8%)	< 0.001
Transverse colectomy	14/593 (2.4%)	236/8740 (2.7%)	
Left hemicolectomy	101/593 (17.0%)	890/8740 (10.2%)	
Sigmoid colectomy	221/593 (37.3%)	3178/8740 (36.4%)	
Surgical approach			
Laparotomy	484/593 (81.6%)	7231/8740 (82.7%)	0.49
Laparoscopy	109/593 (18.4%)	1509/8740 (17.3%)	
Surgical priority			
Elective	528/586 (90.1%)	7920/8593 (92.2%)	0.08
Emergency	58/586 (9.9%)	673/8593 (7.8%)	
Specialization of the surgeon			
Gastrointestinal specialist	440/593 (74.2%)	6362/8738 (72.8%)	0.46
General surgeon	153/593 (25.8%)	2376/8738 (27.2%)	
Hospital case volume, median (range)	50 (5–91)	50 (1–91)	0.27
Resection of nearby organ			
No resection	520/592 (87.8%)	7710/8739 (88.2%)	0.01
Spleen	14/592 (2.4%)	86/8739 (1.0%)	
Other organs	58/592 (9.8%)	943/8739 (10.8%)	
Operative blood loss (ml), median (range)	250 (0–5500)	200 (0–8000)	< 0.001
Perioperative blood transfusion			
No	211/578 (36.5%)	6768/8672 (78.0%)	< 0.001
Yes	367/578 (63.5%)	1904/8672 (22.0%)	

ASA, American Society of Anesthesiologists; UICC, International Union Against Cancer.

statistically insignificant finding in the univariate analysis. Therefore it cannot be concluded that the laparoscopic approach in general increases the risk of AL, but these results prompt future monitoring. A more pronounced impact of laparoscopy may also have been balanced by a positive effect of centralization.

Low hospital case volume was not associated with AL in the present study, whereas data on surgeon case volume were unavailable. Surgeon case volume has previously been related to AL [18]. Moreover, studies have shown an increase in 30-day mortality and a reduced overall survival after operation for colonic cancer in low-volume hospitals [19,20]. In other studies, this conclusion lost statistical significance after adjustment for patient characteristics [21]. The quality of colonic cancer surgery with respect to surgical complications, 30-day mortality, long-term mortality and cancer recurrence should probably be monitored on a surgeon-dependent basis, rather than hospital case volume.

Operative blood loss and perioperative blood transfusion were independently associated with increased risk of AL [6,8,22]. The time of administration and number of units transfused were not recorded in the DCCG database, and could thus have been given at the time of

Table 2 Multivariate analysis of possible factors associated with anastomotic leakage examined by logistic regression with generalized estimating equations.

	Adjusted OR OR (95% CI)	*P*
Age (per year)	0.99 (0.98–0.99)	0.01
Gender		
Female	1.00	0.02
Male	1.41 (1.12–1.75)	
ASA score		
ASA I	1.00	0.19
ASA II	1.14 (0.84–1.55)	
ASA III–V	1.41 (0.95–2.08)	
Tumour stage		
UICC I	1.00	0.14
UICC II	0.76 (0.59–0.98)	
UICC III	0.72 (0.53–0.97)	
Surgical procedure		
Right hemicolectomy	1.00	0.01
Transverse colectomy	1.15 (0.55–2.37)	
Left hemicolectomy	2.02 (1.50–2.72)	
Sigmoid colectomy	1.69 (1.32–2.17)	
Surgical approach		
Laparotomy	1.00	0.03
Laparoscopy	1.34 (1.05–1.70)	
Surgical priority		
Elective	1.00	0.68
Emergency	1.09 (0.74–1.60)	
Specialization of the surgeon		
Gastrointestinal specialist	1.00	0.25
General surgeon	0.81 (0.56–1.17)	
Hospital case volume (per 10 operations)	1.09 (1.00–1.18)	0.07
Resection of nearby organ		
No resection	1.00	0.27
Spleen	0.94 (0.42–2.07)	
Other organs	0.75 (0.53–1.05)	
Operative blood loss (per 100 ml)	1.04 (1.01–1.07)	0.03
Perioperative blood transfusion		
No	1.00	< 0.001
Yes	10.27 (6.82–15.45)	

ASA, American Society of Anesthesiologists; UICC, International Union Against Cancer.

a reoperation following AL. Blood loss may induce ischaemia at the resection line and hence impair anastomotic healing, while blood transfusion could induce immunological suppression increasing the risk of severe postoperative infection including AL [23,24].

Left hemicolectomy or sigmoid colectomy were associated with an increased risk of AL as reported by other groups [12,25]. Impairment in tissue oxygenation, in the case of central ligation of the inferior mesenteric artery, has indicated that the sigmoid resection margins are insufficiently perfused by the marginal artery [26]. Moreover, the vasa recta are spaced further apart and with fewer collaterals in the area from the splenic flexure to the mid descending colon, providing evidence of vascular differences that may lead to an increased risk of AL [27]. A meticulous dissection aiming to preserve the blood supply seems important, especially on the left colon.

Male gender was associated with an increased risk of AL in agreement with previous reports [9]. The difficulties of surgery in the narrow male pelvis have been associated with increased risk of AL in rectal cancer surgery [28], but this factor is irrelevant in the present cohort of colonic cancer patients.

Increasing age was correlated to a lower risk of AL, in contrast to the findings of others [8]. The result may not be clinically relevant, however, as selection bias could account for this occurrence if elderly patients tend to reject operation or are subjected to the safe approach of permanent ostomy instead of a primary anastomosis. Selection may also account for the fact that emergency procedures and patients with a high ASA score were not associated with AL in the present study, opposing the findings by others [3,4,10,22,29].

In agreement with other studies AL increased the 30-day mortality significantly [1] but the true impact should be investigated in multivariate analyses, because mortality may be confounded by factors such as age and lifestyle.

In conclusion, the 6.4% incidence merely reflects the rate of AL in an unselected nationwide cohort. Laparoscopic surgery increased the risk for AL, which should prompt close future monitoring and considerations. Colonic cancer surgery in men, on the left part of the colon, operative blood loss and blood transfusion increased AL and there was no evidence that centralization of surgery to high-volume hospitals reduced the rate of AL.

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ORIGINAL CONTRIBUTION

Association of Comorbidity with Anastomotic Leak, 30-day Mortality, and Length of Stay in Elective Surgery for Colonic Cancer: A Nationwide Cohort Study

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BACKGROUND: Comorbidity has a negative influence on the long-term prognosis in patients with colorectal cancer, whereas its impact on the postoperative course is less clear.

OBJECTIVES: The aim of this study was to investigate the influence of comorbidity on anastomotic leak and short-term outcomes after resection for colonic cancer.

DESIGN: This is a retrospective nationwide cohort study

SETTING: Data were obtained from the Danish Colorectal Cancer Group and the National Patient Registry.

PATIENTS: Patients with colonic cancer undergoing elective resection between 2001 and 2008 were selected.

MAIN OUTCOME MEASURES: The primary outcome was the ability of comorbidity to predict anastomotic leak. Secondary outcomes were 30-day mortality and length of stay. Comorbidity was assessed by the Charlson Comorbidity Index. Multivariable logistic regression and receiver operating characteristics curves were used to adjust for confounding.

RESULTS: The rate of anastomotic leak was 535/8597 (6.2%). The mean (95% CI) Charlson score was 0.83 (0.72–0.94) and 0.63 (0.61–0.66) for patients with and without anastomotic leak, $p < 0.001$. The Charlson score, as assessed in the multivariable analysis (adjusted OR, 1.07; 95% CI, 0.99–1.15; $p = 0.077$) and by receiver operating characteristics curves (area under the curve = 0.548), failed to predict anastomotic leak. Thirty-day mortality was 425/8587 (4.9%). In patients with anastomotic leakage, a Charlson score of ≥2 was associated with increased mortality in comparison with a Charlson score of <2 (adjusted HR, 1.58; 95% CI, 1.00–2.51; $p = 0.047$). Mean length of stay was 8.7 days (95% CI, 8.4–9.2 days) for patients without an anastomotic leak in comparison with 23.3 days (95% CI, 21.5–25.1 days) for patients with anastomotic leak and 25.5 days (95% CI, 21.7–29.3 days) in patients with anastomotic leak and a Charlson score of >2, $p < 0.001$.

LIMITATIONS: This study is limited by the accuracy of the coding used to generate the Charlson Comorbidity Index and the retrospective study design.

CONCLUSION: Comorbidity failed to predict anastomotic leak, but it was associated with an inferior short-term outcome in patients with this surgical complication.

KEY WORDS: Comorbidity; Anastomotic leak; Colon cancer; Charlson Comorbidity Index; Mortality; Length of stay.

Comorbidity has a negative influence on the prognosis for patients with colorectal cancer.[1,2] It is less clear if the impaired outcome in patients with advanced comorbidity is caused by an increased rate of early postoperative complications such as anastomotic

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Table 1. Disease conditions used to calculate the Charlson Comorbidity Index, their weight and ICD-10 codes

Disease condition	*Weight*	*ICD-10 codes*
Acute myocardial infarction	1	I21, I22, I23
Congestive heart failure	1	I50, I110, I130, I132
Peripheral vascular disease	1	I70, I71, I72, I73, I74, I77
Cerebral vascular accident	1	I60, I61, I62, I63, I64, I65, I66, I67, I68, I69,G45, G46
Dementia	1	F00, F04, F02, F051, G30
Pulmonary disease	1	J40, J41, J42, J44, J43, J45, J46, J47, J60, J61, J62, J63, J64, J65, J66, J67, J684, J701, J703, J841, J920, J961, J982, J983
Connective tissue disorder	1	M05, M06, M08, M09, M30, M31, M32, M33, M34, M35, M36, D86
Peptic ulcer	1	K221, K25, K26, K27, K28
Mild liver disease	1	B18, K700, K701, K702, K703, K709, K71, K73, K74, K760
Diabetes mellitus	1	E100, E101, E109, E110, E111, E119
Paraplegia	2	G81 G82
Moderate to severe renal disease	2	I12, I13, N0, N01, N02, N03, N04, N05, N06, N07, N11, N14, N17, N18, N19, Q61
Diabetes complications	2	E102, E103, E104, E105, E107, E108, E109, E112, E113, E114 E115, E116, E117, E118
Malignant tumor	2	C0, C1, C2, C3, C4, C5, C6, C70, C71, C72, C73, C74, C75
Leukemia	2	C91, C92, C93, C94, C95
Lymphoma	2	C81, C82, C83, C84, C85, C88, C90, C96
Severe liver disease	3	B150, B160, B162, B190, K704
Metastatic solid tumor	3	C76, C77, C78, C79, C80
AIDS	6	B21, B22, B23, B24

ICD-10 codes correspond with the ICD-10 classification adopted by the Danish National Board of Health.
ICD-10 = *International Classification of Diseases, 10th Revision*.

leak (AL). Previous data on the relationship between AL and comorbidity are contradictory. Several studies have reported associations between the ASA score and the risk of AL,[3–7] whereas others found no association.[8–10] This divergence is hardly surprising given the fact that the ASA physical status classification was not intended for the prediction of perioperative risk.[11] Prediction of AL is desirable, because patients could potentially be allocated to a tailored surgical treatment, advising against primary anastomosis in high-risk patients to avoid the immense risk of short-term mortality and inferior oncological outcome following AL.[12,13] The Charlson Comorbidity Index (CCI) is a grading system reflecting the cumulative likelihood of 1-year mortality and was originally intended for use in longitudinal studies.[14] The CCI is based on 19 disease conditions, each assigned with a weighed score. A recent study used both the ASA and the CCI system to investigate the association between comorbidity and AL.[15] The authors found no association between CCI and AL, whereas a high ASA score was associated with an increased risk of AL.[15] Although the results were adjusted for age and sex, other well-known risk factors associated with AL such as emergency surgery,[16,17] intraoperative blood loss,[9,10] and blood transfusion[16,18] were not accounted for. Telem et al[9] reported no significant relationship between AL and individual disease conditions including coronary, renal, and liver disease. Trencheva et al[19] investigated CCI as a continuous variable and found no significant association with AL. However, the use of a CCI cutoff score of 3 showed a significant association between comorbidity and AL. Interestingly, the ASA score was not associated with AL in these studies.[9,19] Recent data from the Dutch Surgical Colorectal Audit suggest that the AL rate is not increased in patients with comorbidity.[7] However, most reports were based on single-center studies with limited external validity, and it is difficult to obtain sufficient statistical power from these studies because of their size limitation. The aim of this large-scale nationwide study was to investigate the value of comorbidity in the prediction of AL following curative resection for colonic cancer. In addition, we investigated the impact of comorbidity, in patients with AL, on 30-day mortality and length of hospital stay (LOS).

METHODS

This nationwide cohort study was based on prospectively registered data from the Danish Colorectal Cancer Group (DCCG) merged with data from the National Patient Registry, using the unique personal identification number given to all Danish citizens. The primary outcome was prediction of AL based on CCI stratification. Secondary outcomes were AL prediction of each disease condition of the CCI and the impact of CCI scores on 30-day mortality and LOS in patients with AL. The study population has been described in detail previously.[10] In brief, patients with a first-time diagnosis of colonic adenocarcinoma subjected to curative resection between May 2001 and December 2008 were included. In the present study, all patients underwent elective colon cancer surgery with primary anastomosis without a protective stoma. The curative resection criteria required at least 2 mm from the tumor to the nonperitonealized resection margin at the mesenteric site, as evaluated microscopically, and with no tumor growth or distant disease left after completed surgery. Trained pa-

Table 2. Patient characteristics and data completeness

Variables	Study population, n = 8597	Missing cases, n (%)
Age, y		0 (0)
Median (range)	72.0 (25–99)	
Mean (SD)	71.1 (11.0)	
Sex		0 (0)
Female	4436 (51.6)	
Male	4161 (48.4)	
Charlson Comorbidity index, mean (SD)	0.64 (1.17)	149 (1.7)
Tumor stage		347 (4.0)
UICC I	1338 (15.6)	
UICC II	4119 (47.9)	
UICC III	2793 (32.5)	
Surgical procedure		0 (0)
Right hemicolectomy	4145 (48)	
Transverse colectomy	228 (2.7)	
Left hemicolectomy	919 (10.7)	
Sigmoid colectomy	3325 (38.7)	
Surgical approach		0 (0)
Open	6993 (81.3)	
Laparoscopic	1604 (18.7)	
Surgeons' specialization		2 (<1)
GI	6317 (73.5)	
General	2278 (26.5)	
Organ resection		2 (<1)
No	7591 (88.3)	
Spleen	90 (1.0)	
Other organs	914 (10.6)	
Blood loss (mL)		354 (4.1)
Median (range)	200 (0–8000)	
Mean (SD)	307 (443)	
Blood transfusion		75 (0.9)
No	6507 (75.7)	
Yes	2015 (23.4)	
Anastomotic leak		0 (0)
No	8062 (93.8)	
Yes	535 (6.2)	
30-day mortality		0 (0)
No	8172 (95.1)	
Yes	425 (4.9)	
Length of hospital stay (days)		326 (3.8)
Median (range)	8.0 (1.0–181.0)	
Mean (SD)	9.7 (8.8)	

Values in parentheses are percentages unless indicated otherwise.
UICC = Union for International Cancer Control.

thologists evaluated all excisional specimens according to the guidelines of the DCCG. The proximal and distal resection margins were examined microscopically when the distance from the tumor to these margins was less than 20 mm. The dependent variable, AL, was defined as "Clinical symptoms suggesting AL and confirmed by contrast enema, computerized tomography or surgery" within 60 days from the operation. Patients with AL were identified in the DCCG database or the National Patient Registry by using the codes of the *International Classification of Diseases, 10th Revision* (ICD-10) for diagnosis and reoperation associated with AL (DT813A, KJWF00). Confounding variables, previously shown to be associated with AL in this cohort,[10] were extracted from the DCCG database and included age, sex, surgical procedure, surgical approach, blood loss, and blood transfusion. Surgical approach was defined as open or laparoscopic. Data on conversion were not available; therefore, converted cases were classified as open, and only completed laparoscopic or laparoscopic hand-assisted cases were considered laparoscopic. Additional variables including tumor stage, surgical priority, surgeons' specialization, and resection of neighboring organs were included in the analyses of 30-day mortality and LOS. Comorbidity was assessed according to the 19 disease items constituting the CCI.[14] Associations between the cumulative burden (CCI score) of comorbidity, as well as each disease condition and AL were investigated. Data on comorbidity were extracted from the National Patient Registry by using a validated syntax based on ICD-10 codes.[20] This method has demonstrated a consistently high positive predictive value.[21] The CCI was included as a continuous variable as originally proposed,[14] but because the distribution of CCI scores was right-skewed, a cutoff value of ≥2 was used to stratify subsequent analyses on 30-day mortality and LOS.[22] The data on noncolorectal malignant tumors, leukemia, and lymphoma were grouped as *noncolorectal malignancy* for subsequent analyses excluding the weight of colonic adenocarcinoma.[20] The weight for calculation of the CCI and ICD-10 codes for each disease condition is shown in Table 1. Information on 30-day mortality and LOS was extracted from the National Patient Registry. Length of stay was defined as the number of days at the hospital during the primary admission. For patients dying within 30 days, time to death was used as LOS. None of these patients were discharged before the event of death.

Statistics

The association between CCI and AL was investigated by using χ^2 and Mann-Whitney or *t* tests for categorical and continuous variables.[23] Disease conditions with $p < 0.1$ were included in a multivariable logistic regression analysis adjusting for the confounding variables (age, sex, surgical procedure, surgical approach, blood loss, and blood transfusion).[10] A significant interaction between blood loss and blood transfusion was included in all multivariable analyses. Four models were applied to investigate the predictive power of comorbidity on the occurrence of AL: model I, age, sex, surgical procedure, surgical approach, blood loss, and blood transfusion; model II, CCI alone; model III, model I + model II; model IV, model I + disease conditions ($p < 0.1$ in univariable analyses). The goodness-of-fit was evaluated for each model by using the Hosmer-Lemeshow test.[24] Receiver operating characteristics curves were created for each model by using the predicted probabilities from the logistic regression analyses.[25] The area under the curve (AUC) was applied to determine the prediction

Table 3. Univariable analyses of the association between the Charlson Comorbidity Index or disease conditions with anastomotic leak

Characteristic	*Anastomotic leak*		*OR*	*(95% CI)*	*p*
Charlson Comorbidity Index, mean (SD)[a]	0.83	(1.28)	1.13	(1.06–1.21)	<0.001
Charlson Comorbidity Index score					<0.001
<2	404/7003	(5.8)	1.00		
≥2	125/1445	(8.7)	1.55	(1.26–1.91)	
Acute myocardial infarction					0.865
No	512/8187	(6.3)	1.00		
Yes	17/261	(6.5)	1.04	(0.63–1.72)	
Congestive heart failure					0.080
No	494/8025	(6.2)	1.00		
Yes	35/423	(8.3)	1.38	(0.96–1.97)	
Peripheral vascular disease					0.037
No	509/8243	(6.2)	1.00		
Yes	20/205	(9.8)	1.64	(1.03–2.63)	
Cerebral vascular accident					0.001
No	493/8112	(6.1)	1.00		
Yes	36/336	(10.7)	1.86	(1.30–2.65)	
Dementia					0.325
No	528/9408	(6.3)	1.00		
Yes	1/40	(2.5)	0.38	(0.05–2.79)	
Pulmonary disease					0.116
No	478/7784	(6.1)	1.00		
Yes	51/664	(7.7)	1.27	(0.94–1.72)	
Connective tissue disorder					0.276
No	513/8251	(6.2)	1.00		
Yes	16/197	(8.1)	1.33	(0.79–2.24)	
Peptic ulcer					0.032
No	498/8104	(6.1)	1.00		
Yes	31/344	(9.0)	1.51	(1.03–2.21)	
Mild liver disease					0.008
No	521/8395	(6.2)	1.00		
Yes	8/53	(15.1)	2.69	(1.26–5.73)	
Diabetes mellitus					0.744
No	487/7808	(6.2)	1.00		
Yes	42/640	(6.6)	1.06	(0.76–1.46)	
Diabetes mellitus with complications					0.955
No	513/8189	(6.3)	1.00		
Yes	16/259	(6.2)	0.99	(0.59–1.65)	
Paraplegia					0.381
No	528/8441	(6.3)	1.00		
Yes	1/7	(14.3)	2.50	(0.30–20.79)	
Moderate to severe renal disease					<0.001
No	505/8283	(6.1)	1.00		
Yes	24/165	(14.5)	2.62	(1.69–4.08)	
Noncolorectal malignancy					0.564
No	488/7846	(6.2)	1.00		
Yes	41/602	(6.8)	1.10	(0.79–1.53)	
Noncolorectal metastatic solid tumor					0.642
No	526/8086	(6.3)	1.00		
Yes	3/62	(4.8)	0.76	(0.24–2.43)	
Severe liver disease					0.966
No	528/8434	(6.3)	1.00		
Yes	1/14	(7.1)	1.15	(0.15–8.82)	
AIDS					0.053
No	528/8445	(6.4)	1.00		
Yes	1/3	(33.3)	7.50	(0.68–82.81)	

Values in parentheses are percentages unless indicated otherwise. All analyses were χ^2 tests, with the exception of the Charlson Comorbidity Index.
[a]*t* test.

level of AL. The ability to predict AL was defined as excellent (AUC, 0.90–1.00), good (0.80–0.89), fair (0.70–0.79), poor (0.60–0.69), and failed (0.50–0.59).[26] The impact of comorbidity on 30-day mortality and LOS in patients with AL was investigated by using multivariable Cox regression and multiple linear regression analyses. Anastomotic leak

was entered as a time-dependent variable in the Cox model. Missing values were disregarded and thus not replaced. The number and percentage of missing values are provided in Table 2. All analyses were 2-sided and considered statistically significant if $p < 0.05$. Data were analyzed with SPSS Statistics Version 20 (IBM Corp, Armonk, NY) and results presented as OR or HR with 95% CI.

RESULTS

A total of 8597 patients were included in the study. The incidence of AL was 6.2% (535 patients). Patient characteristics are shown in Table 2. The mean CCI score was 0.83 (95% CI, 0.72–0.94) in patients with AL compared with 0.63 (95% CI, 0.61–0.66) in patients without AL ($p < 0.001$) corresponding to an OR of 1.13 (95% CI, 1.06–1.21) per CCI score point. After the adjustment for risk factors (age, sex, procedure, approach, blood loss, and blood transfusion) the association between AL and CCI lost significance (OR, 1.07; 95% CI, 0.99–1.15; $p = 0.077$). With the use of a CCI cutoff score of 2, a significant association between CCI and AL was demonstrated in the multivariable analysis (OR, 1.33; 95% CI, 1.06–1.66; $p = 0.016$). The dominating comorbidities were pulmonary disease, diabetes mellitus, noncolorectal malignancy, and congestive heart failure. Disease conditions associated with AL in the univariable analyses are shown in Table 3. After adjustment for preexisting risk factors for AL, moderate to severe renal disease (OR, 1.68; 95% CI, 1.02 to 2.78; $p = 0.044$) remained significantly associated with AL (Table 4). Receiver operating characteristics curves were created for each of the 4 models (Fig. 1). Model I, including the factors age, sex, procedure, surgical approach (open/laparoscopic), blood loss, and blood transfusion, displayed an AUC of 0.745. The remaining models II, III, and IV did not increase the level of prediction (Fig. 1).

Thirty-Day Mortality

The 30-day mortality rate was 109/535 (20.4%) in patients with AL compared with 316/8062 (3.9%) in patients without AL, $p < 0.001$. In patients without AL, the mortality rate was 112/1320 (8.5%) for patients with CCI scores ≥2 compared with 194/6599 (2.9%) in patients with a CCI score <2, $p < 0.001$. Considering comorbidity in patients with AL, 30-day mortality increased to 37/125 (29.6%) in patients with a CCI score ≥2 compared with 72/404 (17.8%) in patients with CCI <2, $p < 0.001$. Adjustment for confounding variables confirmed that a CCI score ≥2 was associated with increased mortality in patients without AL (HR, 2.75; 95% CI, 2.14–3.54) compared with patients with CCI <2, $p < 0.001$. AL was associated with further increases in mortality in patients with CCI <2 (HR, 6.24; 95% CI, 4.56–8.53), and in patients with a CCI score ≥ 2 (HR, 7.48; 95% CI, 4.99–11.19), compared with no-leak patients without comorbidity, $p < 0.001$ (Table 5).

Table 4. Multivariable logistic regression analysis of disease conditions associated with anastomotic leak after elective resection for colonic cancer

Disease condition	*OR*	*(95% CI)*	*p*
Congestive heart failure	1.16	(0.79–1.71)	0.439
Peripheral vascular disease	1.16	(0.69–1.97)	0.575
Cerebral vascular accident	1.45	(0.96–2.19)	0.075
Peptic ulcer	1.11	(0.73–1.68)	0.639
Liver disease	1.43	(0.63–3.24)	0.392
Moderate to severe renal disease	1.68	(1.02–2.78)	0.044
AIDS	6.44	(0.46–89.91)	0.166

The model (n = 8046) included disease conditions from the univariable analyses (Table 3) with $p < 0.1$ and was adjusted for age, sex, surgical procedure, surgical approach, blood loss, and blood transfusion.

This corresponds to an increase in mortality of HR of 1.58 (95% CI, 1.00–2.51; $p = 0.047$) for patients with AL.

Length of Hospital Stay

Overall LOS was 9.7 days (95% CI, 9.5–9.9 days). In patients without AL and CCI <2, the average LOS was 8.7 days (95% CI, 8.5–8.9 days). In comparison, comorbidity (CCI ≥2) in non-AL patients was not significantly associated with changes in LOS (8.8 days; 95% CI, 8.5–9.2 days; $p = 0.981$). In patients with AL, mean LOS increased to 25.5 days (95% CI, 21.7–29.3 days) in patients with CCI ≥2, compared with 23.3 days (95% CI, 21.5–25.1 days) in patients with CCI <2, $p = 0.046$. Multiple linear regression analysis confirmed that, in patients without AL, LOS was not significantly different between patients with CCI ≥2 and patients with CCI <2 (reference group) (Table 5). In patients with AL, adjusted LOS increased by 12.9 days (95% CI, 12.2–13.7 days) in patients with limited comorbidity (CCI <2) compared with the reference group, $p < 0.001$. For patients with a CCI ≥2, the adjusted increase in LOS was 15.0 days (95% CI, 13.6–16.4 days) compared with the reference group, $p = 0.001$ (Table 5).

DISCUSSION

This nationwide study on patients undergoing elective resection for colonic cancer demonstrated that comorbidity failed to predict AL. However, comorbidity was associated with a huge impact on 30-day mortality and LOS in patients with AL. The cumulative burden of comorbidity was assessed by the CCI originally designed for use in longitudinal studies such as the present study. The CCI score is a continuous scale ranging from 0 to 34. With the use of this score there was no significant association between comorbidity and AL after adjustment for confounding variables. However, using a cutoff CCI score of 2 resulted in a significant in risk of AL in the multivariable analysis. This finding is in contrast to a recent Dutch study where CCI score ≥2 was not associated with AL.[7] Interestingly the cohorts were very similar with regard of the number of patients with a

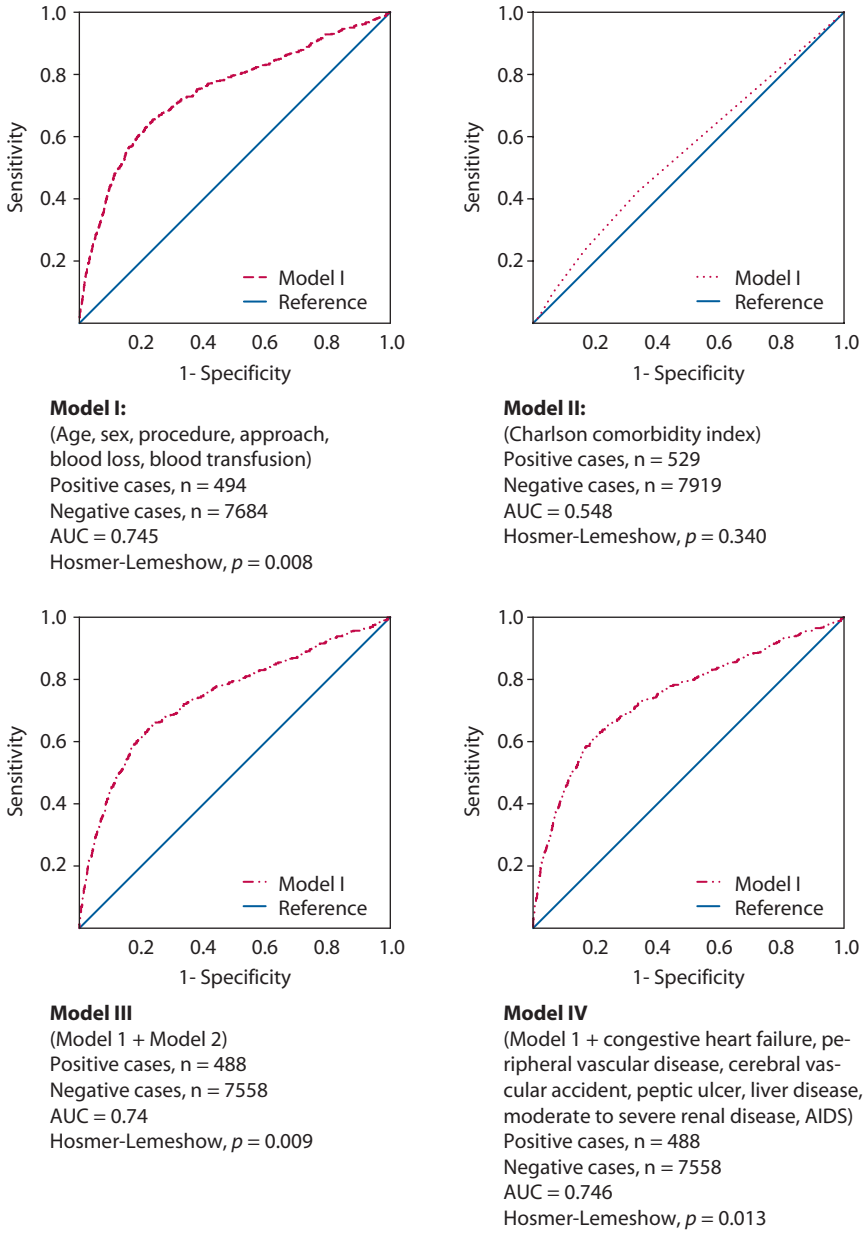

Figure 1. Receiver operating characteristics (ROC) curves for 4 models of anastomotic leak prediction based on predicted probabilities from logistic regression models including the Hosmer-Lemeshow tests. The ability to predict AL was defined as excellent (AUC, 0.90–1.00), good (0.80–0.89), fair (0.70–0.79), poor (0.60–0.69), and failed (0.50–0.59).[26] Model I, age, sex, surgical procedure, surgical approach, blood loss, and blood transfusion; model II, Charlson comorbidity index scores; model III, model I + model II; model IV, model I + disease conditions with $p < 0.1$ (congestive heart failure, peripheral vascular disease, cerebral vascular accident, peptic ulcer, liver disease, renal disease, AIDS). AL = anastomotic leak; AUC = area under the curve.

CCI score ≥2. In addition, neither of the studies adjusted for lifestyle factors such as smoking, which is a known risk factor for both AL and comorbidity. In the present study, the logistic regression analysis for model II (CCI alone) resulted in a proper fit of the model as evaluated with the Hosmer-Lemeshow test. Even so, CCI scores were an inadequate predictor for AL, as indicated by the area under the receiver operating characteristics curve. Combining known risk factors (model I) and CCI scores (model II) did not increase the power to predict AL in comparison with model I, suggesting that additional or other variables are required to predict AL. A recent study including the parameters sex, neoadjuvant therapy, anastomoses below 10 cm from the anus, ligation and level of ligation of the inferior mesenteric artery, intraoperative complications, and CCI scores, reported a good prediction of AL, AUC = 0.807.[19] Several of these factors are specific for rectal resection and do not apply to the present study of patients with colonic cancer. Inclusion of other factors such as smoking, nutritional status, and frailty[27] may have the potential to improve the

Table 5. Multivariable analyses of variables associated with 30-day mortality and length of hospital stay following elective resection for colonic cancer

	30-day mortality[a]			*Hospital stay, days*[b]		
Variable	*HR*	*(95% CI)*	*p*	*Estimated coefficient*	*(95% CI)*	*p*
Anastomotic integrity and comorbidity level			<0.001			<0.001
No AL *and* CCI <2 (reference)	1.00			0.0		
No AL *and* CCI ≥2	2.75	(2.14–3.54)	<0.001	–0.2	(–0.7 to 0.3)	0.376
AL *and* CCI <2	6.24	(4.56–8.53)	<0.001	12.9	(12.1 to 13.7)	<0.001
AL *and* CCI ≥2	7.48	(4.99–11.19)	<0.001	15.0	(13.6 to 16.4)	<0.001
Age (per 10 y)	2.34	(2.04–2.68)	<0.001	0.5	(0.3 to 0.6)	<0.001
Sex			0.109			0.618
Female (reference)	1.00			0.0		
Male	1.20	(0.96–1.49)		0.1	(–0.3 to 0.4)	
Tumor stage			0.943			0.461
UICC I (reference)	1.00			0.0		
UICC II	1.06	(0.76–1.46)	0.733	0.1	(–0.4 to 0.6)	0.777
UICC III	1.05	(0.74–1.47)	0.801	0.2	(–0.3 to 0.7)	0.476
Surgical procedure			0.913			0.435
Right hemicolectomy (reference)	1.00			0.0		
Transverse colectomy	1.14	(0.62–2.10)	0.680	–0.2	(–1.3 to 0.9)	0.713
Left hemicolectomy	1.07	(0.77–1.51)	0.679	0.4	(–0.3 to 0.7)	0.172
Sigmoid colectomy	1.08	(0.84–1.38)	0.550	–0.1	(–0.5 to 0.3)	0.654
Surgical approach			0.016			<0.001
Open (reference)	1.00			0		
Laparoscopic	0.65	(0.46–0.92)		–2.1	(–2.6 to –1.6)	
Surgeons' specialization			0.047			<0.001
GI (reference)	1.00			0		
General	1.27	(1.00–1.61)		0.8	(0.4 to 1.2)	
Organ resection			0.107			<0.001
No (reference)	1.00			0		
Spleen	0.42	(0.17–1.08)	0.071	2.7	(0.9 to 4.6)	0.003
Other organs	1.16	(0.84–1.59)	0.368	1.2	(0.6 to 1.8)	<0.001
Blood loss (per 100 mL)	1.04	(1.02–1.05)	<0.001	0.1	(0.01 to 0.1)	0.002
Blood transfusion			<0.001			<0.001
No (reference)	1.00			0		
Yes	1.91	(1.52–2.43)		3.2	(2.7 to 3.6)	

Multivariable Cox regression and multiple linear regression analyses were used to estimate adjusted associations between variables and 30-day mortality and length of hospital stay. N = 7839. "Anastomotic integrity and comorbidity level" was included as a time-dependent variable. The intercept in the linear regression model was 4.5 days (95% CI, 3.2–5.8 days) and predicts the duration of hospital stay when all variables take the reference value. The coefficients estimate the change in time of hospital stay given a change from the reference value. CCI values <2 indicate no or moderate comorbidity, and values ≥2 indicate severe or very severe comorbidity.

AL = anastomotic leak; CCI = Charlson Comorbidity Index; UICC = Union for International Cancer Control.

[a]Multivariable Cox regression analysis.

[b]Multiple linear regression analysis.

prediction of AL in the risk models. Despite the fact that the CCI comprises 19 disease conditions, the index may inadequately assess comorbidity, because confounding by residual comorbidity cannot be excluded. The calculations of the CCI score from the Danish National Patient Registry have previously produced consistently high positive prediction values.[21] However, the negative predictive value of the CCI has, to our knowledge, not been investigated. The potential inaccuracy of the coding used to generate the CCI is a study limitation.

Moreover, the CCI does not provide information on the pharmacological compensation for each disease condition. Detailed information about the severity of the disease, for instance, the Child-Pugh classification[28] or the Model for End-Stage Liver Disease score[29] for patients with liver disease and the New York Heart Association classification for congestive heart failure,[30] could provide a more dynamic and accurate scoring. There is no evidence that comorbidity influences the occurrence of AL through a common pathway, which is a limitation for the use of the CCI in studies of AL. However, a consistent observation of several of the diseases included in the CCI is reduced levels of serum albumin,[31,32] which is associated with increased risk of AL.[16,33] The relationship between each disease condition of the CCI and the risk of AL was analyzed to elucidate the influence of certain comorbidities. Only 1 item of the CCI, moderate to severe renal disease, was significantly associated with AL in the adjusted analysis. The best of the 4 models in the present study only produced a fair prediction of AL, which is inadequate for preoperative planning of tailored

surgical treatment with or without anastomosis in high-risk patients. The rate of AL was relatively high in the present study, probably reflecting a nationwide unselected population of patients with colonic cancer as reported previously in Dutch and Danish studies.[7,10] The 30-day postoperative mortality was high in patients with AL. The majority of these had a grade C leak requiring reoperation with bowel diversion.[34] The high number of patients presented allowed us to stratify the explaining variable, AL, according to level of comorbidity. Both AL and comorbidity were independently associated with increased 30-day mortality, whereas patients with both AL and a CCI score ≥2 had a 7-fold increase of an early fatal outcome, significantly higher than in AL patients without comorbidity. This striking finding confirms that patients with preexisting comorbidity cannot cope with the immense physical strain associated with AL. Delayed diagnosis and therapy by nonspecialists could be reasons for the excess mortality. However, the analysis is limited by the lack of specific causes of death. Another limitation is the termination of the study period by the end of 2008, excluding patients who would potentially benefit from recently introduced improvements of rescue and intensive care after AL. Recently, a Danish study on mortality after colorectal cancer surgery demonstrated that the rate of patients not subjected to surgery for their colorectal cancer increased from 5.4% to 14.9% between 2001 and 2011. These patients were very frail and had a particularly poor prognosis. Thus, the high resection rate in these patients during the inclusion period of the present study could partly account for the high mortality rate.[35] Age displayed a 2-fold increase in mortality for every 10-year step (Table 5). The inclusion of age in the CCI (1 point per decade above 40 years of age)[14] could thus potentiate the impact of the CCI score on mortality. Here, we chose to analyze comorbidity and age separately to obtain the highest resolution of the explaining variables. The crude LOS increased significantly for patients with AL as previously demonstrated.[36] Interestingly, comorbidity exhibited the same influence on LOS as on mortality, namely increasing the hospital stay for AL patients, whereas comorbidity did not have an impact on LOS for patients without AL. These findings suggest that patients with comorbidity take longer to recover from AL. It is a weakness that data regarding LOS in intensive care units were inaccessible in the present study, because comorbidity may specifically increase the need for such support in patients with AL. Surgical complications, and AL in particular, prolong LOS and increase overall costs. This consequence may be even more pronounced in complex patients with comorbidity developing a leak.

CONCLUSION

This large-scale nationwide study demonstrated that comorbidity failed to predict AL in elective patients with colonic cancer. However, comorbidity increased 30-day mortality and LOS in patients with AL. These findings indicate the importance of including comorbidity in the preoperative planning, especially in patients at increased risk of AL.

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Management of Anastomotic Leakage in a Nationwide Cohort of Colonic Cancer Patients

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BACKGROUND: The mortality associated with anastomotic leakage (AL) after colonic cancer surgery is high and management often results in permanent fecal diversion. Preservation of bowel continuity in combination with proximal loop diversion (salvage) may reduce the number of permanent ostomies without jeopardizing safety.

STUDY DESIGN: This nationwide study used prospective data from the database of the Danish Colorectal Cancer Group, the National Patient Registry, and patient files. Patients with AL requiring surgery (grade C) were categorized according to the type of surgical treatment as anastomotic takedown with an end-ostomy or salvage. Thirty-day mortality, long-term mortality, and permanent ostomy rates were analyzed using multivariable logistic and Cox regression analyses.

RESULTS: Anastomotic leakage occurred in 593 of 9,333 patients (6.4%), of whom 507 with grade C were included. Takedown and salvage were undertaken in 433 (85.4%) and 74 (14.6%) patients, respectively. Salvage was performed more frequently for Hinchey I-II or minor anastomotic defects and resulted in increased likelihood of stoma reversal (adjusted hazard ratio 3.24, 95% CI 2.04 to 5.16, $p < 0.001$), corresponding to a risk of permanent fecal diversion of 16.8%, compared with 54.5% after takedown. Adjusted mortality rates were comparable between the groups. A second episode of AL after stoma reversal occurred more frequently in patients with end-ileostomies (10 of 64) than in patients with end-colostomies (1 of 64) or loop-ileostomies (3 of 36), $p = 0.017$.

CONCLUSIONS: Patients with Hinchey I-II and small anastomotic defect were safely managed by anastomotic salvage, which reduced the risk of permanent fecal diversion. Anastomotic salvage is a viable option for this subset of patients. (J Am Coll Surg 2014;218:940–949. © 2014 by the American College of Surgeons)

Anastomotic leakage (AL) after colonic surgery occurs in 3% to 10% of patients, depending on the location of the anastomosis.[1,2] The consequences of AL are devastating, illustrated by a short-term mortality rate of 19% to 33%.[1-4] In addition, AL contributes to an inferior oncologic outcome leading to decreased long-term survival.[1,4,5]

Surgical management of AL is technically demanding and is associated with high postoperative morbidity,[6] yet the choice of rescue procedure is predominantly based on the attending surgeon's personal experience rather than solid evidence. There are only a few studies investigating the outcomes after different treatment strategies, and the small patient series preclude valid statistical analyses. Furthermore, the lack of a universally used definition of AL makes it difficult to compare outcomes between studies. The definition proposed by Rahbari and colleagues[7] consists of a 3-grade scale based on the following clinical consequences of AL: no therapeutic intervention required (grade A), active therapeutic intervention without a laparotomy (grade B), and laparotomy (grade C). Stratification of AL has proven useful, as the clinical course in patients with major leakage is more severe.[8] Most patients with AL require emergency surgical intervention, categorizing them into grade C.[9]

Various surgical strategies for management of AL are available. Takedown of the anastomosis with creation of an end-ostomy is the most frequently applied approach.[10] This strategy, however, is associated with excessive numbers of patients with permanent fecal diversion[11,12] and reduced quality of life because of ostomy-associated

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Abbreviations and Acronyms

AL = anastomotic leakage
CCI = Charlson comorbidity index
DCCG = Danish Colorectal Cancer Group
HR = hazard ratio
ICD-10 = International Classification of Disease
IQR = interquartile range
OR = odds ratio
reAL = reanastomotic leakage

complications such as leakage, dermatitis, peristomal hernia, and sexual dysfunction.[13] Another viable option includes salvage of the large bowel continuity using a loop-ostomy either alone or in combination with anastomotic repair or redo of the anastomosis.[11,14,15]

In a questionnaire on management of AL sent to 350 members of the Dutch Society of Gastrointestinal Surgery, the answers demonstrated heterogeneous surgical strategies with a tendency toward preservation of left-sided anastomoses in physically fit patients.[16] Recently, anastomotic salvage in 93 patients was associated with lower mortality and an increased likelihood of stoma reversal compared with takedown.[17] However, control for confounding factors was not undertaken, raising the risk that patient selection, in part, could explain the observed benefits of anastomotic salvage.

There is therefore a need for large-scale studies to define the optimal management of AL. The aim of this nationwide study was to investigate the outcomes of anastomotic takedown compared with salvage in a large unselected cohort of patients with grade C AL after curative colonic cancer surgery.

METHODS

Study population and variables

This study was based on prospectively collected nationwide data from 2 population-based Danish registers; the database of the Danish Colorectal Cancer Group (DCCG) and the National Patient Registry. Information from the reoperations for AL extracted from patient records were merged with the 2 databases using the unique personal identification number given all Danish citizens. The primary outcome was 30-day mortality and secondary outcomes were long-term mortality and rate of permanent ostomies in patients subjected to anastomotic takedown or salvage for grade C anastomotic leakage.

Takedown of the anastomosis was defined as interruption of the bowel continuity with resection or transection of the anastomosis in combination with formation of an end-ileostomy, end-colostomy, or both. Anastomotic salvage was defined as preservation of the large bowel continuity with repair or redo-anastomosis either alone or in combination with a proximal loop-ostomy.

Data from patients with a first-time diagnosis of colonic adenocarcinoma were prospectively entered into the DCCG database between May 2001 and December 2008. The DCCG database was approved by The Danish Data Protection Agency (Ref. no. 2000-53-0073) and includes at least 95% of all Danish patients with colorectal cancer.[18] All patients included in the study underwent a curative colonic resection with a primary intraperitoneal anastomosis without a protecting stoma. The curative resection criteria required a colonic excisional specimen with at least 2 mm between the deepest tumor growth and the nonperitonealized resection margin and no tumor growth or distant disease left after completed surgery.

Patients with AL were identified in both the DCCG database and the National Patient Registry using the codes of the International Classification of Disease (ICD-10) for diagnosis and reoperation associated with AL (DT813A, KJWF00). Anastomotic leakage was defined as clinical symptoms suggesting AL and confirmed by contrast enema, CT, or surgery. The AL was then graded according to Rahbari and associates.[7] Information on management of AL was obtained from ICD-10 codes in the National Patient Registry and from the original description of the reoperation in the patient records. Patients dying before treatment or with grade A or B AL were excluded, leaving patients with grade C AL for inclusion. The included patients were subdivided according to the surgical strategy into anastomotic takedown or salvage. The decision to perform a takedown or salvage procedure was taken exclusively by the local surgical staff members. The time to AL was calculated as the duration between the index operation and the primary reoperation for AL.

Short-term mortality was defined as any deaths within 30 days after the reoperation for AL. Long-term mortality encompassed all-cause mortality in patients surviving at least 30 days after the index operation. Information on vital status and complete restoration of bowel continuity was extracted from the National Patient Registry using the ICD-10 codes for stoma reversal (KJFG00-37). The rate of AL after reversal of a temporary ostomy (re-AL) was defined and analyzed as mentioned above.

Potential confounding covariates were extracted from the DCCG database or the National Patient Registry and included age, sex, tumor stage (Union for International Cancer Control, UICC), anastomotic location, surgical priority, surgeon specialization level, surgical approach (open or laparoscopic) at the index operation, time to AL, discharge before detection of AL, and comorbidity as assessed by the Charlson Comorbidity Index (CCI). This parameter reflects the cumulative likelihood

Table 1. Patient Characteristics at the Index Operation and Findings at Reoperation for Anastomotic Leakage According to Treatment

Variable	Anastomotic takedown	Anastomotic salvage	p Value*
Totals, n (%)	433 (85.4)	74 (14.6)	
Age, y, median (range)	73 (36–93)	68 (40–94)	0.002†
Sex			0.305
Female	183 (42.3)	36 (48.6)	
Male	250 (57.7)	38 (51.4)	
Charlson Comorbidity Index (CCI), n (%)			0.970
CCI < 2	324 (76.8)	57 (77.0)	
CCI ≥ 2	92 (21.2)	16 (21.6)	
Missing	17 (3.9)	1 (1.4)	
Tumor stage, n (%)			0.884
UICC I	78 (18.0)	13 (17.6)	
UICC II	207 (47.8)	35 (47.3)	
UICC III	130 (30.0)	25 (33.8)	
Missing	18 (4.2)	1 (1.4)	
Approach at index operation, n (%)			0.463
Open	347 (80.1)	62 (83.8)	
Laparoscopic	86 (19.9)	12 (16.2)	
Priority at index operation, n (%)			0.510
Elective	387 (89.4)	68 (91.9)	
Emergency	46 (10.6)	6 (8.1)	
Anastomosis, n (%)			0.477
Ileocolic	177 (40.9)	27 (36.5)	
Colocolic	256 (59.1)	47 (63.5)	
Year of leakage, n (%)			0.055
2001–2004	171 (39.5)	38 (51.4)	
2005–2008	262 (60.5)	36 (49.6)	
Time to leakage, d, median (range)	7 (0–24)	7 (2–20)	0.957
Discharged before leakage, n (%)			0.420
No	391 (90.3)	69 (93.2)	
Yes	42 (9.7)	5 (6.8)	
Anastomotic defect,§ n (%)			<0.001
Minor	306 (70.7)	65 (87.8)	
Major	109 (25.1)	3 (4.1)	
Missing	18 (4.2)	6 (8.1)	
Hinchey score at reoperation, n (%)			<0.001
I-II	156 (36.0)	53 (71.6)	
III-IV	260 (60.1)	16 (21.6)	
Missing	17 (3.9)	5 (6.8)	
Certified colorectal surgeon, n (%)			0.691
No	248 (59.9)	39 (57.4)	
Yes	166 (40.1)	29 (42.6)	
Missing	19 (4.4)	6 (8.1)	

*Chi-square test, unless otherwise specified.
†Mann-Whitney test.
§Size of the anastomotic defect was categorized as minor (<one-quarter of the anastomotic circumference) or major (≥one-quarter of the anastomotic circumference).
UICC, Union for International Cancer Control.

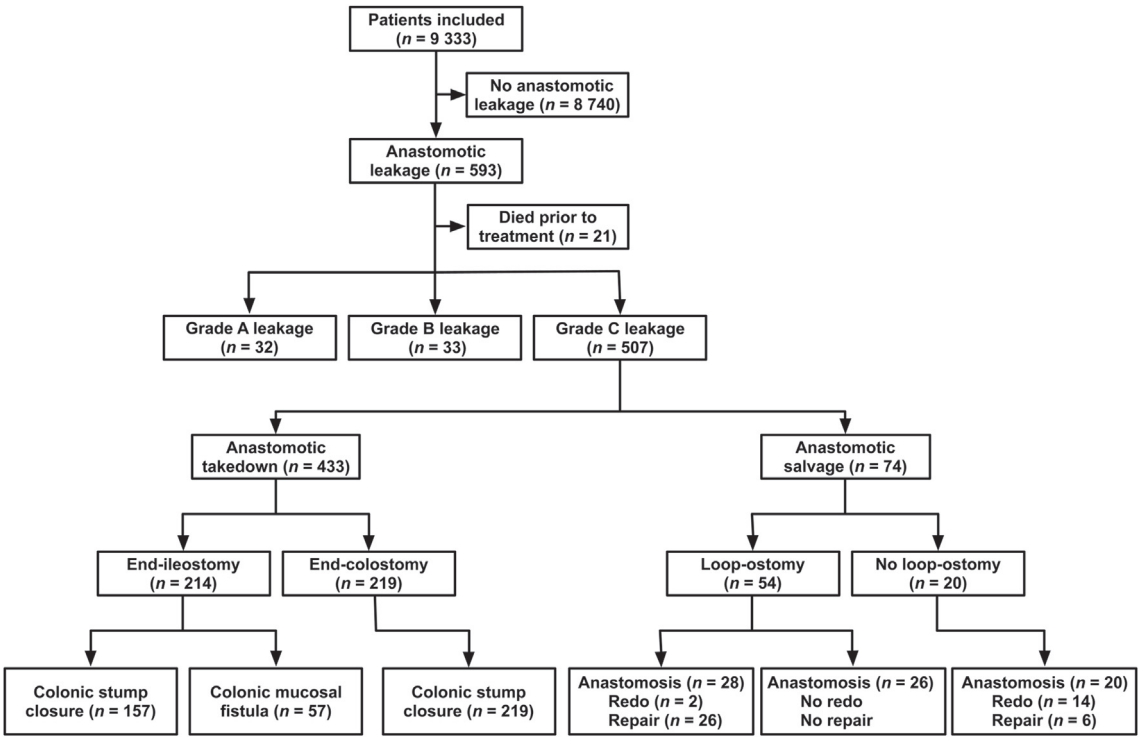

Figure 1. Patient flow chart. Grade A anastomotic leakage, no requirement for therapeutic intervention; grade B leakage, therapeutic intervention not requiring laparotomy; grade C leakage, requiring laparotomy. Anastomotic takedown was defined as interruption of the bowel continuity with resection or transection of the anastomosis in combination with the formation of an end-ostomy. Anastomotic salvage was defined as preservation of the large bowel continuity with repair of the anastomosis or a redo-anastomosis, with or without a proximal loop-ostomy.

of 1-year mortality and was calculated from ICD-10 diagnoses registered in the National Patient Registry before the day of the index operation.[19,20] The use of CCI based on ICD-10 codes in the National Patient Registry has previously been validated.[21] Patients were categorized as CCI < 2 or CCI ≥ 2.[22] Primary anastomoses were defined as ileocolic after right hemicolectomy or colocolic after transverse, left, or sigmoid colectomy.

The severity of grade C AL was stratified according to the Hinchey classification of colonic perforation in acute diverticulitis[23] and the size of the anastomotic defect was determined according to the original descriptions in the patients' medical files. Because the Hinchey classification was used outside its original intention, the grades were dichotomized as Hinchey I-II or III-IV, and the anastomotic defects were categorized as minor (<one-quarter of the anastomosis) or major (≥one-quarter of the anastomosis). Information on the specialization level of the surgeons conducting the reoperations was extracted from the patient records and matched with the list of certified colorectal surgeons according to the Danish Surgical Society. The study period was divided in two: 2001 to 2004 vs 2005 to 2008, to adjust for potential changes over time.

Statistical analyses

Duration of follow-up was calculated from the day of the index operation until November 25, 2010 and analyzed by the reverse Kaplan-Meier method.[24] Kaplan-Meier curves were plotted for stoma reversal and survival after reversal of a temporary ostomy. Patients with loop-ileostomies, end-ileostomies or end-colostomies were compared by the log-rank analysis, as were patients with or without re-AL. Univariable chi-square or Mann-Whitney tests were applied for comparison of groups. Multivariable logistic regression and Cox regression analyses were used to adjust for confounding variables.

Odds ratios (OR) >1 indicated increased likelihood of 30-day mortality. Hazard ratios (HR) >1 indicated increased likelihood of stoma reversal, risk of permanent fecal diversion, or a fatal outcome. Schoenfeld residuals were examined to verify the assumption of proportional hazards. All variables were simultaneously included in the multivariable analyses. Missing values (Table 1) were disregarded and therefore not replaced. All analyses were 2-sided and considered statistically significant if $p < 0.05$. Data were analyzed with SPSS Statistics ver. 20 (IBM Corp).

Table 2. Multivariable Analyses of Factors Associated with 30-Day and Long-Term Mortality after Surgical Treatment of Anastomotic Leakage

	30-day mortality		Long-term mortality	
Variable	**Odds ratio (95% CI)**	**p Value**	**Hazard ratio (95% CI)**	**p Value**
Age (per year)	1.11 (1.07–1.15)	<0.001	1.05 (1.03–1.07)	<0.001
Sex		0.302		0.004
Female	1.00		1.00	
Male	0.76 (0.46–1.28)		1.71 (1.18–2.47)	
Charlson Comorbidity Index (CCI)		0.010		<0.001
CCI < 2	1.00		1.00	
CCI ≥ 2	2.13 (1.20–3.76)		1.96 (1.32–2.92)	
Tumor stage		0.987		0.002
UICC I	1.00		1.00	
UICC II	1.03 (0.52–2.03)		0.83 (0.51–1.35)	
UICC III	1.06 (0.50–2.24)		1.65 (1.01–2.71)	
Approach at index operation		0.268		0.720
Open	1.00		1.00	
Laparoscopic	0.65 (0.30–1.40)		1.09 (0.68–1.75)	
Priority at index operation		0.790		0.132
Elective	1.00		1.00	
Emergency	1.12 (0.49–2.58)		1.53 (0.88–2.65)	
Anastomosis at index operation		0.985		0.038
Ileocolic	1.00		1.00	
Colocolic	1.01 (0.60–1.68)		0.68 (0.48–0.98)	
Year of leakage		0.003		0.196
2001–2004	1.00		1.00	
2005–2008	0.44 (0.25–0.75)		0.77 (0.52–1.14)	
Leakage management		0.323		0.122
Anastomotic takedown	1.00		1.00	
Anastomotic salvage	0.65 (0.27–1.53)		1.44 (0.91–2.28)	
Time to leakage (per day)	0.98 (0.91–1.05)	0.555	1.07 (1.01–1.13)	0.013
Discharged before leakage		0.061		0.560
No	1.00		1.00	
Yes	0.33 (0.10–1.05)		0.83 (0.45–1.54)	
Anastomotic defect*		0.713		0.109
Minor	1.00		1.00	
Major	1.12 (0.62–2.01)		1.40 (0.93–2.10)	
Hinchey score at reoperation		0.047		0.266
I–II	1.00		1.00	
III–IV	1.75 (1.01–3.04)		1.24 (0.85–1.79)	
Certified colorectal surgeon		0.585		0.712
No	1.00		1.00	
Yes	0.87 (0.52–1.45)		1.07 (0.75–1.51)	

Odds ratio or hazard ratio >1 indicates increased likelihood of mortality.
*Size of the anastomotic defect was categorized as minor (<one-quarter of the anastomotic circumference) or major (≥one-quarter of the anastomotic circumference).
UICC, Union for International Cancer Control.

RESULTS

Anastomotic leakage occurred in 593 of 9,333 patients (6.4%). Twenty-one patients (3.5%) with AL died before treatment was initiated, 32 patients (5.4%) with grade A did not receive invasive intervention, and 33 patients (5.6%) with grade B were managed by drainage alone, leaving 507 patients with grade C AL for analysis (Fig. 1).

Table 3. Multivariable Cox Regression Analyses of Factors Associated with Stoma Reversal and Risk for Permanent Fecal Diversion in Patients with Anastomotic Leakage after Curative Surgery for Colonic Cancer

	Stoma reversal		Permanent fecal diversion	
Variable	**Hazard ratio (95% CI)**	**p Value**	**Hazard ratio (95% CI)**	**p Value**
Age (per year)	0.96 (0.95–0.98)	<0.001	1.07 (1.05–1.08)	<0.001
Sex		0.751		0.417
Female	1.00		1.00	
Male	1.05 (0.76–1.46)		1.11 (0.87–1.41)	
Charlson Comorbidity Index (CCI)		0.013		<0.001
CCI < 2	1.00		1.00	
CCI ≥ 2	0.53 (0.32–0.87)		1.76 (1.34–2.32)	
Tumor stage		0.267		0.130
UICC I	1.00		1.00	
UICC II	1.33 (0.83–2.10)		0.81 (0.59–1.10)	
UICC III	1.00 (0.62–1.65)		1.21 (0.87–1.70)	
Approach at index operation		0.051		0.685
Open	1.00		1.00	
Laparoscopic	1.50 (1.00–1.85)		0.94 (0.68–1.29)	
Priority at index operation		0.939		0.377
Elective	1.00		1.00	
Emergency	1.02 (0.57–1.85)		1.20 (0.80–1.78)	
Anastomosis at index operation		0.044		0.897
Ileocolic	1.00		1.00	
Colocolic	0.69 (0.48–0.99)		1.02 (0.79–1.31)	
Year of leakage		0.636		0.105
2001–2004	1.00		1.00	
2005–2008	0.92 (0.64–1.31)		1.27 (0.95–1.69)	
Leakage management		<0.001		<0.001
Anastomotic takedown	1.00		1.00	
Anastomotic salvage	3.24 (2.04–5.16)		0.41 (0.25–0.68)	
Hinchey score at reoperation		0.573		0.208
I–II	1.00		1.00	
III–IV	1.11 (0.78–1.59)		1.18 (0.91–1.51)	
Anastomotic defect*		0.515		0.145
Minor	1.00		1.00	
Major	0.86 (0.56–1.34)		1.23 (0.93–1.62)	
Certified colorectal surgeon		0.616		0.822
No	1.00		1.00	
Yes	1.09 (0.78–1.51)		0.97 (0.76–1.24)	

Hazard ratio (HR) <1 indicates decreased likelihood of stoma reversal or decreased risk of permanent fecal diversion; HR > 1 indicates increased likelihood of stoma reversal and diversion.
*Size of the anastomotic defect was categorized as minor (<one-quarter of the anastomotic circumference) or major (≥one-quarter of the anastomotic circumference).
UICC, Union for International Cancer Control.

The median time from the index operation to diagnosis of AL was 7 days (interquartile range [IQR] 5 to 9 days). Forty-seven patients (9.3%) were discharged before detection of AL and in these, the median time to AL was 8 days (IQR 6 to 8 days) compared with 7 days (IQR 5 to 7 days) in patients who were not discharged before detection, $p < 0.001$. Total median follow-up was 5.0 years (range 4.7 to 5.2 years).

Management and mortality after grade C anastomotic leakage

A total of 6 different management strategies were applied during the study period (Fig. 1). Takedown or salvage after grade C AL was performed in 433 of 507 (85.4%) and 74 of 507 patients (14.6%), respectively. Takedown patients were evenly distributed between end-ileostomies

(n = 214) and end-colostomies (n = 219). In salvage patients, 54 (73%) had a loop-ostomy (loop-ileostomy, n = 48; loop-transversostomy, n = 6). Twenty (27.0%) of the salvage patients underwent an anastomotic repair or redo without a loop-ostomy (Fig. 1). Patients undergoing a salvage procedure were younger, had a lower Hinchey score, and a smaller defect in the anastomotic line than patients subjected to anastomotic takedown (Table 1). Laparoscopic management of AL was attempted in 4 patients (0.8%), 2 of whom were converted to open surgery.

The 30-day mortality was 100 of 433 patients (23.1%) after anastomotic takedown and 13 of 74 (17.6%) after salvage, p = 0.291, There was no significant difference between takedown and salvage after adjustment for confounding variables, OR = 0.65, 95% CI 0.27 to 1.53, p = 0.323. Variables reaching statistical significance for an early fatal outcome were advanced age, a high CCI, leakage in the first period of study, and a high Hinchey score (Table 2).

Patients subjected to anastomotic takedown after leakage of ileocolic anastomoses were managed with either an end-ileostomy in combination with a closed colonic stump (121 of 178) or an end-ileostomy with a colonic mucosal fistula (57 of 121). The 30-day mortality rate was comparable in the 2 groups (adjusted OR 1.14, 95% CI 0.44 to 2.95, p = 0.787).

In the group of salvage patients, the 30-day mortality rate was 7 of 54 (13.0%) after construction of a loop-ostomy compared with 6 of 20 (30.0%) for patients without a loop-ostomy, p = 0.087.

A total of 239 patients (47.1%) were alive at the end of follow-up. Anastomotic salvage had no significant impact on long-term mortality as compared with takedown in the univariable (HR = 1.03, 95% CI 0.68 to 1.56, p = 0.906) or the multivariable analysis (HR 1.44, 95% CI 0.91 to 2.28, p = 0.122); advanced age, male sex, high CCI, stage III tumors, ileocolic anastomoses at the index operation, and time to leakage were associated with increased mortality (Table 2).

Risk of permanent fecal diversion after anastomotic leakage

The rate of any ostomy formation was 487 of 507 (96.1%). Subsequent complete restoration of bowel continuity was achieved in 164 patients (33.7%) after a median of 237 days (IQR 163 to 327 days). The crude overall risk of a permanent ostomy was 323 of 507 (54.5%). Anastomotic salvage was associated with increased likelihood of stoma reversal as compared with anastomotic takedown (adjusted HR 3.24, 95% CI 2.04 to 5.16, p < 0.001, Table 3). Patients with an end-colostomy were less likely to undergo reversal as compared with patients with an end-ileostomy (adjusted HR 0.55, 95% CI 0.31 to 0.97, p = 0.037); patients with a loop-ileostomy were more likely to undergo a reversal procedure compared with patients with an end-ileostomy (adjusted HR 2.30, 95% CI 1.32 to 4.03, p = 0.003, Fig. 2). For takedown patients after a leaking ileocolic anastomosis, there were no significant differences in the reversal rates between patients subjected to colonic stump closure as compared with a colonic mucosal fistula (adjusted HR 1.21, 95% CI 0.65 to 2.24, p = 0.544).

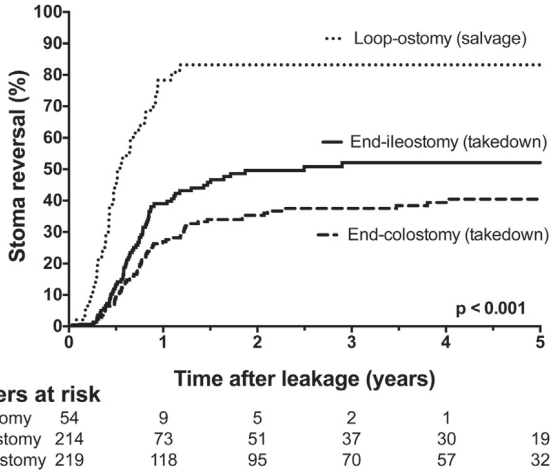

Figure 2. Kaplan-Meier plot depicting the cumulative rate of stoma reversal in patients subjected to an ostomy after anastomotic leakage. The treatment groups are shown in parentheses.

After adjustment for confounding variables, the long-term risk of permanent fecal diversion was significantly reduced in salvage patients (HR 0.41, 95% CI 0.21 to 0.68, p < 0.001). Variables associated with increased risk of permanent diversion were advanced age and high CCI (Table 3).

The risk of re-AL after reversal of a temporary ostomy was 8.5% (14 of 164). Ten of 64 (15.6%) had re-AL after reversal of end-ileostomies, 3 of 36 (8.3%) after reversal of loop-ostomies, and 1 of 64 (1.6%) after colostomy reversal, p = 0.017 (Table 4). The median time to re-AL was 8 days (IQR 6 to 13 days). Overall mortality was higher in patients with re-AL compared with patients without re-AL (HR 2.60, 95% CI 1.15 to 5.87, p = 0.018, Fig. 3).

DISCUSSION

This nationwide study demonstrated that patients with Hinchey I-II and minor defects in the anastomosis after curative resection for colonic cancer were safely managed by anastomotic salvage. For these patients, anastomotic salvage significantly reduced the risk of permanent fecal diversion. Anastomotic salvage was undertaken more frequently in younger patients, corresponding to the approach previously proposed.[25] Attendance of a certified colorectal surgeon at

Table 4. Factors Associated with Reanastomotic Leakage after Stoma Reversal in Patients Treated with an Ostomy after Earlier Anastomotic Leakage

Variable	Releakage	No releakage	p Value*
Totals, n (%)	14 (8.5)	150 (91.5)	
Age, y, median (range)	66 (45, 78)	66 (36, 87)	0.879†
Sex, n (%)			0.145
Female	3 (21.4)	62 (41.3)	
Male	11 (78.6)	88 (58.3)	
Charlson Comorbidity Index (CCI), n (%)			0.243
CCI < 2	11 (78.6)	131 (87.3)	
CCI ≥ 2	3 (21.4)	16 (10.6)	
Missing	0 (0)	3 (2.0)	
Hinchey score at reoperation, n (%)			0.380
I–II	5 (35.7)	71 (47.4)	
III–IV	9 (64.3)	77 (51.3)	
Missing	0 (0)	2 (1.3)	
Leakage management, n (%)			0.961
Anastomotic takedown	11 (78.6)	117 (78.0)	
Anastomotic salvage	3 (21.4)	33 (22.0)	
Time to reversal, d, median (range)	291 (111–626)	235 (12–1467)	0.296
Stoma type, n (%)			0.017
End-ileostomy	10 (71.4)	54 (36.0)	
Loop-ostomy	3 (21.4)	33 (22.6)	
End-colostomy	1 (7.1)	63 (42.0)	

*Chi-square test, unless otherwise specified.
†Mann-Whitney test.

the reoperation for AL did not increase the rate of salvage procedures, contrary to a previous report.[17]

The safety of anastomotic salvage was addressed by Hedrick and coworkers,[14] demonstrating that proximal loop division without anastomotic revision was safe in a small selected group of patients with AL.[14] In another study, anastomotic salvage was associated with a reduction in mortality but the findings were not controlled for confounding.[17] In our study, salvage jeopardized neither short-term nor long-term survival in the univariable analysis, and these observations were consistent after adjustment for potential confounding variables including age, comorbidity, and Hinchey score at the time of AL. There was a trend toward high mortality rates in patients subjected to salvage without proximal diversion, as previously reported.[12,26] It is therefore recommended that anastomotic salvage should always be protected by a loop-ostomy. In patients with leakage of ileocolic anastomoses, takedown with either colonic stump closure or colonic mucosal fistula was equally safe. However, a mucosal fistula is warranted if distal obstruction is suspected. Construction of an end-loop stoma has been demonstrated as an appropriate alternative,[27] but this technique was not reported in this study.

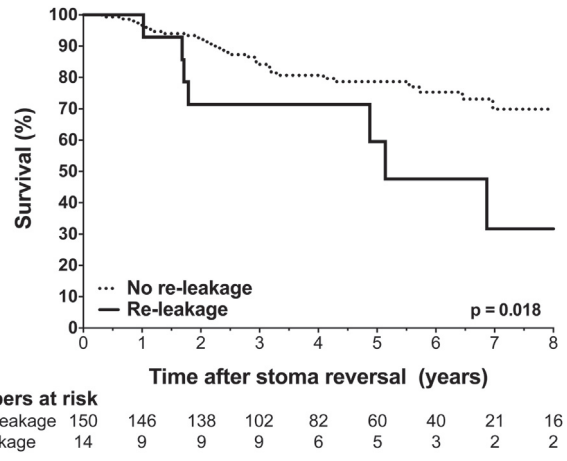

Figure 3. Kaplan-Meier plot depicting the overall cumulative survival after stoma reversal in patients surgically treated for anastomotic leakage.

None of the patients were treated according to the principles of damage control surgery, which, in combination with vacuum-assisted closure, reduces operative time. This novel approach in patients with peritonitis may be reserved for severely ill patients.[28]

Perioperative findings such as the degree of peritonitis and the size of the anastomotic defect clearly influenced the surgeons' choice between salvage and takedown, while the degree of sepsis at the time of the reoperation for AL was

unattainable in this study. The physiologic consequences of AL vary between patients and probably contribute considerably to the choice of operative treatment of the complication. The findings of this study may be subject to bias, because registration of sepsis and other physiologic parameters were not recorded. Future studies on management of AL should include a scoring system such as the APACHE II[29] or the Clavien-Dindo classification[30] in addition to the stratification used in this study.

Only a few patients with AL were managed by a laparoscopic approach. Laparoscopic reintervention in patients initially subjected to laparoscopic resection is a viable option in the subset of AL patients with mild peritonitis and without severe sepsis.[31]

In several centers, fast-track surgery including laparoscopic techniques and Enhanced Recovery After Surgery (ERAS) protocols have accelerated hospital discharge to 5 days or even less after uncomplicated colorectal procedures.[32] This is shorter than the median time to diagnosis and treatment of AL in both this and previous studies,[33] raising concern that AL may go undetected, therefore increasing morbidity and mortality.[10] In this study, median time to AL was significantly prolonged by 1 day without any significant impact on mortality rates in discharged patients, supporting the findings from previous studies.[10]

Restoration of bowel continuity is of great importance because permanent fecal diversion is associated with reduced quality of life.[13] The reported ostomy reversal rate after reoperation for AL varies between 46% and 91% depending on the type of ostomy.[9-12,14,16,23,24,29,30] In our cohort there was a 50% risk of permanent fecal diversion. Patients subjected to salvage with proximal loop diversion had a 3-fold increased likelihood of stoma reversal compared with patients with an end-ileostomy or end-colostomy. The finding persisted after adjustment for confounding variables, in concordance with previous studies.[11,14,17] The long-term overall risk of permanent fecal diversion was dependent on age, comorbidity, and especially on the management of AL. There was a 2.5-fold increase in the adjusted rate of permanent fecal diversion after anastomotic takedown compared with salvage.

A high 8.5% re-AL rate after reversal procedures was observed. Reversal of end-ileostomies more often resulted in re-AL compared with end-colostomies in contrast to a previous study reporting that reanastomoses after reversal of colostomies were more prone to leak.[17] Possibly, the everyday strain associated with an ileostomy could increase surgeons' willingness to re-establish bowel continuity in high-risk patients and could, in part, explain our finding. The high rate of re-AL and the associated significant reduction in overall survival emphasize that closure of a stoma should be a specialist procedure.[34]

This study included patients from all Danish departments conducting colon cancer surgery during the study period. The large number of unselected patients with AL allowed adjustment for confounding variables. The study was further strengthened by the rigid inclusion of patients with grade C AL, allowing comparison of salvage and takedown in a well defined group of patients with AL after surgical treatment of colonic cancer. The obvious selection of patients for salvage procedures was addressed using Hinchey score and the size of the anastomotic defect. This strategy was limited by the retrospective nature of these data. Therefore, a gross categorization of the Hinchey classification and degree of the anastomotic defect reported in the patient files was performed to minimize the risk of interpretation bias. In addition, residual selection bias cannot be ruled out. Furthermore, only 30% of the patients fulfilling the post-hoc criteria for salvage surgery were subjected to this treatment, limiting the external validity of the findings. Because laparoscopic colonic resection was implemented during the study period,[2] a year variable was included in all analyses to adjust for the potential change in strategy over time.

CONCLUSIONS

In conclusion, patients with Hinchey I-II and minor anastomotic defects because of grade C AL after colonic cancer surgery were safely managed with anastomotic salvage in combination with proximal loop diversion; salvage without loop diversion is not recommended. This strategy decreased the rate of permanent fecal diversion significantly compared with a takedown strategy. Further studies with a more rigorous inclusion are needed for a more firm recommendation.

Author Contributions

Study conception and design: Krarup, Jorgensen, Harling
Acquisition of data: Krarup
Analysis and interpretation of data: Krarup, Jorgensen, Harling
Drafting of manuscript: Krarup
Critical revision: Krarup, Jorgensen, Harling

Acknowledgment: Project nurse Tina Lee Brøndum collected patient files. The IM Dæhnfeldt Foundation, the AP Møller Foundation, and the Danish Medical Association supported the study. Dr Steffen Høgskilde extracted data from the DCCG database and the National Patient Registry.

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ORIGINAL ARTICLE

Anastomotic Leak Increases Distant Recurrence and Long-Term Mortality After Curative Resection for Colonic Cancer

A Nationwide Cohort Study

Peter-Martin Krarup, MD, Andreas Nordholm-Carstensen, MD, Lars N. Jorgensen, MD, DMSc, and Henrik Harling, MD, DMSc; on behalf of the Danish Colorectal Cancer Group

Objective: To investigate the impact of anastomotic leak (AL) on disease recurrence and long-term mortality in patients alive 120 days after curative resection for colonic cancer.

Background: There is no solid data as to whether AL after colonic cancer surgery increases the risk of disease recurrence.

Methods: This was a nationwide cohort study of 9333 patients, prospectively registered in the database of the Danish Colorectal Cancer Group and merged with data from the Danish Pathology Registry and the National Patient Registry. Multivariable Cox regression analysis was used to adjust for confounding.

Results: The incidence of AL was 6.4%, 744 patients died within 120 days. Of the remaining 8589 patients, 861 (10.0%) developed local recurrence with no association to AL [adjusted hazard ratio (HR) = 0.78; 95% confidence interval (CI): 0.55–1.12; $P = 0.184$]. Distant recurrence developed in 1281 (14.9%) patients and more frequently after AL (adjusted HR = 1.42; 95% CI: 1.13–1.78; $P = 0.003$). AL was also associated with increased long-term mortality (adjusted HR = 1.20; 95% CI: 1.01–1.44; $P = 0.042$). In 2841 patients with stage III cancer, AL was associated with both decreased likelihood of receiving adjuvant chemotherapy (adjusted HR = 0.58; 95% CI: 0.45–0.74; $P < 0.001$) and a delay to initial administration (16 days; 95% CI: 12–20 days; $P < 0.001$).

Conclusions: AL was significantly associated with increased rates of distant recurrence and long-term all-cause mortality. Cancelled or delayed administration of adjuvant chemotherapy may partly account for these findings.

Keywords: adjuvant chemotherapy, anastomotic leakage, colon cancer, mortality, nationwide, recurrence

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Colorectal cancer is one of the most frequent malignancies worldwide with an estimated 1.2 million new cases and 600,000 deaths annually.[1] Surgical resection is essential to obtain long-term disease-free survival, but postoperative complications have a significant impact on the surgical outcome. Anastomotic leak (AL) is one of the most devastating complications in these patients because of an immense increase in short-term morbidity and mortality.[2,3] The incidence of AL varies between 3% and 12%[2,4,5] depending on the type of surgical procedure. There are only few and contradictory studies on the oncological outcome and long-term mortality in patients with AL after resection of colonic cancer.[2,6–9] In patients undergoing rectal resection for adenocarcinoma, consistent results have demonstrated that AL is associated with an increased rate of local recurrence and long-term mortality, whereas the rate of distant recurrence remains unaffected.[8–11] In contrast, AL after colonic resection has previously not been associated with higher rates of local or distant recurrence.[9] However, there is evidence that AL reduces both disease-free and overall survivals in patients with colonic cancer.[2,6] Accordingly, it is essential to distinguish between colonic and rectal cancer surgery because the influence of AL on long-term outcomes seems to differ. The excess long-term mortality after AL could be mediated by increased rates of disease recurrence, higher comorbidity or the influence from factors associated with AL.[12,13]

In this nationwide study on colonic cancer surgery, the primary objective was to investigate the impact of AL on the rates of local and distant disease recurrence and secondary objective was to do it on long-term all-cause mortality. Furthermore, a subgroup analysis was conducted to evaluate the influence of AL on administration of adjuvant chemotherapy (AC) in patients with stage III cancer.

METHODS

Study Population and Variables

This was a nationwide cohort study with data from 3 Danish registers: the prospective database of the Danish Colorectal Cancer Group (DCCG), the Danish Pathology Registry, and the National Patient Registry. Data from these registers were merged to investigate possible associations between AL and long-term outcomes. The primary and secondary outcomes were disease recurrence and all-cause mortality, respectively.

All patients included in the study were recorded in the DCCG database between May 2001 and December 2008 with a first-time diagnosis of colonic adenocarcinoma. The DCCG database is approved by the Danish Data Protection Agency (Ref no. 2000-53-0073) and includes at least 95% of all Danish patients with colorectal cancer.[4] All patients underwent a curative colonic resection with a primary anastomosis without a protecting ostomy. Trained pathologists evaluated all excisional specimens according to the guidelines of the DCCG. The curative resection criteria required at least 2 mm from the tumor to the nonperitonealized resection margin, as evaluated microscopically, and with no tumor growth or distant disease left after completed surgery. The proximal and distal resection margins were examined microscopically when the distance from the tumor to these margins were less than 20 mm.

Data on disease recurrence and overall survival were obtained from the Danish Pathology Registry and the National Patient Registry on November 25, 2010. Recurrent disease was defined according to the DCCG guidelines as local or distant recurrence diagnosed no earlier than 120 days after the index operation. All cases of recurrent disease were confirmed histologically, by diagnostic imaging or surgery. Patients with both local and distant recurrence were classified as distant recurrence. All-cause mortality was exclusively

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TABLE 1. Patient Characteristics

	AL	No AL
$n = 8589$	420 (5%)	8169 (95%)
Gender		
Female	171 (41%)	4331 (53%)
Male	249 (59%)	3838 (47%)
Age groups, yr		
≤60	80 (19%)	1466 (18%)
>60–70	138 (33%)	2222 (27%)
>70–80	157 (37%)	2865 (35%)
>80	45 (11%)	1616 (20%)
Comorbidity*		
Normal	266 (63%)	5342 (65%)
Moderate	63 (15%)	1274 (16%)
Severe	44 (11%)	799 (10%)
Very severe	33 (8%)	454 (6%)
Missing	14 (3%)	300 (3%)
Tumour stage		
UICC I	74 (18%)	1207 (15%)
UICC II	202 (48%)	3905 (48%)
UICC III	129 (31%)	2712 (33%)
Missing	15 (3%)	345 (4%)
Surgical procedure		
Right hemicolectomy	169 (40%)	4090 (50%)
Transverse colectomy	8 (2%)	214 (3%)
Left hemicolectomy	72 (17%)	823 (10%)
Sigmoid colectomy	171 (41%)	3042 (37%)
Surgical approach		
Open	329 (78%)	6700 (82%)
Laparoscopy	91 (22%)	1469 (18%)
Surgical priority		
Elective	384 (91%)	7607 (93%)
Emergency	36 (9%)	562 (7%)
Surgeons' specialization		
Gastrointestinal	324 (77%)	5976 (73%)
General	96 (23%)	2191 (27%)
Missing	0 (0%)	2 (<1%)
Organ resection		
None	371 (89%)	7243 (89%)
Spleen	9 (2%)	71 (<1%)
Other organs	39 (9%)	855 (11%)
Missing	1 (<1%)	0 (0%)
Blood transfusion		
No	170 (41%)	6468 (79%)
Yes	244 (58%)	1643 (20%)
Missing	6 (1%)	58 (1%)

*Comorbidity according to Charlson comorbidity index scores of 0 (*normal*), 1 (*moderate*), 2 (*severe*), and ≥ 3 (*very severe*).

investigated in patients surviving the first 120 postoperative days after primary surgery.

The outcome in patients with stage III colonic cancer may be predicted by the radicality of surgery, administration of AC, and the time to initial administration in patients who receive AC. A post hoc analysis was therefore undertaken to investigate the impact of AL on administration of AC in the group of patients with stage III cancer. In the subset of patients who did receive AC, the influence of AL on time to initial administration was likewise examined. Patients were regarded as having AC if initial administration was achieved before postoperative day 120 according to data from the National Patient Registry. This minimized the risk of bias associated with recurrence developing before the initial administration of AC. During the study period, the standard AC regimen for patients with stage III colonic cancer was a combination of fluorouracil/leucovorin and oxaliplatin.

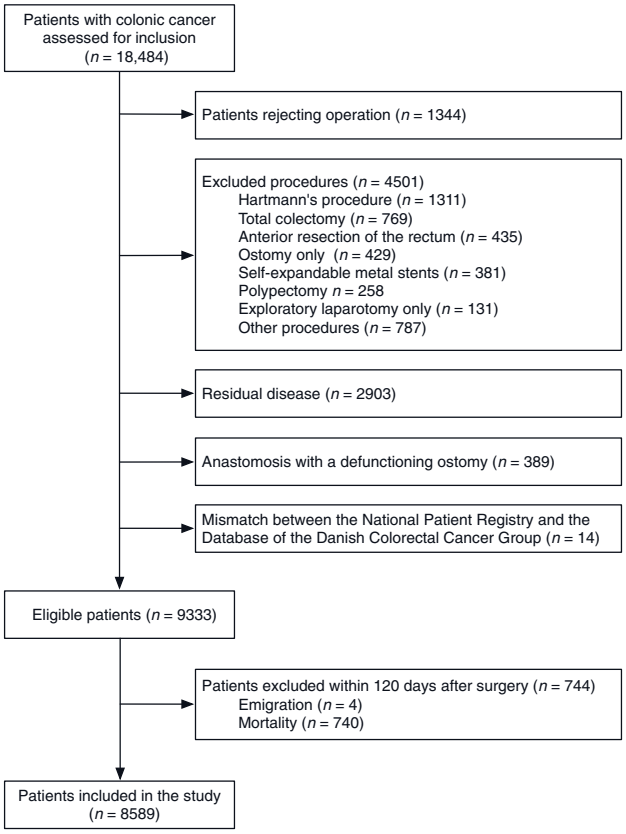

FIGURE 1. Inclusion chart modified from Krarup et al.[5]

The independent variable of interest, AL, was defined according to the guidelines of the DCCG: "Clinical symptoms suggesting AL and confirmed by contrast enema or computerized tomography" within 60 days from the operation. Patients with AL were identified in the DCCG database or National Patient Registry using the *International Classification of Disease* (*ICD-10*) for diagnosis and reoperation codes associated with AL (DT813A, KJWF00).

Relevant and potential confounding covariates were extracted from the DCCG database and the National Patient Registry including age, gender, comorbidity as assessed by the Charlson index, tumor stage (Union for International Cancer control, UICC), type of surgery, surgeon's specialization level, resection of adjacent organs, and perioperative blood transfusion. The Charlson comorbidity index reflects the cumulative increased likelihood of 1-year mortality and was calculated from *ICD-10* diagnoses registered in the National Patient Registry before the day of surgery.[14,15] The comorbidity score was categorized as *normal* (0), *moderate* (1), severe (2), and *very severe* (≥3).[16]

Statistical Analysis

Duration of follow-up was calculated from the day of surgery and analyzed by the reverse Kaplan-Meier method.[17] For long-term outcomes, Kaplan-Meier curves were plotted and patients with and without AL were compared by the log-rank analysis. Univariable and multivariable Cox regression analyses were used to investigate the influence of AL on disease recurrence, all-cause mortality, and administration of AC.

Hazard ratios (HRs) with 95% confidence intervals (CIs) of more than 1 indicated increased likelihood of disease recurrence, fatal

TABLE 2. Multivariable Cox Regression Analyses of Long-Term Outcomes

	Local Recurrence			Distant Recurrence			All-Cause Mortality		
	HR*	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
AL			0.184			0.003			0.042
No	1.00			1.00			1.00		
Yes	0.78	0.55–1.12		1.42	1.13–1.78		1.20	1.01–1.44	
Gender			0.302			0.033			<0.001
Female	1.00			1.00			1.00		
Male	1.08	0.94–1.23		1.13	1.01–1.26		1.35	1.25–1.46	
Age groups, yr			<0.001			<0.001			<0.001
≤ 60	1.00			1.00			1.00		
> 60–70	0.72	0.61–0.86	<0.001	0.93	0.80–1.09	0.380	1.37	1.18–1.60	<0.001
> 70–80	0.55	0.45–0.65	<0.001	0.90	0.77–1.05	0.176	2.29	1.99–2.64	<0.001
> 80	0.35	0.27–0.45	<0.001	0.62	0.50–0.75	<0.001	4.14	3.58–4.78	<0.001
Comorbidity†			0.119			0.004			<0.001
Normal	1.00			1.00			1.00		
Moderate	1.19	0.99–1.44	0.065	1.04	0.88–1.22	0.654	1.39	1.25–1.54	<0.001
Severe	1.14	0.91–1.43	0.266	1.20	1.00–1.44	0.057	1.63	1.44–1.83	<0.001
Very severe	0.83	0.58–1.18	0.293	1.45	1.17–1.81	<0.001	1.99	1.73–2.29	<0.001
Tumour stage			<0.001			<0.001			<0.001
UICC I	1.00			1.00			1.00		
UICC II	0.97	0.79–1.19	0.759	1.90	1.50–2.40	<0.001	1.33	1.16–1.52	<0.001
UICC III	1.50	1.23–1.84	<0.001	4.18	3.31–5.27	<0.001	2.28	1.99–2.61	<0.001
Surgical procedure			0.111			0.993			0.035
Right hemicolectomy	1.00			1.00			1.00		
Transverse colectomy	1.33	0.90–1.96	0.154	1.03	0.71–1.47	0.892	1.09	0.87–1.36	0.441
Left hemicolectomy	0.89	0.70–1.12	0.313	1.00	0.83–1.21	0.984	0.90	0.79–1.04	0.154
Sigmoid colectomy	0.88	0.75–1.03	0.101	0.99	0.87–1.12	0.812	0.90	0.81–0.97	0.010
Surgical approach			0.015			0.789			0.641
Open	1.00			1.00			1.00		
Laparoscopic	1.24	1.04–1.48		0.98	0.83–1.16		0.97	0.86–1.10	
Surgical priority			0.051			<0.001			<0.001
Elective	1.00			1.00			1.00		
Emergency	1.32	1.00–1.74		2.14	1.80–2.54		1.75	1.54–1.99	
Surgeons' specialization			<0.001			0.181			0.023
Gastrointestinal	1.00			1.00			1.00		
General	0.69	0.58–0.82		1.09	0.96–1.24		1.10	1.01–1.20	
Organ resection			0.211			<0.001			<0.001
None	1.00			1.00			1.00		
Spleen	1.16	0.57–2.37	0.690	1.33	0.79–2.24	0.291	1.50	1.07–2.10	0.019
Other organs	1.21	0.98–1.51	0.082	1.59	1.35–1.86	<0.001	1.38	1.23–1.55	<0.001
Blood transfusion			0.467			0.519			0.002
No	1.00			1.00			1.00		
Yes	1.07	0.89–1.28		1.05	0.91–1.20		1.15	1.05–1.26	

The number of patients included in each outcome response was $N = 6900$ for local recurrence, $N = 7315$ for distant recurrence, and $N = 8164$ for all cause mortality.
*HR < 1 indicates decreased likelihood of disease recurrence or mortality, whereas HR > 1 indicates increased likelihood of disease recurrence or mortality.
†Comorbidity according to Charlson comorbidity index scores of 0 (*normal*), 1 (*moderate*), 2 (*severe*), and ≥ 3 (*very severe*).

outcome, and administration of AC. Schoenfeld residuals were examined to verify the assumption of proportional hazards. The adjusted influence of AL on the time to initial administration of AC was calculated with multiple linear regression in the subset of patients who did receive AC. All variables were simultaneously included in the multivariable analyses. Status of disease recurrence was then entered as a time-dependent variable to assess the potential mediating role of disease recurrence on the relationship between AL and all-cause mortality. Missing values were disregarded and thus not replaced. Number and percentage of missing values are provided in Table 1.

The results of the multivariable analyses are presented as hazard ratios (HR) with 95% confidence intervals (CI). All analyses were 2-sided and considered statistically significant if $P < 0.050$. Data were analyzed with SPSS Statistics Version 20 (IBM Corp, Armonk, NY).

Meta-analysis of published data on the relationship between AL and distant disease recurrence was performed using the Mantel-Haenszel random-effects model (Review Manager, Version 5.1, Copenhagen, Denmark: the Nordic Cochrane Centre, the Cochrane Collaboration), updating (September 2013) the systematic review from Mirnezami et al[9] including the present data. A sensitivity analysis was performed removing individual studies in a serial fashion to determine the influence of each study on the combined OR estimate and 95% CI.

RESULTS

A total of 18,484 patients were assessed for inclusion of whom 9333 (50.5%) were eligible (Fig. 1). The overall incidence of AL was 593/9333 (6.4%).[5] Four patients were excluded because of emigration after surgery, and 740 of 9329 (7.9%) patients died within the first 120 days: 173 of 593 (29.2%) with AL and 567 of 8736 (6.5%) without AL, $P < 0.001$. Thus, 8589 patients with a median age of 72 years

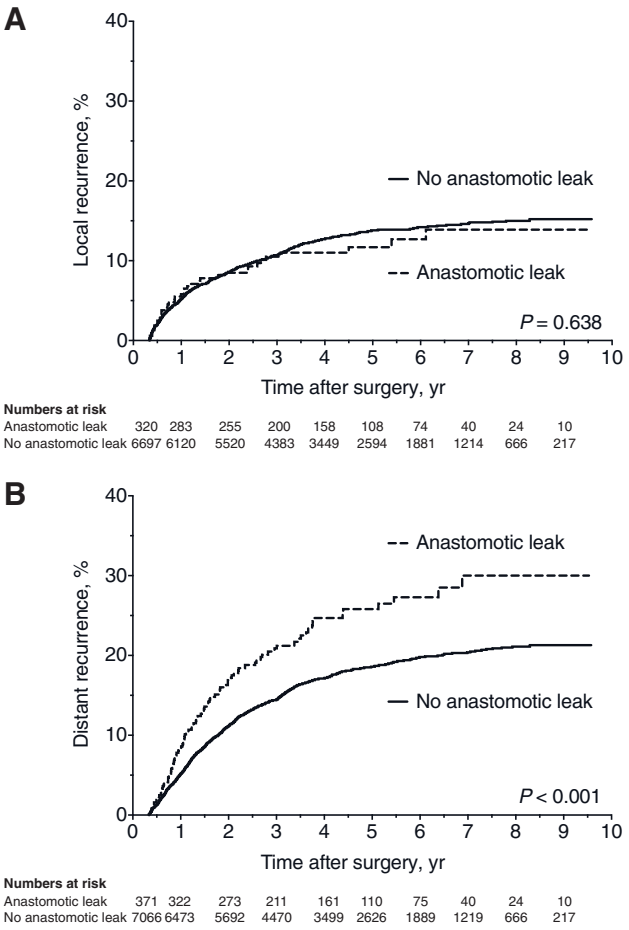

FIGURE 2. Kaplan-Meier plots illustrating the association between AL and the rates of local (A) and distant (B) recurrence in patients alive 120 days after curative colonic cancer surgery.

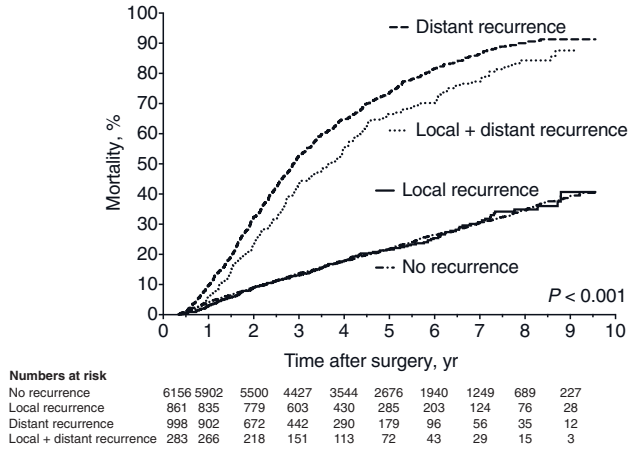

FIGURE 4. Kaplan-Meier plots illustrating all-cause mortality in patients alive 120 days after curative colonic cancer surgery. Mortality was increased in patients with distant recurrence (adjusted HR = 4.07, 95% CI 3.53–4.68, $P < 0.001$), local and distant recurrence (adjusted HR = 3.37, 95% CI: 2.68–4.25, $P < 0.001$), and in contrast to local recurrence (adjusted HR = 1.08, 95% CI: 0.92–1.27, $P = 0.361$).

(range: 23–98 years) were studied (Table 1). Median follow-up was 5.3 years (interquartile range [IQR]: 3.6–7.3 years).

Disease Recurrence

The number of patients with local and distant recurrence were 861 (10.0%) and 1281 (14.9%), respectively. The latter group included 283 (3.3%) patients with both local and distant recurrence. The number of disease-free patients alive at the end of follow-up was 4849 (56.5%). The median time to diagnosis of local and distant recurrence was 1.2 years (IQR: 0.6–2.4 years) and 1.5 years (IQR: 0.9–2.5 years), respectively. In the univariable analysis, AL was not associated with local recurrence [HR = 0.92 (95% CI: 0.66–1.29; $P = 0.638$)], which was confirmed in the multivariable analysis [HR = 0.78 (95% CI: 0.55–1.12; $P = 0.184$)]. Covariates with a significant influence on

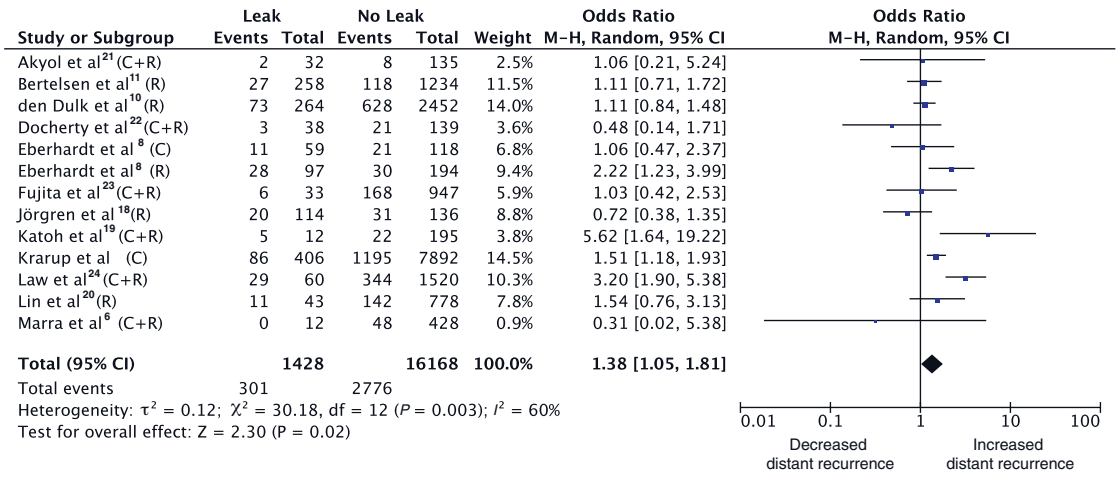

FIGURE 3. Forest plot on the association between AL and the incidence of distant recurrence after colonic (C) and rectal (R) cancer surgery. The meta-analysis was updated from Mirnezami et al[9] in September 2012 adding 4 additional studies[6,18–20] and the present results.

TABLE 3. Univariable and Multivariable Cox Regression Analyses of Adjuvant Chemotherapy in Patients With Stage III Colonic Cancer

	Adjuvant Chemotherapy	No Adjuvant Chemotherapy	Univariable		Multivariable		
			HR*	95% CI)	HR*	95% CI)	*P*
N = 2841	1725 (61%)	1116 (39%)					
AL							<0.001
No	1659 (96%)	1053 (94%)	1.00		1.00		
Yes	66 (4%)	63 (6%)	0.65	0.51–0.83	0.58	0.45–0.74	
Gender							0.449
Female	877 (51%)	650 (58%)	1.00		1.00		
Male	848 (49%)	466 (42%)	1.18	1.08–1.29	1.04	0.94–1.14	
Age groups, yr							<0.001
≤ 60	1275 (74%)	646 (58%)	1.00		1.00		
>60–70	232 (13%)	225 (20%)	0.82	0.73–0.92	0.86	0.76–0.97	
>70–80	142 (8%)	147 (13%)	0.39	0.35–0.45	0.43	0.38–0.49	
> 80	76 (4%)	98 (9%)	0.05	0.04–0.07	0.06	0.04–0.08	
Comorbidity†							<0.001
Normal	523 (30%)	69 (6%)	1.00		1.00		
Moderate	649 (38%)	165 (15%)	0.65	0.56–0.75	0.86	0.74–0.99	
Severe	505 (29%)	444 (40%)	0.61	0.51–0.73	0.72	0.60–0.85	
Very severe	48 (23%)	438 (39%)	0.53	0.42–0.67	0.62	0.49–0.79	
Surgical procedure							0.748
Right hemicolectomy	802 (47%)	626 (56%)	1.00		1.00		
Transverse colectomy	37 (2%)	31 (3%)	0.88	0.63–1.23	0.92	0.66–1.28	
Left hemicolectomy	221 (13%)	109 (10%)	1.33	1.15–1.54	1.06	0.91–1.23	
Sigmoid colectomy	665 (39%)	350 (31%)	1.30	1.17–1.44	0.98	0.88–1.09	
Surgical approach							0.048
Open	1371 (79%)	935 (84%)	1.00		1.00		
Laparoscopic	354 (21%)	181 (16%)	1.22	1.09–1.37	1.13	1.00–1.28	
Surgical priority							0.029
Elective	1589 (92%)	1005 (90%)	1.00		1.00		
Emergency	136 (8%)	111 (10%)	0.80	0.67–0.96	0.82	0.69–0.98	
Surgeons' specialization							<0.001
Gastrointestinal	1391 (81%)	781 (70%)	1.00		1.00		
General	334 (19%)	334 (30%)	0.69	0.62–0.78	0.70	0.62–0.79	
Organ resection							0.454
None	1523 (88%)	986 (88%)	1.00		1.00		
Spleen	13 (1%)	9 (1%)	0.79	0.46–1.36	0.71	0.40–1.23	
Other organs	189 (11%)	121 (11%)	0.98	0.84–1.13	1.02	0.87–1.19	
Blood transfusion							<0.001
No	1445 (84%)	757 (69%)	1.00		1.00		
Yes	268 (16%)	348 (31%)	0.52	0.46–0.59	0.68	0.59–0.78	

*HR < 1 indicates decreased likelihood of receiving AC, whereas HR > 1 indicates increased likelihood of AC. *P*, multivariable analysis.
†Comorbidity according to Charlson comorbidity index scores of 0 (*normal*), 1 (*moderate*), 2 (*severe*), and ≥3 (*very severe*).

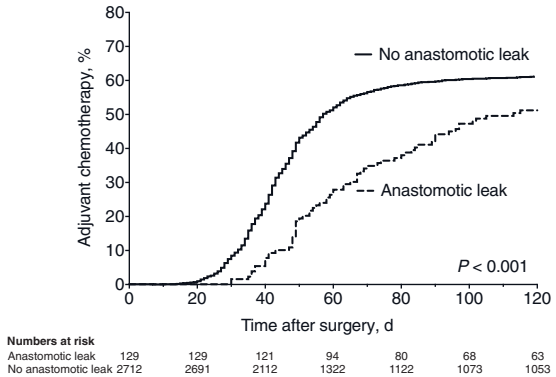

FIGURE 5. Kaplan-Meier plots illustrating the rates of administered AC in stage III colonic cancer patients with and without AL.

local recurrence were age, tumor stage, surgical approach, and surgeon's specialization level (Table 2). In contrast to local recurrence, there was a significant association between AL and distant recurrence in both the univariable analysis [HR = 1.49 (95% CI: 1.20–1.85; $P < 0.001$] (Fig. 2) and the multivariable analysis [HR = 1.42 (95% CI: 1.13–1.78; $P = 0.003$] (Table 2). In addition, gender, age, comorbidity, tumor stage, surgical priority, and extracolonic organ resection were significantly associated with distant recurrence (Table 2).

The association between AL and distant recurrence was put in context of previous published studies, updating a meta-analysis by Mirnezami et al.[9] Four additional studies were included in addition to the present data.[6,18–20] The Forest plot showed a significant association between AL and development of distant recurrence (OR = 1.38; 95% CI: 1.05–1.81; $P = 0.02$; Fig. 3). The sensitivity analysis rendered a nonsignificant association between AL and distant recurrence in the combined estimate after exclusion of the study by Law et al[20] (OR = 1.26; 95% CI: 0.99–1.60; $P = 0.06$) or the present study (OR = 1.35; 95% CI: 0.97–1.89; $P = 0.08$).

TABLE 4. Multiple Linear Regression Analysis of Time to Adjuvant Chemotherapy (Days)

Variables	Estimated Coefficient (95% CI)	*P*
AL		<0.001
No (reference)	0	
Yes	16 (12–20)	
Gender		0.804
Female (reference)	0	
Male	0 (−1 to 2)	
Age groups, yr		0.187
≤ 60 (reference)	0	
> 60–70	0 (−3 to 1)	0.520
> 70–80	1 (0–3)	0.218
> 80	2 (−2 to 7)	0.396
Comorbidity*		0.176
Normal (reference)	0	
Moderate	1 (−1 to 4)	0.205
Severe	3 (0–6)	0.060
Very severe	2 (−3 to 5)	0.546
Surgical procedure		0.153
Right hemicolectomy (reference)	0	
Transverse colectomy	5 (0–11)	0.058
Left hemicolectomy	− 2 (−4 to 1)	0.234
Sigmoid colectomy	0 (−3 to 1)	0.376
Surgical approach		0.026
Open (reference)	0	
Laparoscopic	− 2 (−4 to 0)	
Surgical priority		<0.001
Elective (reference)	0	
Emergency	5 (2–8)	
Surgeons' specialization		0.696
Gastrointestinal (reference)	0	
General	0 (−2 to 2)	
Organ resection		0.012
None (reference)	0	
Spleen	12 (3–21)	0.008
Other organs	2 (−1 to 4)	0.232
Blood transfusion		<0.001
No (reference)	0	
Yes	5 (3–7)	

The analysis included a subset of patients with stage III colonic cancer who received adjuvant chemotherapy within 120 days from the index operation, $N = 1725$. The *Intercept* in this model was 44 days (95% CI: 42–47 days) and predicts the time to AC when all variables take the reference value. The coefficients were rounded to whole numbers (days) and estimate the change in time to AC given a change from the reference value.

*Comorbidity according to the Charlson Comorbidity Index scores of 0 (*normal*), 1 (*moderate*), 2 (*severe*), and ≥3 (*very severe*).

Long-Term All-Cause Mortality

AL was significantly associated with increased all-cause mortality in the multivariable analysis [HR = 1.20 (95% CI: 1.01–1.44; $P = 0.042$)]. Covariates that reached statistical significance were gender, age, comorbidity, tumor stage, surgical procedure and priority, surgeon's specialization level, extracolonic organ resection, and blood transfusion (Table 2).

In contrast to local recurrence, distant recurrence was significantly ($P < 0.001$) associated with all-cause mortality (Fig. 4). Inclusion of recurrence status as a time-dependent variable in the multivariable model eliminated the impact of AL on mortality [HR = 1.10 (95% CI: 0.92–1.32; $P = 0.289$)].

Administration of Adjuvant Chemotherapy in Stage III Cancer Patients

In the subgroup of patients with stage III disease, administration of AC was initiated in 1725 of 2841 (60.7%) patients within 120 days. Patients with AL were less likely to receive AC according to both the univariable [HR = 0.65 (95% CI: 0.51–0.83; $P < 0.001$)] and the multivariable models [HR = 0.58 (95% CI: 0.45–0.74; $P < 0.001$] (Table 3 and Fig. 5). In addition, the time to initial administration of AC was median 59 days (IQR: 48–82 days) after surgery for patients with AL compared with 43 days (IQR: 35–54 days) in patients without AL ($P < 0.001$). Multiple linear regression analysis confirmed that administration of AC was initiated 16 days (95% CI: 12–20 days; $P < 0.001$) later in patients with AL compared with patients without AL (Table 4).

Administration of AC in patients with AL was not associated with a reduction in distant recurrence but a significant reduction in all-cause mortality (Figs. 6A, C). Administration of AC beyond 55 days from primary surgery was not associated with a significant reduction in long-term mortality [HR = 0.88 (95% CI: 0.68–1.02] (Table 5). The rates of distant recurrence and mortality in patients receiving AC within day 55 were comparable between patients with and without AL (Figs. 6B, D).

DISCUSSION

This nationwide study demonstrates an inferior oncological outcome in patients who develop AL after curative resection for colonic cancer and survive the first critical postoperative phase. This is reflected by an increased rate of distant recurrence and therefore a continuing excess mortality.

The distinct finding, that AL was associated with a significant increase in distant recurrence, provides new insight into the negative implications of AL in patients with colonic cancer. The issue was recently addressed in a meta-analysis in which Mirnezami et al were unable to confirm a relationship between AL and distant recurrence (overall OR = 1.38; 95% CI: 0.96–1.99; $P = 0.083$).[9] However, the meta-analysis included pooled data from studies on both colonic[8] and rectal cancer surgery,[8,10,11] as well as mixed colorectal cancer surgery.[21–24] The update of the meta-analysis by Mirnezami et al including the present data suggests a relationship between AL and development of distant recurrence. The sensitivity analysis indicates that the large cohort in the present study may drive the outcome of the meta-analysis to become statistically significant. A fuller meta-analysis including a subgroup analysis of patients with colonic cancer is warranted to address the robustness of the conclusion that AL increases the risk of distant recurrence in patients with colonic cancer. The unadjusted results from the meta-analysis could also be subjected to confounding because advanced tumor stage, emergency surgery, and comorbidity were strong predictors for distant recurrence in the present study. Nevertheless, the association between AL and distant recurrence remained significant in the multivariable analysis. A limitation of this finding is the competing risk of mortality in patients dying before a potential recurrence.

The present study included patients from all Danish departments conducting colorectal surgery during the study period and was further strengthened by an almost complete and unselected compilation of data merged from 3 different population-based national registers. There was a long follow-up period extending beyond the traditional 2 years used for assessing recurrence status. However, radiological and endoscopic follow-up was not performed routinely raising the risk that some patients with recurrence may have remained unrecognized despite the study design with 3 independent sources of data. Another limitation is the allocation of patients with both local and distant recurrence to the group of patients with distant recurrence.

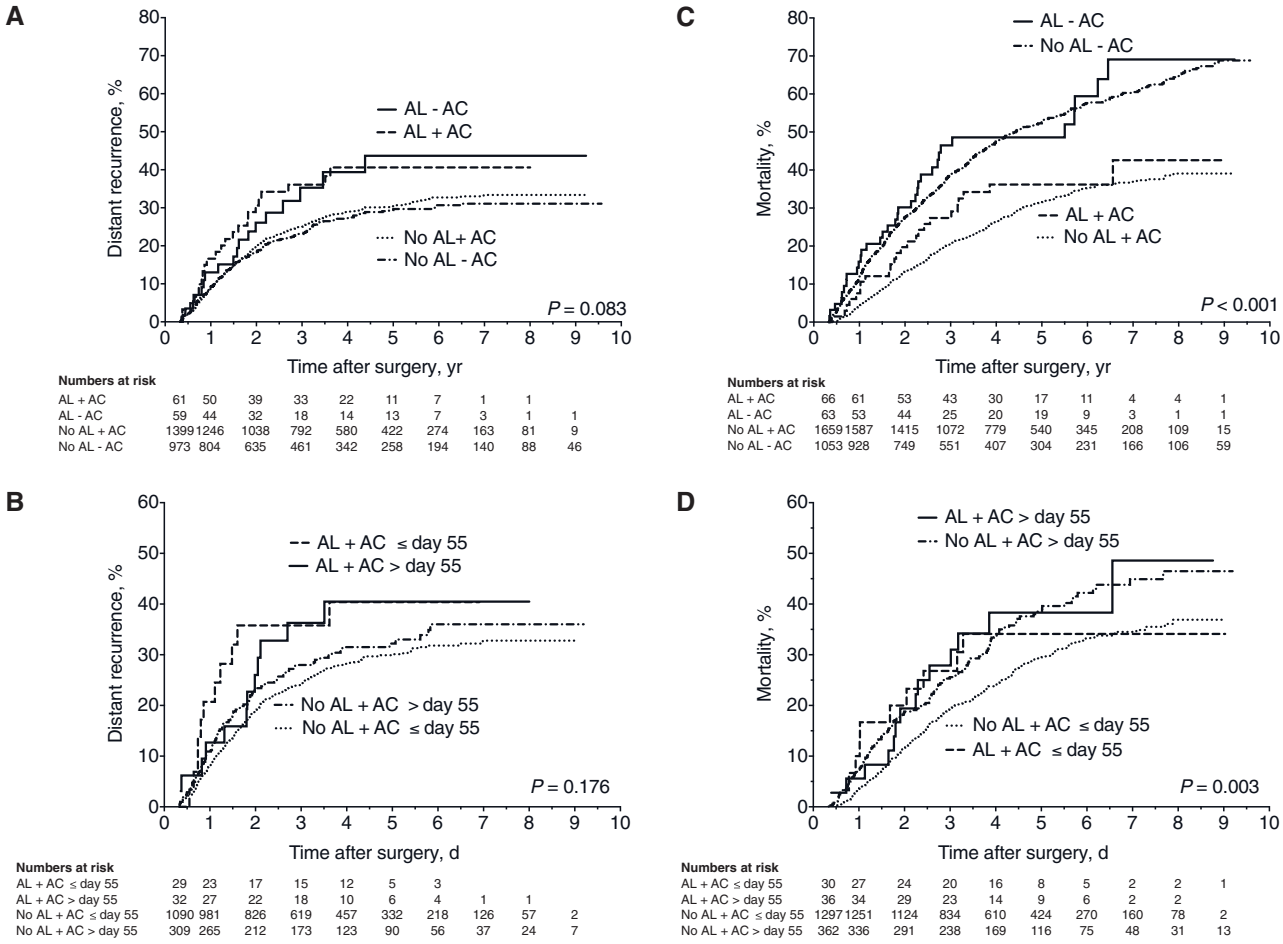

FIGURE 6. Kaplan-Meier plots illustrating the rates of distant recurrence (A, B) and all-cause mortality (C, D) in patients with and without ALs after surgery for stage III colonic cancer. Data were stratified for administration of AC in panels A and C, and for time to initial administration of AC in panels B and D. The *P* value represents the overall log-rank analyses.

TABLE 5. Univariable and Multivariable Analyses of Time to Initial Administration of Adjuvant Chemotherapy and All-Cause Mortality In Patients With Stage III Colonic Cancer

		Univariable		Multivariable	
	n	**HR* (95% CI)**	***P***	**HR* (95% CI)**	***P***
Time to chemotherapy, d			<0.001		<0.001
No chemotherapy	1116	1.00		1.00	
0–35	434	0.51 (0.42–0.61)		0.76 (0.62–0.94)	
36–43	367	0.43 (0.35–0.54)		0.63 (0.50–0.80)	
44–55	424	0.38 (0.31–0.47)		0.53 (0.42–0.66)	
56–120	398	0.62 (0.52–0.74)		0.83 (0.68–1.02)	

Time intervals were based on quartiles. Multivariable Cox regression analysis was adjusted for AL, age, gender, comorbidity, surgical procedure, approach and priority, surgeons' specialization, extracolonic organ resection, and blood transfusion.

*HR < 1 indicates decreased likelihood of fatal outcome.

This could potentially limit the analyses of local recurrence because a subset of these patients was thus not included in the analyses. The purpose was to avoid an overinterpretation of the impact of local recurrence and was supported by the finding of equal mortality rates in any patient with distant metastasis compared with patients with local recurrence (Fig. 4).

Although AL increased the rate of distant recurrence, no influence on local recurrence was observed. This is in agreement with other reports on colonic surgery but differs from rectal surgery.[7–9] The finding illustrates the importance of analyzing long-term outcome in patients with colonic and rectal cancer separately. The incidence of local recurrence is reported lower after resection of colonic cancer

as compared with rectal cancer, possibly because local recurrence in colonic cancer may remain undiagnosed until symptomatic distant metastases develop.[25] In addition, data from a human study demonstrated significantly higher cytokine levels, especially interleukin-8, in the region around rectal anastomoses as compared with colonic anastomoses.[26] interleukin-8 promotes proliferation and metastasis of colorectal cancer cells and may impair anastomotic healing.[26,27]

Interestingly, local recurrence did not affect the overall mortality. This somewhat surprising finding was also reported in a randomized trial on the effect of preoperative radiotherapy in rectal cancer surgery, where a significant reduction in local recurrence from 8.2% to 2.4% was not paralleled by a reduction in mortality.[28] The present cohort of more than 9000 unselected patients with colonic cancer allowed a thorough analysis of the association of AL with local recurrence including adjustment for confounders. The findings confirm that AL does not predict development of local recurrence in patients with colonic cancer.

Previously, the impact of AL on long-term mortality has been analyzed in different ways. Five-year overall survival was decreased in patients with AL,[2,6] but these studies included patients dying within 30 days after surgery. To avoid this potential overlap of short- and long-term mortality, patients who died within the first 120 days were excluded from the present analysis. It was subsequently observed that patients with AL were still at increased risk of a fatal outcome. Adjustment for comorbidity, a known predictor for reduced survival in patients with colorectal cancer,[29] did not alter this conclusion. Interestingly, the influence of AL on mortality did not persist with addition of recurrence status to the multivariable analysis. This suggests that the effect of AL on long-term mortality was mediated by an increased rate of distant recurrence.

It remains to be established which mechanisms promote distant recurrence in patients with AL. To address this, the impact of AL on administration of AC in patients with stage III colonic cancer was analyzed. AC increases overall survival by about 30% compared with surgery alone[30,31] and is thus important in these high-risk patients. Severe inflammation due to peritonitis and septicemia after AL may contribute to the metastatic cascade explaining the association between AL and distant recurrence in the present study.[32] Furthermore, AL was strongly associated with cancelled administration of AC in the subset of patients with stage III colonic cancer. Similar findings were reported in patients with AL after low anterior resection.[33] Recently, El Shayeb et al[34] demonstrated that comorbidity and advanced age were main reasons physicians did not recommend AC. Here, the association between AL and administration of AC was consistent after adjustment for confounding variables including age and comorbidity. The authors did further conclude that reasons for patient refusal were mostly unknown.[34] Postoperative complications including AL could influence these patients' attitude toward AC.

AL was also associated with a significant delay in initial administration of AC. The delay persisted after adjustment for confounding variables and postponed the initial administration to 8 weeks after primary surgery. When AC was initiated beyond day 55 after primary surgery, there was no significant reduction in mortality compared with patients who did not receive AC. Biagi et al[35] recently addressed the consequences of delayed AC, reporting a 14% decrease of both overall and disease-free survival for every 4 weeks' postponement of initial administration. The present data do not support late onset administration of AC in patients with AL after curative surgery for stage III colonic cancer.

CONCLUSIONS

This study demonstrated increased mortality rates in patients surviving the first critical phase after AL. A robust association between AL and distant recurrence was observed suggesting that the poor long-term prognosis in patients with AL was promoted by increased metastatic activity. Cancelled or delayed administration of AC because of AL may partly account for these findings.

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ORIGINAL ARTICLE

Expression and inhibition of matrix metalloproteinase (MMP)-8, MMP-9 and MMP-12 in early colonic anastomotic repair

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Abstract
Purpose Submucosal collagen is paramount for colonic anastomotic integrity. Matrix metalloproteinases (MMPs) mediate collagen degradation that increases the risk of wound dehiscence. Although broad-spectrum MMP inhibitors are beneficial for anastomotic strength, they can cause adverse reactions. Knowledge of specific MMPs responsible for the weakening of anastomoses can be used to optimise MMP inhibition therapy. We aimed to quantify transcript and protein levels of multiple MMPs in colonic anastomoses and evaluate the effect of inhibiting the MMPs that displayed the highest expression levels on anastomotic repair.

Methods Left-sided colonic anastomoses were made in male Sprague-Dawley rats. After 3 days when biomechanical strength is lowest, MMP mRNA and protein levels were measured by quantitative real-time polymerase chain reaction, enzyme-linked immunosorbent assays and gelatin zymography. The effects of the MMP-8, MMP-9 and MMP-12 synthetic inhibitor AZD3342 was also studied.
Results MMP-8, MMP-9 and MMP-12 gene and protein expression increased profoundly ($p<0.01$), and MMP-13 mRNA and MMP-2 mRNA and protein modestly ($p<0.001$) in the anastomoses. MMP-3 mRNA levels were not up-regulated significantly compared with adjacent uninjured colon. Increased anastomotic MMP-12 levels paralleled macrophage infiltration by immunohistochemical analyses. AZD3342 (50 mg/kg) treatment increased the anastomotic breaking strength by 29 % ($p=0.015$) day 3 compared with vehicle. Improved anastomotic strength was not accompanied with alterations of type I or type III procollagen mRNA but was possibly due to inhibition of the concerted digestive action on the existent submucosal collagens by the targeted MMPs.
Conclusion The present findings justify the concept of selective MMP inhibition to enhance anastomotic strength in colon.

Keywords Colonic anastomosis · Matrix metalloproteinase · MMP · Collagen · Experimental

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Introduction

Anastomotic leakage remains a devastating complication following colorectal surgery causing morbidity, permanent stomas and mortality [1, 2].

Anastomotic surgery induces influx of inflammatory cells to the suture-line. These and resident cells in the colon express

multiple matrix metalloproteinases (MMPs). MMPs are a family of 23 human zinc-dependent endopeptidases able to degrade collagens and other components of the extracellular matrix [3].

Submucosal collagen is essential to maintain anastomotic strength by the sutures [4]. Postoperatively, collagen concentration and anastomotic breaking strength decline to a minimum around day 3 [5–7]. Consequently, it has been hypothesised that overexpression of specific MMPs may be responsible for the reduced anastomotic strength via collagen degradation during this critical early phase of healing when leakages are most frequent [1, 8–11]. Excessive local levels of MMP-2, MMP-8 and MMP-9 but not of MMP-1, MMP-3, MMP-7 or MMP-13 were associated with increased risk of anastomotic leakage in humans [12, 13].

Systemic treatment with synthetic compounds that non-selectively inhibit MMPs has consistently shown improvement of anastomotic strength in experimental models [5, 6, 14, 15]. Adverse reactions following administration of these broad-spectrum MMP inhibitors may limit their use in humans [16–18]. Therefore, a detailed understanding of the MMPs indispensable for anastomotic healing will provide the means to selectively inhibit only the MMPs that are detrimental to anastomotic healing [15, 19].

We have previously demonstrated accumulation of macrophages around the suture channels expressing plentiful of MMP-8 and MMP-9 on postoperative day 3 [11]. Interestingly, MMP-8 and MMP-9 combined degrade submucosal collagens synergistically [11]. Macrophages are also the primary source of the macrophage metalloelastase or MMP-12. Increased tissue levels of MMP-12 have been reported during normal wound healing in the skin and in colitis [20–22]. Although elastin is the main substrate [23, 24], it was recently shown that MMP-12 also possesses collagenolytic activity in vitro, i.e. has the capacity to cleave native triple helical collagen [25]. The role of MMP-12 has to our knowledge not been investigated during anastomotic healing in the large bowel.

Because of paucity of quantitative information on MMP expression during anastomotic healing, our aim was to measure a broad panel of MMPs on both the transcriptional and translational levels on postoperative day 3 in colon anastomoses in male rats. The panel included MMP-2, MMP-3, MMP-8, MMP-9, MMP-12 and MMP-13 mRNA, and MMP-2, MMP-8, MMP-9 and MMP-12 proteins. MMP-12 expression was further assessed by immunohistochemistry. To confirm the functional relation to anastomotic strength, the specific MMPs that displayed the largest increase were targeted pharmacologically.

Materials and methods

Animals

Male Sprague–Dawley rats (Taconic, Ry, Denmark) weighing 220–327 g were kept in type III cages at room temperature with a 12-h light cycle [26]. Animals had free access to tap water and pellets. The rats were acclimatised for 7 days prior surgery and then kept in separate cages. The experiments were approved by the Animal Ethics Committee of The Danish Ministry of Justice (2010/561-1775).

Construction of anastomoses and treatment with the MMP inhibitor AZD3342

Anesthesia was introduced with a mixture of isoflurane (Baxter, Deerfield, IL, USA) 3.5 %/O_2 (1 l/min) for 2 min and maintained with isoflurane (2 %)/O_2. Bupivacain (Marcain®; AstraZeneca, London, UK) 2 mg/kg was administered subcutaneously (s.c.) at the incisional site for local analgesia. Preoperative analgesia was provided by s.c. injections of 5 mg/kg carprofen (Rimadyl®; Pfizer Animal Health, New York, NY, USA) and 0.03 mg/kg buprenorphine (Temgesic®; Schering- Plough, Brussels, Belgium). End-to-end left-sided colonic anastomoses were constructed as previously described [11, 15]. Rats were rehydrated with 5 ml saline s.c. and 5 ml saline i.p. Postoperative analgesia was provided by buprenorphine (0.4 mg/kg) p.o. in a hazelnut-butter mixture at 8-h intervals and with daily s.c. injections of carprofen (5 mg/kg).

AZD3342 (AstraZeneca Research & Development, Mölndal, Sweden) is a 403 D, synthetic non-hydroxamate MMP-8, MMP-9 and MMP-12 inhibitor. The half maximal inhibitory concentration (IC_{50}) for MMP-8 was determined to 16 nM, for MMP-9 to 10 nM and for MMP-12 to 6 nM and with greater than three orders of magnitude selectivity over MMP-1 and tumor necrosis factor-α converting enzyme. The in vivo effects of AZD3342 were evaluated in two separate series. In the first series, the plasma AZD3342 concentration and anastomotic breaking strength on postoperative day 3 were investigated in 48 rats recieving vehicle (n=16), 5 mg/kg AZD3342 (n=16) or 50 mg/kg AZD3342 (n=16). In the second series including 31 rats, MMP mRNA and protein profiling was carried out on the anastomoses of the vehicle-treated animals (n=16). The effect of AZD3342 on anastomotic breaking strength and collagen gene expression was also assessed (n=15). The rats were randomised to the groups in blocks of four and given one subcutaneous injection of vehicle composed of 10 % 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich, St. Louis, MO, USA) or AZD3342 in vehicle 2 h prior to surgery, then again on postoperative day 1, and the third and final injection was given on postoperative day 2. Individuals handling the rats were blinded to the groups.

Plasma AZD3342 determination

Blood samples were drawn from the beating heart in heparin-containing tubes 24 h after administration of the last dosage. The blood was centrifuged at 4,000 rpm for 20 min at 4 °C and the plasma stored at −80 °C in polypropylene cryotubes (Greiner Bio-One, Frickenhausen, Germany). AZD3342 was measured by liquid chromatography-mass spectrometry.

Tissue procurement and breaking strength determination

On postoperative day 3, the animals were anesthetised and laparotomised. A 40-mm-long colonic segment with the anastomosis in the middle was resected, examined macroscopically for anastomotic leakage and immersed in Ringer's solution (pH 7.4). The segment was freed of adhesions and feces. The rats were killed by cervical dislocation.

A 5-mm long section was excised 15 mm proximal to the suture line (hereafter named "proximal non-injured colon"). The remaining 35-mm segment was fastened with clamps positioned 10 mm apart in a materials testing machine equipped with 10 N XLC load cell (LF Plus; Lloyd Instruments, Fareham, UK). The specimen was pulled apart vertically at 10 mm/min and the breaking strength in Newton (N) determined from the load-deformation curve [7]. The healing zone (hereafter named "anastomotic wound") was dissected from macroscopically normal colon. Procured tissue samples were bisected. One half was snap-frozen in liquid nitrogen and then transferred to −80 °C for subsequent determination of mRNAs. The other half was placed on dry ice and then kept at −80 °C until extracted for protein analyses.

mRNA determination of MMP-2, MMP-3, MMP-8, MMP-9, MMP-12, MMP-13, type I procollagen (COL1A1) and type III procollagen (COL3A1) by quantitative real-time polymerase chain reaction

Ribonucleic acid (RNA) was extracted from colonic tissue using the TRI-reagent® (Molecular Research Center, Cincinnati, OH, USA) [27]. Following isolation of the aqueous phase, RNA was precipitated with isopropanol. The RNA pellet was washed in ethanol and subsequently dissolved in RNase-free water. RNA concentrations were estimated spectrophotometrically at 260 nm. RNA quality was checked on an agarose gel stained with ethidium bromide.

A total of 200 ng RNA was converted into cDNA in 20 μl using the OmniScript reverse transcriptase (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. For each target mRNA, 0.25 μl cDNA was amplified in a 25 μl SYBR Green polymerase chain reaction (PCR) containing 1× Quantitect SYBR Green Master Mix (Qiagen) and 100 nM of the primers (Table 1). The amplification was monitored in real time using the MX3000P real-time PCR machine (Stratagene, Santa Clara, CA, USA). The real-time PCR conditions were as follows—95 °C for 10 min, followed by 50 cycles of 15 s at 95 °C, 30 s at 58 °C and 90 s at 63 °C, followed by a melting curve analysis consisting of heating from 55 to 95 °C. The threshold cycle (C_t) values were related to a standard curve made with cloned PCR products to determine the relative difference between the unknown samples, accounting for the PCR efficiency. The specificity of the PCR products was confirmed by melting curve analysis after amplification. The large ribosomal protein P0 (RPLP0) was chosen as internal control, as RPLP0 mRNA has been suggested to be constitutively expressed [28] and was found completely stable in relation to GAPDH mRNA.

Tissue protein measurements of MMP-2, MMP-8, MMP-9 and MMP-12

Tissue samples were incubated in buffer (1.0 ml/50 mg tissue) composed of 10 mM cacodylate-HCl (pH 6.0), 1 M NaCl, 1 μM $ZnCl_2$, 0.1 mg/ml Triton X-100 and 0.2 mg/ml NaN_3 for 24 h at 4 °C and centrifuged at 5,000 rpm for 5 min [29]. Resulting pellets were subjected to a second 24-h extraction cycle. Supernatants from the two extractions were combined in equal volumes. The total protein content was determined by the Bradford microassay (TP0100; Sigma-Aldrich) and ranged from 0.5 to 1.4 mg/ml. Tissue extracts were kept at −80 °C until analysed.

MMP-2 and MMP-9 were semiquantified by gelatin zymography using Novex® 10 % minigels (1 mg/ml gelatin), equipment and reagents from Life Technologies (Carlsbad, CA, USA). Extracts were normalised to 4 μg total protein and electrophoresed at constant 125 V. Gels were then renatured in 2.5 % Triton X-100 for 30 min at ambient temperature and incubated for 44 h at 37 °C in developing buffer. Gels were stained with Colloidal Blue Staining kit (Life Technologies), destained and assembled. Densitometry was performed on scanned zymograms using ImageJ (National Institutes of Health, Bethesda, MD, USA) [30].

Rat MMP-8 (RayBiotech, Norcross, GA, USA), rat MMP-9 (Uscn Life Science, Wuhan, China) and rat MMP-12 (Uscn Life Science) levels were quantified using enzyme-linked immunosorbent assays kits according to the manufacturers' manuals and expressed in nanograms per milligram total protein in the tissue extracts.

Immunohistochemistry of MMP-12 and macrophages

Archival paraffin blocks of intact colonic anastomoses from day 0 (n=4) and day 3 (n=10) were used for these studies [11]. Tissue sections (5 μm) were brought to distilled water and boiled 2×5 min in a microwave oven in 10 mM sodium citrate (pH 6.0). The sections were washed in distilled water, endogenous peroxidase quenched with 3 % hydrogen peroxide, washed

Table 1 Primers used for the quantitative real-time PCR

Gene	Sense primer	Antisense primer
MMP-2	CTGGGTTTACCCCCTGATGTCC	AACCGGGGTCCATTTTCTTCTTT
MMP-3	TGGCACAAAGGTGGATGCTGTCT	GGGTCACTTTCCCTGCATTTGG
MMP-8	CCATGGATCCAGGTTACCCCACT	TGTGGTCCACTGAAGAAGAGGAAGA
MMP-9	GGATGTTTTTGATGCCATTGCTG	CCACGTGCGGGCAATAAGAAAG
MMP-12	CAAATCCTGGAAGTCCACCATCAA	CAGGCAGCCTCCACCAGAAG
MMP-13	GCTGCGGTTCACTTTGAGGACAC	TTCTATGAGGCGGGGATAGTCTTTG
COL1A1	ATCAGCCCAAACCCCAAGGAGA	CGCAGGAAGGTCAGCTGGATAG
COL3A1	TGATGGGATCCAATGAGGGAGA	GAGTCTCATGGCCTTGCGTGTTT
GAPDH	CCATTCTTCCACCTTTGATGCT	TGTTGCTGTAGCCATATTCATTGT
RPLP0	CCAGAGGTGCTGGACATCACAGAG	TGGAGTGAGGCACTGAGGCAAC

MMP matrix metalloproteinase, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase, *RPLP0* large ribosomal protein P0

in distilled water and brought to Tris-buffered saline (pH 7.6) containing 1 % bovine serum albumin before they were incubated overnight at 4 °C with rabbit anti MMP-12 polyclonal antibody at 1:400 dilution (ab66157; Abcam, Cambridge, UK) [31] or mouse anti-rat CD68 monoclonal antibody at 1:200 dilution (MCA341R; Serotec, Oxford, UK). The sections were then subjected to treatment with the Vectastain® Elite ABC kits followed by reaction with 3,3'-diaminobenzidine (Vector Laboratories, Burlingame, CA, USA).

A gastrointestinal pathologist (M. E.) evaluated the slides. The number of MMP-12 and CD68-positive cells in the anastomotic wound gap were estimated on a semi-quantitative scale ranging from 0 to 5 where 0=no positive cells, 1=1–19 cells, 2=20–50 cells, 3=51–100 cells, 4=101–200 cells and 5>200 cells per field of view (0.2 mm^2).

Statistical analyses

MMP and collagen mRNA levels were normalised to RPLP0 and MMP protein concentrations to total amount of protein, log-transformed and analysed by the two-sided unpaired or paired *t* test. Anastomotic breaking strength and loss of initial body weight were compared with the unpaired *t*test and presented as mean±SD. Immunohistochemical scores and plasma concentration of AZD3342 were compared with the Mann–Whitney *U* test. Plasma AZD levels are presented as median (interquartile range). Mortality rates were compared using Fisher's exact test. $p<0.05$ was considered statistically significant.

Results

Expression of MMPs on postoperative day 3 in colon anastomoses

We determined the abundance of MMPs in colon anastomoses when the wound strength is the lowest [5–7]. The six target MMPs were detected in non-injured colon and anastomotic wounds by quantitative real-time PCR. The mRNA values were normalised to RPLP0, log-transformed and the data expressed as fold change relative to adjacent proximal non-injured colon. Accordingly, the gene expression of MMP-8 increased 81-fold ($p<0.001$), MMP-9 mRNA 71-fold ($p<0.001$), MMP-12 mRNA 29-fold ($p<0.001$), MMP-13 16-fold ($p<0.001$) and MMP-2 increased 1.5-fold or by 50 % ($p<0.001$) in the anastomotic wounds. MMP-3 mRNA levels did not differ significantly between wounds and non-injured colon (Fig. 1a). Comparing the absolute levels of mRNA for the six MMPs would be relevant. Unfortunately, quantitative real-time PCR is not suitable for direct comparison of absolute expression levels between different targets because the efficiency of converting mRNA into cDNA varies considerably for different mRNA targets. This efficiency is difficult to assess. Nevertheless, in non-injured colon, the median values for the absolute number of detected cDNA molecules in the PCR were 33,145 for MMP-2, 775 for MMP-3, 355 for MMP-8, 48 for MMP-9, 240 for MMP-12 and 266 for MMP-13. Although these absolute cDNA numbers cannot be directly converted to the number of mRNA molecules, the data indicate that MMP-2 is the major MMP expressed in proximal non-injured colon.

To determine the relevance of the relative transcript levels, protein concentrations of the corresponding MMPs were measured for MMP-2, MMP-8, MMP-9 and MMP-12. The MMP-8 level was 439±139 ng/mg protein in the anastomotic wounds compared with 26±12 ng/mg protein in proximal non-injured colon in the same animals. This represents a 17-fold ($p=0.006$) increase (Fig. 1b). The corresponding values for MMP-9 were 6.41±1.43 ng/mg protein and 0.47±0.30 ng/mg protein, a 14-fold ($p<0.001$) increase (Fig. 1b). MMP-9 was also assessed by gelatin zymography. Lysis bands corresponding to the position of active MMP-9 were not detected. This indicates that MMP-9 in the wounds was mainly in its precursor form (Fig. 2a). The MMP-2 protein was present in latent as well as in active forms in non-injured colon as well as in the anastomotic wounds (Fig. 2a). Total amount and the proportion of the active form

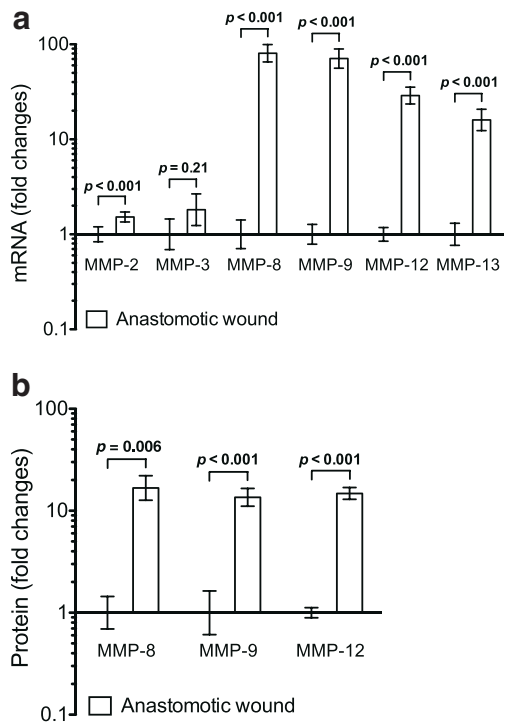

Fig. 1 Expression of indicated MMPs in day 3 colonic anastomoses from vehicle-treated rats was measured by quantitative real-time PCR (**a**) and enzyme-linked immunosorbent assays (**b**). MMP mRNA normalised to RPLP0 (**a**) and MMP proteins to total proteins (**b**) were log-transformed and are presented as fold change relative to proximal non-injured colon (=1) as geometric means±back-transformed SEM

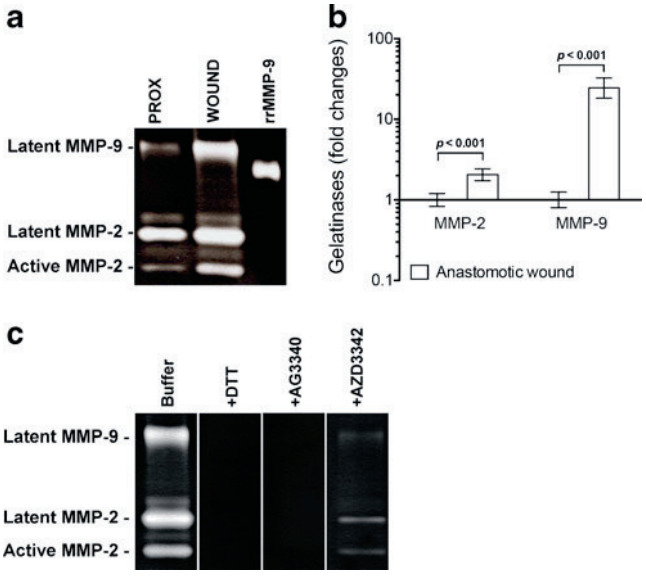

Fig. 2 Zymographic analyses of tissue extracts of non-injured and anastomosed colon in vehicle-treated rats postoperative day 3. **a** Zymogram depicting MMP-2 and MMP-9 in pooled tissue extracts (4 μg total protein per lane) of proximal non-injured colon (PROX) and anastomotic wounds (WOUND). The positive control recombinant rat MMP-9 (rrMMP-9; Uscn Life Science) at 100 pg was run in parallel lane. **b** Total MMP-2 and total MMP-9 were semiquantified by densitometry and presented as fold change relative to proximal non-injured colon (=1) as geometric means±back-transformed SEM. **c** Effect of 50 mM dithiothreitol (DTT) during electrophoresis [32], and the addition of the MMP inhibitors AG3340 [15] and AZD3342 at 10 μM to the renaturing and developing buffers on MMP-2 and MMP-9 in pooled anastomotic wound extracts

of MMP-2 were significantly increased ($p<0.001$) in the wounds compared with proximal non-injured colon (Fig. 2b). The identities of MMP-9 and MMP-2 were indicated by the abolished gelatinase activity with dithiothreitol added to the sample-loading buffer during electrophoresis (Fig. 2c) [32]. Also, the presence of the gelatinase inhibitor AG3340 during incubation blocked MMP-9 and MMP-2 activities completely (Fig. 2c). AZD3342 inhibited MMP-9 activity but also MMP-2 partially (Fig. 2c). The MMP-12 concentration in anastomotic wounds (5.97±0.85 ng/mg protein) was 15-fold ($p<0.001$) higher than in proximal non-injured colon (0.40±0.05 ng/mg protein) (Fig. 1b).

MMP-12 immunohistochemistry

MMP-12 was primarily expressed in the cytoplasm of CD68-positive cells identified as macrophages in the lamina propria and monocytes in the blood vessels in day-0 anastomotic wounds. In addition to the expression of MMP-12 in CD68-positive cells, MMP-12 was also observed in the cytoplasm of lymphocytes, plasma cells and epithelial cells. The intensity of the MMP-12 immunoreaction was more pronounced in macrophages compared with epithelia cells. Three days after surgery, the protein expression of MMP-12 ($p=0.02$) and the numbers of CD68-positive cells ($p=0.008$) had increased in the anastomotic wounds especially around the sutures channels (Fig. 3).

Effect of the MMP inhibitor AZD3342 on plasma AZD3342 concentration, anastomotic breaking strength and collagen synthesis

Three of the 47 AZD3342-treated rats and 3 of the 32 vehicle-treated rats died in conjunction with anesthesia ($p=0.68$). No animal showed signs of anastomotic leakage.

The plasma concentration of AZD3342 at the dosage of 5 mg/kg was 0.29 (0.19–0.64) μM, and at 50 mg/kg, it was 7.69 (4.43–17.60) μM. AZD3342 was undetectable in the plasma from vehicle-treated animals. AZD3342 at 5 mg/kg did not increase anastomotic breaking strength significantly ($p=0.16$), while at 50 mg/kg AZD3342 treatment increased the breaking strength by 29 % ($p=0.015$) compared with the vehicle group. There was no difference ($p=0.57$) in postoperative body weight loss among the groups (Table 2).

AZD3342 at 50 mg/kg was then applied in an independent series to examine the reproducibility of the results on the anastomotic breaking strength and, additionally, the effect of AZD3342 on gene expression of type I and III procollagens.

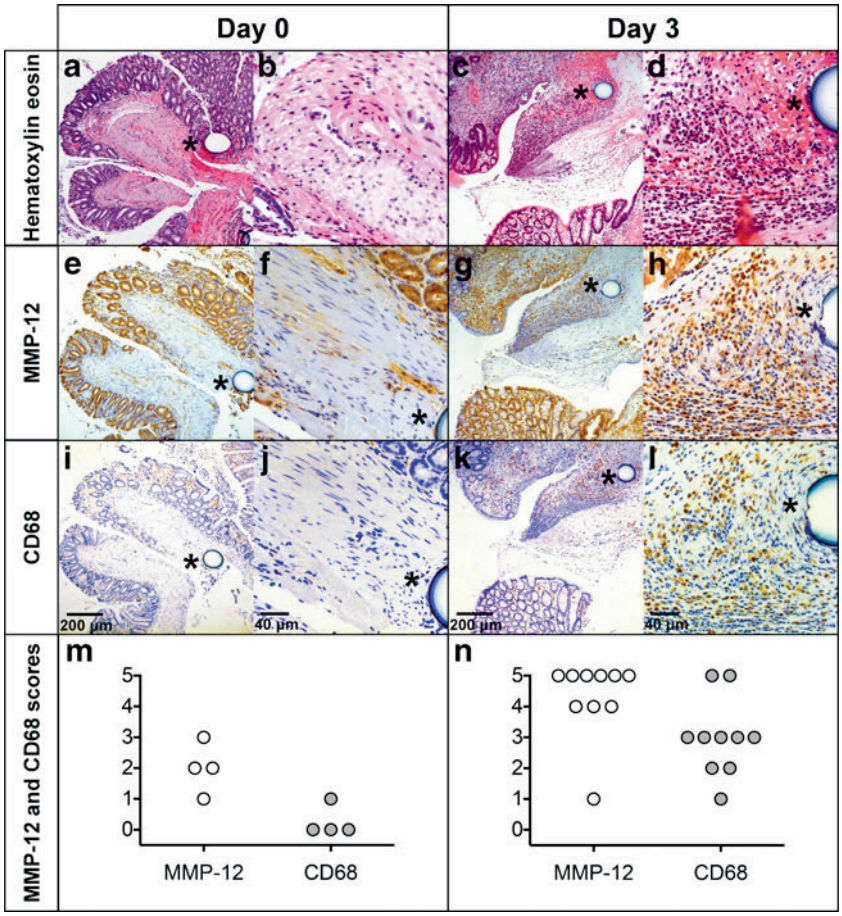

Fig. 3 Colonic anastomoses day 0 (**a**, **b**, **e**, **f**, **i**, **j**) and day 3 (**c**, **d**, **g**, **h**, **k**, **l**) stained with haematoxylin–eosin (**a**–**d**) and immunostained for MMP-12 (**e**-**h**) and for macrophages with CD68 monoclonal antibody (**i**–**l**). Note haemorrhage in the anastomotic wound gap and accumulation of macrophages around the suture channels (**c**, **d**, **g**, **h**). The number of MMP-12 (*open circles*) and CD68-positive (*filled circles*) cells increased in the anastomotic wound day 3 (**n**) compared with day 0 (**m**) evaluated on a scale ranging from 0 to 5 where 0=no positive cells, 1=1–19 cells, 2=20–50 cells, 3=51–100 cells, 4=101–200 cells and 5> 200 cells per field of view (0.2 mm^2). Note MMP-12-positive epithelial cells on days 0 and 3 (**e**, **g**). *Asterisk* suture channel

Again, AZD3342 treatment significantly increased (p=0.024) the anastomotic breaking strength (1.78±0.28 N) compared with vehicle (1.52±0.30 N) (Fig. 4a). AZD3342 treatment did not change the COL1A1 or COL1A3 mRNA levels or the COL1A1/COL3A1 ratio compared with vehicle (Fig. 4b). Furthermore, there was no difference (p=0.39) in the loss of body weight postoperatively between AZD3342-treated rats (9±3 g) and vehicle-treated rats (13±3 g).

Discussion

Broad-spectrum MMP inhibitors produce a robust improvement of anastomotic strength in the colon [5, 6, 14, 15].

Table 2 Effect of AZD3342 on anastomotic breaking strength and weight loss 3 days after surgery

Dosage (mg/kg)	n	Breaking strength (N)	Weight loss (g)
0	15	1.19±0.34	34±18
5	14	1.39±0.41	30±11
50	15	1.54±0.40*	29±7

n=number of animals. Mean±SD

*p=0.015 compared with vehicle (0 mg/kg)

There is a concern of the tolerability and interference with the normal wound healing process of these non-selective compounds [16–19, 33, 34]. Theoretically, these adverse effects are preventable using a rationally designed chemical entity with a more selective MMP inhibitory profile [19].

Our approach was to establish a MMP mRNA fingerprint of colonic anastomotic healing 3 days after surgery. This is the time point when the biomechanical strength is at its minimum [5–7]. The found fingerprint was dominated by MMP-8, MMP-9 and MMP-12 largely reflecting the cellular abundance of neutrophils and macrophages [15, 35]. Importantly, inhibition of these three MMPs with AZD3342 enhanced anastomotic strength significantly. The beneficial effect of AZD3342 was comparable to the broad-spectrum MMP inhibitor BB-94 that increased breaking strength by 27 % at day 3 in a similar animal model [6]. Taken together, these findings indicate pathogenic involvement of MMP-8, MMP-9 and/or MMP-12 in the healing of colonic anastomoses.

Recently, the selective MMP-2, MMP-3, MMP-9, MMP-13 and MMP-14 inhibitor AG3340 (Prinomastat) was also shown to increase day 3 anastomotic biomechanical strength [15]. The comparable results with AZD3342 and AG3340 in improving anastomosis strength, despite their different inhibitory profiles, underscore the complexity of anastomotic healing. Here, MMP-3 showed no increased expression on

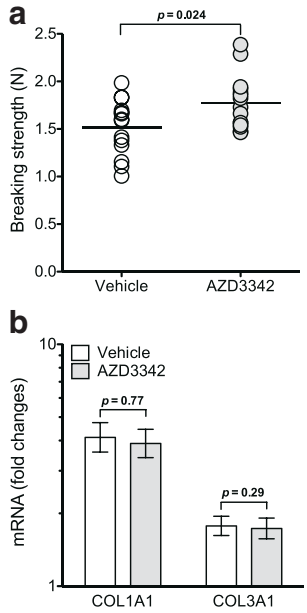

Fig. 4 Effect of the MMP inhibitor AZD3342 ($n=15$) and vehicle ($n=14$) administered subcutaneously at 50 mg/kg on **a** breaking strength and **b** procollagen type I and type III mRNA levels in day 3 colonic anastomoses. **a** Each *symbol* represents one animal, and *horizontal bars* denote group mean values. **b** mRNA levels were normalised to RPLP0, log-transformed and presented as fold change relative to proximal non-injured colon (=1) as geometric means±back-transformed SEM. The gene expression of type I procollagen (COL1A1) increased 4.1-fold ($p<0.001$), type III procollagen (COL3A1) 1.8-fold ($p<0.001$) and the COL1A1/COL3A1 ratio 2.3-fold ($p<0.001$) in the anastomotic wounds compared with proximal non-injured colon of vehicle-treated rats

day 3 and thus is unlikely responsible for impaired anastomotic healing at this early stage.

Existent collagen fibers in the submucosa impart strength to the anastomosis. The initial cut of the native collagen triple-helix is rate-limiting in the collagen degradation process. The true traditional collagenases, MMP-1, MMP-8 and MMP-13, have this property. We could demonstrate a pronounced increase in both MMP-8 mRNA and protein levels in the day 3 anastomoses. This finding is important because overexpression of MMP-8 decreased the amount of type I collagen and the breaking strength of incisional skin wounds in rodents [26]. MMP-13 mRNA was also increased but less than MMP-8. Similar expression pattern was reported for murine primary wounds in the skin [22]. Rat MMP-13 is a functional homologue of human MMP-1. It is possible that inhibition of MMP-13 could improve anastomotic wound healing even further.

Collagenases in cooperation with the gelatinase MMP-9 degrade collagen synergistically [11, 36]. Although MMP-9 mRNA and proteins were increased more than tenfold in the anastomotic wounds only the latent, inactive form of the MMP-9 protein was detected. It is possible though, that active MMP-9 remained bound to the tissue even after the repetitive extractions or that MMP-9 was enzymatically active, despite being in its unprocessed latent form [37]. On the other hand, mRNA of the other gelatinase MMP-2 was elevated, albeit modestly, and the zymographic analysis revealed a significant increase in both total and, importantly, the active form of the MMP-2 protein. Because of the structural and functional similarities between MMP-9 and MMP-2, it is possible that MMP-2 accelerated the degradation of collagenase-generated collagen fragments. This mechanism has actually been demonstrated in vitro [36]. There are no reports on membrane-bound MMP-14 expression in colon anastomoses.

The role of MMP-12 is also unknown. MMP-12 was detected in the full-thickness wall and mucosa of non-injured colon in the rats. This agrees with findings in humans where MMP-12 was demonstrated in the stroma and epithelium of normal colon [38]. Furthermore, MMP-12 mRNA and protein levels were elevated several-fold due to increased macrophage infiltration of the anastomotic wounds. MMP-12 primarily degrades elastin, although it also possesses collagenolytic activity in vitro [23–25]. Thus, overexpression of MMP-12 can potentially damage the extracellular matrix of the submucosa and impair anastomotic wound healing. Surprisingly, in another study, loss of MMP-12 resulted in decreased breaking strength of lacerated ligaments, albeit transiently 7 days postoperatively [39]. The investigators attributed this to decreased synthesis of type I collagen. In our study, inhibition of MMP-12 with AZD3342 neither decreased type I procollagen nor type III procollagen transcription. Moreover, intracellular MMP-12 in macrophages possesses antimicrobial activity, which is a unique property within the MMP family [40]. This finding may be relevant for anastomotic healing, but the consequences of blocking MMP-12 with AZD3342 on the bacterial flora in colon are unknown. Unlike most of the non-selective MMP hydroxamate inhibitors, AZD3342 lacks significant inhibitory activity against tumor necrosis-α converting enzyme [41], which is important in terms of preserved innate immunity. Obviously, more studies are needed to elucidate the biological functions of MMP-12 during anastomotic wound healing.

The present study justifies the concept of selective MMP inhibition to improve anastomotic strength. More studies are needed before AZD3342 can be explored in the clinical setting to reduce anastomotic complications.

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ORIGINAL ARTICLE

Rapid morphological changes and loss of collagen following experimental acute colonic obstruction

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Abstract
Purpose Anastomosis of an acutely obstructed colon is associated with an increased risk of dehiscence. In experimental models, acute obstruction decreases collagen in the colonic wall, but the time course and propagation along the colon of the biochemical changes are unknown. Furthermore, there is a paucity of information on the correlation between these biochemical changes and histological features.
Methods Forty male Sprague Dawley rats were subjected to partial obstruction by placing a silicone ring around the left colon 30 mm above the reflection. Obstruction was maintained for 0, 1, 2, 3 or 4 days. Samples from five different locations along the colon were analysed on circumference, tissue water content, collagen concentration and histomorphology. Neutrophil and macrophage infiltration was characterized immunohistochemically.
Results The colonic circumference and water content increased ($p<0.001$), while the collagen concentration decreased by 48 % ($p<0.01$) proximal to the obstruction already after 1 day. The degree of dilation and collagen reduction did not change significantly over the subsequent 3 days of obstruction, whereas the water content normalized by day 3. Mucosal and submucosal oedema and the relative neutrophil infiltration were highest after 1 day in the colonic segment proximal to the stenosis while the macrophage population continued to increase to day 4. Muscular necrosis in addition to ganglionitis and neuritis in the nervous plexus increased with duration of obstruction.
Conclusions The pronounced and rapid changes of the composition of cells and the extracellular matrix of the colonic wall following acute obstruction may be of guidance for present surgical treatments and future pharmacological interventions.

Keywords Colonic obstruction · Collagen · Histology · Inflammation · Neutrophil · Macrophage

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Introduction

Colorectal cancer is one of the most frequent malignancies worldwide with over 1.2 million new cases annually [1]. Approximately 15 % present as acute obstructing tumours interrupting the bowel continuity [2] and require prompt treatment. These emergency operations are high-risk procedures [3, 4]. Despite progress in surgical techniques, moving from the staged procedures to resection with construction of a primary anastomosis [4, 5], the presence of colonic obstruction is associated with an increased risk of anastomotic leakage [6, 7] and mortality [8]. Unfortunately, Hartmann's procedure [9, 10] and endoscopic placement of a self-expandable metal stent [11–13] are both associated with drawbacks as well. Regardless of surgical technique, a detailed knowledge of the biochemical and structural changes in the obstructed colonic wall is crucial to better understand the pathophysiology behind and the association with increased anastomotic leakage.

In a non-obstructed colon, anastomotic healing comprises a cascade of events, aiming to restore the function and biomechanical strength in the bowel. The submucosal collagen constitutes the suture-holding tissue and ensures anastomotic integrity in the first postoperative week [14]. During this period, collagen degradation predominates over collagen synthesis resulting in decreased concentration of collagen and reduced anastomotic biomechanical strength [15]. In the obstructed colonic wall, reduced collagen has been observed in rodents [16–19], but the time course as well as propagation of changes along the colon following obstruction has not been addressed previously.

The purpose of the present study was to examine the dimensional, biochemical and morphological changes in the colonic wall following 0 to 4 days of obstruction using an established acute model in rats [17–19].

Methods

Forty male Sprague Dawley rats (Taconic M&B, Ry, Denmark) weighing 348±61 g (mean ± SD) were acclimatized for a minimum of 7 days prior to surgery with free access to standard pellets and tap water [15, 17]. The rats were randomized into five groups of eight rats in each group and subjected to partial colonic obstruction for 0, 1, 2, 3 or 4 days. After surgery, four rats were kept per cage. The study was approved by the local ethics committee for animal studies at Lund University (M174-04).

Anaesthesia and analgesics

Anaesthesia was induced with a mixture of 0.14 mg/kg fentanyl citrate and 4.4 mg/kg fluanisone (Hypnorm®; Janssen-Cilag, Beerse, Belgium), and 2.2 mg/kg midazolam (Dormicum®; F. Hoffmann-La Roche, Basel, Switzerland) given subcutaneously. After the surgery was completed, 0.02 mg/kg buprenorphine (Temgesic®; Schering-Plough, Brussels, Belgium) was injected subcutaneously. This regimen was also used for postoperative analgesia.

Surgery

The abdominal cavity was exposed through a 30-mm midline incision. Partial colonic obstruction was established using a 5-mm-wide silicone ring (6.5 mm inner diameter) placed around the left colon between two marginal veins 30 mm above the peritoneal reflection [17–19]. The ring was closed with single 7-0 polypropylene suture (Ethilon®; Ethicon, Johnson & Johnson, Brussels, Belgium). The abdomen was closed in two separate layers using continuous 4.0 sutures (Vicryl®; Ethicon).

The rats were killed by asphyxiation in a carbon dioxide chamber after 0, 1, 2, 3 or 4 days of colonic obstruction. Five-millimetre-wide segments of the colon were excised at five predefined anatomical locations. Segment A was located 10 mm distal to the ileocolonic junction; segment B was 15 mm and segment C 5 mm proximal to the stenotic silicone ring, both segments 5 mm apart, and segment D 5 mm and segment E 15 mm distal to the ring as detailed in Fig. 1. The segments were cut open at the antimesenterial border, and the colonic circumference was measured in millimetres using a slide caliper. Each segment was bisected for determination of water and collagen contents (segments A, B, C, D and E) and for histopathological (segments C, D and E) and immunohistochemical examination (segments C and D).

Water content and collagen (hydroxyproline) analyses

Tissues were weighed (m_{Fresh}), dried to constant weight at 100 °C and weighed (m_{Dry}). Tissue water content was calculated as: $(1-(m_{Dry}/m_{Fresh}))\times 100$ %.

Tissues were hydrolyzed in 6 M hydrochloric acid for 18 h at 110 °C. The acid hydrolysates were evaporated and the washed, acid-free residues dissolved in acetate–citrate buffer [19]. The hydroxyproline content was determined colourimetrically [19].

Histological evaluation

Colonic specimens were fixed in 4 % phosphate-buffered paraformaldehyde for 24 h and embedded in paraffin. Sections (5 μm) were stained with haematoxylin–eosin. A consultant specialised in gastrointestinal pathology evaluated the sections without prior knowledge of group affiliation. Oedema was graded as no, slight or pronounced. The degree of inflammatory cell infiltration was evaluated on a four-graded scale [20].

Immunohistochemical double labelling of neutrophils and macrophages

Tissue sections (5 μm) were deparaffinized, rehydrated and microwaved for 10 min in Tris–ethylenediaminetetraacetic

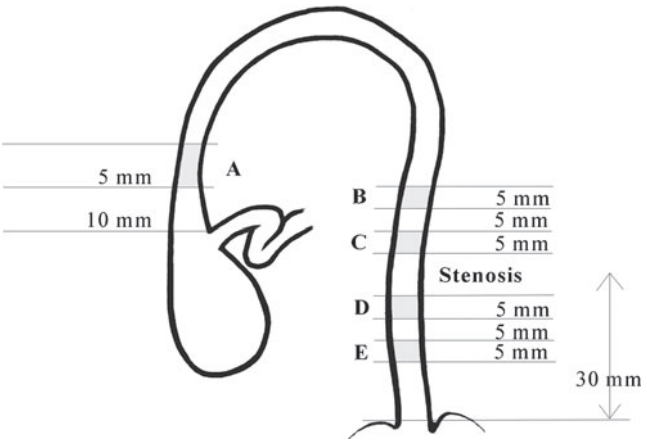

Fig. 1 Colonic sampling sites

acid (pH 9.0). Double labelling of neutrophils and macrophages was performed using reagents and protocols from Vector Laboratories (Burlingame, CA, USA) unless stated otherwise. Each incubation step was carried out at ambient temperature and was followed by washing in phosphate-buffered saline (pH 7.2) for 5 min.

Endogenous peroxidase activity was quenched with 3 % hydrogen peroxide for 5 min. Sections were then incubated with 2.5 % normal horse serum for 20 min. The rabbit anti-rat neutrophil adsorbed antiserum (AIAD51140; Accurate Chemical & Scientific Corporation, Westbury, NY, USA) [21] was diluted 1:10,000 in 1 % bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) and applied for 60 min. The section was then incubated with the anti-mouse/rabbit Ig ImmPRESS peroxidase universal reagent for 30 min. The bound antibody complex was visualized with 3,3′-diaminobenzidine supplemented with nickel chloride for 4 min resulting in grey/black-labelled neutrophils. The tissue section was blocked again with 2.5 % normal horse serum for 20 min. The mouse monoclonal anti-rat CD68 antibody (clone ED1; Serotec, Oxford, UK) [22] was applied at 1:400 dilution in 1 % bovine serum albumin for 60 min. Following 30-min incubation with the anti-mouse/rabbit Ig ImmPRESS peroxidase universal reagent, ImmPACT NovaRED was applied to the section for 2 min resulting in red-labelled macrophages. Sections were counterstained with Mayer's haematoxylin (blue) for 40 s, cleared and mounted using Pertex mounting medium (Histolab Products, Göteborg, Sweden).

Statistics

Hydroxyproline levels were log-transformed to obtain normal distribution. One-way ANOVA with Newman–Keuls was applied for comparisons with day 0. A $p<0.05$ was considered statistically significant. Hydroxyproline data are given as geometric mean ± back-transformed standard error (SE) and the other variables as mean ± SD.

Results

We have characterized the temporal and histological changes in the colonic wall following acute mechanical obstruction using a model developed in our laboratory [17–19]. There were no differences in preoperative body weight ($p=0.45$) among the five groups. One day-3 rat died during recovery from anaesthesia leaving 39 rats for the analyses. The partially obstructed colon allowed passage of flatus but not faeces, and animals continuously lost weight throughout the study period from 8±9 g after day 1 to 33±6 g after 4 days of obstruction.

Colonic circumference

After 1 day of obstruction, the colonic circumference increased in segment B from 8±1 to 17±2 mm ($p<0.001$) and in segment C from 8±2 to 19±2 mm ($p<0.001$) (Fig. 2). The colonic dilation did not increase further with duration of obstruction. The colonic circumference of segment A increased from 6±1 mm before obstruction to 12±3 mm ($p<0.01$) after 4 days of obstruction. The circumference of distal segment D did not change significantly over time whereas the circumference of segment E decreased after 2 days of obstruction from 11±3 day 0 to 8±1 mm on day 4 ($p<0.01$).

Colonic water content and collagen

The percentage of water in the colonic wall of segment B increased from 80±2 % on day 0 to 86±3 % on day 1 ($p<0.001$) and to 83±1 % on day 2 ($p<0.05$) but returned to day-0 levels days 3 and 4 (Fig. 2). A similar pattern was observed in segment C, whereas the water content in segments A, D and E did not change with time.

The collagen concentration decreased in segment B after 1 day of obstruction by 39 % from 11.3±1.4 to 6.9±0.9 μg hydroxyproline/mg dry weight ($p<0.01$) (Fig. 2). The corresponding value for segment C was 48 % from 12.5±1.3 to 6.5±0.9 μg/mg dry weight ($p<0.01$). The collagen concentration did not significantly change further after day 1 in either segment B or C. In segment E, collagen levels increased from days 0 to 3 by 58 % from 11.2±1.7 to 17.7±2.5 μg/mg dry weight ($p<0.05$) and from days 0 to 4 by 102 % to 22.7±2.0 μg/mg dry weight ($p<0.01$). The collagen levels did not change significantly with duration of obstruction in segment A or D.

Histological and immunohistochemical examinations

These analyses are presented in relation to day 0 (Fig. 3a). Immunohistochemical staining was applied to specifically identify neutrophils and CD68-positive macrophages in selected sections (Fig. 4a and b).

Day 1 The mucosa of the proximal segment C was slightly oedematous and infiltrated with neutrophils. The CD68 immunoreactivity in the mucosa did not change appreciably with obstruction. The muscularis mucosa was thickened with scattered necroses. The submucosa exhibited a pronounced oedema and a moderate infiltrate composed mostly of neutrophils with fewer macrophages, lymphocytes and plasma cells (Figs. 3b and 4c). The submucosa of distal segment D was slightly oedematous but less populated by neutrophils than the C segment (Fig. 4e and g).

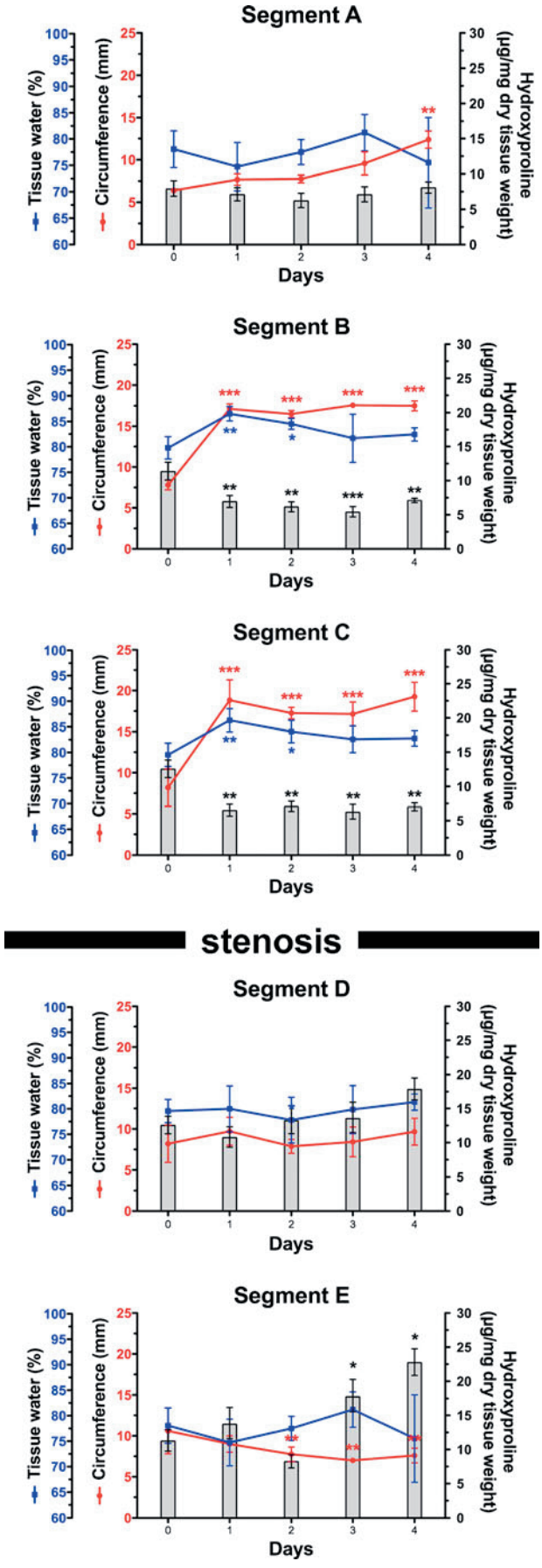

Fig. 2 Colonic inner circumference (red curves, mean ± SD), tissue water in percent (blue curves, mean ± SD) and hydroxyproline concentration (bars, geometric mean ± back-transformed SE) in the five segments of the colonic wall (see Fig. 1) after 0 to 4 days of obstruction. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to day 0

Day 2 The mucosal oedema had diminished, while the inflammation persisted. In the submucosa, the oedema was unaltered from day 1, and the inflammatory cell infiltration was moderate. At this stage of obstruction, scattered fibrinoid necroses and thrombosed arteries were apparent. The longitudinal layer of the tunica muscularis was moderately inflamed with scattered necroses. In the myenteric plexus of Auerbach, lymphocytes were found in the ganglions. Ganglionitis was evident in the proximal segment C and, to a lesser degree, in the distal segment D.

Days 3 and 4 Occasional neutrophils were observed in the mucosa of the proximal segment. The submucosal oedema decreased leading to apparently increased cell densities. The inflammatory cell infiltrate peaked on day 3 (Fig. 3c). Although the proportional number of neutrophils declined progressively proximally to the stenosis, it still exceeded that of the distal D segment on day 4 (Fig. 4g). In contrast, the proportion of CD68-positive macrophages was increased and encompassed about half of the cell count in the submucosa both above and below the obstruction on day 4 (Fig. 4d, f and g). Pronounced neuritis with neutrophils and lymphocytes in close vicinity to the neurons in the circular layer of the tunica muscularis was noted on either side of the stenosis. Ganglionitis persisted, and the neurons began to degenerate although necrotic neurons were only demonstrated in the proximal segment C (Fig. 3d). Severe peritonitis was present in the serosa of segment C. There were no obvious histological changes with time in segment E.

Discussion

The collagen-rich submucosa of the colonic wall constitutes the suture-bearing capacity and is paramount for the biomechanical strength of an anastomosis [14]. The present experimental study demonstrates that acute colonic obstruction dramatically and rapidly decreases collagen levels within the first day of obstruction. Interestingly, the kinetics of this collagen loss is analogous to that of the rat uterus during postpartum involution [23]. The early changes in the colon correlated with bowel dilation and pronounced oedema proximal to the obstruction.

Furthermore, neutrophils accumulated proximal as opposed to distal to the stenosis in the submucosa. We did not anticipate this rapid cellular response, and it is possible that the influx of neutrophils peaked even before 1 day of obstruction. On the other hand, the finding is consistent with the significantly higher

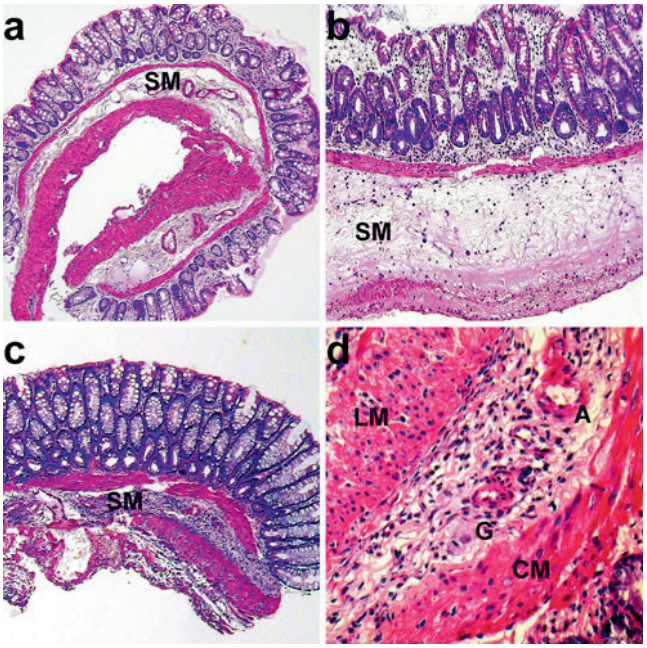

Fig. 3 Morphologic changes of the proximal segment of the colon with time of obstruction from day 0 (**a**), 1 (**b**), 3 (**c**) and to day 4 (**d**). Note mucosal and submucosal oedema with inflammatory cell infiltrate on day 1 (**b**), while at day 3 (**c**), inflammation was severe in the submucosa with serosal peritonitis but with less submucosal oedema. **d** Pronounced ganglionitis was observed on day 4. *A* artery with adjoining extravasated neutrophils, *G* ganglionitis, *LM/CM* longitudinal/circular layers of the tunica muscularis, *SM* submucosa. Original magnifications: **a** ×100; **b** and **c** ×200; **d** ×600. Haematoxylin–eosin stain

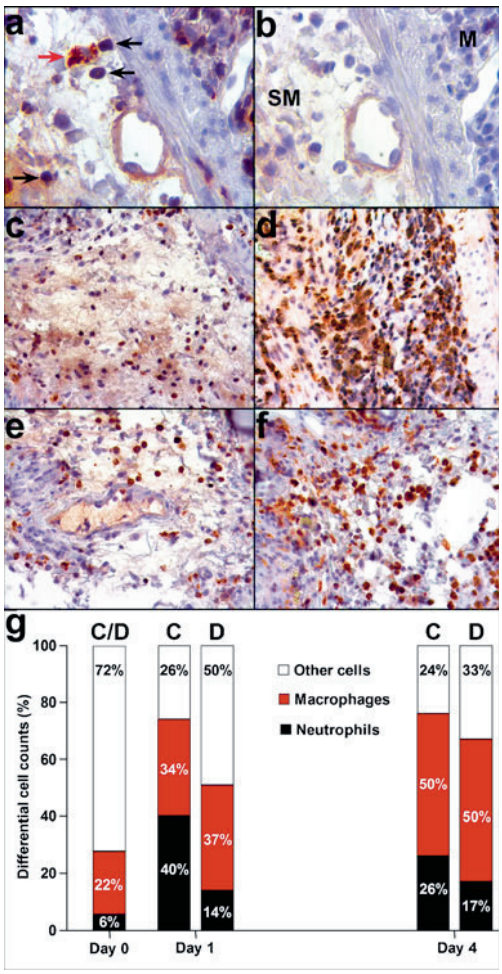

Fig. 4 Immunohistochemical double labelling of neutrophils (*black*) and macrophages (*red*) in rat colon proximal (**a**–**d**) and distal (**e**, **f**) to the obstructive ring applied for 1 day (**a**–**c**, **e**) or for 4 days (**d**, **f**). **a**, **b** High-resolution (oil) images of adjacent tissue sections treated with (**a**) or without (**b**) the primary antibodies directed against neutrophils (**a** *black arrows*) and macrophages (**a** *red arrow*). **b** Submucosa (*SM*) and mucosa (*M*) are indicated for the purpose of orientation. **c**–**f** Note the apparent sparse cellularity on day 1 due to extensive oedema but increased cell density in proximal versus distal segments. Original magnifications: **a** and **b** ×1,000, **c**–**f** ×400. Mayer's haematoxylin counterstain. **g** Differential counts of neutrophils, macrophages and other cells (endothelial cells excluded). Cells were counted manually in digital images (×400) of representative areas of the entire thickness of the submucosa in comparison with day 0. *C* and *D* above bars refer to the proximal and distal segments to the obstruction

myeloperoxidase levels previously demonstrated in the colonic wall proximal to the stenosis on day 4 [17].

Collagen degradation is accomplished by the action of one or more matrix metalloproteinases (MMPs). We have previously shown that the reduced collagen in an obstructed colon correlates with MMP activity [17], which tended to increase with the degree of colonic dilation [19]. Neutrophils primarily express MMP-8 and MMP-9, which collectively degrade the colon and type I collagens synergistically [24]. Neutrophils were probably the major cellular sources of MMPs here. To test this hypothesis, the recruitment of neutrophils could be blocked experimentally by giving specific antibodies against the adhesion molecule CD18 [25] or perhaps against responsible chemoattractants such as lipopolysaccharide-induced CXC chemokine [26]. Intuitively, the high mechanical load would increase the susceptibility of collagen to degradation by MMPs, although the opposite has been reported in vitro [27].

We suggest that the colonic wall proximal to an obstructing lesion is depleted of collagen, which impairs the healing conditions of anastomoses [14, 28]. Broad-spectrum MMP inhibition restores anastomotic integrity in a non-obstructive colon [15, 20], but it remains to be demonstrated whether this therapeutic approach is beneficial for anastomotic repair in the obstructed colon as well.

With time, the macrophage infiltration increased in the submucosa of the stenotic area. This may reflect an increased demand for macrophages in protective and reparative processes [29].

It is striking that, after the initial reduction, the collagen concentration proximal to the stenosis was maintained at the same low level. This finding suggests that degraded collagen was compensated for by the synthesis of new collagen molecules. In general, mechanical tension induces a synthetic fibroblast phenotype [30] under experimental conditions

that mimic the increased strain exerted on the fibroblasts and myofibroblasts in the dilated colon.

Colonic dilation progressed to the most proximal segment of the colon after 4 days of obstruction. In contrast, the circumference of the most distal segment examined decreased, resulting in increased collagen concentration. The mechanisms are elusive because, in experimental studies on diverting stoma, the collagen concentration in the offloaded colon did not change [31]. Moreover, the chronically obstructed small intestine exhibits increased collagen levels and muscle hypertrophy [32], none of which was demonstrated in the present study.

We did not investigate the ultrastructural arrangements of the collagen fibrils [33] or the specific types of collagen although the submucosa is composed primarily of type I and III collagens [34, 35]. Apart from collagen, hydroxyproline is also found in elastin, but the contribution of elastin-derived compared with collagen-derived hydroxyproline is negligible [36]. It would be interesting to study the changes of other extracellular components such as fibronectin and laminin during colonic obstruction [34].

The scattered necroses observed after 4 days of obstruction in the muscular layer of the colonic wall might inhibit the return of bowel function as we have previously observed [19]. Neural damage including neuritis and ganglionitis in the tunica muscularis, shown here, may impair bowel peristaltic movement and prolong postoperative ileus.

In conclusion, the early and dramatic loss of structural collagen in the large bowel may hint that caution should be taken regarding primary anastomosis, also in patients with only a short history of colonic obstruction. Moreover, our results indicate that it would be worthwhile to test if the loss of connective tissue elements is preventable by pharmacologic means to reduce the risk of anastomotic leakage.

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ORIGINAL ARTICLE

Selective matrix metalloproteinase inhibition increases breaking strength and reduces anastomotic leakage in experimentally obstructed colon

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Abstract
Purpose Colonic obstruction causes loss of collagen and impairment of anastomotic integrity by matrix metalloproteinases (MMPs). Unexpectedly, pharmacological MMP inhibition increased anastomotic leakage (AL) in obstructed colon possibly due to the non-selective nature of these compounds and the experimental model applied. We therefore studied the effects of selective MMP inhibition on the healing of anastomoses in colon obstructed by a novel laparoscopic technique.
Methods Left colon was obstructed in 38 male Sprague-Dawley rats (226–284 g). After 12 h, stenoses were resected and end-to-end anastomoses constructed. Baseline breaking strength was determined in 6 animals on day 0. The remaining 32 rats were randomized to daily treatment with the selective MMP-8, MMP-9, and MMP-12 inhibitor AZD3342 (n = 16) or vehicle (n = 16). On day 3, anastomoses were evaluated for AL and breaking strength. Isolated anastomotic wound tissue was analyzed on total collagen and pepsin-insoluble and pepsin-soluble collagen by hydroxyproline. The soluble collagens were further differentiated into native, measured by Sircol, and fragmented forms.
Results Baseline breaking strength was maintained with AZD3342 but decreased by 25% (P = 0.023) in the vehicle group. The anastomotic breaking strength of AZD3342-treated rats was 44% higher (P = 0.008) than the vehicle-treated rats. Furthermore, the AL rate was reduced (P = 0.037) with AZD3342 compared with vehicle treatment. AZD3342 treatment influenced neither the total or insoluble collagen concentrations nor the degree of fragmentation of the soluble collagen triple helices.
Conclusion Selective MMP inhibition increased anastomotic breaking strength and reduced AL after resection of colonic obstruction.

Keywords Anastomosis · Anastomotic leak · Breaking strength · Colonic obstruction · Collagen

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Background

Anastomotic leakage (AL) is a devastating complication after colorectal surgery. The risk of AL increases in the presence of colonic obstruction to about 15% of the patients operated for colorectal cancer [1–3].

Obstruction changes the morphology of the colonic wall rapidly after onset with pronounced edema and infiltration of the mucosa and submucosa first by neutrophils then by macrophages, lymphocytes, and plasma cells [4]. Submucosal collagen is also markedly reduced leading to a destabilized anastomosis [4–6]. These cellular and biochemical changes of the obstructed bowel wall may be responsible for the increased AL rate.

The degradation of collagen has been attributed to the action of matrix metalloproteinases (MMPs) [6, 7]. The MMP family comprises more than 20 different enzymes with both physiological and pathophysiological functions [8]. Treatment

with non-selective MMP inhibitors improves the biomechanical strength of elective anastomoses [9–13]. The mechanisms for the beneficial effects are poorly understood. Paradoxically, the total collagen concentration of the anastomoses seems unaffected by the treatment with these drugs [9, 11]. This observation indicates that MMP inhibitors possibly influence the quality rather than the quantity of collagen during anastomotic healing. One non-selective MMP inhibitor increased the amount of soluble but not insoluble collagen in colonic anastomoses [10]. However, both native and fragmented collagens were included in their analyses [10]. In another study, the non-selective MMP inhibitor BB-94 protected the recently deposited collagen triple helices from MMP-mediated damage [14].

We recently investigated the effect of the non-selective MMP inhibitor GM6001 on anastomotic healing in a rat model complicated by colonic obstruction. Contrary to our expectations, GM6001 treatment *increased* AL [15]. Because epithelial coverage of wounds depends on the activity of specific MMPs [16], inhibition of these obligatory MMPs may have impaired epithelialization. In addition, the use of non-selective MMP inhibitors is associated with musculoskeletal adverse effects, bone destruction, and Dupuytren-like disease [17–19].

Another explanation for the unexpected results with GM6001 is perhaps the animal model. In this model, the breaking strength of anastomoses in obstructed colon *increases* compared with anastomoses in non-obstructed colon [20]. This is remarkable considering that the complications were, as anticipated, more common in anastomoses made in the obstructed colon than in the non-obstructed colon. This contradictory finding can be explained by the priming effect of the inflammatory reaction elicited by the laparotomy. Blood levels of the pro-inflammatory cytokine TNF-α were also higher in rats undergoing laparotomy compared with rats subjected to laparoscopy [21]. Another important finding is that pneumoperitoneum itself does not seem to influence the strength of colonic anastomoses [22].

Our primary objective was to investigate the effect of selective MMP inhibition on anastomotic repair in the obstructed colon. To circumvent the disadvantage with the previous experimental models [15, 20], we developed a novel laparoscopic method to induce obstruction. For the intervention studies, we chose the selective MMP-8, MMP-9, and MMP-12 inhibitor AZD3342 with proven efficacy in the repair of anastomoses in the normal, non-obstructed colon [23]. To study the effect of the AZD3342 on collagen metabolism specifically, total as well as fractionated collagens present in the anastomoses were analyzed.

Methods

The study adhered to the ARRIVE guidelines for reporting animal research [24].

Animals Thirty-eight inbred male Sprague–Dawley albino rats (Taconic, Ry, Denmark), weighing 226–284 g, were acclimatized for at least 7 days prior to surgery and kept in type III cages at room temperature with a 12-h light cycle. The rats were transferred to individual cages after the initial surgical procedure. The animals had free access to tap water and a highly digestible diet (TransWean; Special Diets Service, Essex, UK). The experiments were approved by the Animal Ethics Committee of the Danish Ministry of Justice (2010/561-1775).

Study design Acute colonic obstruction was induced by a novel laparoscopic technique. After 12 h of obstruction on day 0, the stenotic segment was resected and a primary anastomosis constructed. In 6 of the 38 rats, the anastomotic breaking strength was measured immediately after construction of the anastomosis day 0. The remaining 32 rats were assigned to daily treatment with AZD3342 (n = 16), or with vehicle (n = 16) by randomization. Three days after anastomotic surgery (day 3), the anastomoses were evaluated for AL, breaking strength, and total and fractionated collagens.

Selective MMP inhibitor AZD3342 (AstraZeneca Research and Development, Mölndal, Sweden) is a 403 D, synthetic non-hydroxamate MMP-8, MMP-9, and MMP-12 inhibitor [23]. The half-maximal inhibitory concentration (IC_{50}) for AZD3342 with respect to MMP-8 was determined to 16 nM, for MMP-9 10 nM and for MMP-12 6 nM. Accordingly, AZD3342 presents a greater than three orders of magnitude selectivity for MMP-8, MMP-9, and MMP-12 over MMP-1 and tumor necrosis factor-α (TNF-α) converting enzyme (TACE) [23].

AZD3342 at 50 mg/kg in vehicle (10% 2-hydroxypropyl-β-cyclodextrin; Sigma-Aldrich, St. Louis, MO) or vehicle alone were injected subcutaneously (s.c.) daily for three consecutive days starting day 0 directly after construction of anastomoses. All individuals handling the rats were blinded to the group allocation.

Anesthesia and analgesics Anesthesia was introduced with a mixture of isoflurane (Baxter, Deerfield, IL) 3.5%/O_2 (1 l/min) for 2 min and maintained with isoflurane (2%)/O_2. Bupivacaine (Marcain®; AstraZeneca, London, UK) 2 mg/kg was injected s.c. at the incisional site for local analgesia. Preoperative analgesia was provided by s.c. injections of 5 mg/kg carprofen (Rimadyl®; Pfizer Animal Health, New York, NY) and 0.03 mg/kg buprenorphine (Temgesic®; Schering-Plough, Brussels, Belgium). After completion of the anastomoses, rats were given 5 ml saline s.c. and 5 ml saline intraperitoneally. Postoperative analgesia in the treated rats was provided by buprenorphine (0.4 mg/kg) per os in a

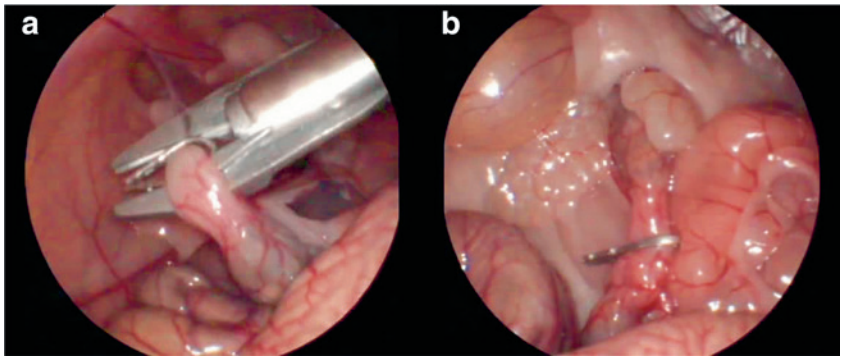

Fig. 1 Laparoscopic induction of experimental colonic obstruction in rats. **a** Application of a titanium clip on the left colon 30 mm from the peritoneal reflection using a 5-mm clip applier. **b** The titanium clip in situ on the left colon just after its application

hazelnut butter mixture at 8-h intervals and with daily s.c. injections of carprofen (5 mg/kg).

Induction of colonic obstruction and construction and evaluation of the anastomoses A 2.7-mm, 30° TrueView II arthroscope (A70963A; Olympus Danmark, Ballerup, Denmark) with a working distance of 70 mm was used to induce acute colonic obstruction. The scope was connected to a VISERA OTV-S7 N-H camera head, OTV-S7 video processor, and a CLV-S40 light-source (Olympus Danmark). A 2-mm skin incision was made in the epigastrium, and a suture was placed tangentially through the abdominal wall. Pneumoperitoneum was established with a 21-gauge needle as the holding suture was used to retract the abdominal wall. The abdominal cavity was then inflated with CO_2 to a maximal pressure of 4 mmHg using the UHI-2 insufflation unit (Olympus Danmark). Hereafter, a sheet and trocar were introduced through a 1-mm incision in the linea alba. The videoscope was inserted through the sheet. In the right lower quadrant, a 3-mm skin incision was made and the muscle fibers were split with a straight hemostat. The hemostat was forced through a 1-mm incision in the peritoneum where, after the laparoscopic, instruments were inserted directly into the abdominal cavity between the branches of the hemostat. The incision was tightened with a purse string suture (Ethicon, Somerville, NJ). A titanium clip was applied around the colon 30 mm from the peritoneal reflection, between two marginal veins using a 5-mm EndoClip (Autosuture; Covidien, Dublin, Ireland) shown in Fig. 1. Feces in the distal colon were cleared. The abdominal wall at the lateral incision was closed with two single Ethilon® 3/0 sutures (Ethicon). The skin incision was closed with titanium clips (Appose ULC 35 W; Covidien).

After 12 h of colonic obstruction (day 0), the peritoneal cavity was exposed through a 30-mm midline incision. A 15-mm colonic segment with the obstructive clip in the middle was resected. The colon was opened at the anti-mesenteric border and the inner circumference of the colon measured 10 mm proximal and distal to the stenosis. An end-to-end single-layer anastomosis was constructed with nine interrupted Ethilon® 6/0 polyamide sutures (Ethicon). The abdominal muscles and the transverse fascia were closed with a continuous Ethilon® 3/0 suture (Ethicon) and the skin incision with titanium clips (Appose ULC 35 W).

The return of bowel function was taken as the time to first defecation determined through daily inspection of the cages.

On day 3, a re-laparotomy was commenced, and the anastomoses were evaluated macroscopically and meticulously. AL was defined as a visible defect in the anastomotic suture line or an abscess in conjunction with the suture line [25]. The anastomoses were then freed of adhesions, excised with a 20-mm margin on each side of the suture line, and placed in saline. The colonic segment was fastened with clamps positioned 10 mm apart in a material testing machine equipped with a 10 N XLC load cell (LF Plus; Lloyd Instruments, Bognor Regis, UK). The specimen was pulled apart within 10 min after excision and vertically at 10 mm/min and the breaking strength in Newton (N) determined from the load-deformation curve [23]. The healing zone of the anastomoses was dissected from macroscopically uninjured colon. Two excisional biopsies of the anastomotic line were snap-frozen on dry ice and stored at −80 °C until analyzed for total collagen concentration and collagen fractions.

Collagen assessments Total anastomotic collagen was determined by hydroxyproline. One of the two anastomotic wound biopsies was lyophilized (11.4 ± 4.6 mg dry tissue), hydrolyzed in 6 N hydrochloric acid at 110 °C for 18 h, and assayed for hydroxyproline colorimetrically [26]. Hydroxyproline was converted into collagen by multiplying with 7.46 [27].

To further characterize the collagen present in the anastomotic wounds, collagens were isolated into pepsin-insoluble

Table 1 Colonic circumference (mm) after 12 h of obstruction

	Controls	Vehicle	AZD3342	*P*
Proximal	18.3 ± 2.4	18.6 ± 2.0	19.2 ± 2.0	.597[a]
Distal	10.3 ± 2.1	10.4 ± 1.3	10.8 ± 1.2	.603[a]
	P < .001[b]	*P* < .001[b]	*P* < .001[b]	

Mean ± SD

[a] One-way ANOVA

[b] Paired *t* test

Table 2 Study flow (number of rats)

	Vehicle	AZD3342	*P*
Starting population	16	16	
Withdrawn from study[a]	0	1	.999
Intention-to-treat population	16	15	
Mortality	3	4	.685
Per-protocol population	13	11	

[a] Trocar hernia day 0. Fisher's exact test

and pepsin-soluble collagen. Technically, the other anastomotic wound biopsy (46.7 ± 17.3 mg wet tissue) was finely dispersed for 10 s at 30,000 r.p.m. using a T10 Ultra-Turrax® instrument (IKA-Werke, Staufen, Germany) equipped with a 5-mm dispersing tool (S10 N-5G) in 5 ml of 0.5 N acetic acid with 1 mg pepsin (P7012; Sigma-Aldrich) per 10 mg wet tissue [28]. A pre-study showed that the amount of solubilized collagen determined by the Sircol collagen assay (Biocolor, Carrickfergus, Northern Ireland, UK) doubled in homogenized colonic tissue compared without prior homogenization. The homogenate was incubated for 24 h at 4 °C with continuous stirring at 100 r.p.m., then centrifuged at 16,000×*g* for 10 min and supernatant aspirated. One aliquot (0.5 ml) of the supernatant representing the total soluble collagen fraction was mixed with 0.5 ml 12 N hydrochloric acid, hydrolyzed at 110 °C for 18 h, and assayed for hydroxyproline [6]. Another aliquot (1.0 ml) representing intact soluble collagen was analyzed by Sircol per the manufacturer's collagen isolation and concentration protocol and with the rat type I collagen standard. The addition of AZD3342 (100 nM) [23] during the 24-h incubation period did not influence the soluble collagen levels by Sircol. A third aliquot (0.5 ml) was lyophilized, reconstituted in 0.05 ml NuPAGE® LDS sample buffer (Thermo Fisher Scientific, Carlsbad, CA) without or with 50 mM dithiothreitol (70 °C for 10 min), and electrophoresed on NuPAGE® 4–12% Bis–Tris gels (Thermo Fisher Scientific). The gels were stained with Colloidal Blue (Thermo Fisher Scientific). The pellet, representing insoluble collagen, was analyzed by hydroxyproline after hydrolysis in 6 N hydrochloric acid at 110 °C for 18 h.

Table 3 Body weight (g) after obstruction but before anastomotic surgery day 0 and 3 days after anastomotic surgery (per-protocol)

	Controls	Vehicle	AZD3342	*P*
Day 0	269 ± 12	262 ± 15	264 ± 10	.958[a]
Day 3		244 ± 19	252 ± 16	.240[b]
		P < .001[c]	*P* < .001[c]	

Mean ± SD

[a] One-way ANOVA

[b] Unpaired *t* test

[c] Paired *t* test

Sample size calculation and statistical analyses The sample size was based on the results of a previous study on colonic obstruction in rats, where each group comprised 12 animals [15]. Because of the unknown effects of the laparoscopic technique, we decided to include 16 animals in the vehicle group and 16 animals in the AZD3343 group.

Colonic circumference and complication rates were analyzed according to the intention-to-treat principle using one-way analysis of variance (ANOVA) or Fisher's exact tests. Body weight, anastomotic breaking strength, and collagen levels were evaluated per-protocol with the unpaired or paired *t* test or ANOVA. Collagen levels were log-transformed before performing the statistical analyses. All analyses were two-sided and carried out using IBM® SPSS® Statistics Version 20 (IBM Corporation, Armonk, NY). Data are presented as mean ± standard deviation (SD) unless stated otherwise. The level of statistical significance chosen was $P < 0.05$.

Results

Effects of acute colonic obstruction After 12 h of colonic obstruction, the circumference of the colon proximal to the obstruction increased 1.8-fold in the three groups (Table 1). The body weight after 12 h of obstruction increased from 263 ± 10 to 272 ± 12 g ($P < 0.001$) in the control group, from 250 ± 10 to 261 ± 15 g ($P < 0.001$) in the vehicle group, and from 250 ± 17 to 261 ± 13 g ($P < 0.001$) in the AZD3342 group.

Mortality and bowel function following anastomotic surgery and treatments One rat in the AZD3342 group was excluded because of a postoperative trocar hernia. Three of the 16 vehicle-treated rats and 4 of the 15 AZD3342-treated rats died prematurely (Table 2). One vehicle-treated animal died on postoperative day 3 with a completely dehisced anastomosis, which disqualified this animal from breaking strength determination. One animal in the AZD3342 group died in conjunction with anesthesia on day 0. The remaining 5 rats, 2 from the vehicle group and 3 from the AZD3342 group, died between days 1 and 2. Autopsy of these animals did not reveal any obvious causes.

The bowel function had returned in 8 of the 13 vehicle-treated animals compared with 8 of 11 animals treated with AZD3342 ($P = 0.562$) that survived the 3-day postoperative period.

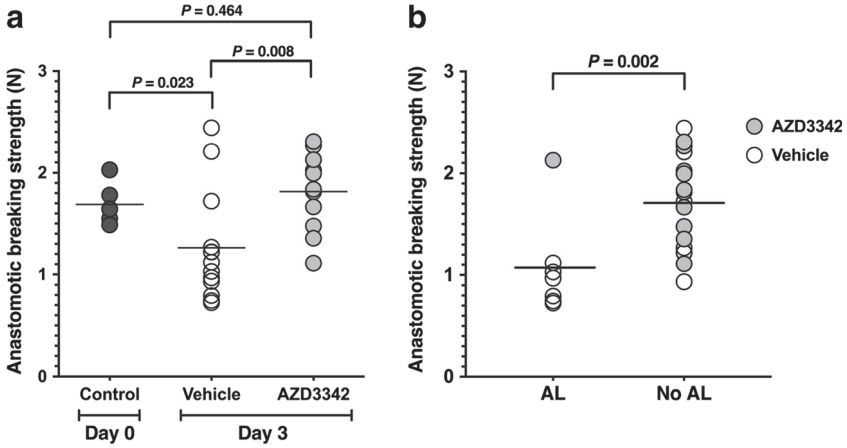

Fig. 2 Anastomotic breaking strength directly after construction of the anastomosis in obstructed colon day 0, and 3 days later in vehicle and AZD3342-treated rats (**a**). **b** Anastomotic breaking strength in rats with (n = 7) or without (n = 17) anastomotic leakage (AL). *Horizontal bars* indicate group mean values

Effect of AZD3342 on body weight, anastomotic breaking strength, AL, and anastomotic collagen

Body weight After anastomotic surgery, the body weight decreased in both groups (Table 3).

Breaking strength Anastomotic breaking strength was analyzed by the per-protocol principle because of the animal deaths before day 3 (Fig. 2a). Breaking strength day 3 was lower (P = 0.023) in the vehicle-treated rats (1.26 ± 0.54 N) but not (P = 0.464) in the AZD3342-treated rats (1.82 ± 0.38 N) compared with initial breaking strength day 0 (1.69 ± 0.19 N). AZD3342 treatment increased (P = 0.008) anastomotic breaking strength by 44% compared with vehicle treatment day 3.

Anastomotic leak To account for all animals included in this high-risk model, the intention-to-treat principle was applied for the statistical assessment of complications. One animal of the 15 animals in the AZD3342 group developed AL compared with 7 of 16 vehicle-treated (P = 0.037) animals (Fig. 3). The anastomotic breaking strength was lower in rats showing AL (P = 0.002) compared with non-AL rats (Fig. 2b).

Analyses of different collagens The total collagen concentration in the anastomotic wounds decreased from day 0 to day 3 with no statistical difference between the vehicle and AZD3342 groups day 3 (Fig. 4). After fractionation of the anastomotic wound tissue with pepsin treatment, the resulting insoluble collagen fraction of the anastomoses did not differ significantly between the vehicle and the AZD3342-treated animals either. Furthermore, the total concentration of soluble collagens and the proportion of intact collagen to total collagen in the soluble fraction was not significantly different between the vehicle and AZD3342 groups either (Table 4). The soluble collagen fractions were also analyzed after

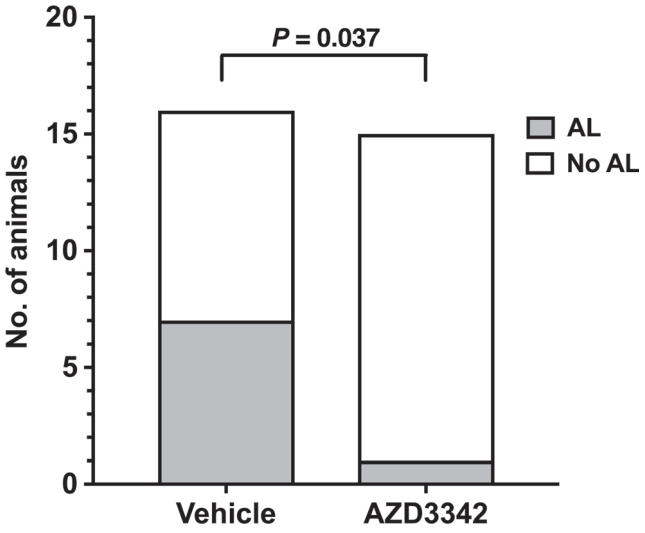

Fig. 3 Effect of the selective MMP inhibitor AZD3342 on the occurrence of anastomotic leakage (AL) on day 3

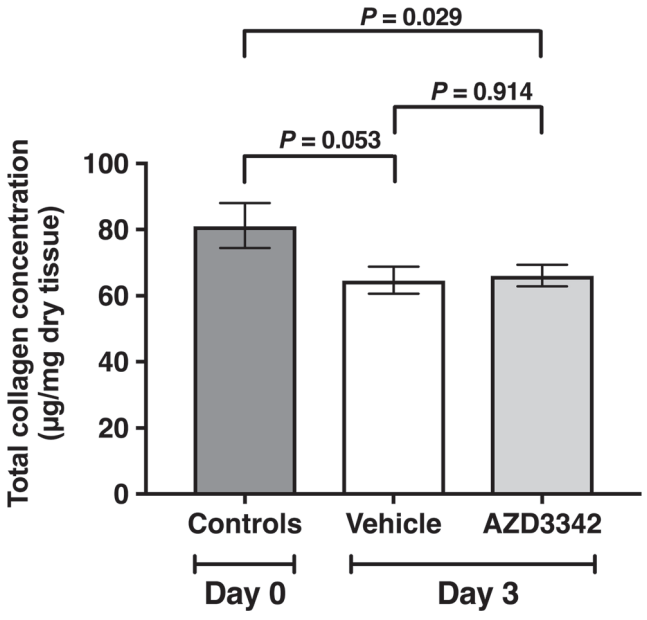

Fig. 4 Total collagen (converted from hydroxyproline) concentration in the anastomosis. Geometric mean ± backtransformed standard error

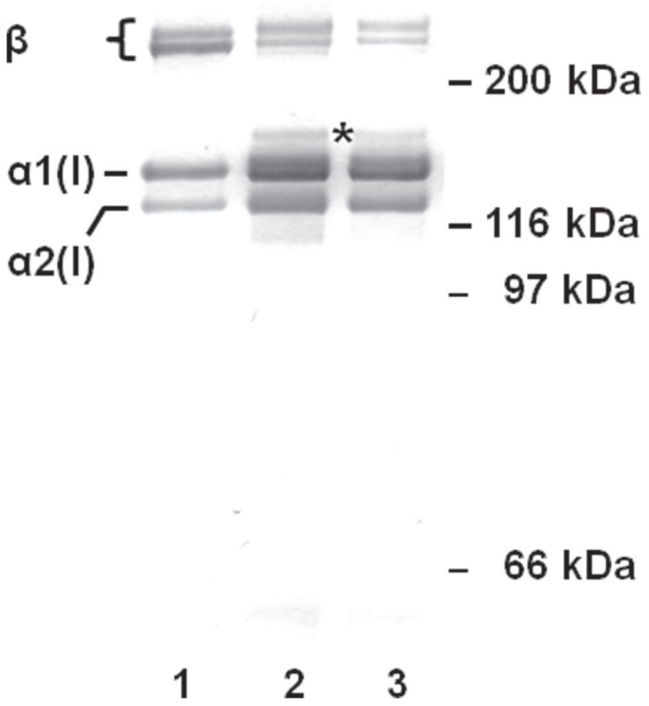

Fig. 5 Analysis of the pepsin-soluble collagen fraction of anastomotic wound tissue by electrophoresis (non-reduced). Lane *1*, rat type I collagen standard (1 μg, Biocolor); *2*, pepsin extract from the vehicle group; *3*, pepsin extract from the AZD3342 group. The corresponding determinations of collagen by the Sircol assay were 1.1 μg (lane 2) and 0.7 μg (lane 3). *Asterisk* (*) indicates unidentified band

electrophoretic separation. Sircol and electrophoretic collagen determinations showed good concordance. The pepsin extracts contained primarily monomers of type I collagen. The band above α1 chains indicated with asterisk (*) in Fig. 5 was also present when gel was run at reduced conditions indicating that it was not the α1 chain of type III collagen but possibly the α1 chain of type I procollagen. No apparent differences were found between anastomotic pepsin extracts of vehicle and AZD3342-treated animals.

Discussion

AL is a serious complication following emergency surgery for colonic obstruction. There are no available therapeutic agents to prevent AL [13, 29]. Although overexpression of certain MMPs is detrimental to anastomotic wound healing indiscriminate MMP inhibition *increases* AL in experimental models of colonic obstruction [15]. In the present study, we have demonstrated that by using a selective MMP inhibitor, AL is *reduced* perhaps by increasing the anastomotic biomechanical strength following experimental colonic obstruction.

In established experimental models of acute colonic obstruction, typically in animals undergoing laparotomy, suture materials or silicone rings are placed around the colon for periods of 24 h or longer [4, 7, 20, 29]. One drawback with these procedures is that the laparotomy per se increases MMP levels in the colon [7]. To correct for this model artifact, we developed a novel laparoscopic model of acute colonic obstruction. To avoid complications from prolonged obstruction by the titanium clip, the duration of obstruction was reduced to 12 h. This approach produced almost a 2-fold increase in the colonic circumference, corresponding to the circumference of using a silicone ring for 24 h [4]. Anastomoses constructed in the 12-h obstructed colon showed the anticipated high AL rate (44%). These observations suggest that by reducing the systemic response to trauma, the expected complications were manifested clinically using our laparoscopic technique.

The increased breaking strength with AZD3342 treatment was not accompanied by increased total collagen concentration of the anastomoses. Moreover, AZD3342 treatment increased neither the ratio of insoluble collagen to total soluble collagen nor the intact forms of soluble collagen. These findings suggest that AZD3342 did not alter the cross-linked, existent collagen of the anastomoses [30] or protected the susceptible soluble collagens from MMP-mediated fragmentation. This disagrees with the effects of non-selective MMP inhibition [14] indicating that other MMPs than MMP-8, MMP-9, and MMP-12 are responsible for collagen remodeling in the anastomoses. Taken together, these findings indicate that AZD3342 improved anastomotic healing by mechanisms other than by influencing collagen metabolism. It should be emphasized though, that AZD3342 possibly impacted other parameters important for tissue strength such as the type I collagen to type III collagen ratio, and/or collagen fiber diameter or orientation [31].

We speculated that the unfavorable effects of GM6001 [15] were due not only to inhibition of epithelialization-requiring MMPs [16] but also to reduced levels of TNF-α via inhibition of TACE. AZD3342 is a poor inhibitor of TACE and primarily inhibits inflammation-associated MMPs directly but spares the MMPs required for epithelialization [16].

The optimal MMP selectivity for AL prevention is unknown. The concept of selective MMP inhibition to improve complicated anastomotic wound healing was recently applied to an ischemic anastomotic rat model [32]. Shogan et al. observed a substantial reduction in AL from 50% in non-treated animals to almost zero in animals treated with an MMP-9 inhibitor [32]. Our results with AZD3342 suggest that by targeting up-regulated MMP-8, MMP-9, and MMP-12, the anastomotic wound healing complicated by an acute obstruction is significantly improved. We cannot exclude that inhibition of additional MMPs would produce a superior effect compared with AZD3342. For example, MMP-13 mRNA levels are highly up-regulated in day-3 colonic anastomoses [23]. From an overall clinical benefit-risk perspective, an additional advantage with selective MMP inhibitors is the expected reduced occurrence of adverse effects as compared with non-selective MMP inhibitors [17–19].

Table 4 Collagen forms in the day-3 anastomoses (per-protocol)

	Vehicle	AZD3342	*P*
Insoluble collagen (μg/mg wet tissue)	5.1 ± 1.5	3.9 ± 1.4	.071
Total soluble collagen (μg/mg wet tissue)	11.0 ± 0.5	10.7 ± 0.7	.320
Intact soluble collagen/total soluble collagen	0.12 ± 0.11	0.12 ± 0.10	.878
Insoluble collagen/total soluble collagen	0.46 ± 0.14	0.37 ± 0.14	.131

Geometric mean ± back-transformed standard error. Unpaired *t*-test

At least four different compounds have previously been investigated in experimental models of obstructed colon [29]. Iloprost, a synthetic prostacycline analog, was the most promising compound [29, 33]. Iloprost treatment decreased MMP-13 protein levels, more than doubled anastomotic bursting pressure but did not reduce the AL rates of 4-day-old anastomoses in obstructed colon [33].

In conclusion, selective MMP inhibition increased anastomotic breaking strength and decreased the AL rate in the acutely obstructed colon. The mechanism for the beneficial effects appears unrelated to the quantity or MMP susceptibility of existent or nascent collagen molecules and thus requires further elucidation. Nevertheless, it seems worthwhile exploring this class of therapeutics further for AL prevention after acute colonic obstruction.

Compliance with ethical standards The experiments were approved by the Animal Ethics Committee of the Danish Ministry of Justice (2010/561-1775).

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