Novel insights in the pathophysiology of colorectal anastomotic leakage

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Novel insights in the pathophysiology of colorectal anastomotic leakage

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Novel insights in the pathophysiology of colorectal anastomotic leakage

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Chapter 1

General introduction

Partially adapted from:

Joanna WAM Bosmans, Audrey CHM Jongen, Nicole D Bouvy, Joep PM Derikx

Colorectal anastomotic healing: why the the biological processes that lead to anastomotic leakage should be revealed prior to conducting intervention studies

BMC Gastroenterol. 2015;15;180

and

Audrey CHM Jongen, Victor van Woerden, Jeroen L van Vugt, Patrick A de Hoogt,
Elisabeth M de Wijkerslooth de Weerdesteijn, Juul J Tegels, Jan H Stoot

Improving outcome in gastrointestinal and hepatopancreaticobiliary surgical oncology by preoperative risk assessment and optimization of perioperative care

Book chapter – Oncology Critical Care

General introduction

Colorectal anastomotic leakage (CAL) is the most feared complication following colorectal surgery, with incidence rates between 7-10%. It is associated with high rates of morbidity and mortality^{1,2}, poor health-related quality of life (HRQoL)^{3,4} and an increase of healthcare costs⁵. Recent reports have even found an association between CAL and a poor oncological outcome, which reflects in increased local recurrence rates and decreased disease-specific survival⁶⁻⁸. Studies that have been conducted on this topic have revealed several risk factors for the occurrence of CAL such as: male gender, old age, smoking, corticosteroid and NSAID use, diabetes mellitus, pre-operative radiotherapy and low level of the anastomosis^{9,10}. Several others have examined options to decrease the incidence rates of CAL after surgery, however only a few of the developed preventive methods indeed made it into the clinical setting, including luminal devices such as the C-seal¹¹, alternative closure devices such as the (magnetic) compression ring^{12,13} and patches (e.g. TachoSil®)¹⁴. In the experimental setting, several tissue glues have been investigated for their ability to strengthen the anastomosis. The most often tested glues include fibrin or cyanoacrylate glues¹⁵, of which the fibrin glues yield the most promising results¹⁶. However, none of the investigated preventive techniques are currently being used in routine clinical practice, and have failed to decrease the incidence rates of CAL⁹. An explanation for the failure of implementation of these innovative techniques could be that they are not specifically aimed at the healing process of the gut, as the mechanisms behind this are largely unknown. We believe that the pathophysiological processes involved in the occurrence of CAL need to be elucidated first and foremost in order to find specific targets for new preventive techniques.

An important aspect of the pathogenesis is the composition of the luminal content near the site of the anastomosis, of which the microbiome comprises a substantial part. Several studies have shown that different microbes can change into a more virulent phenotype when exposed to factors such as (surgical) stress and systemic opioids^{17,18}. These more virulent bacteria have been shown to increase their production of collagenases, which weakens the anastomotic strength and consequently increases the CAL risk¹⁹. Other studies have found a relationship between composition of the microbiome and leakage, such as diversity and the balance between bacteroides and firmicutes²⁰. More insight in the exact role and composition of the microbiota on the processes of intestinal healing would also aid in the choice of antimicrobial prophylaxis or bowel preparation, something that varies substantially between hospitals.

During the past decade, more attention has been paid to assessing and optimizing the preoperative condition of our patients. Especially in the light of the increasing age of our general population, it is important to consider the impact of surgery on the functional reserve of our patients. Even though improvements in the perioperative care over the past decades reflect positively on the outcome of the overall patient population, the older patient (octo- and nonagenarians) has benefited much less from these improvements²¹. Identifying the patient at risk for an adverse outcome is therefore of great importance, and several tools can be used to do this. Sarcopenia for example is considered a hallmark sign of frailty, and is characterized by a loss of skeletal muscle mass and strength²². Multiple studies have shown an association between the presence of sarcopenia and adverse outcome after surgery, for example for patients undergoing surgery for colorectal liver metastases²³. Optimizing the patient's condition before surgery has been proven to be beneficial for short and long-term morbidity and mortality rates in other surgical fields, and is currently being investigated in colorectal patients²⁴.

Another preoperative issue that is still under debate is whether patients should be subjected to bowel preparation. Although the evidence for mechanical bowel preparation is still inconclusive, the use of (oral) antibiotics can be supported by several studies and meta-analyses^{25,26}. A meta-analysis published in 2016 concludes that bowel preparation alone does not decrease the risk for CAL, but should be used in combination with oral (and not only systemic) antibiotics in order to decrease the bacterial load in the gut²⁵.

The surgical procedure itself has proven to be important as well, and parameters such as mean arterial pressure (MAP), body temperature, duration of surgery and the amount of blood loss have been identified as risk factors for CAL²⁷⁻³⁰. It has been suggested that the perfusion of the intestinal tissue is one of the most important aspects of successfully constructing an anastomosis. Near infrared fluorescence imaging is therefore being used in operation theatres more often, as it allows surgeons to visualize the microperfusion of the intestine in real-time. It has been shown to significantly improve safety of colorectal surgery^{31,32}.

The question whether or not to construct a (temporary) colostomy or ileostomy remains debatable. For example, it seems advisable to construct a temporary ileostomy when a low anterior resection (LAR) is indicated, but some surgeons opt for a colostomy instead. It is debatable however to routinely construct a deviation in all patients undergoing a LAR, as it is unclear when the risks outweigh the benefits of such a deviation. It has been shown that although the presence of a deviating ostomy mitigates the clinical consequences of a leak, the risk of developing a leak does not decrease³³⁻³⁵.

Furthermore, stoma complications have a reported incidence of up to 30%, ranging from skin complications and high output to herniation and stoma prolapse, which often requires reoperation^{33,36}. Temporary stoma reversal exposes the patient to the risks of another operation, including anastomotic leakage. Finally, a large proportion of all ostomies that were initially planned to be only temporary will never be reversed, which severely affects QoL^{36,37}. For the more proximal resections, evidence and guidelines are even less unambiguous.

Aim and outline of this thesis

PART I: increasing international consensus on the definition of colorectal anastomotic leakage

Several definitions have been proposed for CAL during the past decades, but none of these have been implemented in clinical practice yet. The fact that authors use different definitions that include a smaller or larger variety of clinical presentations of the complication makes comparison of study results between studies rather difficult, and meta-analyses nearly impossible. The use of different definitions could also explain for the variance in reported incidence rates between studies and even between continents.

Aims

- Increasing insight of what is considered colorectal anastomotic leakage by surgeons in different continents
- Increasing insight in what types of definitions of CAL are being used in literature and how these definitions influence reported incidence rates

The first part of this thesis describes the first steps of a process to develop a generally accepted definition for CAL. In **Chapter 2**, a survey among Chinese and Dutch colorectal surgeons is described that investigated the different views regarding what should be considered colorectal anastomotic leakage. The results of this chapter partly explain the difference in reported incidence between Western and Eastern countries and give more insight in the views of colorectal surgeons as to what should be included in a general definition for CAL. **Chapter 3** is a systematic review that focuses on the use of different CAL definitions in literature. In this chapter, we also give recommendations regarding the reporting of data for studies with colorectal anastomotic leakage as one of their main outcomes.

PART II: Developing a predictive algorithm for anastomotic leakage

Despite the numerous studies that have been conducted in the past focusing on risk factors for colorectal anastomotic leakage, accurate prediction of the occurrence of such a leak in the individual patient remains impossible. In fact, a study conducted by Karliczek et al. has shown that the assessment of the risk of a complicated postoperative course by the colorectal surgeon during surgery and immediately after the construction of an anastomosis is highly inaccurate³⁸. Several other studies have focused on finding biomarkers for colorectal anastomotic leakage, in order to be able to set the diagnosis at an earlier stage. Indeed, mean time of diagnosis AL varies greatly, from postoperative day 3 to beyond 30 days, with a mean of 12.7 days postoperatively³⁹. It has been shown that early recognition of CAL substantially reduces the morbidity and mortality associated with the complication⁴⁰.

Aims

- formulating a predictive algorithm that can be used to assess the risk of CAL in every individual patient
- diagnosing the development of a leakage in earlier stages
- quantification of the microperfusion of the intestine during surgery

Part II of this thesis focuses on the development of adequate prediction of the risk of CAL and diagnosis of the complication in early stages. **Chapter 4** is a study protocol for a large observational study that is currently being conducted in three hospitals in the Netherlands: Zuyderland Medical Centre in Sittard-Geleen, VieCuri Medical Centre in Venlo, and the Maastricht University Medical Centre. In **Chapter 5**, we have investigated the use of near infrared fluorescence (NIRF) imaging after intravenous indocyanine green (ICG) administration during colorectal surgery, and the correlation between fluorescence intensity and postoperative inflammatory markers.

PART III: Considerations regarding perioperative care of the colorectal surgical patient

The perioperative care has undergone substantial changes over the past decade. Some of these involve the preoperative phase. For example, physicians have aimed to improve the condition of the patient prior to undergoing surgery by means of so called prehabilitation programmes. In these programmes, a multidisciplinary team works together to focus on fitness, nutritional status and body composition. During this perioperative period, several decisions have to be taken by the surgical team for every individual

patient on a daily basis. These include for example whether or not to prepare the bowel by decontamination by means of enemas and antibiotics, and whether or not to construct a deviating ostomy. For most of these dilemmas, the surgical community has failed to reach consensus. The need to assess the integrity of the anastomosis prior to temporary stoma reversal, and which method is most accurate to do so, is a good example of such a dilemma. In part III of this thesis, we will focus on this question in more detail.

As mentioned in the general introduction, CAL is associated with high rates of comorbidity and mortality, and has been identified as a risk factor for cancer recurrence and reduced disease-specific survival⁴¹. The reason for this correlation remains largely unknown, but several mechanisms have been proposed to explain these findings. One theory states that anastomotic leakage could lead to the mechanical spreading of viable cancer cells into the peritoneal cavity, others believe that the increased postoperative inflammatory response stimulates growth and proliferation of tumour cells. Because of the impact that colorectal anastomotic leakage has on the oncological outcome in the long-term, we have also focused on this question in part III of this thesis. Indeed, better understanding of the prognostic relevance of the inflammatory parameters might assist in patient selection for adjuvant therapy in the future.

Aims

- Comparing the accuracy of contrast enema and colonoscopy for the assessment of the integrity of the anastomosis prior to temporary stoma reversal
- Investigating the relationship between the inflammatory response and the oncological prognosis after colorectal cancer surgery

In **Chapter 6**, we investigated which type of diagnostic method is most suitable for the assessment of integrity of the anastomosis before temporary stoma reversal. We have investigated this in a large cohort of patients that underwent rectal surgery in 2011. **Chapter 7** focuses on the association between an increased perioperative inflammatory status and the risk of recurrence and disease-specific mortality after colorectal cancer surgery.

PART IV: Novel insights in the (patho)physiology of anastomotic healing and leakage

As our research group has discussed in our debate paper, very little is known about the exact mechanism underlying the pathophysiology of CAL. Moreover, little research has been done on normal intestinal healing. We know that the general phases of healing also

occur in the intestine (inflammation, proliferation, remodelling), and that an important interaction exists with the mucosal and luminal microbiome and the intestinal wall and mucosa. Several other studies have focused on the role of possible beneficial compounds such as the short-chain fatty acid butyrate, which is the most important source of energy for the colonocyte, and has been shown to increase proliferation rates both in vitro and in vivo. However, many questions regarding the exact mechanistic pathways remain unanswered. We have stressed the need for studies investigating these processes before more intervention studies are to be conducted.

Aims:

- Investigating the optimal application method for butyrate in a rat model
- Investigating the role of the mucus layer in the intestinal healing process in a murine model
- Investigating the influence of diclofenac and surgery on the composition of the microbiome in a rat model

In **Chapter 8**, we have investigated different application routes (patch, injection, enema) for butyrate to improve intestinal healing in a rat model. Previous animal studies showed that butyrate enemas had a beneficial effect on the strength of (distal) colonic anastomoses⁴². In our experiments, the glycosaminoglycan hyaluronan was added to the mixture, which is known to promote neovascularization, has beneficial effects on cell proliferation, and diminishes the inflammatory response⁴³. In **Chapter 9**, we have focused on the role of the mucus layer, the primary line of defence in the gastrointestinal tract and the home of a large variety of microbial species. The amount of mucin secreted by the goblet cells influences the ability of the mucus layer to protect the colon wall, and several conditions have been shown to alter the mucin secretion such as the presence of SCFA's and certain types of bacteria⁴⁴. Prostaglandin E2 (PGE2), which is an enzymatic product of cyclo-oxygenase (COX) 1 and 2 has also been shown to positively influence the mucus secretion⁴⁵. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX, and therefore cause a decrease in PG synthesis⁴⁶. We constructed an anastomosis in the distal colon of muc2 knockout mice and wild-type controls, and investigated whether the administration of prostaglandin E2, an important inflammatory mediator, exerted an effect on the thickness of the mucus layer and/or on the leakage rates in this model. **Chapter 10** describes another animal study in which the effect of surgery and a non-steroidal anti-inflammatory drug (NSAID) – in this case diclofenac – was studied on the composition of the microbiome in the rat before and after the construction of an anastomosis in different sites of the intestinal tract.

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Part I

Increasing international consensus on the definition of
colorectal anastomotic leakage

Chapter 2

Definition of colorectal anastomotic leakage:
consensus survey among Dutch and Chinese
colorectal surgeons

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Gerrit D Slooter, Rudy MH Roumen, Nicole D Bouvy

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Abstract

Introduction

Colorectal anastomotic leakage (CAL) is the most feared complication after colorectal surgery. Despite increased awareness of the complication, no consensus exists for a general definition for CAL. The large variety in applied definitions may partly explain differences in reported CAL incidence between Asian and Western studies (1.5-23%) and complicates the interpretation of reported outcomes.

Aim

The purpose of this study was to determine the level of consensus on the definition of CAL among Dutch and Chinese colorectal surgeons.

Methods

Dutch and Chinese colorectal surgeons were asked to partake in an online questionnaire. Consensus in the online questionnaire was defined as >80% agreement between respondents on various statements regarding a general definition of CAL, and regarding clinical and radiological diagnosis of the complication.

Results

Fifty-nine Dutch and 202 Chinese dedicated colorectal surgeons participated in the online survey. Consensus was found on only one of the proposed elements of a general definition of CAL in both countries: 'extravasation of contrast after rectal enema at the CT-scan'. Another two were found relevant according to Dutch surgeons: 'necrosis of the anastomosis found during reoperation', and 'a radiological collection with percutaneous drainage'. No consensus was found for all other proposed elements that may be included in a general definition.

Conclusion

There is no universally accepted definition of colorectal anastomotic leakage in the Netherlands and China. Diagnosis of CAL based on clinical manifestations remains a point of discussion in both countries. Dutch surgeons are more likely to report 'subclinical' leaks as CAL, which partly explains the higher reported Dutch CAL rates.

Introduction

Colorectal anastomotic leakage (CAL) remains gastrointestinal surgeons' most feared complication, despite important improvements in perioperative care and the development of novel surgical techniques. It is associated with high rates of morbidity and mortality^{1,2}, poor patient quality of life³ and an increase of healthcare costs^{4,5}. Since CAL influences the direct postoperative course and has recently been proven to impact oncological outcome as well⁶⁻⁸, it is frequently used as an outcome measure in clinical studies. However, the CAL rates vary considerably in international literature: from 1.5 to 23%^{9,10}. Large variations in leakage rates have been reported between studies published by Western and Asian research groups, in which the reported incidence of CAL in Asian publications is substantially lower^{11,12,5,13,14}. Such differences can be partly explained by the variations of operation technique, tumor location and patient characteristics^{15,16}. However, little attention has been paid to potential differences in the CAL definition and the available methods of diagnosis.

Although CAL is sometimes defined as "a defect in the bowel wall at the anastomotic site, leading to communication of intra- and extraluminal compartments"¹⁷, this definition translates rather difficult to the clinical situation. Therefore, many authors formulate new definitions or diagnostic criteria in their studies, which usually include clinical and radiological features¹⁸, and the impact of a leak on the treatment plan. However, since the pathophysiology of anastomotic leakage is multifactorial, the manifestation of a clinical leak can be rather variable¹⁵. Furthermore, due to the increased use of (routine) diagnostics such as CT or contrast enema, "radiological" leaks that do not eventually influence patient management are diagnosed more often. These factors complicate comparison of study results, and weaken the reliability of further analyses. This in turn hampers the construction of evidence-based guidelines on patient management and surgical technique.

Clearly, there is a need for a generally accepted and practical definition for CAL and its diagnostic criteria to serve as a template for future research on CAL and the clinical decision-making process¹⁹. Several surveys have been performed to reach consensus regarding the definition of CAL, however, most of them were restricted to a single country¹⁹. We hypothesize that the aforementioned reported differences in incidence rates between Asian and Western countries can partly be explained by differences in the definitions and diagnostic methods used. The aim of this study is therefore to determine the level of consensus regarding different aspects of a general definition of colorectal anastomotic leakage within and between populations of Chinese and Dutch colorectal

surgeons, who can be considered good representatives of the East and West, respectively.

Materials and methods

An online survey was performed among colorectal surgeons from the Netherlands and China. In the Netherlands, the survey was constructed and run through an online database using SurveyMonkey™ Palo Alto, CA, USA. Colorectal surgeons in the Netherlands were identified from the contacts section of the colorectal subdivision of the Dutch Society of Gastro Intestinal Surgery (NVGIC): the Taskforce Coloproctology (WCP). Within this subdivision, 141 both senior and junior colorectal surgeons were identified. Respondents were invited to partake in the online survey by email. Dutch surgeons completed the questionnaire between May and June 2015.

In China, the survey was conducted on the platform provided by DXY (www.dxy.cn), which is the largest medical website in China with more than one million registered medical users. An invitation was sent to all the registered colorectal surgeons to invite them to participate a five-minute survey. Due to a relatively large number of registered users, the survey was designed to be terminated when 200 replies were received. Surgeons from Hong Kong, Macao, and Taiwan were not invited in this survey, since to the application of different medical systems in those areas. A demographic chart of the regions represented by the respondents can be observed in Figure 2.1.

The survey was divided into three major categories with questions addressing the general definition, and the clinical and radiological diagnosis of CAL. It was partly adapted from a previous study of Adams et al.²⁰ and was initially constructed in English, and then translated to Dutch and Chinese by surgeons fluent in both English and Dutch and English and Chinese for the Dutch and Chinese by surgeons fluent in both English and Dutch, and English and Chinese for the Dutch and Chinese versions respectively, and checked for interpretation bias. Details of the English questionnaire are shown in Table 2.1 (See supplementary data for Dutch and Chinese version).

Category I mainly focused on the agreement of general definitions used in international literature²⁰. Surgeons were asked to state whether 10 different clinical situations should or should not be included a general definition. Category II focused on clinical manifestations and its predictive value on CAL. A numeric scale with a grading from 1 (not predictive at all) to 10 (very predictive) was used to assess the agreement of the

respondents' views on the clinical parameters. The parameters used were partially adapted from the Dutch Leakage Score (DULK)¹¹. Category III consisted of four questions regarding the use of radiological examination and the influence of this diagnostic method on patient care. This third category was also partially adapted from Adams et al.²⁰. The last general question focused on surgeons' views regarding the cause of very **early** anastomotic leakage.

Figure 2.1 Demographic chart of the Chinese regions this survey covers. The gray scale reflects the number of participants in each region, varying from 21 from Zhejiang to 1 from Hainan. Correlating with the number of colorectal surgeons in each region, more surgeons from the east regions participated this survey. Tibet and Ningxia had no participants, which also corresponds to the fact that the number of surgeons is very limited compared to the east provinces. Due to the application of different medical systems, doctors from Hongkong, Macao, and Taiwan were not invited in this survey.

Table 2.1 English questionnaire definition colorectal anastomotic leakage.

General definition		
Do we have to consider the following findings as anastomotic leakage?		
	Yes	No
1 Extravasation of contrast after rectal enema at the CT-scan		
2 Radiological collection around the anastomosis and no treatment		
3 Radiological collection around the anastomosis treated with antibiotics		
4 Radiological collection around the anastomosis treated with percutaneous drainage		
5 Abdominal sepsis and reoperation needed		
6 Necrosis of the anastomosis seen at reoperation		
7 Necrosis of the blind loop seen at reoperation		
8 Signs of peritonitis during reoperation		
9 Air bubbles around the anastomosis seen on a CT scan		
10 Free intra-abdominal air seen on a CT scan		
Clinical diagnosis		
In what extent do the following clinical parameters contribute to the suspicion of colorectal anastomotic leakage? Please note the relevance on a numeric scale of 0-10:		
1 Increased C- reactive protein (CRP)		
2 Increased leukocytes		
3 Tachycardia		
4 Increased respiratory rate		
5 (Sub-) febrile temperature		
6 Postoperative ileus (> 4 days)		
7 Deterioration in clinical condition		
8 Abdominal pain, other than wound pain		
Radiological diagnosis		
Answer the following questions using percentages (0% = never, 100% = always)		
1 In how many percent of patients with clinical suspicion of anastomotic leakage do you perform radiodiagnostics?	%	
2 In how many percent of patients with clinical suspicion of anastomotic leakage do radiodiagnostics change your treatment policy?	%	
3 In how many cases did the CT scan report no anastomotic leakage while there finally was an anastomotic leakage.	%	
4 In how many percent of cases do you consider a reoperation without previous radiodiagnostics?	%	
Early anastomotic leakage		
In your opinion, is 'very early (<3 days) anastomotic leakage the result of technical failure?		
1 Yes		
2 No		

Definitions

Very early anastomotic leakage was defined as leakage occurring within the first 3 days post surgery. Postoperative ileus was defined as an interval of more than 4 days from surgery until passage of flatus or stool and the tolerance of an oral diet²¹. Blind loop was defined as a bypassed loop of bowel after the construction of an end to end or end to side bowel anastomosis.

Statistics

Basic descriptive statistics were used to summarize data for the online survey. Consensus was defined as >80% agreement between respondents on various statements, as described by Duncan et al.²². If less than 80% of respondents deemed the statements important, it was stated that no consensus was reached. Graphical depictions of information were used where appropriate to facilitate interpretation of data. Chi square test or Mann-Whitney test were applied with proper indications. A p-value smaller than 0.05 was considered to indicate statistical significance.

Results

Of the 141 colorectal Dutch surgeons that were invited to partake in the online survey, 62 respondents accepted the invitation, and 59 completed the survey, resulting in a 42% response rate and 95% survey completion. In total, 100% of 201 questionnaires received from Chinese surgeons were completed. A demographic chart of the regions represented by the respondents can be observed in Figure 2.1, as it shows that this survey covers 96.8% (30/31, Hong Kong, Macao, Taiwan not included) of provinces and areas of China.

Consensus was found on only one clinical situation proposed as an element of a general definition in both countries: 'extravasation of contrast on enema' (Figure 2.2), and in the Netherlands on two additional elements: radiological collection for which percutaneous drainage was needed (50/59 respondents, 85%) and necrosis of the anastomosis visible upon reintervention (51/59 respondents, 86%). For all other items on the available general definitions, clinical and radiological diagnosis of CAL, no consensus was found. Scores were significantly different between China and the Netherlands for the following elements: radiological collection treated conservatively (21% vs. 39% respectively $P=0.010$), necrosis of the blind loop on reintervention (41% vs. 69% respectively, $P<0.001$), and for air surrounding the anastomosis on CT-scan (65% vs. 44% respectively, $P=0.004$).

Figure 2.2 Percentage of respondents in agreement to general definitions of colorectal anastomotic leakage in the Netherlands (white bars) and China (dark grey bars). The dotted line indicates the 80% consensus threshold for the different statements. An asterisk indicates a significant ($P<0.05$) difference between percentages of agreement of Dutch and Chinese surgeons.

Grades given for the clinical parameters are shown in Figure 2.3 for both China and the Netherlands. Clinical deterioration, increased CRP, tachypnea and tachycardia were seen as most contributory for the clinical suspicion of CAL in the Netherlands, and were given a weighed score of 7.83, 7.45 7.13 and 7.13 respectively (Table 2.2). In China clinical deterioration and abdominal pain other than wound pain were deemed most attributable for the suspicion of anastomotic leakage in the direct postoperative period, with scores of 6.67 and 6.61 respectively. Increased plasma concentration of C-reactive protein received the lowest score of all parameters in China: 4.35, while in the Netherlands this was deemed more sensitive (7.45, $P<0.001$). Upon categorization of the grades for the value of clinical parameters into different categories of the numeric scale: disagree (0-3), neutral (4-6) and agree (7-10), most surgeons from both countries (45-59% of surgeons for each parameter) remained neutral towards the added value of specific clinical parameters during the postoperative course.

The data on radio diagnostics are shown in Table 2.3. The majority of Chinese and Dutch surgeons perform radio diagnostics upon clinical suspicion of a leak. The distribution of the answers over the different classifications, however, was significantly different between the two nationalities (Chi square test, $P=0.020$). Expected false-negative rates for CT-scans were equal for surgeons in both countries. A significantly larger portion of the Chinese colorectal surgeons (25.4% compared to 13.6%, $P<0.001$) would consider performing a reoperation for the suspicion of colorectal anastomotic leak without

performing radiological diagnostics. The distribution of the scores differs significantly between countries as to in how many cases a reoperation is considered without previous radio diagnostics (Chi square test, $P=0.002$).

Concerning the question about early CAL, 90.6 percent of the Chinese surgeons agreed that the cause of such should be considered a technical failure, whilst only 70,4% of the Dutch colorectal surgeons agreed to this statement ($P<0.001$).

Figure 2.3 Distribution of categorized scores for the value clinical parameters in the direct postoperative phase. Comparison between the Netherlands (A) and China (B). Scores are divided into three categories: numeric scales ranging from 0-3 are depicted in grey (disagree), 4-6 depicted in black (neutral), and numeric scales ranging from 7-10 (agree) are depicted in white.

Table 2.2 Sensitivity scores of clinical parameters for the suspicion of anastomotic leakage in the direct postoperative period in China and The Netherlands.

Clinical Parameter	China		The Netherlands		P-value
	Score	SD	Score	SD	
Increased CRP	4.35	±2.466	7.45	±1.871	<0.001
Leukocytosis	5.96	±2.596	6.53	±1.824	0.095
Tachycardia	4.55	±2.411	7.13	±1.937	<0.001
Tachypnea	4.46	±2.244	7.13	±1.937	<0.001
Febrile temperature	6.23	±2.281	5.86	±1.963	0.207
Postoperative ileus	4.47	±2.363	5.76	±1.679	<0.001
Clinical deterioration	6.67	±2.033	7.83	±1.205	<0.001
Abdominal pain	6.61	±2.247	6.74	±1.835	0.659

Table 2.3 Surgeons' opinion regarding the value of radio diagnostics in the diagnosis of colorectal anastomotic leakage.

	China (%)	The Netherlands (%)	P-value
In how many percent of patients with clinical suspicion of anastomotic leakage do you perform radio diagnostics?			
Responders, no. (%)	202 (100%)	55 (93%)	
0-20%	3.0	0	
21-40%	6.4	0	
41-60%	6.9	1.8	
61-80%	24.3	16.4	
81-100%	59.4	81.8	
Average	83.3	91.5	0.285
In how many percent of patients with clinical suspicion of anastomotic leakage do radio diagnostics change your treatment policy?			
Responders, no. (%)	202 (100%)	54 (91.5%)	
0-20%	10.9	13.0	
21-40%	9.9	5.6	
41-60%	27.7	44.4	
61-80%	30.2	25.9	
81-100%	26.7	11.1	
Average	63.6	55.9	0.028
In how many cases did the CT scan report no anastomotic leakage while there finally was an anastomotic leakage?			
Responders, no. (%)	202 (100%)	52 (88.1%)	
0-20%	40.6	51.9	
21-40%	29.2	28.8	
41-60%	25.2	15.4	
61-80%	4.0	1.9	
81-100%	1.0	1.9	
Average	31.8	28.7	0.221
In how many percent of cases do you consider a reoperation without previous radio diagnostics?			
Responders, no (%)	202 (100%)	53 (89.8)	
0-20%	58.4	84.9	
21-40%	18.8	13.2	
41-60%	17.3	0	
61-80%	4.5	0	
81-100%	1.0	1.9	
Average	25.4	13.6	<0.001

Discussion

Despite extensive research in the field of colorectal anastomotic leakage, no international consensus regarding a practical definition exists, which limits transparency and comparison of study outcomes. Several definitions of CAL have been proposed during the last decades^{18,23}, but review of the literature shows that newly published papers fail to adopt these definitions²⁴. Instead, authors seem to prefer to use their own

definitions or no definition at all²⁴. It could be postulated that these previously proposed definitions were not yet implemented in clinical practice and (retrospective) research because of limited awareness of the existence of such a definition and/or lack of support from a large expert group.

Reports from Asian studies show CAL rates that are substantially lower than those reported by Western research groups²⁵. This could partially be explained by demographic differences that exist in patient population, availability and use of diagnostic tools, or how perioperative care is structured. Another explanation could be that Chinese surgeons only report a leak as such in case a reintervention is required.

On the other hand, despite the lower prevalence of obesity in Asian countries, rates of diabetes mellitus type II and metabolic syndrome are relatively high due to ethnic and genetic factors. Indeed, Asians account for 60% of diabetes mellitus cases worldwide²⁶, which is considered an important risk factor for CAL^{27,28}. Furthermore, the most common location of colorectal tumors in the Asian population is the left hemicolon^{16,29}, compared to the Western population, in which the predominant side is the right^{29,30}. Literature shows a significantly higher CAL risk for surgeries on colorectal tumors located in the left hemicolon³¹. These regional differences therefore fail to completely explain the variation in reported CAL rates. It is very likely that important regional differences exist as to what is considered an anastomotic leak, i.e. Asian surgeons may report a leak mostly when a reintervention is required, while the Western surgeons may report latent leaks. In order to gain more insight in these differences in views, the present survey was conducted both in China and in the Netherlands, countries that are considered to be representative for their continent.

In the first part of the survey, surgeons were asked whether different statements including clinical and radiological signs and interventions regarding CAL should be considered anastomotic leakage. Of ten statements, only one was deemed as CAL by more than 80% of respondents in both countries: 'Extravasation of contrast after rectal enema visible on CT-scan'. This is generally considered a radiological hallmark sign for anastomotic leakage after left sided colorectal surgery and should naturally be included in a general definition. Moreover, other important and evident CAL signs including "Radiological collection around the anastomosis treated with percutaneous drainage" and "Necrosis of the anastomosis seen at reoperation" received more than 80% positive responses in the Netherlands, however, not in China, and thus were not considered as consent according to the predetermined criteria. Despite this, the majority of the Chinese surgeons also agreed on these items and their answers did not differ

significantly from those of their Dutch colleagues. In conclusion, it seems that for the evident signs of CAL, the majority of surgeons from both countries have quite similar views.

“Radiological collection treated conservatively” is only considered as CAL in 21% of the Chinese surgeons (versus 39% in the Netherlands), which is almost a consensus of NOT diagnosing CAL. On the contrary, it is at least remarkable that in current grading systems, a radiological collection is considered anastomotic leakage. As such this is reported as a Grade-A CAL according to the International Study group of Rectal Cancer (ISREC)¹⁸ and Grade I-II CAL according the Clavien-Dindo Scale.

Despite the fact that only a minority of Dutch surgeons consider conservatively treated radiological collections as CAL, these numbers are higher than the Chinese. These differences in views regarding the subclinical signs of CAL may eventually lead to a significantly higher reported CAL rate in the Dutch studies than the Chinese ones. However, considering the fact that more than 30%³² of the CAL do not require invasive intervention, the treatment provided by surgeons from both countries may eventually be similar, i.e. leading to a similar intervention rate for the complication. To rule out the reporting difference in this regard, one solution is to report complications with a Clavien-Dindo score higher than IIIa, which actually is also commonly accepted and applied in recent studies.

The second part of the survey focused mainly on clinical markers and parameters for CAL. Early clinical diagnosis of CAL remains a challenge for surgeons worldwide. Many clinical symptoms and biomarkers have been suggested as early signs of CAL³³⁻³⁶. However, previous studies of these parameters have shown that almost none of these parameters yield sufficient diagnostic accuracy that allows a confirmative diagnosis³⁷. This explains our findings that most surgeons do not rely their diagnosis of CAL based on these parameters, which results in a relatively low score of their contribution to the suspicion of CAL. Surgeons from both countries deemed ‘deterioration of clinical condition’ as an important symptom of CAL, which further accentuates the complexity of CAL diagnosis based on its clinical manifestations. We believe the surgeons’ opinions indeed reflect the unsatisfactory status of CAL diagnosis, which stresses the need for further research in this field³⁸. However, important differences exist between the two countries. Although surgeons from both countries agreed about the predictive value of higher temperature, abdominal pain other than wound pain and increased leukocytes, more than half of the clinical parameters scored significantly lower in China than the Netherlands. Although these abnormal clinical manifestations are indeed very common

after gastrointestinal surgery³⁹, it seems they are considered less suggestive by the Chinese surgeons.

The third part of the survey focused on radiological tools used in the diagnosis of CAL. Based on the present data, the majority of the surgeons in both countries would perform radiological examination on patients in whom CAL was suspected (these numbers are slightly higher in the Netherlands), and more than half of the treatment plans would be changed after the imaging. In this regard, although differences have been found in the views of Chinese and Dutch surgeons regarding the definition of CAL, the treatment they provide is similar. However, our data also show that surgeons from both countries do not blindly rely on the results from radio diagnostics. Instead, they state that in approximately 30% of the cases in which CAL is suspected, CAL is eventually diagnosed even though the radiological report is negative. This correlates with previously reported false negative rates of CT scans⁴⁰. Experience with inaccurate CT scan reports may be a reason for surgeons to consider reoperation without affirmative CT results, which according to the data, is being done in about 25% of cases.

Further research and education may facilitate the achievement of international consensus. However, definition without considerations of the practical issues in different regions is unlikely to gain sufficient popularity. In 2010, The international Study Group of Rectal Cancer (ISREC) proposed a graded system for the diagnosis and treatment of CAL¹⁸. Grade A CAL refers to anastomotic leakage for which no active therapeutic intervention is required. It seems that this grade correlates with the second statement “Radiological collection surrounding the anastomosis treated conservatively” that, according to the majority of both Dutch and Chinese surgeons is not classified as CAL. This discrepancy between an established definition and the views of colorectal surgeons could partly explain why the ISREC definition has not been adopted in practice and science. In accordance with that, our survey clearly demonstrates how different practice may influence surgeons’ opinion.

For example, in the Netherlands the Enhanced Recovery After Surgery (ERAS) program has been widely adapted for over years, and recommends no abdominal drainage after surgery. In China, on the contrary, ERAS is less commonly implemented, while intra-abdominal drainage is often left in situ for longer period after surgery. Moreover, CT imaging is less commonly used for radio-diagnostics of CAL, and laboratory analysis by means of CRP is not yet included into a routine practice in many rural areas. This could explain why increased CRP was deemed least contributory in the diagnostic process in

the present survey. These points, though small, significantly influenced the results, and would certainly influence the applicability of a proposed CAL definition.

To embed a successful definition in clinical practice, research on CAL would greatly benefit from establishing a uniform definition and recording in national databases. We will therefore continue to perform an extensive and systematic literature review. The results from that review and the consensus assessment described in this paper will lead to an international Delphi analysis, that will allow us to reach consensus on a new definition proposal that will be supported by a large panel of experts. We sincerely welcome others to participate in this further research, in order to formulate a new definition based on joint experience and opinions.

The most important limitations of the study are the following. The content of questionnaires is always susceptible to researcher imposition and there may be a level of subjectivity in the answers given. Furthermore, the relatively low numbers of respondents from both countries will have a negative influence of the generalizability of study results. Finally, the original questionnaire was constructed in English and translated into Dutch and Chinese, which could introduce bias and weaken the validity of comparisons between the countries. Finally, as some of the clinical parameters used in the questionnaire were derived from the DULK-score, which was constructed and validated in the Netherlands, it is plausible that the Dutch participants scored similarly on these items because they were familiar with the content of the DULK-score, or because they have been (in)directly involved in the construction of the scoring system. However, the use of the DULK-score has not remained limited to the Netherlands, and it is unknown whether the subset of Dutch surgeons familiar with the DULK-score is higher than the number of Chinese surgeons who use this score routinely, and whether this difference is large enough to alter the data significantly.

Conclusion

No international consensus of a practical definition of CAL is yet available, which limits transparency and comparison of published results. The present international online survey proves the inconsistent views as to what is considered CAL among surgeons in the Netherlands and China, and shows large differences between countries. Dutch surgeons are more likely to report ‘subclinical’ leaks as CAL, which partly explains the higher reported Dutch CAL rates. Surgeons from both countries rely on radiological diagnostics and laboratory parameters in the decision-making process, but are well

aware of the limitations of these diagnostic aids. A Delphi analysis within a representative panel of colorectal surgeons is desired to develop a widely accepted definition of colorectal anastomotic leakage.

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Chapter 3

Use of colorectal anastomotic leakage definitions in
literature: results of a systematic review and
recommendations for future reporting

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Submitted

Abstract

Background

Anastomotic leakage is the most feared complication following colorectal surgery, but a common definition is still lacking. This complicates comparison of study outcome.

Objective

The study aimed to gain insight in the use of different definitions in the literature and how this affects leakage rates. The study further focuses on availability of data in the included papers.

Data sources

A systematic literature search was performed in Medline and Embase databases.

Study selection

All retrospective and prospective papers discussing colorectal surgery with the construction of an anastomosis, published between January 1990 and January 2016, were reviewed for the presence of a definition of CAL and/or details of clinical or radiological assessment.

Main Outcome Measures

A total of 361 manuscripts containing a definition on colorectal anastomotic leakage were used. Definitions were clustered based on the description of clinical and radiological signs and the consequences of a leak.

Results

A vast number (73,9%) of papers published on this subject did not include a definition of colorectal anastomotic leakage. Leakage rates vary strongly between rectal and colon anastomoses (7,48 and 5,32%, respectively) and with the type of definition used. Nineteen percent of authors used a grading system to illustrate the severity of the anastomotic leaks. Reporting of data regarding the operative procedure and the postoperative period varied substantially between studies.

Limitations

The most important limitation of this study is the heterogeneity between the included studies and the varying presentation of data, which makes comparing of data rather difficult.

Conclusion

The present review shows the lack of a commonly used definition of colorectal anastomotic leakage. This systematic review provides insight in the different components of definitions that might serve as the basis of a Delphi-based proposal for a novel definition. The authors believe that implementation of a graded system with treatment recommendations will enhance uniformity of patient care and may lead to decreased treatment delay.

Introduction

Colorectal anastomotic leakage (CAL) remains the most feared complication after colorectal surgery with a reported incidence of 1.5-23%^{1,2}. CAL substantially adds to the morbidity and mortality rates and thereby decreases patients' health-related quality of life (HRQoL)³⁻⁷. Compared to a normal postoperative course, the high number of reoperations and the associated increased length of hospital stay lead to a fivefold increase in expenditures on health care⁸. Furthermore, evidence arises from recent literature that CAL impacts the oncological prognosis, leading to an increased local recurrence and a decreased disease-specific survival^{9,10}. Despite the large number of experimental and clinical studies conducted on this subject, and although CAL is commonly used as an outcome measure in these reports, no consistent definition of CAL is applied in literature¹¹.

General definitions such as 'a defect of the intestinal wall at the anastomotic site leading to a communication between the intra and extra luminal compartments'¹² or 'the leak of luminal content from a surgical joining between 2 hollow viscera'¹³ can be applied in case the leak is evident. However, this definition is more difficult to apply when a patient presents with atypical symptoms, which is often the case¹⁴. Due to the lack of a generally accepted definition of CAL, reported leakage rates vary significantly, which complicates comparison of study outcomes and comparison of quality of care in individual institutions. The use of (inter)national registration systems could potentially yield very large patient populations for database studies, and would therefore positively influence the impact of the presented results. However, the application of these registration systems for scientific research is also limited because of the aforementioned issues. Reinke et al. evaluated the ability of two different registration systems, an administrative and a clinical registry, to identify anastomotic leaks, and found that both types of databases had acceptable specificity but poor sensitivity for diagnosis of CAL¹⁵. There is an obvious need for an internationally accepted definition for both colon and rectal anastomotic leakage that will serve as a template for future research and to improve clinical decision-making.

Several definitions have been proposed in the past decades^{11,16}. However, none of the suggested definitions functions as leading in current literature. We believe that this lack of acceptance is mainly due to the lack of an expert panel supporting the construction of these definitions. Our research team performed a consensus assessment among Dutch and Chinese colorectal surgeons and concluded there was no national or international consensus on what should comprise a general definition of CAL¹⁷. The aim of the present

systematic review is therefore to gain insight in the use of different components of definitions in the literature, and how this effects reported leakage rates. Furthermore, the review focuses on the availability of data in the included papers, as this is also a large contributing factor as to why it is difficult to compare results from different studies. The findings of this study will eventually serve as the basis of a future Delphi-based proposal for a novel and widely accepted definition of CAL.

Materials and methods

An extensive systematic literature search in the biomedical databases of Medline and Embase was performed in January 2016 in accordance with the guidelines as presented in the PRISMA statement. All retrospective and prospective papers discussing colorectal surgery with the construction of an anastomosis, published between January 1990 and January 2016, in which anastomotic leakage was a primary or secondary outcome measure, were reviewed for the presence of a definition of CAL and/or details of clinical or radiological assessment. No restrictions were placed on the type of surgery or the indication of surgical resection (i.e., malignant or benign disease). The following papers were excluded from review: reviews, meta-analyses, articles discussing upper GI-surgery and animal studies.

The search that was used was as follows: Search (((((((("Anastomotic Leak"[Mesh]) OR colorectal anastomotic leakage) OR colorectal anastomotic leak) OR anastomotic dehiscence colorectal) OR anastomotic leakage) OR anastomotic leak) OR anastomotic dehiscence)) AND (((rectum) OR colon) OR colorectal).

Definitions that were found were assessed for the presence of clinical and radiological diagnostic methods, grading systems and the clinical usability of the definition. The definitions were clustered into 11 categories based on the description of clinical and radiological signs and the consequences of a leak (i.e. whether radiological or surgical intervention was required) as part of the definition, in order to clarify the differences between the identified definitions. The categories listed in Table 3.1 can be appreciated to increase in completeness, varying from, category 1, which consists of reports that only include clinical symptoms in their definition of CAL, to category 8, which includes all possible aspects of a presentation of CAL. The papers included in category 9 only deemed the CAL that was observed during reoperation to be proven, papers referring to the ISREC classification were clustered in category 10, whereas category 11 contains definitions that did not fit any of the aforementioned descriptions.

Additional information was extracted from the included papers, regarding the construction of the anastomosis (e.g. type of surgery, height of the anastomosis,

presence of deviating ostomy), timing and method of diagnosis of CAL, treatment and outcome after diagnosis of CAL, as well as signs and symptoms that were described in the diagnosis of CAL (fever, abdominal pain, ileus, fecal discharge from drain or wound, abnormal laboratory results etcetera).

Two researchers (AJ and SvR) conducted the selection process, and a third researcher (VS) was consulted in case of disagreement on the inclusion or exclusion of papers. A complete list of the included papers in this review can be found in the Supplementary Table S3.1. A two-sample test for proportions was used to calculate the difference in CAL rates between colon and rectal anastomoses.

Results

A total of 2938 abstracts and 1382 full-text articles were reviewed for the presence of a definition of colorectal anastomotic leakage and/or a description of clinical and radiological signs used for the diagnosis of CAL. 1021 papers (73,9% of 1382) were excluded from analysis as they did not contain a definition of CAL. 361 papers met the inclusion criteria and were further used for data analysis. A total of 344 papers were used for the calculation of reported incidence rates, after the exclusion of papers using the same study population, protocol papers and manuscripts using matched cohorts. See Figure 3.1 for the flow diagram of the inclusion process. Figure 3.2 renders all included peer reviewed papers published between 1990 and 2015 on colorectal anastomotic leakage with and without the description of a definition. It shows that although CAL was a primary or secondary outcome in these studies, a vast number of publications did not include a definition for CAL. A total of 30 randomized controlled trials, 165 prospective studies and 152 retrospective studies were included. The following data were extracted from the included articles: study type, surgical procedure or intervention, definition of CAL, reported leakage rates for colon and/or rectal anastomoses, median days postoperative of diagnosis of CAL, means of assessment of anastomotic integrity during surgery, number and percentages of deviating ostomies, and finally the description of clinical symptoms associated with CAL (e.g. fever, abdominal pain, leukocytosis). In order to clarify the differences between the identified definitions, they were then subclassified into 11 categories, based on combinations of the presence or absence of several components of the definition: clinical symptoms, radiological signs and/or findings during reintervention. See Table 3.1 for examples of the categorized definitions and the mean reported incidence rates of CAL associated with their classification. It can clearly be appreciated that these leakage rates vary strongly with the type of definition used for the diagnosis of CAL (leakage rates for rectal

anastomoses varying between 2.4 and 12.3% for categories 9 and 11, respectively, and colonic leakage rates between 4.4 and 6.6% for categories 1 and 4, respectively).

Figure 3.1 PRISMA flow diagram of selection process.

Signs and symptoms

A total of 238 papers (65.9%) reported clinical signs and symptoms associated with colorectal anastomotic leakage, either as part of the formulated definition, or in the description of the method of diagnosis. The most commonly described clinical signs and symptoms were as follows: purulent or fecal discharge from drain, peritonitis, fever, purulent discharge from the rectum and abdominal pain (Table 3.2).

Figure 3.2 Papers published on the subject of colorectal anastomotic leakage with and without the formulation of a definition.

Grading systems

A small proportion of included papers (69 out of 361 papers, 19%) explicitly stated the use of a grading system when formulating a definition (Table 3.3). The authors of 15 papers referred to the International Study group of Rectal Cancer (ISREC)-classification¹⁸⁻³⁰, whereas 11 authors used the Clavien-Dindo classification³¹⁻⁴¹. The grading systems used in literature were further clarified in Table 3.3, and mainly divided the clinical cases based on clinical presentation, means of diagnosis or the resulting management of the complication.

Colon versus rectum

A comparison was made between leakage rates of rectal versus colon anastomoses, which showed that the rectal anastomoses were at significantly higher risk of developing a leak (7.48% vs. 5.32%, Odds Ratio: 0.71 (95%CI: 0.693-0,736), $Z=-21.72$, $P\text{-value}<0.001$). Only papers providing separate numbers for colonic and/or rectal anastomotic leakage were taken into account for this analysis ($n=84$ for colonic CAL, $n=223$ for rectal CAL). Comparison of the reported rates of CAL in both anatomical regions further showed that the incidence rates have remained stable over the past decades (Figure 3.3).

Table 3.1 Classification of definitions of colorectal anastomotic leakage (CAL); examples and % CAL per classification.

Classification (n) papers	Descriptions	% CAL mean (range)
1. Clinical diagnosis (n=23)	The definition of anastomotic leakage was clinical and included: peritonitis caused by leakage, discharge of feces from the abdominal drain, rectovaginal fistula, leakage from all staple lines, pelvic abscess without radiologically proven leakage mechanism. ⁶⁷ Anastomotic leakage was defined clinically by peritonitis resulting from the leakage, with signs of acute abdomen, anal bleeding, or suspicious quality of drainage. ⁶⁸	C= 4.4 (0 – 7.6%) R= 8.1 (2.3 – 33)
2. Imaging diagnosis (n=11)	Colorectal anastomotic leakage was defined as a radiographically confirmed extravasation of contrast at the anastomosis. ⁶⁹ An anastomotic leak was verified either by a radiographic enema performed with computed tomography (CT) scan (standard for the inpatient setting), X-ray, or by endoscopy. ⁷⁰	C=N.A. R=6.4 (5.7 – 12.5)
3. Requiring reintervention (n=34)	Anastomotic leakage was further defined, in line with recent proposals, as a defect of the intestinal wall at the site of the anastomosis requiring surgical or radiological intervention. ³¹ Anastomotic leak was defined as minor leak requiring percutaneous intervention or major leak requiring laparotomy. ⁷¹	C=5.4 (0.7 – 7.1) R=6.6 (1.2 – 17.6)
4. Theoretical (n=20)	Anastomotic leakage defined as a defect in the intestinal wall at the anastomotic site, which led to a linkage between intra- and extraluminal compartments. ⁷² Anastomotic leakage was defined as the leak of luminal content from a surgical join between two hollow viscera. ⁷³	C=6.6 (2.3 -10) R=8.2 (0 – 21.6)
5. Clinical suspicion confirmed by imaging and/or reoperation (n=71)	Anastomotic leakage was suspected due to fecal discharge from wound or drain, fever, pelvic abscess, local peritonitis, discharge of pus per anus, a CT scan was performed to confirm diagnosis. ⁷⁴ Anastomotic leakage was defined as the presence of clinical symptoms such as fever or septicemia combined with the occurrence of pelvic abscess, presence of rectal pus discharge, formation of a rectovaginal fistula, or presence of peritonitis within 30 days after surgery, leading to a clinical and/or radiological examination or repeat surgery to confirm the diagnosis. ⁷⁵	C=6.1 (1.8 – 13.2) R=10.1 (0.6 – 30.4)
6. Clinical and imaging diagnosis (n=96)	To define anastomotic leak both the clinical signs, symptoms (signs of localized or generalized peritonitis, fecal discharge from the wound and/or drain) and radiologic investigations are used ⁷⁶ The definition of anastomotic leakage included any clinical or radiologic evidence of anastomotic dehiscence (such as discharge of intestinal content through a drain), regardless of whether reoperation or any other intervention was required. Additional tests such as chest and abdominal computed tomography were performed to assess leakage-related complications. ⁷⁷	C=5.6 (2.3 – 7.0) R=8.2 (1.5 – 22.5)

Table 3.1 (continued)

Classification (n) papers	Descriptions	% CAL mean (range)
7. Imaging diagnosis or at reoperation (n=24)	A leak was defined as a breakdown of a colonic anastomosis associated with an intra-abdominal collection identified either by contrast radiographs or by the surgeon at the time of a subsequent operation. ⁷⁸ In case of imaging, leakage was considered to be present when free air or contrast was visible around the anastomosis. In case of relaparotomy, leakage was considered to be present when a dehiscence anastomosis was visualized. ⁷⁹	C=4.6 (0 -21) R=9.0 (5.2 – 21.1)
8. Clinical and/or imaging and/or at reoperation (n=64)	Anastomotic leakage was defined by at least one of the following criteria: 1) an anastomotic defect noted on physical examination, 2) an anastomotic defect confirmed in the operating room, 3) an anastomotic defect seen on proctoscopy, 4) radiologic evidence of a leak consisting of either a defect in the anastomosis and an adjacent fluid collection, or stranding or the extravasation of rectal contrast into the extraluminal space, or 5) clinical evidence of a leak such as feculent output from a pelvic drain. ⁸⁰ Clinical leak was defined as an anastomotic dehiscence verified by any of the following: imaging [computed tomography (CT) or barium enema], change in drainage material, operative intervention showing drainage, endoscopic evidence of anastomotic dehiscence, or signs of sepsis. ³²	C=5.4 (1.7 – 20.3) R=8.7 (2.2 – 28.5)
9. Diagnosis only at reoperation (n=3)	Anastomotic leakage was defined as the presence of bowel content leaking from a defect in the anastomosis, identified at repeat operation. ⁸¹ Patients with operatively confirmed anastomotic leak. ^[82]	C=N.A. R=2.4 (2.4- 2.4)
10. ISREC (n=8)	Leakages were classified into three groups: A) CAL requiring no active therapeutic intervention B) AL requiring active therapeutic intervention but manageable without re-laparotomy C) CAL requiring re-laparotomy. Patients presented symptoms and signs of leakage such as sepsis, fever, elevated white cell count and C-reactive protein, perineal or pelvic pain, localized or generalized peritonitis and discharge of blood or puss per rectum. CAL was diagnosed by computed tomography (CT) with intravenous contrast. ²⁵	C=N.A. R=7.8 (5.6 – 17.7)
11. Other (n=7)	Anastomotic leaks were categorized as either major clinical leaks with signs of generalized peritonitis requiring emergency abdominal reoperation or minor leaks diagnosed on clinical signs, generally confirmed radiologically, and managed expectantly without abdominal reoperation. ⁸³	C=N.A. R=12.3 (7.5–13.9)

C=Colon, R=Rectum, N.A.=Not applicable. * % leakage calculated with pooled means from all papers included in specific category. The range of the reported incidence of the different articles included in a specific category is given.

Table 3.2 Symptoms associated with colorectal anastomotic leakage as reported in definition or means of diagnosis.

Symptom	Reported in papers: n (%)
Peritonitis	115 (48.3)
Discharge from drain	115 (48.3)
Fever	97 (40.8)
Discharge from rectum	68 (28.6)
Rectovaginal fistula	68 (28.6)
Abdominal pain	66 (27.7)
Dehiscence identified upon palpation	59 (24.8)
Leukocytosis	55 (23.1)
Sepsis	43 (18.1)
Tachycardia	26 (10.9)
Tachypnea	19 (8.0)
Discharge from wound	18 (7.6)
Increased C-reactive protein (CRP)	14 (5.9)
Ileus	13 (5.5)

Table 3.3 Use of various grading systems for the severity of colorectal anastomotic leakage.

Reference	Grading terms	Description
Midura et al. 2015 ⁷¹ Snijders et al. 2015 ⁸⁴ Garcia et al. 2013 ⁸⁵	Minor / Mild	Can be treated conservatively or by radiologic intervention
Van 't Sant et al. 2012 ⁸⁶ Frye et al. 2009 ⁸³ O'Grady et al. 2007 ⁸⁷ Oguz et al. 2007 ⁸⁸ Fa-Si-Oen et al. 2005 ⁸⁹ Raveenthiran et al. 2004 ⁹⁰ Eckmann et al. 2004 ⁹¹ Kanellos et al. 2004 ^{92,93} Marusch et al. 2002 ⁹⁴ Galandiuk et al. 1991 ⁹⁵ Tuson et al. 1990 ⁹⁶	Major / Severe	Requires surgical reintervention
Vermeer et al. 2014 ²²	Presacral abscess	Abscess on the anterior side of the of the sacrum without extravasation of enteral contrast on CT-scan or signs of generalized peritonitis due to CAL
	Anastomotic leakage	Extravasation of enteral contrast on CT-scan, the presence of a PA in combination with a defect in the anastomosis on palpation or the presence of fecal peritonitis when performing a laparotomy.
Hua et al. 2014 ⁹⁷ Walker et al. 2004 ⁹⁸	Minor / Local	Can be treated conservatively or by radiologic intervention
	Major / General	Requires surgical reintervention
	No clinical / Radiological	Diagnosed radiologically, without clinical symptoms

Table 3.3 (continued)

Reference	Grading terms	Description
Ashraf et al. 2013 ⁹⁹	i peritoneal	Requires laparotomy, washout and formation of stoma
	ii contained pelvic	Managed with EUA with pelvic washout, with or without VAC endosponge therapy
	iii localized perianastomotic	Managed conservatively by percutaneous radiological drainage and/or antibiotics
Shiomi et al. 2015 ¹⁸	ISREC: Grade A	No change in patients' management
Yun et al. 2015 ¹⁹		
Dauser et al. 2014 ²⁰		
Placer et al. 2014 ²¹	Grade B	Requires active therapeutic intervention but is manageable without re-laparotomy
Reilly et al. 2014 ²⁹		
Kawada et al. 2014 ³⁰		
Krarup et al. 2014 ¹⁰⁰		
Vermeer et al. 2014 ²²	Grade C	Requires re-laparotomy.
Boesen et al. 2013 ²³		
Kulu et al. 2013 ²⁴		
Morks et al. 2013 ⁵⁵		
Nerup et al. 2013 ²⁵		
Saber et al. 2013 ²⁸		
Bakker et al. 2012 ²⁶		
Penninckx et al. 2012 ²⁷		
Garcia-Granero et al. 2013 ⁸⁵	Minor	Conservative medical treatment, Clavien-Dindo grades I and II
Asoglu et al. 2013 ³⁴	Major	Need of reoperation or percutaneous radiological drainage, Clavien-Dindo grades III to V
Mongin et al. 2014 ¹⁰¹	Symptomatic / Clinical	The presence of peritonitis, fever, or when gas, pus, or feces were discharged from the abdominal drain or from the vagina.
Ogilvie et al. 2012 ¹⁰²		
Piessen et al. 2012 ⁴¹		
Fong et al. 2011 ¹⁰³		
Lim et al. 2006 ¹⁰⁴		
Millan et al. 2006 ¹⁰⁵		
Rudinskaite et al. 2005 ¹⁰⁶	Asymptomatic / Radiological / Occult	Considered if CAL was assessed on the systematic CT scan with contrast enema performed before diverting stoma reversal, without any relevant clinical symptom
Bell et al. 2003 ¹⁰		
Dehni et al. 1998 ¹⁰⁷		
Petersen et al. 1998 ¹⁰⁸		
Moore et al. 1996 ¹⁰⁹		
Bokey et al. 1995 ¹¹⁰		
Bugiantella et al. 2014 ¹¹¹		
Leroy et al. 2004 ¹¹²		
Brown et al. 2001 ¹¹³		
Merad et al. 1998 ¹¹⁴		
Akyol et al. 1992 ¹¹⁵		

Table 3.3 (continued)

Reference	Grading terms	Description
Hellan et al. 2015 ³² Richards et al. 2015 ³¹ Yamaguchi et al. 2015 ³³ Asoglu et al. 2013 ³⁴ Nakajima et al. 2013 ³⁶ Nakajima et al. 2012 ³⁵ Gorissen et al. 2012 ³⁷ Lagoutte et al. 2012 ³⁸ Manceau et al. 2012 ³⁹ Piessen et al. 2012 ⁴¹ Park et al. 2011 ⁴⁰	Clavien Dindo Classification	Grade I: Any deviation from the normal postoperative course without the need for pharmacological treatment or surgical, endoscopic and radiological interventions Grade II: requiring pharmacological treatment Grade III: Requiring surgical, endoscopic or radiological intervention Grade IV: life-threatening complication Grade V: Death of a patient
Platell et al. 2005 ¹¹⁶	i ii iii	No treatment Antibiotics Drainage
Killingback et al. 2002 ¹¹⁷	iv Clinical grade I Clinical grade II	Reoperation Requiring reoperation Not requiring reoperation
Ogilvie et al. 2012 ¹⁰²	Subclinical grade III Small Medium	No clinical consequences 5 mm or less 6-19 mm
Smith et al. 2007 ¹¹⁸	Large Intraoperative Postoperative	20 mm or larger Visualization of methylene blue dye outside of the colon (in the operative field) Defined clinically as a constellation of fever, abdominal pain/peritonitis, and leukocytosis, radiographically, or by the institution of treatment indicating that the patient had a leak (such as the creation of a diverting ostomy)
Lim et al. 2015 ¹¹⁹	Type I Type II Fistula or chronic sinus	Generalized peritonitis Localized peritonitis with or without abscess Any type of fistula-associated leakage or chronic sinus formation

Table 3.3 (continued)

Reference	Grading terms	Description
Caulfield et al. 2013 ¹²⁰	Gross	defined as a disruption of the anastomosis identified at reoperation or extravasation of contrast medium at the anastomotic site on an imaging study, irrespective of the presence of symptoms.
	Occult	An abscess or free pelvic fluid collection without extravasation of contrast medium was considered an occult anastomotic leak

Figure 3.3 Reported mean incidence rates for colorectal anastomotic leakage for colon and rectal anastomoses over the past 25 years.

Uniformity in data reporting

Information that had been taken into account in this review also included specific information regarding the operative procedure and the period directly following surgery. The median time to diagnosis of CAL was only sparsely described in the investigated papers: a total of 56 papers (15.5%) reported a mean or median interval to CAL diagnosis, ranging from 3.5 to 43 days. Furthermore, standard testing of the integrity of the primary anastomosis was underreported in most papers: only 71 authors (20%) reported data on standard intraoperative testing with e.g. insufflated air or water, contrast enema, donut assessment, palpation of the anastomosis or flexible sigmoidoscopy. The information regarding the construction of a deviating ostomy was described much better in the included papers: a total of 241 papers (66.8%) reported on the number of cases in which a deviating ostomy was created during the index surgery,

however, hardly any papers reported whether these consisted of colostomies or ileostomies.

Discussion

The present literature review shows that there is no commonly used definition of both colon and rectal anastomotic leakage available. The numbers of papers published on this subject have increased tremendously over the past years. Surprisingly, our study showed that more than half of these papers do not report on what is considered CAL, despite the fact that CAL was their primary outcome measure. Another important finding of this study was the large variety of definitions used, which also impacted reported CAL rates between categorized definitions. This systematic review provides insight in the different components of definitions used that might serve as the basis of a future Delphi-based proposal for a novel definition that will be used by clinicians and researchers worldwide.

Several definitions of CAL have been proposed in the past, ranging from a theoretical to an extensive clinical description, and definitions including grading systems to differentiate between stages of clinical severity and their impact on treatment^{42,43}. The Dutch Surgical Colorectal Audit, a registration database for colorectal surgery patients, described CAL as a leakage of bowel content or abscess formation near the anastomosis for which a relaparotomy and/or radiologic drainage was conducted⁴⁴. Descriptions in all reviewed papers are in some way similar and mostly describe the general entity of CAL. However, as the clinical presentation of this specific complication can differ significantly with regards to severity, such a general definition of CAL is limited in its clinical usability and comparison of data.

The international study group of rectal cancer (ISREC) introduced a grading system in which the clinical impact of rectal anastomotic leakage is included^{42,45,46}. Grade A severity indicates no change in patient management, whereas grade B requires active therapeutic interventions but is manageable without relaparotomy. Grade C leakage has the largest clinical impact and requires relaparotomy. The most commonly used other grading systems in this systematic review based their severity grading either on clinical presentation, means of diagnosis or the resulting management of the complication. Grading the severity of CAL is of importance as it may give an indication of the required intervention, prognosis and consequences for the individual patient. A confined anastomotic leak with mild clinical symptoms may be treated conservatively or with oral antibiotics alone, while an abscess surrounding the anastomosis may be drained

radiologically. Although the ISREC definition and grading system is rather complete and has been used by a number of authors, it has not yet been fully implemented in daily clinical practice and research. Some authors believe that by the use of this definition, no distinction can be made between anastomotic leakage and a presacral abscess, which is not necessarily an indication for a leak, as it could be formed due to peroperative spill or an infected hematoma^{22,47}. The fact that others did indeed classify presacral abscess formation as indicative for a leak^{48,49}, clearly shows that several issues need to be resolved in order to come to complete agreement regarding a new definition for CAL.

With the formation of this new definition, we may even discuss whether CAL is the right description and if it were not more appropriate to focus on the period of intestinal healing directly following surgery instead. An impaired healing process may result in clinical consequences such as a prolonged hospital stay, administration of antibiotics, radiological drainage of an abscess, a reoperation including a prolonged hospital stay or even intensive care stay, and in worst case scenario long lasting sepsis with substantial additional consequences for the patient (e.g. significant duration of hospitalization, delayed return to functional capacity and work or additional treatment [chemotherapy/surgery for metastasis], and/or decreased health related quality of life), or even death. We may consider identifying and modifying all risk factors that are potentially related to the healing process. These modifiable risk factors may be patient related and present preoperatively⁵⁰, or may be part of the intraoperative approach such as vascularization, technique of anastomosis construction, blood loss and use of inotropes⁵¹⁻⁵³. We may consider *impaired healing* as part of a future definition but should also include the effects that these outcomes have on our patients. From the patient's point of view, prolonged hospital stay or even a re-intervention could be seen as being more important than the burden of living with a temporary or permanent stoma. For another patient, however, the impact on quality of life that a stoma has may not outweigh the risk of developing CAL and its associated clinical consequences. We would therefore advise to include information regarding the presence and type of a deviating ostomy in future reports on CAL.

Before we are able to formulate a definition for colorectal anastomotic leakage that will be accepted by both clinicians and research groups worldwide, several issues need to be addressed. First, the terminology of a leak varies widely as shown in this review (See Table 3.1), which makes interpretation of study outcomes rather difficult. Secondly, timing of CAL occurrence has also been described as part of the definition. This present study shows that there is a wide range within the postoperative course to diagnose CAL. It is unclear whether the duration of follow-up taken into account in the different papers

has had a significant influence on this range, as a considerable number of CAL cases are diagnosed after the direct postoperative period of 30 days^{54,55}. In fact, when also taking into account cases of CAL that are diagnosed after discharge and/or more than 30 days after low anterior resection, incidence rates show an up to 40% increase^{54,56,55}. Interestingly, some studies found that the construction of a deviating ostomy reduced the numbers of early anastomotic leak. This was, however, not the case for the late anastomotic leaks suggesting that in the presence of a temporary ostomy CAL diagnosis is delayed^{56,54}. Some authors may consider early and late anastomotic leakage as separate entities, and may therefore underreport late anastomotic leaks as such⁵⁷. It was indeed shown that the clinical course differs considerably between early and late CAL, with early CAL requiring more often a relaparotomy and the construction of a permanent ileostomy. Finally, preoperative radiotherapy increases the risk of late, but not early CAL^{55,58,57}.

Furthermore, colon and rectal anastomotic leakage may be seen and described as separate entities. Reasons to consider rectal and colonic anastomotic leakage as two separate problems include the different reported incidence rates, which were again confirmed by the results of this review, the different anatomy, surgical techniques and microbial composition⁵⁹. However, the studies included in this review that reported separate incidence rates for rectal and colon anastomoses did not specify what was considered rectum and colon. This is interesting, as specialists involved in the care of the colorectal patient such as radiologists, surgeons and pathologists, define the rectosigmoid junction differently. In radiology, the rectosigmoid junction is somewhat arbitrarily defined as 15 cm above the anal verge, or, in some cases, 15 cm above the dentate line. As surgeons and pathologist can directly view the anatomical landmarks, the rectosigmoid junction is determined differently: where the teniae coli coalesce to form a continuous outer longitudinal muscle layer⁶⁰. In contrast to rectal anastomotic leakage, an exact definition of colonic anastomotic leakage is only sporadically found in literature^{45,61}. It is highly recommended that these aforementioned issues are resolved in collaboration with an expert panel prior to the construction of a novel definition.

Several important other factors should be taken into account when comparing results from different studies, such as methodology, duration of follow-up and method of collection and reporting of data. Uniform reporting of data has gained attention over the past years, with the CONSORT Statement for RCT's being one of the first to provide guidance about what should be reported in a research article⁶². Several other guidelines have been developed since, a trend that is welcomed and supported by a number of organizations such as the Council of Science Editors and the International Committee of Medical Journal Editors^{63,64}. Based on the findings (and the lack thereof) regarding the

reporting of data in this extensive literature review, the authors believe the research field of colorectal anastomotic leakage would greatly benefit from increased guidance on the reporting of data. We strongly feel that both uniformity in the use of a CAL definition as well as equality in the reported information would positively influence comparability of study data and would allow for the reliable interpretation of meta-analyses. In collaboration with an expert working group, the Dutch Taskforce for Anastomotic Leakage, a set of guidelines has been drafted to address this issue. The authors therefore recommend that newly published papers in this field adhere to the standard reporting of the following 11 points: type of surgery and height of anastomosis, standard testing of integrity of anastomosis both during and after surgery (prior to temporary stoma reversal), construction of deviating ostomies and type of ostomy (primary and secondary), median interval of CAL diagnosis from day of surgery, diagnostic tools used to establish diagnosis of CAL, clinical symptoms associated with the diagnosis of CAL, separate leakage numbers for colon and rectal anastomoses, the use of a grading system and associated treatment plan. A checklist describing these 11 points in more detail can be found in the Supplementary Table S3.2.

The need for an all-embracing definition of colorectal anastomotic leakage continues to exist, so that comparability of study results and understanding between all members of the medical team increases. The authors strongly believe that implementation of a graded system with treatment recommendations for the different severity grades will enhance uniformity of patient care and may lead to decreased delay in treatment^{65,66}. The results of this paper and those of a consensus assessment among surgeons in the Netherlands and China¹⁷ will be used to initiate a Delphi round, that will eventually lead to the formulation of a uniform definition of CAL supported by a large panel of experts.

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Supplementary Data

Table S3.1 Items suggested by the Taskforce Colorectal Anastomotic Leakage to be considered in standard reporting.

Item	Report
Construction of anastomosis	
Type of surgery	Colon/rectum
Height of anastomosis	In cm from the anal verge
Stoma construction	Primary/secondary Deviating ileostomy/deviating colostomy/end ileostomy/end colostomy
Test of integrity during surgery	Air leak test/...
Clinical symptoms	
DULK score	Yes/no, if yes score: ...
Leukocytosis	> $10 \times 10^9/l$
Sepsis	Yes/no
Tachycardia	> 100/minute
Tachypnea	> 20/minute
Increased C-reactive protein	> 250
Discharge from wound	Yes/no
Abdominal pain (other than wound pain)	Yes/no
Fever	> 38 degrees Celsius
Discharge from drain	Yes/no
Discharge from rectum	Yes/no
Rectovaginal fistula	Yes/no
Ileus	No stool > 4 days postoperatively
Diagnosis	
Moment of diagnosis	Median interval in days from surgery
Diagnostic tools	CT/X-thorax/laboratory/...
Dehiscence identified upon palpation	Yes/no
Radiological diagnosis	No leakage, infiltration anastomosis, abscess around anastomosis, free abdominal air, free fluids abdomen
Outcome	
Hospital stay	Number of days until discharge
Prolonged hospital stay	> 5 days
Intensive care stay	Yes/no
Treatment	Conservative, ileus treatment, antibiotics, percutaneous drainage, reoperation with extra suture and deviating stoma, reoperation with reconstruction new anastomosis and deviating stoma, reoperation with end stoma
	Yes/no
Delayed functional recovery	Yes/no
Delayed start of additional therapy (chemo/surgery for metastasis)	Yes/no
Delayed return to work	

Table S3.2 List of included articles in systematic review

Author	Year	Title
Zerhouni	2015	Severity of inflammation as a risk factor for ileo-anal anastomotic leak after a pouch procedure in ulcerative colitis
Yun	2015	Clinical manifestations and risk factors of anastomotic leakage after low anterior resection for rectal cancer
Yamaguchi	2015	Robotic-assisted laparoscopic versus open lateral lymph node dissection for advanced lower rectal cancer
Vestweber	2015	Single-port laparoscopic resection for diverticular disease: experiences with more than 300 consecutive patient
Turrentine	2015	Morbidity, mortality, cost, and survival estimates of gastrointestinal anastomotic leaks
Torba	2015	The influence of the risk factor on the abdominal complications in colon injury management
Tanaka	2015	Analysis of anastomotic leakage after rectal surgery: A case-control study
Snijders	2015	Optimal treatment strategy in rectal cancer surgery: should we be cowboys or chickens
Shiomi	2015	Effects of a Diverting Stoma on Symptomatic Anastomotic Leakage after Low Anterior Resection for Rectal Cancer: A Propensity Score Matching Analysis of 1,014 Consecutive Patients
Scarborough	2015	Combined Mechanical and Oral Antibiotic Bowel Preparation Reduces Incisional Surgical Site Infection and Anastomotic Leak Rates After Elective Colorectal Resection: An Analysis of Colectomy-Targeted ACS NSQIP
Richards	2015	Surgical outcome in patients undergoing reversal of Hartmann's procedures: a multicentre study
Ramanathan	2015	The Impact of Open Versus Laparoscopic Resection for Colon Cancer on C-Reactive Protein Concentrations as a Predictor of Postoperative Infective Complications
Pittet	2015	Rectal enema is an alternative to full mechanical bowel preparation for primary rectal cancer surgery
Midura	2015	Risk factors and consequences of anastomotic leak after colectomy: a national analysis
Strangio	2015	Endo-sponge therapy for management of anastomotic leakages after colorectal surgery: A case series and review of literature
Reisinger	2015	Functional compromise reflected by sarcopenia, frailty, and nutritional depletion predicts adverse postoperative outcome after colorectal cancer surgery
Paulasir	2015	Nonsteroidal Anti-inflammatory Drugs: Do They Increase the Risk of Anastomotic Leaks Following Colorectal Operations?
Matsuda	2015	Transanal drainage tube placement to prevent anastomotic leakage following colorectal cancer surgery with double stapling reconstruction
Martin	2015	Validation of a score for the early diagnosis of anastomotic leakage following elective colorectal surgery
Maeda	2015	Rarity of late anastomotic leakage after low anterior resection of the rectum
Lim	2015	The types of anastomotic leakage that develop following anterior resection for rectal cancer demonstrate distinct characteristics and oncologic outcomes
Krarpup	2015	Association of Comorbidity with Anastomotic Leak, 30-day Mortality, and Length of Stay in Elective Surgery for Colonic Cancer: A Nationwide Cohort Study
Komatsu	2015	Efficacy of perioperative synbiotics treatment for the prevention of surgical site infection after laparoscopic colorectal surgery: a randomized controlled trial
Kiran	2015	Combined Preoperative Mechanical Bowel Preparation With Oral Antibiotics Significantly Reduces Surgical Site Infection, Anastomotic Leak, and Ileus After Colorectal Surgery
Kin	2015	Equivocal effect of intraoperative fluorescence angiography on colorectal anastomotic leaks

Table S3.2 (continued)

Author	Year	Title
Kim	2015	Anastomotic Leakage After Low Anterior Resection for Rectal Cancer Is Different Between Minimally Invasive Surgery and Open Surgery
Kauv	2015	Anastomotic leakage after colorectal surgery: diagnostic accuracy of CT
Kanso	2015	Perineal or Abdominal Approach First During Intersphincteric Resection for Low Rectal Cancer: Which Is the Best Strategy?
Hu	2015	A Clinical Parameters-Based Model Predicts Anastomotic Leakage After a Laparoscopic Total Mesorectal Excision: A Large Study With Data From China
Hellan	2015	Robotic Rectal Cancer Resection: A Retrospective Multicenter Analysis
Hayden	2015	Patient factors may predict anastomotic complications after rectal cancer surgery: Anastomotic complications in rectal cancer
Gustafsson	2015	Higher frequency of anastomotic leakage with stapled compared to hand-sewn ileocolic anastomosis in a large population-based study
Frasson	2015	Risk Factors for Anastomotic Leak After Colon Resection for Cancer: Multivariate Analysis and Nomogram From a Multicentric, Prospective, National Study With 3193 Patients
Espin	2015	Oncological outcome following anastomotic leak in rectal surgery
Ebinger	2015	Anastomotic leakage after curative rectal cancer resection has no impact on long-term survival: a propensity score analysis
d'Hoore	2015	COMPRES: a prospective postmarketing evaluation of the compression anastomosis ring CAR 27/ColonRing
Cho	2015	Morphologic change of rectosigmoid colon using belly board and distended bladder protocol
Buscail	2015	Surgery for rectal cancer after high-dose radiotherapy for prostate cancer: is sphincter preservation relevant?
Bostrom	2015	High arterial ligation and risk of anastomotic leakage in anterior resection for rectal cancer in patients with increased cardiovascular risk
Baucom	2015	Smoking as dominant risk factor for anastomotic leak after left colon resection
Alonso	2015	Postoperative intra-abdominal infection and colorectal cancer recurrence: a prospective matched cohort study of inflammatory and angiogenic responses as mechanisms involved in this association
Allaix	2015	Laparoscopic colon resection: To prep or not to prep? Analysis of 1535 patients
Akladios	2015	Is ileostomy always necessary following rectal resection for deep infiltrating endometriosis?
Ye	2014	Use of Valtrac-secured intracolonic bypass in laparoscopic rectal cancer resection
Vermeer	2014	Anastomotic leakage and presacral abscess formation after locally advanced rectal cancer surgery: Incidence, risk factors and treatment
Stey	2014	Outcomes and cost of diverted versus undiverted restorative proctocolectomy
Snijders	2014	High 1-year complication rate after anterior resection for rectal cancer
Senagore	2014	Bioabsorbable staple line reinforcement in restorative proctectomy and anterior resection: a randomized study
Salvans	2014	Postoperative peritoneal infection enhances migration and invasion capacities of tumor cells in vitro: an insight into the association between anastomotic leak and recurrence after surgery for colorectal cancer
Reisinger	2014	Accurate prediction of anastomotic leakage after colorectal surgery using plasma markers for intestinal damage and inflammation
Reilly	2014	Incidence, risks and outcome of radiological leak following early contrast enema after anterior resection
Placer	2014	Preventing complications in colorectal anastomosis: results of a randomized controlled trial using bioabsorbable staple line reinforcement for circular stapler

Table S3.2 (continued)

Author	Year	Title
Nishigori	2014	Effectiveness of a transanal tube for the prevention of anastomotic leakage after rectal cancer surgery
Nason	2014	Early rise in C-reactive protein is a marker for infective complications in laparoscopic colorectal surgery
Mongin	2014	Does anastomotic leakage impair functional results and quality of life after laparoscopic sphincter-saving total mesorectal excision for rectal cancer? A case-matched study
Matsubara	2014	Mortality after common rectal surgery in Japan: a study on low anterior resection from a newly established nationwide large-scale clinical database
Martellucci	2014	Laparoscopic total mesorectal excision for extraperitoneal rectal cancer: long-term results
Maggiori	2014	Does pathologic response of rectal cancer influence postoperative morbidity after neoadjuvant radiochemotherapy and total mesorectal excision?
Liu	2014	A scoring system to predict the risk of anastomotic leakage after anterior resection for rectal cancer
Liang	2014	Surgical technique of robotic D3 lymph node dissection around the inferior mesenteric artery with preservation of the left colic artery and autonomic nerves for the treatment of distal rectal cancer
Leahy	2014	What is the risk of clinical anastomotic leak in the diverted colorectal anastomosis?
Kwag	2014	Niti CAR 27 Versus a Conventional End-to-End Anastomosis Stapler in a Laparoscopic Anterior Resection for Sigmoid Colon Cancer
Krarup	2014	Anastomotic leak increases distant recurrence and long-term mortality after curative resection for colonic cancer: a nationwide cohort study
Krarup	2014	Management of anastomotic leakage in a nationwide cohort of colonic cancer patients
Kornmann	2014	Compromised visceral circulation does not affect the outcome of colorectal surgery
Kornmann	2014	Beware of false-negative CT scan for anastomotic leakage after colonic surgery
Komen	2014	Polymerase chain reaction for <i>Enterococcus faecalis</i> in drain fluid: the first screening test for symptomatic colorectal anastomotic leakage. The Appeal-study: analysis of parameters predictive for evident anastomotic leakage
Komen	2014	Acute phase proteins in drain fluid: a new screening tool for colorectal anastomotic leakage? The APPEAL study: analysis of parameters predictive for evident anastomotic leakage
Kobayashi	2014	Risk factors for anastomotic leakage and favorable antimicrobial treatment as empirical therapy for intra-abdominal infection in patients undergoing colorectal surgery
Kawada	2014	Risk factors for anastomotic leakage after laparoscopic low anterior resection with DST anastomosis
Kaser	2014	Could hyponatremia be a marker of anastomotic leakage after colorectal surgery? A single center analysis of 1,106 patients over 5 years
Hua	2014	Defunctioning cannula ileostomy after lower anterior resection of rectal cancer
Hicks	2014	Does intramesorectal proctectomy with rectal eversion affect postoperative complications compared to standard total mesorectal excision in patients with ulcerative colitis?
Henneman	2014	Safety of elective colorectal cancer surgery: non-surgical complications and colectomies are targets for quality improvement
Hammond	2014	The burden of gastrointestinal anastomotic leaks: an evaluation of clinical and economic outcomes
Giaccaglia	2014	Procalcitonin, as an early biomarker of colorectal anastomotic leak, facilitates enhanced recovery after surgery

Table S3.2 (continued)

Author	Year	Title
Ganschow	2014	Early postoperative complications after stapled vs handsewn restorative proctocolectomy with ileal pouch-anal anastomosis in 148 patients with familial adenomatous polyposis coli: a matched-pair analysis
Eyvazzadeh	2014	Outcomes after transanal endoscopic microsurgery with intraperitoneal anastomosis
Ellebaek	2014	Cytokine response in peripheral blood indicates different pathophysiological mechanisms behind anastomotic leakage after low anterior resection: a pilot study
Dinc	2014	Comparing methods of ileostomy closure constructed in colorectal surgery in Turkey
De Raffe	2014	Intermittent clamping of the hepatic pedicle in simultaneous ultrasonography-guided liver resection and colorectal resection with intestinal anastomosis: is it safe?
Dauser	2014	Diagnosis, management and outcome of early anastomotic leakage following colorectal anastomosis using a compression device: is it different?
Cooper	2014	Outcomes of the use of fully covered esophageal self-expandable stent in the management of colorectal anastomotic strictures and leaks
Bugiantella	2014	Traditional lateral ileostomy versus percutaneous ileostomy by exclusion probe for the protection of extraperitoneal colo-rectal anastomosis: the ALPPI (Anastomotic Leak Prevention by Probe Ileostomy) trial. A randomized controlled trial
Boelens	2014	Reduction of postoperative ileus by early enteral nutrition in patients undergoing major rectal surgery: prospective, randomized, controlled trial
Blumetti	2014	Management of anastomotic leak: lessons learned from a large colon and rectal surgery training program
Binnebosel	2014	Influence of CD68+ macrophages and neutrophils on anastomotic healing following laparoscopic sigmoid resection due to diverticulitis
Banaszkiewicz	2014	Colorectal cancer with intestinal perforation - a retrospective analysis of treatment outcomes
Bakker	2014	High complication rate after low anterior resection for mid and high rectal cancer; results of a population-based study
Bakker	2014	Risk factors for anastomotic leakage and leak-related mortality after colonic cancer surgery in a nationwide audit
Anjaria	2014	Management of colonic injuries in the setting of damage-control laparotomy: one shot to get it right
Ahmed Ali	2014	Actual versus estimated length of stay after colorectal surgery: which factors influence a deviation?
Adams	2014	Creation of an effective colorectal anastomotic leak early detection tool using an artificial neural network
Zhao	2013	Use of a transanal drainage tube for prevention of anastomotic leakage and bleeding after anterior resection for rectal cancer
Trencheva	2013	Identifying important predictors for anastomotic leak after colon and rectal resection: prospective study on 616 patients
Tan	2013	American Society of Anesthesiologists class and Charlson's comorbidity index as predictors of postoperative colorectal anastomotic leak: a single-institution experience
Snijders	2013	An increasing use of defunctioning stomas after low anterior resection for rectal cancer. Is this the way to go?
Snijders	2013	Anastomotic leakage as an outcome measure for quality of colorectal cancer surgery
Smith	2013	Anastomotic leak following low anterior resection in stage IV rectal cancer is associated with poor survival
Sirois-Giguere	2013	Transanal drainage to treat anastomotic leaks after low anterior resection for rectal cancer: a valuable option

Table S3.2 (continued)

Author	Year	Title
Seo	2013	The Role of Diverting Stoma After an Ultra-low Anterior Resection for Rectal Cancer
Seo	2013	Characteristics and risk factors associated with permanent stomas after sphincter-saving resection for rectal cancer
Saber	2013	Efficacy of protective tube cecostomy after restorative resection for colorectal cancer: a randomized trial
Pasic	2013	Predictive score for anastomotic leakage after elective colorectal cancer surgery: a decision making tool for choice of protective measures
Park	2013	Multicenter analysis of risk factors for anastomotic leakage after laparoscopic rectal cancer excision: the Korean laparoscopic colorectal surgery study group
Ortiz	2013	Impact of a multidisciplinary team training programme on rectal cancer outcomes in Spain
Okoshi	2013	Efficacy of transanal drainage for anastomotic leakage after laparoscopic low anterior resection of the rectum
Nerup	2013	Promising results after endoscopic vacuum treatment of anastomotic leakage following resection of rectal cancer with ileostomy
Nakajima	2013	Efficacy of the predicted operation time (POT) strategy for synchronous colorectal liver metastasis (SCLM): feasibility study for staged resection in patients with a long POT
Morse	2013	Determination of independent predictive factors for anastomotic leak: analysis of 682 intestinal anastomoses
Morks	2013	Late anastomotic leakage in colorectal surgery: a significant problem
Lai	2013	Risk factors for anastomotic leakage following anterior resection for colorectal cancer: the effect of epidural analgesia on occurrence
Kulu	2013	Validation of the International Study Group of Rectal Cancer definition and severity grading of anastomotic leakage
Fischer	2013	Vasopressor use after initial damage control laparotomy increases risk for anastomotic disruption in the management of destructive colon injuries
Kim	2013	Comparison of intracorporeal single-stapled and double-stapled anastomosis in laparoscopic low anterior resection for rectal cancer: a case-control study
Kang	2013	A nationwide analysis of laparoscopy in high-risk colorectal surgery patients
Jafari	2013	The use of indocyanine green fluorescence to assess anastomotic perfusion during robotic assisted laparoscopic rectal surgery
Genser	2013	Postoperative and long-term outcomes after redo surgery for failed colorectal or coloanal anastomosis: retrospective analysis of 50 patients and review of the literature
Garude	2013	Single layered intestinal anastomosis: a safe and economic technique
Garcia-Granero	2013	Procalcitonin and C-reactive protein as early predictors of anastomotic leak in colorectal surgery: a prospective observational study
Floodeen	2013	Early and late symptomatic anastomotic leakage following low anterior resection of the rectum for cancer: are they different entities?
Dumont	2013	Options and outcome for reconstruction after extended left hemicolectomy
Dinnewitzer	2013	Cumulative incidence of permanent stoma after sphincter preserving low anterior resection of mid and low rectal cancer
den Dulk	2013	The DULK (Dutch leakage) and modified DULK score compared: actively seek the leak
Caulfield	2013	Anastomotic leak after low anterior resection: a spectrum of clinical entities
Boesen	2013	Perioperative fluid infusion and its influence on anastomotic leakage after rectal cancer surgery: implications for prevention strategies
Bisgard	2013	Perioperative statin therapy is not associated with reduced risk of anastomotic leakage after colorectal resection
Biondo	2013	Long-term functional results from a randomized clinical study of transverse coloplasty compared with colon J-pouch after low anterior resection for rectal cancer

Table S3.2 (continued)

Author	Year	Title
Aytac	2013	Impact of stress dose steroids on the outcomes of restorative proctocolectomy in patients with ulcerative colitis
Aytac	2013	Impact of obesity on operation performed, complications, and long-term outcomes in terms of restoration of intestinal continuity for patients with mid and low rectal cancer
Asoglu	2013	Laparoscopic surgery for rectal cancer: outcomes in 513 patients
Ashraf	2013	The economic impact of anastomotic leakage after anterior resections in English NHS hospitals: are we adequately remunerating them?
Ashburn	2013	Consequences of anastomotic leak after restorative proctectomy for cancer: effect on long-term function and quality of life
Smith	2012	Anastomotic leak is not associated with oncologic outcome in patients undergoing low anterior resection for rectal cancer
Van't Sant	2012	The influence of mechanical bowel preparation in elective colorectal surgery for diverticulitis
Slieker	2012	Long-term and perioperative corticosteroids in anastomotic leakage: a prospective study of 259 left-sided colorectal anastomoses
Schietroma	2012	Colorectal Intraoperative anastomosis: the effects of perioperative supplemental oxygen administration on the anastomotic dehiscence
Rutegard	2012	High tie in anterior resection for rectal cancer confers no increased risk of anastomotic leakage
Ruffo	2012	Laparoscopic rectal resection for severe endometriosis of the mid and low rectum: technique and operative results
Rondelli	2012	Temporary percutaneous ileostomy versus conventional loop ileostomy in mechanical extraperitoneal colorectal anastomosis: a retrospective study
Richards	2012	Smoking is a major risk factor for anastomotic leak in patients undergoing low anterior resection
Post	2012	Intraoperative blood pressure changes as a risk factor for anastomotic leakage in colorectal surgery
Platt	2012	C-reactive protein as a predictor of postoperative infective complications after curative resection in patients with colorectal cancer
Piessen	2012	Previous transanal full-thickness excision increases the morbidity of radical resection for rectal cancer
Penninckx	2012	Risk adjusted benchmarking of clinical anastomotic leakage rate after total mesorectal excision in the context of an improvement project
Pedersen	2012	Increased levels of C-reactive protein and leukocyte count are poor predictors of anastomotic leakage following laparoscopic colorectal resection
Ogilvie	2012	Anastomotic leak after restorative proctosigmoidectomy for cancer: what are the chances of a permanent ostomy?
Nisar	2012	Influence of neoadjuvant radiotherapy on anastomotic leak after restorative resection for rectal cancer
Nakajima	2012	Predictive factors for anastomotic leakage after simultaneous resection of synchronous colorectal liver metastasis
Millan	2012	Risk factors for prolonged postoperative ileus after colorectal cancer surgery
Manceau	2012	Right colon to rectal anastomosis (Deloyers procedure) as a salvage technique for low colorectal or coloanal anastomosis: postoperative and long-term outcomes
Leichtle	2012	Risk factors for anastomotic leakage after colectomy
Lagoutte	2012	C-reactive protein and procalcitonin for the early detection of anastomotic leakage after elective colorectal surgery: pilot study in 100 patients
Krurup	2012	A nationwide study on anastomotic leakage after colonic cancer surgery

Table S3.2 (continued)

Author	Year	Title
Klein	2012	Postoperative use of non-steroidal anti-inflammatory drugs in patients with anastomotic leakage requiring reoperation after colorectal resection: cohort study based on prospective data
Kanazawa	2012	Risk factors for postoperative intra-abdominal septic complications after bowel resection in patients with Crohn's disease
Gorissen	2012	Risk of anastomotic leakage with non-steroidal anti-inflammatory drugs in colorectal surgery
Fauno	2012	Low complication rate after stoma closure. Consultants attended 90% of the operations
Blumetti	2012	Delayed transanal repair of persistent coloanal anastomotic leak in diverted patients after resection for rectal cancer
Biondo	2012	Risk factors for surgical site infection after elective resection for rectal cancer. A multivariate analysis on 2131 patients
Bakker	2012	The C-seal trial: colorectal anastomosis protected by a biodegradable drain fixed to the anastomosis by a circular stapler, a multi-center randomized controlled trial
Almeida	2012	Elevated serum C-reactive protein as a predictive factor for anastomotic leakage in colorectal surgery
Xiao	2011	Can transanal tube placement after anterior resection for rectal carcinoma reduce anastomotic leakage rate? A single-institution prospective randomized study
Warschcow	2011	Epidural analgesia in open resection of colorectal cancer: is there a clinical benefit? a retrospective study on 1,470 patients
Trenti	2011	Generalized peritonitis due to perforated diverticulitis: Hartmann's procedure or primary anastomosis?
Thornton	2011	Management and outcome of colorectal anastomotic leaks
Sule	2011	Adult large bowel obstruction: a review of clinical experience
Shiomi	2011	Diverting stoma in rectal cancer surgery. A retrospective study of 329 patients from Japanese cancer centers
Shiomi	2011	The indications for a diverting stoma in low anterior resection for rectal cancer: a prospective multicentre study of 222 patients from Japanese cancer centers
Schnuriger	2011	Crystalloids after primary colon resection and anastomosis at initial trauma laparotomy: excessive volumes are associated with anastomotic leakage
Penninckx	2011	Anastomotic leakage: a disaster or a challenge with an impact on survival after rectal cancer surgery?
Park	2011	S052: a comparison of robot-assisted, laparoscopic, and open surgery in the treatment of rectal cancer
Maggiori	2011	Conservative management is associated with a decreased risk of definitive stoma after anastomotic leakage complicating sphincter-saving resection for rectal cancer
Maggiori	2011	Laparoscopic colorectal anastomosis using the novel Chex((R)) circular stapler: a case-control study
Lindgren	2011	What is the risk for a permanent stoma after low anterior resection of the rectum for cancer? A six-year follow-up of a multicenter trial
Lindgren	2011	Does a defunctioning stoma affect anorectal function after low rectal resection? Results of a randomized multicenter trial
Lin	2011	The influence of fecal diversion and anastomotic leakage on survival after resection of rectal cancer
Levack	2011	Laparoscopy decreases anastomotic leak rate in sigmoid colectomy for diverticulitis
Lehmann	2011	Does sacrifice of the inferior mesenteric artery or superior rectal artery affect anastomotic leak following sigmoidectomy for diverticulitis? a retrospective review
Kumar	2011	Anterior resection for rectal carcinoma - risk factors for anastomotic leaks and strictures

Table S3.2 (continued)

Author	Year	Title
Kruschewski	2011	Management and results of complications after anterior resection with colonic pouch reconstruction for rectal cancer
Katoh	2011	Anastomotic leakage contributes to the risk for systemic recurrence in stage II colorectal cancer
Jurado	2011	Low colorectal anastomosis after pelvic exenteration for gynecologic malignancies: risk factors analysis for leakage
Jorgren	2011	Anastomotic leakage after surgery for rectal cancer: a risk factor for local recurrence, distant metastasis and reduced cancer-specific survival?
Gulla	2011	Ghost ileostomy after anterior resection for rectal cancer: a preliminary experience
Good	2011	Laparoscopic surgery for rectal cancer: a single-centre experience of 120 cases
Gadiot	2011	Reduction of anastomotic failure in laparoscopic colorectal surgery using antitraction sutures
Frago	2011	Differences between proximal and distal obstructing colonic cancer after curative surgery
Fouda	2011	Early detection of anastomotic leakage after elective low anterior resection
Fong	2011	Chronic anastomotic sinus after low anterior resection: when can the defunctioning stoma be reversed?
Dekker	2011	Predicting the risk of anastomotic leakage in left-sided colorectal surgery using a colon leakage score
Cerroni	2011	Ghost Ileostomy with or without abdominal parietal split
Buchberg	2011	The use of a compression device as an alternative to hand-sewn and stapled colorectal anastomoses: is three a crowd?
Antolovic	2011	Restoration of intestinal continuity after Hartmann's procedure--not a benign operation. Are there predictors for morbidity?
Woeste	2010	Increased serum levels of C-reactive protein precede anastomotic leakage in colorectal surgery
Wang	2010	Risk factors for symptomatic anastomotic leakage after low anterior resection for rectal cancer with 30Gy/10f/2w preoperative radiotherapy
Vergara-Fernandez	2010	Assessing outcomes following surgery for colorectal cancer using quality of care indicators
Telem	2010	Diagnosis of gastrointestinal anastomotic dehiscence after hospital discharge: Impact on patient management and outcome
Telem	2010	Risk factors for anastomotic leak following colorectal surgery: a case-control study
Shin	2010	Delayed anastomotic leakage following sphincter-preserving surgery for rectal cancer
Pramateftakis	2010	The handsewn anastomosis after colon resection due to colonic cancer
Pasternak	2010	Elevated intraperitoneal matrix metalloproteinases-8 and -9 in patients who develop anastomotic leakage after rectal cancer surgery: a pilot study
Ortega-Deballon	2010	C-reactive protein is an early predictor of septic complications after elective colorectal surgery
Matthiessen	2010	Symptomatic anastomotic leakage diagnosed after hospital discharge following low anterior resection for rectal cancer
Matthiessen	2010	Anastomotic-vaginal fistula (AVF) after anterior resection of the rectum for cancer--occurrence and risk factors
Kim	2010	Complications after sphincter-saving resection in rectal cancer patients according to whether chemoradiotherapy is performed before or after surgery
Kanellos	2010	Anastomotic leakage following low anterior resection for rectal cancer
Kanellos	2010	Anastomotic leakage after colonic resection

Table S3.2 (continued)

Author	Year	Title
Jeong	2010	Cefotetan versus conventional triple antibiotic prophylaxis in elective colorectal cancer surgery
Hidalgo	2010	Laparoscopic rectal surgery: does immediate outcome differ in respect to sex?
El-Gazzaz	2010	Risk of clinical leak after laparoscopic versus open bowel anastomosis
Ciga	2010	Comparative study of one-stage colectomy of the descending colon in emergency and elective surgery without mechanical preparation
Choi	2010	Risk factors for anastomotic leakage after laparoscopic rectal resection
Biondo	2010	Impact of surgical specialization on emergency colorectal surgery outcomes
Bertelsen	2010	Anastomotic leakage after anterior resection for rectal cancer: risk factors
Bertelsen	2010	Anastomotic leakage after curative anterior resection for rectal cancer: short and long-term outcome
Akasu	2010	Risk factors for anastomotic leakage following intersphincteric resection for very low rectal adenocarcinoma
Ulrich	2009	Diverting stoma after low anterior resection: more arguments in favor
Szynglarewicz	2009	The impact of pre- or postoperative radiochemotherapy on complication following anterior resection with en bloc excision of female genitalia for T4 rectal cancer
Saha	2009	Morbidity and mortality after closure of loop ileostomy
Roos	2009	Perioperative selective decontamination of the digestive tract (SDD) in elective colorectal surgery
Ricciardi	2009	Anastomotic leak testing after colorectal resection: what are the data?
Cheung	2009	Endolaparoscopic Approach vs Conventional Open Surgery in the Treatment of Obstructing Left-Sided Colon Cancer
Frye	2009	Anastomotic leakage after resection of colorectal cancer generates prodigious use of hospital resources
Li	2009	Use of routine intraoperative endoscopy in elective laparoscopic colorectal surgery: can it further avoid anastomotic failure?
Komen	2009	Detection of colon flora in peritoneal drain fluid after colorectal surgery: Can RT-PCR play a role in diagnosing anastomotic leakage?
Marra	2009	Anastomotic leakage as a risk factor for the long-term outcome after curative resection of colon cancer.
Akiyoshi	2009	Factors affecting the difficulty of laparoscopic total mesorectal excision with double stapling technique anastomosis for low rectal cancer
Komen	2009	After-hours colorectal surgery: a risk factor for anastomotic leakage
Cong	2009	Influencing Factors of Symptomatic Anastomotic Leakage After Anterior Resection of the Rectum for Cancer
Karlizcek	2009	Surgeons lack predictive accuracy for anastomotic leakage in gastrointestinal surgery
Park	2009	Multidimensional analysis of the learning curve for laparoscopic colorectal surgery: lessons from 1,000 cases of laparoscopic colorectal surgery
Damrauer	2009	Contained Anastomotic Leaks After Colorectal Surgery
Dulk	2009	Improved diagnosis and treatment of anastomotic leakage after colorectal surgery
Bellows	2009	Early predictors of anastomotic leaks after colectomy
Park	2009	Multidimensional Analysis of the Learning Curve for Laparoscopic Resection in Rectal cancer
Kingham	2009	Colonic Anastomotic Leak: Risk Factors, Diagnosis, and Treatment
Choptra	2009	The effect of endoscopic treatment on healing of anastomotic leaks after anterior resection of rectal cancer
Brisinda	2009	End-to-End Versus End-to-Side Stapled Anastomoses After Anterior Resection for Rectal Cancer

Table S3.2 (continued)

Author	Year	Title
Kim	2009	Risk Factors for Anastomotic Leakage after Laparoscopic Intracorporeal Colorectal Anastomosis with a Double Stapling Technique
Eberhardt	2009	The Impact of Anastomotic Leak and Intra-Abdominal Abscess on Cancer-Related Outcomes After Resection for Colorectal Cancer: A Case Control Study
Lian	2009	Outcomes for Patients Developing Anastomotic Leak After Ileal Pouch-Anal Anastomosis: Does a Handsewn vs. Stapled Anastomosis Matter?
Joh	2009	Anastomotic Leakage after Laparoscopic Proctectomy can be Managed by a Minimally Invasive Approach
Klein	2009	Increased Risk of Anastomotic Leakage with Diclofenac Treatment after Laparoscopic Colorectal Surgery
Ulrich	2008	Early results from a randomized clinical trial of colon J pouch versus transverse coloplasty pouch after low anterior resection for rectal cancer
Chessin	2008	Septic Complications after Restorative Proctocolectomy do not Impair Functional Outcome: Long-Term Follow-Up from a Specialty Center
Jestin	2008	Risk factors for anastomotic leakage after rectal cancer surgery: a case-control study
Doeksen	2008	Radiological evaluation of colorectal anastomoses
Martel	2008	Neoadjuvant Therapy and Anastomotic Leak After Tumor-Specific Mesorectal Excision for Rectal Cancer
Weidenhagen	2008	Endoscopic vacuum-assisted closure of anastomotic leakage following anterior resection of the rectum: a new method
Ito	2008	Relationship between multiple numbers of stapler firings during rectal division and anastomotic leakage
Iancu	2008	Host-Related Predictive Factors for Anastomotic Leakage Following Large Bowel Resections for Colorectal Cancer*
Khan	2008	The management and outcome of anastomotic leaks in colorectal surgery
Lee	2008	Risk Factors and Clinical Outcome for Anastomotic Leakage After Total Mesorectal Excision for Rectal Cancer
Jung	2008	Risk Factors and Oncologic Impact of Anastomotic Leakage after Rectal Cancer Surgery
Williams	2008	The outcome of loop ileostomy closure: a prospective study
Lim	2008	Outcomes of colorectal anastomoses during pelvic exenteration for gynaecological malignancy
Tsujinaka	2008	Pelvic drainage for anterior resection revisited: use of drains in anastomotic leaks
Wilson	2008	Comparative Costs of Ertapenem and Cefotetan as Prophylaxis for Elective Colorectal Surgery
Buchs	2008	Incidence, consequences, and risk factors for anastomotic dehiscence after colorectal surgery: a prospective monocentric study
Glitsch	2008	Endoscopic transanal vacuum-assisted rectal drainage (ETVARD): an optimized therapy for major leaks from extraperitoneal rectal anastomoses
Steffen	2008	Safety and morbidity after ultra-low coloanal anastomoses: J-pouch vs end-to-end reconstruction
Ye	2008	Use of Intracolonic Bypass Secured by a Biodegradable Anastomotic Ring to Protect the Low Rectal Anastomosis
Matthiessen	2008	Increase of serum C-reactive protein is an early indicator of subsequent symptomatic anastomotic leakage after anterior resection
Contant	2007	Mechanical bowel preparation for elective colorectal surgery: a multicentre randomised trial
Wind	2007	Laparoscopic reintervention for anastomotic leakage after primary laparoscopic colorectal surgery

Table S3.2 (continued)

Author	Year	Title
Welsch	2007	C-reactive protein as early predictor for infectious postoperative complications in rectal surgery
Veyrie	2007	Anastomotic Leakage after Elective Right Versus Left Colectomy for Cancer: Prevalence and Independent Risk Factors
Ptok	2007	Impact of anastomotic leakage on oncological outcome after rectal cancer resection
O'Grady	2007	Colorectal cancer management in the provincial New Zealand setting of Nelson
Samson	2007	Colorectal resection in peripheral New Zealand: workload, outcomes and its future
Bretagnol	2007	Rectal cancer surgery without mechanical bowel preparation
Matthiessen	2007	Is Early Detection of Anastomotic Leakage Possible by Intraperitoneal Microdialysis and Intraperitoneal Cytokines After Anterior Resection of the Rectum for Cancer?
Jones	2007	Low anastomotic leak rate after colorectal surgery: a single- centre study
Staudacher	2007	Laparoscopic vs. Open Total Mesorectal Excision in Unselected Patients with Rectal Cancer: Impact on Early Outcome
Vermaas	2007	Introduction of preoperative radiotherapy in the treatment of operable rectal cancer in the Southwest region of the Netherlands
Kruschewski	2007	Risk factors for clinical anastomotic leakage and postoperative mortality in elective surgery for rectal cancer
Kong	2007	Selective treatment of rectal cancer with single-stage coloanal or ultralow colorectal anastomosis does not adversely affect morbidity and mortality
Matthiessen	2007	Defunctioning Stoma Reduces Symptomatic Anastomotic Leakage After Low Anterior Resection of the Rectum for Cancer
Huh	2007	A Diverting Stoma Is Not Necessary when Performing a Handsewn Coloanal Anastomosis for Lower Rectal Cancer
Oguz	2007	L-Alanin-L-glutamine supplementation improves the outcome after colorectal surgery for cancer
Pena-Soria	2007	Mechanical Bowel Preparation for Elective Colorectal Surgery with Primary Intraperitoneal Anastomosis by a Single Surgeon: Interim Analysis of a Prospective Single-Blinded Randomized Trial
Marecik	2007	Single-stapled double-pursestring anastomosis after anterior resection of the rectum
Brennan	2007	Routine Mobilization of the Splenic Flexure is not Necessary During Anterior Resection for Rectal Cancer
Rickard	2007	Ileal pouch-anal anastomosis: the Australasian experience
Nicksa	2007	Anastomotic Leaks: What is the Best Diagnostic Imaging Study?
Law	2007	Anastomotic Leakage is Associated with Poor Long-Term Outcome in Patients After Curative Colorectal Resection for Malignancy
Platell	2007	The incidence of anastomotic leaks in patients undergoing colorectal surgery
Smith	2007	The efficacy of intraoperative methylene blue enemas to assess the integrity of a colonic anastomosis
Shrikhande	2007	Outcomes of resection for rectal cancer in India: The impact of the double stapling technique
Rodriguez-Ramirez	2006	Risk factors for anastomotic leakage after preoperative chemoradiation therapy and low anterior resection with total mesorectal excision for locally advanced rectal cancer
Richardson	2006	Risk factors for anastomotic leak after recto-sigmoid resection for ovarian cancer
Kim	2006	Laparoscopic resection for rectal cancer: a prospective analysis of thirty-month follow-up outcomes in 312 patients
Zmora	2006	Is mechanical bowel preparation mandatory for left-sided colonic anastomosis? Results of a prospective randomized trial
Bulow	2006	Transanal stent in anterior resection does not prevent anastomotic leakage

Table S3.2 (continued)

Author	Year	Title
Lim	2006	Clinical and Subclinical Leaks After Low Colorectal Anastomosis: A Clinical and Radiologic Study
Byrn	2006	The Management of 38 Anastomotic Leaks After 1,684 Intestinal Resections
Law	2006	Laparoscopic and Open Anterior Resection for Upper and Mid Rectal Cancer: An Evaluation of Outcomes
Choi	2006	Leakage After Resection and Intraperitoneal Anastomosis for Colorectal Malignancy: Analysis of Risk Factors
Lipska	2006	Anastomotic leakage after lower gastrointestinal anastomosis: men are at higher risk
Millan	2006	Early Prediction of Anastomotic Leak in Colorectal Cancer Surgery by Intramucosal pH
Matthiessen	2006	Population-based study of risk factors for postoperative death after anterior resection of the rectum
Konishi	2006	Risk Factors for Anastomotic Leakage after Surgery for Colorectal Cancer: Results of Prospective Surveillance
Korkolis	2006	Short-term preoperative radiotherapy is a safe approach for treatment of locally advanced rectal cancer
Bernardshaw	2006	Treatment of Rectal Cancer: Reduction of Local Recurrence after the Introduction of TME – Experience from One University Hospital
Fa-Si-Oen	2005	Mechanical Bowel Preparation or Not? Outcome of a Multicenter, Randomized Trial in Elective Open Colon Surgery
Biondo	2005	Anastomotic Dehiscence After Resection and Primary Anastomosis in Left-Sided Colonic Emergencies
McArdle	2005	Impact of anastomotic leakage on long-term survival of patients undergoing curative resection for colorectal cancer
Breukink	2005	Laparoscopic versus open total mesorectal excision: a case-control study
Patrioti	2005	One-stage resection without colonic lavage in emergency surgery of the left colon
Bucher	2005	Randomized clinical trial of mechanical bowel preparation versus no preparation before elective left-sided colorectal surgery
Platell	2005	Clinical utility of a de-functioning loop ileostomy
Resegotti	2005	Side-to-Side Stapled Anastomosis Strongly Reduces Anastomotic Leak Rates in Crohn's Disease Surgery
Peeters	2005	Risk factors for anastomotic failure after total mesorectal excision of rectal cancer
Stumpf	2005	Changes of the extracellular matrix as a risk factor for anastomotic leakage after large bowel surgery
Eriksen	2005	Anastomotic leakage following routine mesorectal excision for rectal cancer in a national cohort of patients
Yeh	2005	Pelvic drainage and other risk factors for leakage after elective anterior resection in rectal cancer patients: a prospective study of 978 patients
Rudinskaite	2005	risk factors for clinical anastomotic leakage following the resection of sigmoid and rectal cancer
Matthiessen	2004	Risk factors for anastomotic leakage after anterior resection of the rectum
Kanellos	2004	Anastomotic leakage following anterior resection for rectal cancer
Kanellos	2004	The failed intraperitoneal colon anastomosis after colon resection
Raveenthiran	2004	Restorative resection of unprepared left-colon in gangrenous vs. viable sigmoid volvulus
Schwandner	2004	Laparoscopic colectomy for recurrent and complicated diverticulitis: a prospective study of 396 patients
Eckmann	2004	Anastomotic leakage following low anterior resection: results of a standardized diagnostic and therapeutic approach
Leroy	2004	Laparoscopic total mesorectal excision (TME) for rectal cancer surgery

Table S3.2 (continued)

Author	Year	Title
Garcia-Botello	2004	A Prospective Audit of the Complications of Loop Ileostomy Construction and Takedown
Walker	2004	Anastomotic leakage is predictive of diminished survival after potentially curative resection for colorectal
Law	2004	Anterior resection for rectal cancer with mesorectal excision: a prospective evaluation of 622 patients
Bell	2003	Anastomotic leakage after curative anterior resection results in a higher prevalence of local recurrence.
Makela	2003	Risk factors for anastomotic leakage after left-sided colorectal resection with rectal anastomosis
Schmidt	2003	Anastomotic leakage after low rectal stapler anastomosis: significance of intraoperative anastomotic testing
Zmora	2003	Colon and rectal surgery without mechanical bowel preparation: a randomized prospective trial.
Bertram	2003	Peritoneal release of TNFalpha and IL-6 after elective colorectal surgery and anastomotic leakage
Killingback	2002	Elective resection and anastomosis for colorectal cancer: a prospective audit of mortality and morbidity 1976-1998
Marusch	2002	Value of a protective stoma in low anterior resections for rectal cancer
Kuzu	2002	Emergent resection for acute sigmoid volvulus: results of 106 consecutive cases
Leester	2002	Septic complications after low anterior rectal resection--is diverting stoma still justified?
Z'graggen	2001	A new surgical concept for rectal replacement after low anterior resection: the transverse coloplasty pouch.
Read	2001	Neoadjuvant external beam radiation and proctectomy for adenocarcinoma of the rectum.
Tang	2001	Risk factors for surgical site infection after elective resection of the colon and rectum: a single-center prospective study of 2,809 consecutive patients.
Brown	2001	A prospective randomised study of drains in infra-peritoneal rectal anastomoses.
Hartley	2001	
Poulin	2001	Septic complications of elective laparoscopic colorectal resection
Tocchi	2000	Prospective evaluation of omentoplasty in preventing leakage of colorectal anastomosis
Dehni	1998	Influence of a defunctioning stoma on leakage rates after low colorectal anastomosis and colonic J pouch-anal anastomosis
Breen	1998	Functional results after perineal complications of ileal pouch-anal anastomosis
Merad	1998	Omentoplasty in the prevention of anastomotic leakage after colonic or rectal resection: a prospective randomized study in 712 patients. French Associations for Surgical Research
Petersen	1998	Anastomotic leakage: impact on local recurrence and survival in surgery of colorectal cancer
Mella	1997	Population-based audit of colorectal cancer management in two UK health regions. Colorectal Cancer Working Group, Royal College of Surgeons of England Clinical Epidemiology and Audit Unit
Vignali	1997	Factors associated with the occurrence of leaks in stapled rectal anastomoses: a review of 1,014 patients
Moore	1996	Morbidity and mortality after single- and double-stapled colorectal anastomoses in patients with carcinoma of the rectum
Hansen	1996	Colorectal stapled anastomoses. Experiences and results
Hallbook	1996	Anastomotic leakage and functional outcome after anterior resection of the rectum

Table S3.2 (continued)

Author	Year	Title
Bokey	1995	Postoperative morbidity and mortality following resection of the colon and rectum for cancer
Kessler	1993	Operative mortality in carcinoma of the rectum. Results of the German Multicentre Study
Akyol	1992	Early postoperative contrast radiology in the assessment of colorectal anastomotic integrity
Akyol	1991	Anastomotic leaks in colorectal cancer surgery: a risk factor for recurrence?
Galandiuk	1991	Postoperative irrigation-suction drainage after pelvic colonic surgery. A prospective randomized trial
Tuson	1990	A retrospective study of colostomies, leaks and strictures after colorectal anastomosis
Cornwell	1998	The Fate of Colonic Suture Lines in High-Risk Trauma Patients: A Prospective Analysis
Docherty	1995	Comparison of Manually Constructed and Stapled Anastomoses in Colorectal Surgery
Fingerhut	1995	Supraperitoneal colorectal anastomosis: hand-sewn versus circular staples - a controlled clinical trial
Kracht	1993	Ileocolonic anastomosis after right hemicolectomy for carcinoma: stapled or hand-sewn?
Miller	1996	Early Detection of Anastomotic Leaks After Low Anterior Resection of the rectum

Part II

Developing a predictive algorithm for
anastomotic leakage

Chapter 4

Predictive factors for anastomotic leakage after colorectal surgery: study protocol for a prospective observational study (REVEAL Study)

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Abstract

Background

Anastomotic leakage (AL) remains the most important complication following colorectal surgery, and is associated with high morbidity and mortality rates. Previous research has focused on identifying risk factors and potential biomarkers for AL, but the sensitivity of these tests remains poor.

Objective

This prospective multicenter observational study aims at combining multiple parameters to establish a diagnostic algorithm for colorectal AL.

Methods

This study aims to include 588 patients undergoing surgery for colorectal carcinoma. Patients will be eligible for inclusion when surgery includes the construction of a colorectal anastomosis. Patient characteristics will be collected upon consented inclusion, and buccal swabs, breath, stool, and blood samples will be obtained prior to surgery. These samples will allow for the collection of information regarding patients' inflammatory status, genetic predisposition, and intestinal microbiota. Additionally, breath and blood samples will be taken postoperatively and patients will be strictly observed during their in-hospital stay, and the period shortly thereafter.

Results

This study has been open for inclusion since August 2015.

Conclusions

An estimated 8-10% of patients will develop AL following surgery, and they will be compared to non-leakage patients. The objectives of this study are twofold. The primary aim is to establish and validate a diagnostic algorithm for the pre-operative prediction of the risk of AL development using a combination of inflammatory, immune-related, and genetic parameters. Previously established risk factors and novel parameters will be incorporated into this algorithm, which will aid in the recognition of patients who are at risk for AL. Based on these results, recommendations can be made regarding the construction of an anastomosis or deviating stoma, and possible preventive strategies. Furthermore, we aim to develop a new algorithm for the post-operative diagnosis of AL at an earlier stage, which will positively reflect on short-term survival rates.

Introduction

Surgery remains the predominant curative treatment for patients with colorectal cancer (CRC), but can lead to severe post-operative complications, of which anastomotic leakage (AL) is the most feared. AL occurs in 8-15% of patients undergoing colorectal surgery and is associated with high morbidity and short-term mortality rates of up to 39%¹⁻⁴. AL often requires one or more re-interventions, leading to a significant increase in length of hospital stay and subsequently to high health care costs³. Some authors even suggest that AL is associated with an impaired oncological prognosis⁵⁻⁸. Both decreased disease-specific survival (odds ratio [OR] 1.75)^{9,10} and an increased local recurrence rate of CRC (OR 2.9)¹⁰ have been reported.

In the past decades, important risk factors for AL such as male gender⁴, neo-adjuvant chemotherapy¹¹, tumour size¹², malnutrition¹³, smoking¹⁴, steroid treatment^{1,15}, and the use of non-steroidal anti-inflammatory drugs (NSAIDs)¹⁶ have been identified. Despite the determination of these risk factors for AL, surgeons' prediction of the risk to develop AL for the individual patient remains inaccurate¹⁷. Despite decades of extensive research on preventive methods, the introduction of innovative surgical techniques, and fast-track protocols, incidence rates of AL have remained stable¹⁸.

A recently conducted study in one of the participating centres, that can be considered a pilot study, suggests that pre-operative levels of a certain plasma marker (intestinal fatty acid binding protein, I-FABP) can predict the development of AL in patients undergoing CRC surgery. I-FABP is present in mature enterocytes of the small and large intestine, and is released upon intestinal damage¹⁹. It is hypothesized that increased plasma levels of I-FABP are the result of an underlying clinical condition of the gastrointestinal tract, predisposing the patient to AL²⁰.

Cumulative evidence in the literature suggests that perioperative pain treatment with NSAIDs, commonly prescribed analgesics that inhibit cyclo-oxygenase 2 (COX-2) expression, increase the risk of AL^{16,21,22}. COX-2 knockout mice have an increased risk of developing AL, and this risk can be reduced by the administration of prostaglandin E2, a product of COX-2²³. Another study conducted by our research group has demonstrated that decreased COX-2 expression, due to a polymorphism in the COX-2 gene, leads to an increased risk of the development of AL²³. Furthermore, it has been shown that patients with different genotypes of mannose-binding lectin (MBL), an important complement factor of the immune system, respond differently to intestinal damage²⁴.

In the pilot study mentioned previously, it was shown that a combination of C-reactive protein (CRP) and calprotectin levels in plasma provides high diagnostic accuracy for AL during the post-operative period. Finally, we have shown that functional compromise (characterized by malnutrition, frailty, and sarcopenia) is associated with post-operative morbidity and mortality²⁵. Malnutrition, as indicated by high Short Nutritional Assessment Questionnaire (SNAQ) scores, and sarcopenia were predictive for, or showed a trend towards, prediction of AL.

Based on previous literature and both our experimental and clinical data, we hypothesize that the occurrence of AL is partly due to patient-derived factors such as a compromised immune response, sarcopenia, genetic predisposition, and an aberrant intestinal microbiome composition, and that the post-operative course can be further influenced by surgical stress, ischemia, and a derailed systemic response. Our hypothesis is that, based on these parameters, individual risks for the development of AL can be assessed pre-operatively.

Besides the obvious improvements that can be made regarding pre-operative risk assessment, the post-operative recognition and management of AL has also proven to be challenging. The timing of AL diagnoses varies greatly, from post-operative day 3 to beyond 30 days, with a mean of 12.7 days post-operatively²⁶. Abnormal vital signs and biochemical tests are quite common following the construction of a colorectal anastomosis, and can therefore not be used adequately in the diagnosis of AL. The presentation of AL can vary from abdominal pain and low-grade fever to peritonitis and severe sepsis. This nonspecific course of AL often delays radiologic imaging, and even if this test is undertaken, the diagnoses frequently remain uncertain. Previous research has presented false-negative rates varying from 17-52% for both contrast enemas and computerized tomography (CT) scans²⁷, resulting in a significant delay of re-intervention²⁸.

Since a delayed diagnosis of AL is associated with poor outcome²⁹ and premature re-intervention could lead to a high number of negative re-explorations, one should outweigh the risks of a delayed intervention and the morbidity of re-intervention. Physicians are armed with a restricted number of parameters contributing to risk analysis for the development of AL, with only limited specificity and sensitivity.

Study objectives

The objectives of the Predictive Factors of Anastomotic Leakage after Colorectal Surgery (REVEAL) study are twofold. The primary aim of this study is to establish a risk assessment tool for the pre-operative prediction of the development of AL based on a combination of inflammatory, immune-related, and genetic parameters. This algorithm should enable surgeons to make an adequate estimation for every individual patient's risk of AL, and will eventually aid in the decision-making process regarding the construction of anastomoses and deviating ostomies. In addition, this study aims to provide additional post-operative biomarkers for AL diagnosis at earlier stages, thereby reducing the clinical impact of this feared complication.

Methods

This is a study protocol for a multicenter, prospective observational study. This study is approved by the Medical Ethical Committee of the Maastricht University Medical Centre. A written informed consent is required from all participating patients and the trial will be conducted in compliance with the rules of Good Clinical Practice.

Study population

All patients aged >18 years undergoing elective colorectal surgery for colorectal carcinoma, with the construction of an anastomosis, in one of the three participating centres are eligible for inclusion. Patients with large adenomas (large tubular, tubulovillous, or sessile) that cannot be resected radically by means of endoscopy will be included as well, provided that the adenoma is removed surgically with the construction of a colorectal anastomosis. Sample size analysis revealed that 588 consecutive patients have to be included at the outpatient department, prior to surgery. Patients undergoing colorectal surgery for benign conditions, those with permanent stomata without anastomosis, or patients that are unable to give informed consent will be excluded from participation in this study, as well as pregnant patients. If no anastomosis is constructed during surgery, the patient will be withdrawn from the study. Eligibility for inclusion will be determined during a visit at the outpatient department. Upon approval, the patient will receive oral and written information regarding the study, after which he/she will have ample time to reconsider participation. Informed consent will be signed in the presence of the surgeon or researcher.

Participating centers

This study will be conducted at Maastricht University Medical Centre (MUMC+, Maastricht, The Netherlands), Zuyderland Medical Centre (Sittard-Geleen and Heerlen, The Netherlands), and Máxima Medical Centre (MMC, Veldhoven, The Netherlands).

Study outline

Patients will be admitted to the ward one day prior to surgery, and perioperative care will be performed according to Enhanced Recovery After Surgery guidelines for elective colonic surgery³⁰. Due to the compelling evidence regarding the detrimental effects of NSAIDs on intestinal wound healing, these drugs will not be administered in this patient population during the perioperative phase. Instead, adequate pain treatment will be provided by means of acetaminophen and opioids if necessary. A Data Safety Monitoring Board has been commissioned to evaluate the quality of data collection and monitor patient safety. A web-based system was constructed in order to facilitate the collection of standardized and coded patient data.

The primary end point of this study is post-operative AL, occurring during the first 30 days after surgery, either during hospital admission or following discharge. AL is defined as a communication between the intra- and extra-luminal compartments, resulting from a defect in the integrity of the intestinal wall. Leakage from the suture or staple line from a neorectal reservoir, as well as an abscess near the anastomosis, are also considered leaks³¹. The impact of AL on clinical management is recorded, together with the presence of subclinical (radiological) leaks.

Upon hospital admission, a buccal swab will be performed in order to collect DNA to screen for MBL and COX-2 polymorphisms. In addition, composition of volatile organic compounds (VOCs) in exhaled air will be measured both pre- and post-operatively. Various metabolic processes produce markers that are released into the circulation and, upon passage through the pulmonary system, can be identified as volatile products in exhaled air. The occurrence of (chronic) inflammation and/or oxidative stress can result in the excretion of volatile compounds that generate unique VOC patterns³². Furthermore, several baseline characteristics will be acquired prior to surgery, and stool will be collected on three occasions (pre-operatively, post-operatively and at the outpatient department during a control visit) in which the intestinal microbiome can be identified. It has been suggested that the microbiota play an important role in the pathogenesis of AL³³ and that the composition of the intestinal flora can be altered by surgical stress³⁴. Plasma will be collected prior to surgery and on post-operative days 1,

3, and 5, in order to determine the concentration of markers for enterocyte damage (eg, I-FABP, citrullin, and calprotectin), transmural ischemia (e.g, smooth muscle protein 22), and general inflammation markers (eg, CRP and leukocyte count).

In addition, SNAQ and Malnutrition Universal Screening Tool scores are obtained to assess the nutritional status of the patient, and CT-images are analyzed for total skeletal muscle cross-sectional area in order to distinguish sarcopenic patients from those in good nutritional health²⁵. Generalized atherosclerosis will be assessed using abdominal CT-scans, as this is a proposed risk factor for AL^{35,36}. Finally, a tissue sample will be obtained from the resected specimen, on which conventional and immunohistochemistry staining will be performed.

Statistical analyses

The sample size is calculated with a power analysis and is aimed at our main study outcome, AL. From literature, we know that approximately 10% of all colorectal patients receiving an anastomosis will develop AL^{14,37}. The aim of the study is to detect significant and clinically relevant differences between the AL group and the non-AL group. Therefore, we used data from a previous study undertaken by our group to determine sample size²⁰. We chose our least significant finding, to avoid the study being underpowered. Based on an effect size of 0.41 (calculated with mean calprotectin levels and SD on day 1), with a power of 0.80 and a 95% confidence interval, the total sample size will be 560, of whom an estimated 51 patients will develop AL. Assuming a 5% dropout rate, the total number of patients that needs to be included in this study is 588.

After data collection, normality will be tested by the D'Agostino-Pearson test. Student's t-tests will be used for between-group comparisons for continuous data. Dichotomous variables will be compared using Pearson's chi-squared test. All data will be presented as mean and standard error of the mean. The area under the curve of receiver operating characteristic (ROC) curves will be used to calculate the diagnostic ability of the studied markers predicting AL. The area under the ROC curve is a summary measure of accuracy, lying in the range from 0.5 to 1, with 1 indicating perfect discrimination and 0.5 indicating no discrimination capacity. To determine the most ideal combination of markers, logistic regression analyses will be performed.

Results

This study has been open for inclusion since August 2015.

Discussion

Despite important improvements in peri-operative care for CRC surgeries and increased awareness of AL, incidence rates of this dreaded complication have remained stable for several decades. Most strikingly, leakage continues to occur in patients treated under the most expert care, without the presence of any known risk factors³⁸. The lack of knowledge regarding the pathophysiological process of AL and the process of normal intestinal healing hampers the development of novel predictive and preventive methods. AL has significant impacts on morbidity and mortality, quality of life, and health care costs, and is suggested to negatively interfere with oncological prognoses⁵⁻⁸. Although large numbers of clinical trials have identified important patient-related and technical factors that aid in the prediction of AL^{38,39}, the search for a predictive biomarker for AL has hitherto remained unsuccessful. Almost all previously conducted studies reported a single risk factor for the development of AL in CRC patients undergoing surgery. The multi-modal design of the current study enables us to bypass the limitations encountered by previous studies.

In order to minimize the potentially life threatening clinical consequences of a leak, some surgeons opt to perform a deviating ostomy directly following the initial operation⁴¹. Although it is generally accepted that the presence of a colostoma or ileostoma reduces the sequelae of AL and the need for reoperation in case of a leak^{42,43}, a decrease in incidence of post-operative mortality has not yet been proven⁴²⁻⁴⁶. The presence of selection bias, in which an ostomy is performed in high-risk patients, should be considered when interpreting these results. Surgeons perform elective deviating ostomy in approximately 70% of cases of low rectal carcinoma, of which a significant percentage (19-40%) will never have their temporary ostomy reversed^{47,48}. Possible benefits of a deviating stoma should be weighed against stoma-related morbidity, the impact on quality of life, and the mortality rates after stoma closure^{49,50}. An adequate pre-operative risk analysis could aid surgeons and their patients in the decision-making process regarding the construction of temporary ostomies. The successful implementation of risk assessment tools would have a positive influence on morbidity and mortality rates, duration of hospital stays, and number of readmissions, re-interventions, and admissions to the ICU, leading to a significant increase in quality of life

for the general patient population³⁸. Anastomoses are also constructed in gastrointestinal surgery for other purposes than malignancies, such as inflammatory bowel disease or diverticulitis^{51,52}. We have chosen to exclude these patients since their inflammatory status can be a confounding factor in the early detection and/or risk assessment of AL⁵³⁻⁵⁵.

This study outline is based on the hypothesis that AL is partly due to patient-derived factors such as a derailed immune response, genetic predisposition, and a deficient microbiome composition, and that the clinical course can be further influenced by surgical stress, ischemia, and a compromised systemic response. This study aims at broadening our understanding of the pathophysiological process of AL by introducing novel biomarkers of intestinal damage and function, and to decrease the clinical burden of AL by both individual pre-operative risk assessment and early post-operative detection in the future.

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Chapter 5

Near-infrared fluorescence imaging-guidance in
anastomotic colorectal cancer surgery and its relation to
serum markers of anastomotic leakage:
a clinical pilot study

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Abstract

Objective

Near-infrared fluorescence (NIRF) imaging using indocyanine green (ICG) might help reduce anastomotic leakage (AL) after colorectal surgery. This pilot study aims to analyze whether a relation exists between measured fluorescence intensity (FI) and postoperative inflammatory markers of AL, C-reactive protein (CRP), Intestinal fatty-acid binding protein (I-FABP), and calprotectin, to AL, in order to evaluate the potential of FI to objectively predict AL.

Methods

Patients scheduled for anastomotic colorectal cancer surgery were eligible for inclusion in this prospective pilot study. During surgery, at three time points (after bowel devascularization; before actual transection; after completion of anastomosis) a bolus of 0.2 mg/kg ICG was administered intravenously for assessment of bowel perfusion. FI was scored in scale from 1 to 5 based on the operating surgeon's judgment (1=no fluorescence visible, 5=maximum fluorescent signal). The complete surgical procedure was digitally recorded. These recordings were used to measure FI postoperatively using OsiriX imaging software. Serum CRP, I-FABP, and calprotectin values were determined before surgery and on day 1, 3, and 5 postoperative; furthermore, the occurrence of AL was recorded.

Results

Thirty patients (n=19 males; mean age 67 years; mean BMI 27.2) undergoing either laparoscopic or robotic anastomotic colorectal surgery were included. Indication for surgery was rectal-(n=10), rectosigmoid-(n=2), sigmoid-(n=10), or more proximal colon carcinomas (n=8). Five patients (16.7%) developed AL (n=2 (6.6%) grade C according to the definition of the International Study group of Rectal Cancer). In patients with AL, the maximum fluorescence score was given less often ($P=0.02$) and a lower FI compared to background FI was measured at 1st assessment ($P=0.039$). However, no relation between FI and postoperative inflammatory parameters could be found.

Conclusion

Both subjective and measured FI seem to be related to AL. In this study, no relation between FI and inflammatory serum markers could yet be found.

Introduction

Anastomotic leakage (AL) is the most feared complication in colorectal (cancer) surgery. The severity of leakage may vary from a contained peri-anastomotic fluid collection to generalized peritonitis. Furthermore, long-term morbidity including stricture formation, bowel dysfunction, and an increased chance of locoregional cancer recurrence has been described¹⁻³.

Clinically apparent anastomotic insufficiency occurs in 5.6–11.9% of all colorectal resections⁴⁻⁶. As reported in literature, up to 32% of patients with AL die because of this postoperative complication⁴. These numbers show the urgent need to prevent AL. Prevention starts with eliminating provoking factors. Decreased blood perfusion at the anastomosis is such a factor that contributes to anastomotic leakage⁷⁻⁹. Therefore, it is hypothesized that a better visualization of the vascularization of the anastomosis will aid in preventing anastomotic leaks.

Intra-operatively, the selection of an optimal site for anastomosis with adequate perfusion is now dependent on subjective clinical indicators such as colour of the bowel, bleeding of the resection margins, and palpable or visible pulsations of the mesenteric arterial vessels (i.e., the clinical judgement of the surgeon). Karliczek et al.⁸ discussed that there is a lack of a reliable intraoperative predictive test for AL by the operating surgeon.

Earlier studies used Doppler ultrasound and laser Doppler to assess colorectal anastomotic vascularization⁹. Peri-anastomotic tissue oxygenation has been evaluated, but has not been incorporated in routine practice due to the complexity and limited effectivity of the technique¹⁰. There is need for a technique that can accurately and consistently assess bowel perfusion at the anastomotic site in real time.

Near-infrared fluorescence (NIRF) imaging allows surgeons to visualize the micro-perfusion of the bowel in real time¹¹. This technique, with perioperative indocyanine green (ICG) administration, has been shown feasible in assessing the vascularization of the bowel to be anastomosed. Fluorescence angiography with perioperative ICG administration seems to improve the outcome of laparoscopic anastomotic bowel surgery, in terms of safety and efficiency^{11,12}. However, in studies confirming the lower incidence of AL, no objective quantification of the signal was performed^{1,2,7,13-15}. Such quantification is desired to make the technique a reliable tool to accurately and consistently assess bowel perfusion at the anastomotic site in real time.

C-reactive protein (CRP) is an early predictor of postoperative infection and has been shown to correlate with AL¹⁶⁻¹⁸. Intestinal fatty-acid binding protein (I-FABP) is a marker for enterocyte damage^{19,20}. Earlier research showed a significant higher I-FABP at day 3 postoperatively in patients with AL²¹. In the same study, calprotectin levels were elevated on day 2 up to day 5 in patients with AL.

In this pilot study, we aim to analyze whether a relation exists between the objectively quantified signal of ICG fluorescence angiography during laparoscopic or robotic colorectal surgery and the levels of the serum markers related to anastomotic leakage: CRP, I-FABP, and calprotectin, in order to evaluate the potential of FI to objectively predict AL. To our knowledge, this is the first effort to assess the existence of such a correlation.

Materials and methods

All patients provided written informed consent. This study was approved by the Medical Ethical Committee of Maastricht University Medical Centre and conducted according to the Declaration of Helsinki (October 2013, Fortaleza). Included patients were part of the REVEAL study²², which is registered at clinicaltrials.gov (NCT02347735).

Study subjects

Colorectal cancer patients scheduled for either laparoscopic or robotic anastomotic surgery were eligible for inclusion. Patients with inflammatory bowel disease were excluded because this could influence plasma levels of the inflammatory markers. Additional exclusion criteria were known iodine or indocyanine green hypersensitivity, impaired renal function (eGFR<45), pregnancy or breast-feeding (all are contraindications for the use of ICG), and inability to give informed consent. None of the included patients used non-steroidal anti-inflammatory drugs (NSAIDs) or corticosteroids. Patient characteristics (sex, age, BMI, medical history, tumour location) and surgical characteristics (type of surgery, anastomotic technique, ostomy, operation time, conversion rate) were collected.

Surgical technique

The surgical procedures were performed by experienced laparoscopic colorectal surgeons and started as usual in white light. In laparoscopic cases, a system equipped with fluorescence imaging (Karl Storz GmbH & CO. KG, Tuttlingen, Germany) with a

xenon-based light source (D-light P) was used and in robotic surgeries, the da Vinci® System with Firefly fluorescence imaging (Surgical Intuitive, Sunnyvale, United States of America) was used. Surgery was performed as usual except for three time points, at which a bolus of 0.2 mg/kg ICG (Verdye; Diagnostic Green Aschheim, Germany) was administered. These three set time points were:

1. Directly after transection of all the vessels supplying the bowel segment to be resected (i.e., after devascularization of the segment).
2. Just before actual transection.
3. Directly after the anastomosis is made.

After each gift of ICG, the system was switched to NIRF mode and the surgeon was asked to rate the fluorescence intensity of the bowel both proximal and distal to the planned proximal transection line or stapler line on a scale from 1 to 5. Herein, a score of 1 meant no fluorescent signal, while a score of 5 meant 'maximum fluorescent signal.' Also, the surgeon was asked whether he or she changed the location of transection based on the visualized fluorescent signal.

When proximal transection was performed extra-corporally, the operative room was completely darkened in order to capture the fluorescent signal with the respective imaging device.

Fluorescence intensity measurement

Postoperatively, the images of the bowel were assessed with OsiriX imaging software (Pixmeo, Geneva, Switzerland) to quantify the FI. This software measures the gray value in arbitrary units (AU). At the aforementioned three time points, the FI was measured approximately 1 cm proximal and 1 cm distal from the proximal transection point or anastomosis and of the background (abdominal wall or fat directly surrounding the relevant bowel part) in a screenshot taken approximately 1 min after ICG administration. In the right hemicolectomies, the distal transection point was used. In case of extracorporeal proximal transection, also three points in the direct surrounding of the bowel were measured as background. The target-to-background ratio (TBR) was defined as the mean FI in arbitrary units (AU) of the bowel wall 1 cm proximal or distal from the planned proximal (or in right hemicolectomies distal) transection location or 1 cm from the staple line of the anastomosis minus the FI of the background divided by the mean FI in the background (abdominal wall or fat directly surrounding the relevant bowel part).

Definition anastomotic leakage

The patients were followed up to 30 days after surgery, during which the occurrence of complications and especially AL was recorded. Clinically relevant AL was defined and classified according to the definition from the International Study group of Rectal Cancer²³. In this definition, AL is a defect of the anastomotic wall integrity at the colorectal or colo-anal anastomotic site leading to a communication between the intra-and extraluminal compartments. A pelvic abscess close to the anastomosis is also considered AL. We applied this definition also to the included colocolic and ileocolic anastomoses in our study. A distinction is made between the severity of AL based on the intervention required:

- Grade A: Anastomotic leakage requiring no active therapeutic intervention;
- Grade B: Anastomotic leakage requiring active therapeutic intervention but manageable without relaparotomy;
- Grade C: Anastomotic leakage requiring relaparotomy²³.

Analysis of inflammatory markers

Venous blood was drawn at hospital admission (preoperative sample) and on day 1, 3, and 5 after surgery, unless the patient was discharged home earlier. C-reactive protein (CRP) plasma levels were determined by using Immunoturbidimetric assay for the in vitro quantitative determination of CRP in human serum and plasma (Roche diagnostics, Germany). Serum calprotectin concentration was determined using a commercially available calprotectin ELISA (Hycult Biotechnology, Uden, The Netherlands). Intestinal fatty-acid binding protein (I-FABP) plasma levels were determined using an in-house ELISA that selectively detects human I-FABP²¹.

Statistical analysis

Basic descriptive statistics were applied to calculate means and standard deviation of the baseline characteristics. Because of the small sample size, non-parametric tests were used to analyze the data retrieved in this study. To compare continuous variables between two groups, the Mann–Whitney test was performed. To compare categorical outcomes between two groups, a Pearson Chi-square crosstab was used. The relation between the measured fluorescence intensity and the values of the inflammatory markers was evaluated using a regression analysis. $P < 0.05$ was considered to denote statistical significance. All statistical analyses were performed with SPSS version 23 (IBM, Armonk, NY, USA).

Results

Study population

Thirty patients were included with a mean age of 67.2 (SD 8.2) years of whom 19 (63%) were male. All underwent elective surgery for colorectal cancer, of which 10 (33.3%) concerned sigmoid carcinoma, 10 (33.3%) rectum carcinoma, 5 (16.7%) colon carcinoma in the right hemi-colon, 3 (10%) cecum carcinoma, and 2 (6.7%) rectosigmoid carcinoma. Five patients were treated with concomitant chemoradiotherapy before surgery, 4 with only chemotherapy (CAPOX–bevacizumab), and 3 received only radiotherapy (5 × 5 Gy). A conversion to open surgery was needed in 7 patients in whom a laparoscopic procedure was started, due to unclear anatomy in obese patients. Further baseline characteristics and surgical characteristics are summarized in Table 5.1.

Table 5.1 Baseline characteristics and surgical characteristics.

	n (%)	Mean (SD)
Male	19 (63.3)	
Age, years		67.2 (8.2)
Weight, kg		82 (16.4)
BMI, kg/m ²		27.2 (4.9)
Smoking	12 (40)	
Both preoperative chemo- and radiotherapy	5 (16.6)	
Preoperative chemotherapy	4 (13)	
Preoperative radiotherapy	3 (10)	
Location of suspected tumor		
Cecum	3 (10)	
Right hemi colon	5 (16.7)	
Sigmoid	10 (33.3)	
Recto-sigmoid	2 (6.7)	
Rectum	10 (33.3)	
Type of surgery		
Laparoscopic right hemicolectomy	8 (26.7)	
Laparoscopic sigmoid resection	11 (36.7)	
Laparoscopic low anterior resection	4 (13.3)	
Robot-assisted low anterior resection	7 (23.3)	
Deviating stoma	11 (36.7)	
Type of anastomosis		
Side-to-end	16 (53.3)	
End-to-end	9 (30)	
Side-to-side	5 (16.7)	
Conversion to open surgery	7 (23.3)	

Near-infrared fluorescence imaging

A bolus of 0.2 mg/kg ICG was administered intravenously. Thus, the total dose ranged between 10.3 and 21.8 mg with a mean of 16.4 mg (SD 3.3). NIRF imaging was aimed to be performed at three time points. In 19 patients, fluorescence imaging was achievable at all three time points. In 10 patients, the first time point was very close to the second time point; therefore in these patients only just before transection (second time point) and after completion of the anastomosis, the bowel was assessed using NIRF imaging, in order to prevent potential accumulation of the dye at the second time point. In two patients, the anastomosis was too low to visualize the NIRF signal. The time between the first and second time point was on average 68.6 min (range 9-201) and between the second and third time point on average 40 min (range 11-84).

Intraoperative decision making based on fluorescence imaging

In six patients, the dissection location was changed, based on the fluorescent signal. One of these patients developed AL; the remaining 5 patients did not.

Anastomotic leakage (AL)

In 5 patients (16%), AL occurred. In 2 of these cases, only a small abscess around the anastomosis was seen on CT scan and could be treated non-surgically with intravenous administration of antibiotics and CT-guided drainage. These leakages were considered as grade B AL. In 1 patient, AL could be treated with an endo-sponge applied via endoscopy and was also considered as Grade B AL. The remaining 2 patients (6% of the total study population) needed relaparotomy for AL treatment (grade C). All patients with AL underwent a low anterior resection of a rectum or rectosigmoid carcinoma. Two of the AL patients had undergone preoperative chemoradiotherapy: one preoperative radiotherapy and one preoperative chemotherapy. The remaining patient was not preoperatively treated with neoadjuvant radiotherapy or chemotherapy. There were no statistical differences in age at the time of surgery, preoperative chemotherapy, BMI, administered dose of ICG per bolus (mg), conversion to open surgery, type of anastomosis, TNM classification after surgery, and difficulty of surgery between the patients who developed AL and the patients who did not. There were significantly more smokers in the AL group ($P=0.046$). The postoperative hospital stay was on average 16 days (median 12 days) in the patients with AL, which was significantly longer compared to the patients without this complication, who stayed on average 5 days ($P=0.004$). A deviating ileostomy had been constructed more often in the AL group. See Table 5.2 for an overview of these characteristics.

Other postoperative complications were postoperative ileus in one patient, which resolved without further consequence. The two patients who needed relaparotomy due to anastomotic leakage both developed pneumonia after the second surgery, which was treated with antibiotics.

Table 5.2 Characteristics of patients with anastomotic leakage compared to those without.

	No AL (n=25)	AL (n=5)	P-value
Male n	15 /25	4 /5	0.397
Age, years (median (IQR))	65.5 (11.5)	65.0 (17)	0.787
BMI, kg/m ² (median (IQR))	28.3 (5.27)	24.4 (14.1)	0.627
Smoking n	8 /25	4 /5	0.046*
Preoperative chemoradiation	3/25	2/5	0.183
Preoperative chemotherapy	3 /25	1/5	0.538
Preoperative radiotherapy	2/25	1/5	0.433
Indication for surgery			0.058
Cecum carcinoma	3	0	
Right hemi colon carcinoma	5	0	
Sigmoid carcinoma	10	0	
Rectosigmoid carcinoma	1	1	
Rectum carcinoma	6	4	
Type of surgery			0.107
Laparoscopic right colectomy	8	0	
Laparoscopic sigmoid resection	10	1	
Laparoscopic low anterior resection	2	2	
Robotic low anterior resection	5	2	
Change of dissection place	5 /25	1 /5	1.000
Ileostomy	7/25	4/5	0.028*
Type of anastomosis			
Side-to-end	13	3	
Side-to-side	9	0	
End-to-end	3	2	
Conversion to open surgery	6 /25	1 /5	0.847
Surgical time, minutes (median (IQR))	203.5 (95)	287.0 (358)	0.344
Postoperative hospital stay, days (median (IQR))	4 (2.25)	12 (22)	0.004*

Subjective fluorescence intensity (FI)

The FI was scored by the surgeon on a scale from 1 to 5. In all cases, in all three assessments, this score ranged between 3 and 5 for the bowel proximal to the proximal transection site, or distal transection site in right hemicolectomies. In the second assessment, the subjective score given for the FI was 4 in four of the anastomotic leakage cases. In one of the anastomotic leakage cases, a score of 5 was given; this was one of the cases in which only an abscess close to the anastomosis was present. In the patients who did not develop AL the maximum score of 5 was given in 16 cases (73%), which was significantly more often compared to the AL group ($P=0.02$).

In both the first and third assessment, no significant differences in subjective fluorescence intensity between the patients with and without anastomotic leakage were seen. See Table 5.3 for the given scores.

Table 5.3 Subjective fluorescence intensity (FI) scores and anastomotic leakage (AL).

		FI score 3	FI score 4	FI score 5	Total no of patients assessed
1 st assessment	No AL	1	5	10	16
	AL	0	1	3	4
2 nd assessment	No AL	3	4	18	25
	AL	0	4	1	5
3 rd assessment	No AL	2	11	11	24
	AL	1	1	2	4

The fluorescence intensity on first and second assessment is scored on the distal side of the distal anastomosis in laparoscopic right hemicolectomies, whilst on the proximal site of the proximal anastomosis in all remaining procedures.

Measured fluorescence intensity

Next to subjective scoring by the surgeon, the FI of the bowel proximal and distal from the proximal transection or distal in right hemicolectomies and the background was measured by image quantification. As shown in Tables 5.4, 5.5, 5.6, and 5.7, the FI in patients without AL was higher at almost all measurements compared to the FI in patients who develop AL. This difference is however only statistically significant when we take the background into account and assess the bowel distal from the proximal anastomosis in the 1st assessment ($P=0.039$) (see Table 5.7).

Table 5.4 Fluorescence intensity (FI) bowel proximal from the proximal transection line and anastomotic leakage (AL) (distal from distal transection line in right hemicolectomies, thus side from the transection line that stays in the patient).

	Median FI no AL	Median FI AL	P-value
1 st assessment	38.8 (61.2)	34.2 (28.7)	0.494
2 nd assessment	49.2 (45.0)	44.1 (94.6)	0.385
3 rd assessment	86.5 (54.8)	99.5 (68.1)	0.336

Median fluorescence intensity (interquartile range).

Table 5.5 Fluorescence intensity (FI) bowel distal from the proximal transection-line and anastomotic leakage (AL) (proximal from the distal transection line in right hemicolectomies, thus side from the transection line that will be resected).

	Median FI no AL	Median FI AL	P-value
1 st assessment	31.7 (23.7)	36.7 (27.1)	0.064
2 nd assessment	40.8 (32.4)	64.5 (72.8)	0.448
3 rd assessment	59.9 (37.6)	55.9 (30.5)	0.952

Median fluorescence intensity (interquartile range).

Table 5.6 Target to background ratio (TBR) bowel proximal from the proximal transection line and anastomotic leakage (AL) (distal from distal transection line in right hemicolectomies, thus side from the transection line that stays in the patient).

	Median TBR no AL	Median TBR AL	P-value
1 st assessment	2.8 (4.7)	1.1 (1.4)	0.820
2 nd assessment	2.2 (13)	0.5 (2.1)	0.229
3 rd assessment	1.4 (2.6)	1.4 (1.0)	0.669

Median fluorescence intensity (Interquartile range).

Table 5.7 Target to background ratio (TBR) bowel distal from the proximal transection line and anastomotic leakage (AL) (proximal from distal transection line in right hemicolectomies, thus the side from the transection line that will be resected).

	Median TBR no AL	Median TBR AL	P-value
1 st assessment	3.4 (6.9)	1.2 (1.3)	0.039
2 nd assessment	2.6 (13.5)	1.8 (4.0)	0.482
3 rd assessment	1.4 (2.9)	0.9 (0.1)	0.364

Median fluorescence intensity (interquartile range).

Inflammatory serum markers

As shown in Table 5.8, CRP was significantly higher on postoperative day 5 in anastomotic leakage patients. As is shown in Table 8, all other measured markers do not show a statistical difference between the occurrence of anastomotic leakage and serum levels on day 1, 3, or 5 (Table 5.8).

Table 5.8 Serum markers anastomotic leakage versus no anastomotic leakage.

	No anastomotic leakage	Anastomotic leakage	P-value
Mean CRP			
Pre-operative (mg/ml) $\pm$ SD	2.9 $\pm$ 2.3	2.1 $\pm$ 0.9	0.69
Postoperative day 1 (mg/ml) $\pm$ SD	87.4 $\pm$ 35.8	87.4 $\pm$ 35.3	0.90
Postoperative day 3 (mg/ml) $\pm$ SD	101.1 $\pm$ 48.0	183.6 $\pm$ 123.5	0.09
Postoperative day 5 (mg/ml) $\pm$ SD	48.5 $\pm$ 24.66	152.8 $\pm$ 90.7	0.01*
Mean Calprotectin			
Preoperative (ng/ml) $\pm$ SD	822.9 $\pm$ 765.4	3451.6 $\pm$ 3987.1	0.41
Postoperative day 1 (ng/ml) $\pm$ SD	7587.3 $\pm$ 17589.9	7743.5 $\pm$ 12026.8	1.0
Postoperative day 3 (ng/ml) $\pm$ SD	2467.0 $\pm$ 1430.3	1683.3 $\pm$ 1549.4	0.15
Postoperative day 5 (ng/ml) $\pm$ SD	3326.7 $\pm$ 2783.0	2756.1 $\pm$ 1974.3	0.68
I-FABP			
Preoperative (pg/ml) $\pm$ SD	805 $\pm$ 376.6	520.1 $\pm$ 516.1	0.16
Postoperative day 1 (pg/ml) $\pm$ SD	644.7 $\pm$ 370.4	383.2 $\pm$ 160.4	0.14
Postoperative day 3 (pg/ml) $\pm$ SD	574.8 $\pm$ 431.4	373.9 $\pm$ 335.3	0.31
Postoperative day 5 (pg/ml) $\pm$ SD	496.9 $\pm$ 291.4	143.0 $\pm$ 49.6	0.05

Fluorescence intensity in relation to inflammatory serum markers

The relation between the respective serum markers CRP, Calprotectin and I-FABP, and the fluorescent signal was assessed using a regression analysis. For none of the serum markers on any time point, a significant relation could be found with the fluorescence both proximal and distal or measured as TBR, with one exception. At the third time point, after the anastomosis is made, a higher TBR of the distal part of the anastomosis was related to a lower serum calprotectin level on postoperative day 5 ($P=0.046$).

Discussion

NIRF angiography with ICG is used more and more in colorectal surgery as this technique holds the potential to lower the risk for anastomotic leakage¹¹. However, it is unknown when the bowel is fluorescent enough to actually predict optimal anastomotic healing. In this respect, a cut-off value is desirable. To come to such consensus, we first need to know whether a “more fluorescent bowel” actually is indicative for a lower risk for anastomotic leakage and accompanying lower postoperative inflammatory parameters. Therefore, we performed this clinical pilot study, in which we aimed to correlate the signal strength of fluorescence angiography with the occurrence of AL and with the level of the serum markers that are thought to be predictive of AL: CRP, calprotectin, and I-FABP. The fluorescence intensity was measured after administration of 0.02 mg/kg ICG intravenously at three time points. CRP, calprotectin and I-FABP were determined preoperatively and on day 1, 3, and 5 after surgery. Follow-up for anastomotic leakage was until 30 days postoperatively.

In the current study, 5 (16.7%) patients developed AL. Three of these leakages were considered a grade B leakage, while the remaining two were grade C anastomotic leakages which needed relaparoscopy. The number of patients with AL is somewhat higher compared to the literature (5.6–11.9%)^{4–6}, and especially when compared to other studies in which NIRF angiography is used during colorectal surgery¹¹. This could be explained by the fact that both grade B and grade C anastomotic leakage were taken into account in this study, while other studies may have only considered grade C leakage only. In addition, 40% of patients smoked, which is a risk factor for developing AL^{4,24}. Remarkably, preoperative radiotherapy, which is also a known risk for leakage was not identified as such in our study. The limited number of patients may have contributed to this discrepancy.

In all cases, the subjective score for FI proximal from the (planned) proximal transection line or distal from distal transection line (in right hemicolectomies), ranged between 3 and 5 on a scale from 1 to 5. For the bowel proximal from the transection line, the maximum score of 5 was given significantly more often in the patients that healed without a leak. This is in line with the study by Ris et al.² in which the fluorescence score ranges from 'good,' 'average,' to 'bad.' In all patients, the fluorescence was scored 'good' by the surgeon and none of the patients developed AL. Other studies scored the fluorescence intensity as 'good enough' or 'not good enough' based on subjective score of the surgeon. In these studies, if the fluorescence intensity was considered not good enough, the dissection place was changed^{3,25-29}. The anastomotic leakage rate in patients with changed dissection level was equal^{13,25-27,29} or higher²⁸ compared to the patients in which no change was needed after NIRF imaging. A possible explanation for a higher leak rate after change of transection location may be the fact that a clearly insufficiently perfused bowel end may be indicator of a longer segment of moderate perfusion. This emphasizes the need for cut-off values for level of perfusion¹¹. In the current study, revision of surgical plan based on the FI of the bowel occurred in six patients. One of these developed AL. These numbers are too low to draw conclusions.

In the present study, the objectively measured FI was higher in the patients who did not develop AL. This difference was only significant when taking the background into account and expressing data as TBR. By using TBR, a correction is made for the distance between the endoscope and the bowel. A shorter distance between the laparoscope and the target causes a higher FI³⁰. By taking the background into account, this effect is minimized, since the background will also show increased fluorescence intensity when holding the laparoscope closer to the tissue. Such correction of the signal seems necessary, as in practice, it is not always possible to exactly measure and standardize the distance to the bowel.

According to literature, the CRP trajectory is highly predictive for AL⁶. In the current series, CRP on postoperative day 5 was significantly higher in patients who developed AL. This observation is in line with findings reported in literature. An earlier study by Reisinger et al.²¹ showed a rise in postoperative CRP in patients with an anastomotic leak, which was only significant on day 4. Bigin et al.³¹ showed a significant higher CRP on postoperative day 3, but unfortunately CRP was not measured on postoperative day 5. Reynolds et al.¹⁷ found a significantly higher CRP level in patients with grade C AL on day 5 up to day 7. Unfortunately, in the current study, no relation of CRP and subjective or measured fluorescence intensity or TBR could be found.

Calprotectin is a non-specific marker for acute inflammation³². An earlier study by Reisinger et al.²¹ showed higher calprotectin levels on postoperative day 2, 3, 4, and 5 in patients who developed AL. Similar results were found by Cikot et al.³³. In the current study however, no statistical difference in serum calprotectin levels could be found between patients with and without anastomotic leakage on postoperative day 1, 3, or 5. Interestingly, serum calprotectin levels on postoperative day 5 were negatively correlated with TBR of the distal part of the anastomosis. This relation needs to be confirmed in a larger study to determine its clinical relevance.

I-FABP is an intestinal cell damage marker²¹. An earlier study by Reisinger et al.²¹ showed significantly higher I-FABP levels preoperatively in patients who developed anastomotic leakage. The I-FABP levels remained higher in these patients after surgery, but only statistically significant on postoperative day 3. In the current study however, I-FABP levels were lower at all time points in the patients who developed anastomotic leakage, although this difference was not significant. Moreover, we could not detect a significant relation between FI and serum I-FABP values in this study.

A possible explanation for this discrepancy might be the low specificity of I-FABP. This marker for enterocyte damage is not only elevated in case of inflammation, but also in patients with colorectal cancer compared to patients without colorectal cancer³⁴. When only taking the patients with a grade C anastomotic leakage into account, a (non-statistically significant) higher I-FABP value is found in the leakage patients. More research is needed to determine its place in the management of the colorectal operative patient.

Higher fluorescence intensity was found in all cases in the bowel at the side that will remain in the patients after surgery. This means that the devascularized bowel results in a lower signal, indicating that the fluorescent signal does reflect bowel perfusion.

This study has some limitations. First, it was designed as a pilot study to explore whether there is a potential relation between FI and inflammatory serum markers that correspond with AL. A relatively small and heterogenous group of patients was included. With the markers and the patient group chosen here, it was not yet possible to identify a cut-off value of fluorescent signal that indicates an optimal anastomotic healing potential. A more homogenous patient group (e.g., only right hemicolectomies or rectal resections) might have given different results. Another factor of influence could be that only the measurements at single time points were performed, and not an evaluation of the signal intensity over time after administration of the respective boluses of ICG. The

small number of included patients can be an explanation for finding few statistical significant differences for most outcome parameters.

In this study, several time points of ICG administration were chosen. Although arterially the dye washes out very quickly—which is evident when observed with the naked eye—it cannot be ruled out that some retention of the dye occurred. Although this phenomenon will take place in both the bowel wall and the background, this may have influenced the objective measurements. The time between the first and second dose administration was on average 68.6 min (range 9-201) and between the second and third bolus of ICG 40 min (range 11-84). A cut-off value for the optimal interval is not known in literature.

In conclusion, both subjective and measured FI seem to be related to the occurrence of AL. In this pilot study, no relation between FI and inflammatory serum markers could yet be found nor a cut-off value of FI predicting AL. A larger study based on the current study, with a single time point of measurement in a homogenous population seems preferable to enhance the chance that an objective cut-off value of the fluorescent signal to predict AL will be identified. Ideally, future research identifies such cut-off value, but also leads to presenting the objective result of fluorescence measurement real time to the surgeon, thereby supplying the surgeon with essential per-operative information for the optimal transection point to prevent anastomotic leakage.

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Part III

Considerations regarding perioperative care of the
colorectal surgery patient

Chapter 6

Contrast enema vs. colonoscopy prior to temporary
stoma reversal in rectal surgery; results of a national
snapshot study

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In preparation

Abstract

Introduction

Colorectal anastomotic leakage (CAL) is the most feared complication following surgery of the colon and rectum. Surgeons may choose to construct a deviating ostomy to diminish the clinical sequelae of a leak. Prior to temporary stoma closure, it is common practice to assess the integrity of the anastomosis by either x-ray with contrast enema (CE) or colonoscopy. The aim of this study was to investigate whether CE or colonoscopy is the most accurate tool to assess anastomotic integrity prior to temporary stoma reversal.

Methods

The present study included all patients who underwent rectal cancer resection with the construction of an anastomosis and deviating ostomy in 2011, from a collaborative cross-sectional study performed in 71 Dutch hospitals. From a total of 2095 patients, 997 patients underwent a Low Anterior Resection (LAR) with the construction of an anastomosis. Of these, 260 cases were excluded as no defunctioning ileostomy was constructed. In 584 cases information about the assessment of anastomotic integrity was reported. When taking into account only the deviating ostomies that were reversed, a total of 420 patients were included in the study, of which 285 had their anastomoses assessed using CE, whereas 135 assessments were done by means of colonoscopy.

Results

The majority of constructed deviating stomas were ileostomies (74.3%). The number of anastomotic leaks diagnosed by either modality within 30 days after employment was not significantly different (4.2 vs. 5.2% for CE and colonoscopy, respectively, OR 3.79, 95%CI 0.468-30.606, P=0.21).

Conclusion

No significant difference in the number of CAL cases detected by either CE or colonoscopy could be detected prior to temporary stoma reversal in patients who underwent rectal cancer resection.

Introduction

Colorectal anastomotic leakage (CAL) is a frequently occurring complication after colorectal surgery with an incidence of 8-20% in the Netherlands¹. CAL results in increased morbidity, mortality rates of up to 40% and is even associated with a poor oncological outcome that reflects in a shorter disease free survival and a higher risk of local tumor recurrence²⁻⁷. CAL leads to a fivefold increase in health care costs due to high numbers of reoperation and Intensive Care Unit-admission as compared to a normal postoperative course⁸. Risk factors for anastomotic leakage include: male gender, smoking, obesity, distal anastomosis, tumor size, ASA grade higher than II, diabetes and other comorbidities (including vascular, pulmonary and renal disease), preoperative use of steroid- and non-steroidal anti-inflammatory drugs, longer duration of the operation, emergency surgery and contamination of the operative field^{9,10}. In order to minimize the clinical consequences of such a leak the surgeon may opt to perform a temporary deviating ostomy, which is usually reversed after 8 to 12 weeks³.

Before removal of the temporary ostomy it is common practice to assess the integrity of the previously created anastomosis in order to decrease the risk to develop anastomotic leakage. However, some controversy exists as to which method is the most suitable for this assessment. This is partially due to a paucity of research on the subject. Another complicating factor is a lack of consensus about the definition of anastomotic leakage, making it difficult to compare study results^{11,12}. Two methods used to evaluate anastomotic integrity prior to stoma reversal are contrast enema and colonoscopy¹².

The primary aim of this study is to investigate whether X-ray with contrast or colonoscopy is the most accurate tool to assess anastomotic integrity prior to temporary stoma reversal.

Methods

The data used in this study was obtained from the database of the Dutch Snapshot Research Group (DSRG). In 2015 the DSRG assembled a multi-center, retrospective, cross-sectional database of all the patients undergoing rectal cancer resections in 2011 in collaboration with the Dutch Surgical Colorectal Audit (DSCA). 71 out of 94 hospitals registering in the DSCA participated in the data collection. The retrospectively collected long-term data was subsequently combined with the short-term data that was available from the DSCA registration to form the final dataset.

A total of 2095 patients were included in the original dataset. From this dataset 997 patients were selected who underwent a low anterior resection (LAR) for further

analysis. Of those patients, 737 had received a deviating ostomy either prior to or during the operation. Patients with a primary anastomosis without a defunctioning stoma were excluded. For this specific research question, only patients were included who had undergone assessment of the integrity of the anastomosis prior to temporary stoma reversal, either by means of colonoscopy or x-ray with contrast enema (CE). One hundred and thirty patients of these did not undergo assessment of anastomosis integrity, and information regarding this was lacking in 23 cases. A total of 584 patients did undergo integrity assessment of their anastomosis, of which 34 patients underwent CT with rectal contrast shortly after the index surgery because of clinical symptoms of CAL and were excluded. The final study population existed of a total of 420 patients who had their temporary deviating ostomies reversed (see Figure 6.1 for a flowchart of the selection process).

Figure 6.1 Flowchart of the selection process.

In order to control for the possibility that the diagnosis of CAL was established in any other manner than with the diagnostic modalities that are the focus of this study, only cases of CAL that were diagnosed within 30 days following the diagnostics were included.

The following data were collected: patient characteristics, type of operation and anastomosis, presence of deviating ostomy, type of ostomy, method of assessing integrity anastomosis, occurrence of postoperative or stoma-related complications, and date of diagnosis CAL.

The mean follow-up after the index surgery was 40,41 months (11,50 months SD).

CAL was defined as the presence of any of the following factors: contrast extravasation on imaging studies, presacral collection requiring surgical or radiological or endoscopic intervention, or a presacral collection that either led to delay in stoma reversal or let to resection or reconstruction of the anastomosis.

Statistics

Statistical significance was defined as $P < 0.05$. A Student's t -test was used to control for baseline differences of continuous variables, whereas the χ^2 test was used for categorical variables. The reported incidence of anastomotic leakage was determined after examination through either colonoscopy or CE prior to stoma reversal. Because the outcomes (i.e. AL or no AL) of the diagnostics tests were not reported in the database, it was not possible to directly calculate the specificity and sensitivity of the tests. Instead, it was chosen to compare the post-test incidence of both diagnostic methods using Odds Ratio. In this particular study, the Odds Ratio is used to examine the correlation between the test used and the incidence of anastomotic leakage. If the Odds Ratio is 1, this indicates that both tests diagnose the same number of cases of anastomotic leakage, thus the tests are of equal value for detecting anastomotic leakage. An Odds ratio that is either larger or smaller than 1 means that during the period following one of the tests groups, a higher incidence of anastomotic leakage was reported. If the confounders are balanced between groups this difference is the result of a difference in diagnostic value, in other words: one of the tests is detecting more cases of anastomotic leakage than the other.

Demographics

A total of 997 patients were identified from the original dataset ($n=2095$) that underwent LAR for rectal carcinoma. The total leakage rate in this cohort was 25.2%. Cases of anastomotic leakage ($n=164$) as well as cases of chronic sinus ($n=87$) were included in these numbers. 737 of the patients from the original database had received a

deviating ostomy either prior to or during the operation. For further analysis, a total of 420 patients were included who received a colorectal anastomosis with construction of a diverting ostomy during the initial operation, and who had undergone assessment of the integrity of the anastomosis prior to stoma reversal.

285 out of these 420 anastomoses (70.3%) were assessed for integrity using radiodiagnostics with contrast enema, while 135 assessments (29.7%) were done by means of colonoscopy. A number of patients were diagnosed with CAL before assessment of integrity of the anastomosis, either by CE (n=16 leaks) or by means of colonoscopy (n=7 leaks). These cases were not taken into account for the analyses regarding the use of the diagnostic tools, as they do not contribute to answering the research question (see Table 6.1 for an overview of timing of diagnosis of the anastomotic leaks).

Table 6.1 Timing of CAL diagnosis.

Timing of CAL diagnosis	Study group	
	Contrast enema n=285 n(% of total leakage)	Colonoscopy n=135 n(% of total leakage)
Prior to integrity assessment	16 (57.1)	7 (50)
<30 days after assessment	8 (28.6)	1 (7.1)
>30 days after assessment	4 (14.3)	6 (42.9)
Total number of leakage	28	14

Of the 285 patients in the contrast enema group, 8 (2.8%) were diagnosed with anastomotic leakage within 30 days after employment of either diagnostic modality, the number of leaks increased to 12 (4.2%) when taking into account all leakages that were diagnosed before stoma reversal. In the colonoscopy group, one out of 135 patients (0.7%) was diagnosed with CAL within 30 days after colonoscopy, these numbers increased to 7 (5.2%) before stoma reversal (Odds ratio within 30 days: 3.79, 95% CI 0.469-30.606, $P=0.21$, Odds ratio total leakages: 0.812; 95% CI 0.313-2.109, $P=0.67$). See Table 6.1 for an overview of the demographics of both study groups.

The mean duration of the interval between assessment of the anastomotic integrity and stoma reversal was 11.2 weeks (SD 13,00) for the CE group, compared to 15,7 weeks (SD 31.73) for the colonoscopy group.

Most of the anastomoses created during the index operation in both groups were side-to-end anastomoses (208 cases; 73.0% vs. 95 cases; 70.1% for the CE and colonoscopy group, respectively), followed by end-to-end anastomoses (62 cases; 21.8% vs. 35 cases 26.0%) and colon pouches in 2 patients of the CE group (0.7%), none of these differences were significant. The majority of these anastomoses were stapled:

267 anastomoses (96.8%) in the enema group and 132 anastomoses in the colonoscopy group (97.8%).

Table 6.2 Patient demographics.

Characteristic	Study group		P
	Contrast enema (n= 285)	Colonoscopy (n= 135)	
Sex			0.931
Male	187 (65.6%)	88 (65.2%)	
Female	98 (34.4%)	47 (34.8%)	
Age (SD, yr)	67.4 (10.6)	70.1 (9.9)	0.013
Follow-up (SD, months)	40.4 (11.6)	40.4 (11.0)	0.991
ASA n (%)			0.887
I	99 (34.7%)	43 (31.9%)	
II	150 (52.6%)	72 (53.3%)	
III	30 (10.5%)	15 (11.11%)	
Comorbidity n (%)	182 (63.9%)	81 (60.0%)	0.597
Anastomosis type n (%)			0.425
Side-to-end	208 (73.0%)	95 (70.4%)	
End-to-End	62 (21.8%)	35 (26.0%)	
Colon pouch	2 (0.7%)	-	
Stoma type n (%)			0.403
Ileostomy	239 (83.9%)	73 (77.8%)	
Colonostomy	26 (9.1%)	17 (12.6%)	
Anastomosis technique n (%)			0.959
Stapled	276 (96.8%)	132 (97.8%)	
Hand-sewn	4 (1.4%)	2 (1.5%)	
Laparoscopy/laparotomy n (%)			0.015
Laparoscopy	154 (54.0%)	75 (55.5%)	
Laparotomy	125 (43.9%)	55 (40.7%)	
Conversion n (%)	21 (7.4%)	9 (6.7%)	0.807
Peroperative complications n (%)			0.949
Bleeding	3 (1.1%)	-	
Intestinal defect	2 (0.7%)	2 (1.5%)	

Results

We examined potential confounders by examining correlation of age, sex, comorbidity and open versus laparoscopic operation with the incidence of anastomotic leakage in both groups. All the variables with a P-value <0.20 at univariate analysis (in this case age and open/laparoscopic surgery) were taken into account in a multivariable analysis. Neither showed a statistically significant correlation with the occurrence of anastomotic leakage in our cohort (results not shown).

Univariate logistic regression revealed an Odds Ratio (OR) of 0.812 (in favor of the colonoscopy) when comparing the odds of anastomotic leakage in the CE group to the

colonoscopy group for the full duration of follow-up (95% CI: 0.313-2.109, $P=0.667$). When taking into account only the cases diagnosed within 30 days after employment of either diagnostic modality, an OR of 3.78 was found in favor of the contrast enema (95% CI: 0.469-30.606, $P=0.21$) (Table 6.3).

Table 6.3 Univariate analysis (Logistic Regression).

Factor	Univariate analysis		P
	Odds ratio	95% CI	
Contrast enema/colonoscopy			
CAL diagnosed < 30 days	3.790	0.469-30.606	0.210
Total CAL cases diagnosed after assessment	0.812	0.313-2.109	0.667

Discussion

Anastomotic leakage remains one of the most dreaded complications after colorectal surgery. Due to patient expectations and technological advancement in the field of colorectal surgery, low rectal and anal anastomoses are constructed in increasing numbers. This has led to the fact that in a rising number of cases, a temporary deviating ostomy is constructed proximal to the anastomotic site¹³. Routine usage of such a deviating ostomy has long been subject to discussion and the general consensus is that this practice does not decrease the incidence of anastomotic leakage¹⁴⁻¹⁶. However, several studies indicate that construction of a temporary ostomy may mitigate the consequences of an anastomotic leak¹⁵⁻¹⁷. Others advise to refrain from the creation of temporary deviating ostomies, because of the complications associated with this procedure¹⁸⁻²⁰. Stoma complications have a reported incidence of up to 30% and range from skin complications and high stoma output to parastomal herniation and stoma prolapse, which often requires reoperation^{14,21}. Furthermore, closure of an ostomy exposes the patient to another operation and its associated complications, including anastomotic leakage. Lastly, up to 20% of all the temporary ostomies will never be reversed, which severely affects Quality of Life (QoL)^{14,21,22}.

Besides the risk of abdominal sepsis that exists upon closure of a temporary stoma in the presence of an incompletely healed anastomosis, Killeen et al. showed that even clinically occult leaks could severely impact bowel-specific QoL²³. A variety of diagnostic modalities can be used to assess the quality of the anastomosis after colorectal surgery. Despite the frequent use of these diagnostic tools prior to temporary stoma reversal, no clear consensus exists on whether routine assessment of the integrity of the

anastomosis is necessary, and which diagnostic tool is most suitable^{3,24-27}. Besides contrast imaging, which is most commonly used, colonoscopy is also employed to assess the quality of the anastomosis prior to stoma reversal, but very little scientific evidence exists for the use of colonoscopy for means other than intraoperative assessment or assessment of cancer recurrence²⁸⁻³⁰. Another important indication for the performance of these tests is to determine the presence of a stenosis at the anastomotic site. In this study we retrospectively compared two most commonly used techniques of assessing anastomotic integrity after colorectal surgery: contrast enema and colonoscopy.

Contrast enema has been reported to have relatively high sensitivity and specificity for colorectal anastomotic leakage in the presence of clinical signs^{12,31}. However, there are several drawbacks to this diagnostic method. It has been shown that with a negative test, reversal is unlikely to result in abdominal sepsis or anastomotic leakage, but, on the other hand, that many cases with an abnormal test result could also be safely reversed^{12,32,33}. Indeed, CE detects a large number of radiological leaks without clinical symptoms - up to 41% - that may not require intervention³¹. These cases may be caused by the accumulation of contrast in the 'dog ears' of the staple line³⁴, or may represent leaks with only very minor anastomotic sinuses, that do not cause any clinical symptoms³². Some authors raised concerns regarding the pressure increase and associated risk of mechanical disruption¹¹, but the use of water-soluble contrast enema is generally considered to be a safe measure to evaluate anastomotic integrity^{11,12,26}. Its routine use prior to ileostomy closure, on the other hand, remains a matter of debate³². Recent evidence suggests that CE has little additional value next to digital rectal examination for the very low anastomoses or next to colonoscopy³².

As stated before, very little literature exists on the routine use of colonoscopy as a means to evaluate the quality of the anastomosis, although it was used relatively frequent in this study population. Several authors however describe its application after LAR to assess the anastomoses that are too high to be examined by digital palpation²⁷. Endoscopic evaluation is generally regarded safe in use, readily available and associated with low costs. An additional benefit of the use of colonoscopy is the detection of – although rare – intraluminal colorectal cancer recurrence, as well as metachronous (or missed synchronous) cancers and advanced adenomas³⁰. It has been shown that these lesions can be found within two years of surgery³⁵. The mean time in this specific patient population between primary surgery and the use of either diagnostic method was relatively long, which would support the use of colonoscopy as a screening tool. Colonoscopy could also aid in the assessment of (benign) anastomotic stenosis, which

occurs in up to 30% of cases³⁶, and the modality can also be deployed to resolve short stenoses by means of balloon dilation³⁷.

One of the main limitations of our study is that, as stated before, the outcomes of the diagnostic tests were not available for analysis. This was due to the retrospective nature of the study. Therefore, to ensure that the diagnosis was established based only on the results from the colonoscopy or contrast enema without any following diagnostic measures, only patients who were diagnosed with colorectal anastomotic leakage within 30 days after examination were included in the study. It cannot be completely ruled out that other additional diagnostic tools were used to establish the diagnosis, which could interfere with the observed differences between our tests of interest, especially when taking into account the full duration of follow-up after either examination. Information regarding complications following the two diagnostic modalities (bleeding, perforation or sensitivity to contrast) was unfortunately not included in the database and could therefore not be taken into account in this study. Another limitation is the lack of insight in the motivation of surgeons to choose for a particular test in a specific case; preferred use of one of the diagnostic tests to examine high-risk cases would lead to an overestimation of the effect of the diagnostic method in our study.

No significant difference was found in the odds of the diagnosis of anastomotic leakage between the two imaging modalities. This may be partially due to the small number of patients that developed anastomotic leakage in both groups (4,2% in the enema group and 5,2% in the colonoscopy group). Univariate analysis did not reveal a significant influence of other risk factors. An Odds ratio of 3,790 for detecting colorectal anastomotic leakage in favor of the contrast enema ($P=0,21$) within 30 days after the examination is a relatively small difference. Furthermore, when taking into account all of the cases of CAL that were diagnosed after use of both diagnostic tools, an OR of 0,667 ($P=0,667$) was found in favor of the colonoscopy. From these results, no preference arises for one of these modalities over the other, leaving the choice between the two diagnostic options to the preference of the surgeon. However, colonoscopy has several additional advantages over contrast enema, as it can also detect intraluminal cancer recurrence, metachronous cancer and anastomotic stricture. As mentioned before, it has been suggested that assessment of the integrity of the anastomosis should be reserved for selected patients with for example a positive intraoperative air leak test and/or complications during the postoperative period. To strengthen these suggestions, the performance of a four-armed RCT with and without routine integrity assessment and with colonoscopy or contrast enema is needed, which would unfortunately require rather large sample size.

Conclusion

No significant difference could be found in the ability of detecting colorectal anastomotic leakage between contrast enema and colonoscopy prior to temporary stoma reversal. An RCT is needed before strong recommendations can be made regarding routine use of either diagnostic tool.

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Appendix S6.1

Supplementary information according to ARRIVE guidelines²⁴

- Muc2^{-/-} mice backcrossed on C57BL/6 background between 8 and 16 weeks of age were used. Muc2^{-/-} mice on the C57BL/6 background were bred as Muc2^{+/-} × Muc2^{+/-} at the Experimental Biomedicine animal facility at the University of Gothenburg, and offspring were genotyped.
- Mice were kept under standard conditions and provided with food and water ad libitum. Mice were housed together with their littermates, with a maximum of six companions per cage. The general health of mice was monitored several times per week for signs of inflammation, and animals were weighed once a week. During the experiment, animals were weighed daily and scored for discomfort twice daily.
- Scoring was performed according Pommergaard *et al.*²⁵:

Parameter	Grading	Score
Activity	Normal/medium/low	2/1/0
Fur	Smooth/fluffy/erect	2/1/0
Eyes	Clean and open/clean and closed/dirty and closed	2/1/0
Able to stand straight	Yes/no	1/0
Posture	Normal/modestly curled/fully curled up	2/1/0
Position on feet	Normal/high	1/0
Solitary	Yes/no	0/1
Shivering	Yes/no	0/1

Scoring was done by one researcher who was blinded for treatment at the time of scoring, while the other researcher performed preparation of the prostaglandin (PG) E₂ or placebo syringes for peritoneal injection.

- Protocols were approved by the government animal ethics committee, and institutional animal use and care guidelines were followed (permit 63-2014).
- Mice were killed by intracardial puncture after isoflurane anaesthesia using an airstream of 2 l/min and 3.5 per cent isoflurane.
- A sample size calculation was conducted before the experiment based on an expected reduction in the anastomotic leakage rate of 20 per cent. To detect a relevant difference in leakage reduction of 20 per cent (δ) between the different experimental groups with a variance of ± 16 per cent (σ), the number of animals per group should be: (power analysis $\alpha = 0.05$, $\pi = 0.8$, 2-sided testing):

$$n = 2(z\alpha/2 - z\pi)^2 * (\sigma/\delta)^2 \text{ (Sachs)}$$

$$n = 2(1.96 + 0.84)^2 * (16/20)^2$$

$$n = 15.7 * 0.64 = 10.03 \approx 11/\text{group}$$

- In total, 52 mice were used in this study. There were 11 animals per experimental group, and four groups (knockout *versus* control mice, with and without 16,16-dimethyl PGE₂ (dmPGE₂) supplementation). In addition, eight mice were killed to obtain control tissue (mesenteric lymph nodes, spleen) for bacterial translocation analysis.
- Animals were matched for age and sex where possible, making it impossible to use randomization procedures.

Part IV

Novel insights in the physiology of anastomotic
healing and leakage

Chapter 7

Comparison of three different application routes of butyrate to improve colonic anastomotic strength in rats

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Abstract

Introduction

Despite extensive research, anastomotic leakage (AL) remains one of the most dreaded complications after colorectal surgery. Since butyrate enemas are known to enhance anastomotic healing, several administration routes have been explored in this study.

Methods

Three intraluminal approaches involving butyrate were investigated: (1) butyrate-elucidating patch, (2) a single injection of hyaluronan-butyrate (HA-But) prior to construction of the proximal anastomosis and (3) rectal hyaluronan-butyrate (HA-But) enemas designed for distal anastomoses. The main outcome was AL and secondary outcomes were bursting pressure, histological analysis of the anastomosis, zymography to detect MMP activity and qPCR for gene expression of MMP2, MMP9, MUC2 and TFF3.

Results

Neither the patches nor the injections led to a reduction of AL in experiments 1 and 2. In experiment 3, a significant reduction of AL was accomplished with the (HA-But) enema compared to the control group together with a higher bursting pressure. Histological analysis detected only an increased inflammation in experiment 2 in the hyaluronan injection group compared to the control group. No other differences were found regarding wound healing. Zymography identified a decreased proenzyme of MMP9 when HA-But was administered as a rectal enema. qPCR did not show any significant differences between groups in any experiment.

Conclusion

Butyrate enemas are effective in the enhancement of colonic anastomosis. Enhanced butyrate-based approaches designed to reduce AL in animal models for both proximal and distal anastomoses were not more effective than were butyrate enemas alone. Further research should focus on how exogenous butyrate can improve anastomotic healing after gastrointestinal surgery.

Introduction

Despite extensive research, anastomotic leakage (AL) remains one of the most dreaded complications after colorectal surgery. It still results in high morbidity and mortality, and causes prolonged hospital stay and high healthcare costs^{1,2,3}. Many studies have been performed regarding this subject, all aiming at improvement of anastomotic healing and thereby preventing leakage. Since the incidence of anastomotic leakage has not declined over the years, the need for a solution continues to exist^{4,5}.

Previous animal studies have shown that rectal administration of butyrate—one of the predominant short chain fatty acids (SCFAs)—results in a higher bursting pressure in colonic anastomoses, thereby claiming an enhanced strength which may prevent anastomotic leakage^{6,7,8}.

These positive results did not change current practice, nor have human studies been reported in which patients received postoperative butyrate enemas to enhance anastomotic strength. This is not surprising, when you consider that clinicians are not keen on enemas directly after surgery, especially when a fragile rectal anastomosis is involved. Water-soluble contrast enemas are often used to determine the presence of colorectal AL, mostly after rectum resection. However, these enemas are never given in the very early postoperative phase (<postoperative day 5) due to the potential risk of complications⁵. Another disadvantage of the use of enemas is the low compliance rate and the short and discontinuous exposure of butyrate to the colonic mucosa.

A recent study however showed that butyrate has remarkable anti-inflammatory and regenerative effects improving the repair of intestinal mucosa in rats with colitis⁹. Butyrate stimulates re-epithelisation and may influence collagen lysis by reducing matrix metalloproteinase (MMP) release¹⁰. Butyrate is a product of the fermentation process by bacteria that occurs mainly in the distal colon. Because it serves as the most important energy source for colonocytes¹¹, our hypothesis is that increasing the concentration of butyrate will lead to an enhanced anastomotic healing in the colon. A number of studies indicate that butyrate affects the composition and thickness of the colonic mucus layer through alteration of mucin gene expression¹² and nutritional deficiency of the colonic epithelium and that butyrate also can act as a signalling molecule through specific G-protein-coupled receptors that are involved in the immune surveillance of the colonic mucosa towards microbial activity¹³. More recently, it has been shown that mice with an insufficient mucus layer on the colonic mucosa (Muc2^{-/-}) suffered more often from anastomotic leakage compared to control mice¹⁴.

Furthermore, animal studies showed that butyrate enemas have a significant positive effect on the left colonic anastomoses, but this effect was weaker on the right side of the colon⁸. Obviously, not the same volume will reach the proximal anastomotic site compared to the distal anastomosis, which may explain these findings. Another explanation might be that the butyrate-producing bacteria mainly reside in the mucus layer at the distal part of the colon, that the proximal colon does not respond to a higher amount of butyrate, simply because the proximal enterocytes are not familiar to having that energy source available and are not able to use it. To increase the beneficial effect of butyrate, we added hyaluronan (HA), a glycosaminoglycan that is known to promote neovascularization, to enhance the process of scarring, and it has beneficial effects on cell proliferation in several tissues^{15,16}. Hyaluronan also plays a role in the downregulation of the inflammatory response. Its free-radical scavenging and antioxidant properties and its supposed inhibiting effect on proteinases such as MMPs seem to be responsible for this downregulation, stabilizing the granulation tissue during the healing process¹⁶.

In this study, we investigated several ways to improve the proximal anastomotic strength by applying butyrate near this anastomosis: using a patch, injections and enemas. Our aim was to develop a method that resulted in a lower anastomotic leakage rate and more practical methods that can be applied in daily clinical practice.

Methods

Animals

Eighty-four Wistar rats with a body weight of 250-300 g were used. Animals were housed and cared for at the Central Animal Facility of Maastricht University. All animals were provided ad libitum access to food and water and were cared for according to local standards. Postoperatively, welfare assessment was performed twice daily using a standardized method and animals were given pain medication in case of discomfort. The experimental protocol complied with the Dutch Animal Experimental Act and was approved by the Animal Experimental Committee of Maastricht University Medical Centre. The ARRIVE guidelines for reporting in vivo experiments were followed¹⁷ (see also Supplementary Data S7.1).

Study design

Primary objective of the study is to investigate if anastomotic leakage can be reduced in a leakage model by application of either an intraluminal butyrate-eluting patch, a single-time intraluminal administration of butyrate or a daily HA-But enema. For the proximal anastomoses, we used both an intraluminal butyrate-eluting patch that covered the anastomosis and a single-time intraluminal administration of hyaluronan-butyrate (HA-But). Since most research is performed in the distal part of the colon, we also used a left-colon anastomosis model to test the HA-But enemas. This resulted in the following three experiments:

- Experiment 1:
Butyrate-eluting patches (20 mass% tributyrin) versus placebo patches (n=12/group)
- Experiment 2:
HA-But injection (5% HA, 60 mM butyrate) versus HA injection (5% HA) versus saline injection (0.9% NaCl) (n=8/group)
- Experiment 3:
Five millilitres of HA-But enema (5% HA, 60 mM butyrate) versus 5 mL sodium butyrate (60 mM) enema versus a control group that received no enemas (n=12/group)

Patches were kindly provided by Polyganics (Groningen, the Netherlands), and enemas and injections were produced by Sigea (Trieste, Italy).

In all experiments, follow up was 7 days.

Surgical procedure

All rats received 0,05 mg/kg buprenorphine as analgesic and were anaesthetised using isoflurane. To acquire access to the abdominal cavity, a 5-cm craniocaudal midline incision of the skin and abdominal musculature was made in all experiments. The cecum was then identified and moved outside of the peritoneal cavity and onto sterile gauzes that were hydrated with sterile saline solution to prevent dehydration. For proximal anastomoses, the colon was transected two centimetres distal from the cecum and an end-to-end anastomosis was created using four interrupted polypropylene 6/0 sutures (Prolene, Ethicon, Johnson & Johnson) (Exp 1& 2, see Supplementary Data S7.1). In experiment 3, the anastomosis was performed in similar fashion but at 4 cm ab ani. After performing the anastomosis, the intestines were repositioned and the abdomen was closed in two layers, a running suture for the muscle layer (Vicryl 4-0, Ethicon, Inc) and interrupted sutures for the skin (Monocryl 4-0, Ethicon, Inc).

Macroscopic endpoints

Anastomotic leakage was defined as (1) no anastomotic leakage, (2) small abscess at the anastomotic site $<1\text{ cm}^3$, (3) large ($>1\text{ cm}^3$) abscess at the anastomotic site or (4) complete dehiscence with peritonitis/death due to faecal peritonitis. Adhesions to the anastomotic site were assessed according to van der Ham et al.¹⁹.

Bursting pressure

Bursting pressure was measured as previously described¹⁹. In short, a 5-cm segment of intestine including the anastomosis with adherent organs was resected en bloc and the part distal of the anastomosis was clamped. A plastic tube was inserted in the proximal end and ligated with a single polyglactine 4/0 suture (Vicryl, Ethicon). The complete anastomosis was immersed in phosphate-buffered saline, while air was infused using a manometer (IDEE, Maastricht University, the Netherlands) and pressure was manually increased by inflating the colon. Bursting pressure was defined as the intraluminal pressure at which air leakage was initially observed from the anastomosis (mBar).

Tissue preparation

In anaesthetised rats, the anastomotic site was dissected (after measuring bursting pressure) with a 0.5-cm margin at each site of the anastomosis. Tissue samples were divided in three equal pieces: one for qPCR, one for zymography purposes and one for immunohistochemistry purposes. This latter one was cut in longitudinal direction and tissue was stretched and pinned onto a cork layer in order to secure a straight anastomotic line and improve quality of histological assessment prior to fixation in formalin (supplementary data S2). Sections were deparaffinized in xylene and rehydrated in graded ethanol to distilled water prior to performing histological staining. The part dedicated for qPCR analysis was snap frozen in liquid nitrogen and stored at $-80\text{ }^{\circ}\text{C}$ until required for mRNA isolation.

Histology and immunohistochemistry

Sections were stained with standard haematoxylin-eosin and Picro Sirius Red using Direct Red 80 and Picric Acid solution (both Sigma-Aldrich, the Netherlands).

An independent, experienced animal pathologist performed blinded histological assessment on the obtained tissue, scoring (from 0 to 4) histological sections on inflammation, granulocyte influx, fibroblast activity and collagen deposition. Collagen

ration was calculated using an in-house software program on pictures obtained with a polarized light microscope (Leica DM5000B, Leica Microsystems, Wetzlar, Germany).

MMP2, pro-MMP9 and MMP9 activity analysis using zymography

MMP2 and MMP9 activities were assessed using gelatin zymography since its increase of MMP activity has been implicated in the pathogenesis of AL²⁰. Samples were lysed from tissue and loaded on a polyacrylamide gel containing gelatin (10% Ready Zymogram Gel, Bio-Rad, the Netherlands). Gel electrophoresis took place at 100 V for 30 min. After that, the gel was incubated with 2.5% Triton X-100 in MQ for 30 min at room temperature. The gel was then placed in developing buffer at 37°C overnight. Colouring of the gel took place the following night with PAGE-Blue protein staining solution (Thermo Scientific, the Netherlands). At day 3, the staining was decoloured with MQ, revealing MMP2 and MMP9 activity. The gels were scanned using a Bio-Rad GS-800 Densitometer and digital quantification was done using ImageJ software (ImageJ Software, U.S. National Institutes of Health, Bethesda, Maryland, USA).

MMP2, MMP9, TFF3 and Muc2 expression analysis using qPCR

The expression of matrix metalloproteinases 2 (MMP2) and 9 (MMP9) as well as mucin 2 (MUC2) and trefoil factor 3 (TFF3) was determined using qPCR to see if exogenous butyrate had an effect on the expression of these MMPs and the abundant secretory mucin MUC2 as well as its associated trefoil factor. All qPCR-experiments were carried out by a dedicated technician (BB) and are reported as detailed as possible according to the MIQE guidelines [21]. RNA was isolated from the tissue sample using TRI reagent (Sigma-Aldrich, the Netherlands) and treated with RNase-Free DNase Set (Qiagen). The purity of RNA was tested using Nanodrop Spectrophotometer (Thermo Scientific) by 260/280 absorbance (A260/A280) ratios, ranging from 1.89 to 2.02. Therefore, the purity of the isolated RNA was satisfactory for further studies. RNA of 750 ng was converted to DNA with sensifast cDNA Synthese kit (Bioline, London, UK). qPCR was performed on 10 ng cDNA with 0.3 µM primers in Sensimix SYBR & Fluorescein Kit (Bioline, London, UK) using white 384-wells qPCR plate. Primer sequences are listed in Table 7.1. Reference genes included were glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and B-actin. Reactions were run on the LightCycler480 (Roche). PCR conditions used were 10 min at 95°C, followed by 40 amplification cycles of 15 s at 95°-60°C-72°C followed by a melting curve to validate the amplifications. The cycle threshold (Ct) value of each reference gene was obtained from qPCR analysis. The obtained Ct values were applied to evaluate the expression of the genes of interest. The mean Ct value of the reference genes ranged from 16 to 23 cycles, with HA-injection having the highest

transcript levels in MMP9, and butyrin patch was associated with having the lowest transcript levels in TTF3. For analysis purposes, the level of MMP2 expression of the saline group was set at 1 and used as a control.

Statistical analyses

Statistical analysis was performed using Prism 5.0 for Mac (Graphpad software, San Diego, CA). Normality was tested using Kolmogorov-Smirnov. All continuous variables are presented as mean and standard error of the mean (SEM) and compared using student's t test or Mann Whitney U when appropriate. Dichotomous variables were compared using the Chi-square test.

Table 7.1 Primer sequences used for qPCR experiments.

r-MMP9-F1	AGCCGACGTCACTGTAAGT
r-MMP9-R1	AACAGGCTGTACCCTTGGTC
r-MMP2-F1	ACAACAGCTGTACCACCGAG
r-MMP2-R1	GGACATAGCAGTCTCTGGGC
R-MUC-F1	CGAAGTGAAGAGTGAGCACG
R-MUC-R1	GATCCGGGTGGTATTCAGCA
R-Tff-F1	GGCCTATCTCCAAGCCAATGT
R-Tff-R1	TGCAGAGGTTTGAAGCACCA
GAPDH FW	GGAAGCTCACTGGCATGGC
GAPDH RV	CCTGCTTCACCACTTCTTG
Beta-actin FW	GACAGGATGCAGAAGGAGATTACTG
Beta-actin RV	CCACCGATCCACACAGAGTACTT

Results

Macroscopical results

In experiment 1, two rats in the butyrin patch group died prior to completing follow-up due to faecal peritonitis. All animals in experiment 2 completed the 7-day follow up. In experiment 3, three rats (one in the HA-But group, three in the But group) were euthanized because they had reached humane endpoints, due to faecal peritonitis. An overview of the types of anastomotic leakage within each experimental group can be found in Figure 7.1a. Adhesions occurred in every experiment, and there were no significant differences between experiments (proximal versus distal anastomoses) nor within each experiment (Figure 7.1b). It became evident that animals receiving a patch (either butyrin eluting or placebo) had lower welfare scores and significant more weight

loss compared to animals receiving enemas (Figure 7.1C), possibly due to an extended bowel proximal to the location of the patch upon sacrifice.

Figure 7.1 Butyrate-enriched enemas reduced AL in Exp. 3, no other butyrate-based intervention caused a decrease in AL rates (A). Adhesion scores did not differ significantly between groups in Exp. 1, 2 or 3 (B). Weight loss was most prominent in Exp. 1 where placement of the patches caused a sudden change in calibre of the colon; animals receiving enemas had the lowest percentage of weight loss but also received a different type of anastomosis compared to animals in Exp. 1 and 2 (distal vs proximal respectively) (C). The administration of butyrate enemas caused a significant higher bursting pressure of the anastomosis in Exp. 3 compared to the control group. No differences were found in Exp. 2 and in Exp. 1; no BP was measured due to porous, distended intestines

Bursting pressure

Bursting pressure (BP) was not measured during experiment 1 because of evident extended intestines in the majority of the rats at the place where the patches were placed. This abrupt change in luminal calibre made the dilated bowel very porous, disabling accurate BP measurements. In experiment 2, BP was measured and compared to a historical control group since the control group from experiment 3 received a distal

anastomosis. No differences were found in experiment 2 between saline injections, HA injections and HA-But injections. In experiment 3, however, a significant difference could be found between the control group (104.1 ± 40.8) and HA-But (250.7 ± 23.1) and But enemas (256.4 ± 44.4 , $P<0.05$).

Histology and collagen ratio

Inflammatory parameters were scored as a surrogate marker of anastomotic healing, as indicated appropriately by a recent Delphi consensus method²². Histological analysis (Figure 7.2) only detected an increased inflammation in experiment 2 in the hyaluronan injection group (2.18 ± 0.22) compared to the control group (3.27 ± 0.27 , $P<0.05$). No other differences were found regarding wound healing. Picro Sirius red staining, which depicts the level of collagen maturity, of the anastomotic region showed comparable percentages of collagen for all groups (Table 7.2).

Figure 7.2 Histological analysis only detected an increased inflammation in experiment 2 in the hyaluronan injection group (2.18 ± 0.22) compared to the control group (3.27 ± 0.27 , $P<0.05$). No other differences were found regarding wound healing. Values are mean ranking (S.E.M.).

Pro-MMP9, MMP2 and MMP9 activities measured by zymography

Zymography revealed no differences between MMP2 and MMP9 activity within each experiment between control and intervention groups (Figure 7.3). There was relatively large variability within the samples of each group as can be seen by the error bars/whiskers in the different graphs. In experiment 3, the addition of hyaluronan to the

butyrate enema did decrease the activity of pro-MMP9 (40.6 ± 8.2) compared to the butyrate enema alone (102.6 ± 26.9) and the control group (114 ± 20.9 , $P=0.01$).

Table 7.2 Total collagen and mature/immature collagen ratios.

Experiment 1	Butyrin patch	Placebo patch	P-value	
Collagen percentage (%)	32.25 (± 2.69)	28.64 (± 2.00)	0.239	
Red/green ratio	464.83 (± 278.36)	361.42 (± 207.96)	0.861	
Experiment 2	Saline injection	HA-But injection	HA injection	P-value
Collagen percentage (%)	53.22 (± 2.04)	45.29 (± 4.51)	46.50 (± 4.83)	0.361
Red/green ratio	66.89 (± 16.76)	39.66 (± 7.88)	95.64 (± 44.43)	0.340
Experiment 3	Control	HA-But enema	Butyrate enema	P-value
Collagen percentage (%)	42.84 (± 4.75)	35.57 (± 4.23)	35.93 (± 2.12)	0.343
Red/green ratio	321.50 (± 71.60)	282.00 (± 154.85)	176.54 (± 46.35)	0.355

Figure 7.3 Zymography revealed no differences between MMP2 and MMP9 activity within each experiment between control and intervention groups. The addition of hyaluronan to the butyrate enema did decrease the activity of pro-MMP9 (40.6 ± 8.2) compared to the butyrate enema alone (102.6 ± 26.9) and the control group (114 ± 20.9) in experiment 3 ($P=0.01$). Values are given in arbitrary units and data represent mean + S.E.M..

qPCR showed no significant differences in the three experiments regarding gene expression

In experiment 1, a trend was found in the expression of MMP2 between the group that received a butyrin patch (0.35 ± 44.4) and the placebo patch group (1.14 ± 0.34 , $P<0.07$, Figure 7.4). The other tested matrix-metalloproteinase MMP9 also showed a trend between these two groups, 0.39 ± 0.21 versus 1.14 ± 0.34 respectively, $P<0.08$. No differences were found regarding MUC2 and TFF3. In experiments 2 and 3, no significant differences were found in gene expression between the intervention and control groups regarding all genes of interest.

Figure 7.4 Overview of relative gene expression where the level of MMP2 expression of the saline group was set at 1 and used as a control. No significant differences were found in gene expression between the intervention and control groups regarding all genes of interest in the separate experiments.

Discussion

This study is the first study as far as we know that aims to improve butyrate supplementation in animal models to overcome the reservation of surgeons to optimize their patients with butyrate prior or during gastrointestinal surgery. We investigated different administration routes besides enemas that could enable surgeons to incorporate butyrate in the surgical procedure. Unfortunately, a single dosage of butyrate or a butyrin-eluting patch that we used in this study did not seem promising. Addition of hyaluronan to butyrate did not improve the effect of an enema on the healing of distal anastomoses. Unfortunately, this study did not lead to promising tools to transport butyrate to the proximal anastomotic site, but it did confirm that butyrate can lead to better anastomotic healing and this should be further investigated in the future.

Butyrate is known to have a critical mediating role in the colonic inflammatory response; for example, it can induce the differentiation of colonic regulatory T cells and thereby ameliorate the development of colitis as shown by Furusawa et al.²³. More recently, it has been shown that exogenous butyrate restoration improved intestinal epithelial cell junctional integrity, decreased apoptosis and mitigated graft-versus-host-disease after allogeneic bone marrow transplant²⁴. Especially the restorative effect of butyrate on the epithelial integrity and, consequently, a decreased translocation of luminal contents are of great interest in the field of anastomotic leakage. It was often assumed that butyrate increases collagen synthesis and maturation and that this reduced the risk of anastomotic leakage^{6,7,8}; however, with these new insights, it can be hypothesised that

increased epithelial integrity leads to a lesser amount of (clinically relevant) anastomotic leakage.

As stated before, enemas seem to be a promising tool to deliver butyrate in the colon, especially in more distal anastomoses; however, it remains difficult to reach the same levels of butyrate in proximal anastomoses. In addition, butyrate is metabolized rapidly as soon as it enters the colonocyte via its active transport system. This could be the reason that experiment 2 was not successful; the amount of available butyrate by a single injection was simply not sufficient to cause any effect on the anastomotic site.

It was suggested that a prodrug of natural butyrate, tributyrin, could overcome the pharmacokinetic drawbacks of natural butyrate as a drug²⁵. Since it was very difficult to incorporate butyrate in an eluting patch, butyrin was used instead, making sure that there were high concentrations available at the site of release. Unfortunately, the attachment of a patch into the colon caused extension of the bowel proximal to the location of the patch, indicating colonic stenosis. This effect of patches or fleeces has been previously described²⁶; however, in that study, the authors used an external fleece and in experiment 1, the film-like resorbable patches were attached intraluminally. This stenosis effect—clinically translatable to ileus—could be the explanation of why the animals in experiment 1 had lower welfare scores and lower weight compared to the other groups.

Hyaluronan is generally known to be associated with tissue repair and wound healing, and its concentration is high in healing tissues²⁷. Its biological functions are the result of both its physicochemical properties and its biological interactions, depending on molecular weight²⁸. Despite the logical hypothesis that the addition of hyaluronan to butyrate would enhance anastomotic healing, this was not observed in this study, possibly due to a low concentration or not having the appropriate molecular weight.

One of the limitations of this study — even if the newly tested approaches were successful — is the translation to the human setting. While butyrate can be administered as a rectal enema, this is rather invasive and not the preferred choice in drug administration. The risk of anastomotic dehiscence in a recent constructed anastomosis remains, which could be an explanation of the faecal peritonitis that was found in the enema groups in experiment 3. However, the very unpleasant odour and taste of butyrate makes oral administration extremely difficult. For this reason, Raso et al. have studied a derivative of butyrate, N-(1-carbamoyl-2-phenyl-ethyl) butiramide (FBA) as a successful therapeutic alternative to butyrate, sharing a comparable efficacy, but a

better palatability and compliance, unfortunately so far only in a rat model²⁹. Other articles that describe oral administration of butyrate were also designed as animal studies^{30,31}. Nevertheless, there are a few studies in which oral butyrate or its prodrug was given in humans and was considered safe and well tolerated³². The question remains if butyrate can be administered as a local agent at the site of the anastomosis, without potential harmful effects when it enters the systemic circulation. Van Beek et al. have recently demonstrated that splanchnic butyrate release was prevented in patients after colonic butyrate administration, indicating that rectal enemas even with high therapeutic dosage (up to 100 mmol/L) of butyrate can be given safely³³.

Conceivably new approaches may become available as nano-based drug delivery systems for encapsulation and release of drugs are currently being investigated in different fields of medicine^{34,35}. Imaginably in the future, butyrate tablets will become standard perioperative treatment and due to nanotechnology, the release of butyrate will only take place at the site of interest, the colonic anastomosis.

In conclusion, butyrate can enhance the colonic anastomosis, making it less prone to lead to leakage. The most effective method thus far is by rectal enemas and the addition of hyaluronan did not ameliorate the effect of butyrate on the AL rate. Further research should be done to identify the optimal way of butyrate delivery to the anastomotic site where it can enhance anastomotic healing.

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Chapter 8

Functional mucous layer and healing of proximal colonic anastomoses in an experimental model

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Abstract

Introduction

Anastomotic leakage (AL) is the most dreaded complication after colorectal surgery, causing high morbidity and mortality. Mucus is a first line of defence against external factors in the gastrointestinal tract. In this study, the structural mucus protein Muc2 was depleted in genetically engineered mice and the effect on healing of colonic anastomoses studied in an experimental model.

Methods

Mice of different Muc2 genotypes were used in a proximal colonic AL model. Tissues were scored histologically for inflammation, bacterial translocation was determined by quantitative PCR of bacterial 16S ribosomal DNA, and epithelial cell damage was determined by assessing serum levels of intestinal fatty acid-binding protein.

Results

Of 22 Muc2-deficient (Muc2^{-/-}) mice, 20 developed AL, compared with seven of 22 control animals (P<0.001). Control mice showed normal healing, whereas Muc2^{-/-} mice had more inflammation with less collagen deposition and neo-angiogenesis. A tendency towards higher bacterial translocation was seen in mesenteric lymph nodes and spleen in Muc2^{-/-} mice. Intestinal fatty acid-binding protein levels were significantly higher in Muc2^{-/-} mice compared with controls (P=0.011).

Conclusion

A functional mucous layer facilitates the healing of colonic anastomoses.

Introduction

Anastomotic leakage (AL) is one of the most dreaded complications of colorectal surgery, and leads to high morbidity and mortality^{1,2}. AL rates remain high, with reported rates ranging from 1 to almost 20 per cent, despite extensive research, improvement of surgical techniques and the development of fast-track programmes^{3,4}. Identified risk factors for AL development, such as male sex, steroid use and location of the tumour, as well as concurrent disease and malnutrition, can help clinicians to determine which patients are at risk^{5,6}. However, the underlying pathophysiological mechanisms for AL remain unclear⁷. A better insight to these mechanisms, and the factors promoting anastomotic healing, is needed to reduce rates of AL⁸.

The colon is protected by a two-phase mucous layer built around the gel-forming MUC2 mucin and a limited number of other components secreted from the goblet cells^{9,10}. MUC2 mucin polymers form net-like sheets that, when layered on top of one another, form an inner colonic mucous layer that is impenetrable to bacteria¹¹. This layer is about 50 µm thick in mice and 200 µm in humans, and is quickly renewed¹². The inner layer is converted to a non-attached outer mucous layer, which is penetrable for colonic bacteria that use this layer as their habitat^{9,10}. Muc2 gene-deficient mice lack a functional mucous layer, and have bacteria in direct contact with the epithelial cells in the intestine⁹. These mice develop colonic inflammation with a severity that depends on the bacterial flora in the animal housing facility.

Among the numerous factors contributing to healing of surgical anastomoses, the intestinal mucus system has been largely overlooked. This is despite the fact that numerous studies have shown that mucus is essential for protection of the epithelium from luminal challenge. For example, it was recently demonstrated that colonic ischemia resulted in detachment of mucus, which allowed bacteria to come into contact with the epithelium¹³. When blood flow was restored, crypt goblet cells secreted stored mucus, which cleared the bacteria in contact with the epithelium, and re-established homeostasis. Thus, goblet cell mucous secretion plays a central role in regenerating a functional mucous layer that protects the host epithelium¹⁴.

Mucin secretion influences the capacity of the mucous layer to protect the colon, and altered secretion may result in different physiological consequences¹⁵. Barcelo and colleagues¹⁶ demonstrated that mucin secretion can be modulated by luminal factors such as certain algal polysaccharides, uronic acids and short-chain fatty acids (SCFAs). Other factors influencing mucous secretion are prostaglandins and certain bacteria, such

as *Listeria monocytogenes* and *Akkermansia muciniphila*^{17,18}. In addition, prostaglandin (PG) E₂, produced by the two isoforms of cyclo-oxygenase (COX) 1 and 2, can stimulate mucous secretion in both small intestine and proximal colon *ex vivo*¹⁹. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX enzymes, leading to a decrease in prostaglandin synthesis²⁰. An association between the use of NSAIDs and AL has been described in both experimental and observational studies^{21–23}, suggesting an interaction between prostaglandins and anastomotic healing. PGE₂ could thus have a positive influence on anastomotic healing, mediated by its effect on mucous secretion.

In the present study, the protective role of a functional mucous layer on AL rates after colonic surgery in mice, and the influence of PGE₂ administration on anastomotic wound healing were investigated.

Methods

Animals

All animal experiments were approved by the Gothenburg Animal Experiments Ethics Committee (permit 63-2014), and all experiments were performed in compliance with the directive on the protection of animals used for scientific purposes (2010/63/EU). This study followed the ARRIVE guidelines²⁴; Appendix S8.1 (supporting information) provides more detailed information. Forty-four mice (mean(s.d.) weight 22·3(3.8) g) of different genotypes (*Muc2*^{-/-}, *Muc2*^{+/-} and *Muc2*^{+/+}) were matched for age (8–20 weeks) and sex when possible, in order to obtain an equal distribution within experimental groups (2 groups: *Muc2*^{-/-} versus control (*Muc2*^{+/-} and *Muc2*^{+/+}); 22 mice per group). Additionally, eight *Muc2*^{-/-} mice served as control animals in the quantitative PCR (qPCR) experiment for bacterial translocation, to distinguish between effects induced by surgery and the genotype. The mice had unlimited access to standard mouse chow and water. Welfare of the animals was assessed twice daily using extensive scoring according to Pommergaard and colleagues²⁵. Postoperative pain relief with buprenorphine 0.1 mg/kg subcutaneously (Buprecare®; AST Farma, Oudewater, The Netherlands) was administered when needed.

Study design

Muc2^{-/-}, *Muc2*^{+/-} and wild-type (WT; *Muc2*^{+/+}) mice underwent laparotomy with colonic anastomosis and were killed on at day 3 after the operation. The heterozygous and WT mice were considered control animals (*Muc2*⁺), as both of these genotypes determine a

normal mucous layer. The primary outcome was AL, defined by either faecal peritonitis or abscess formation at the anastomotic site. Macroscopic AL was diagnosed by two independent observers, who were blinded to the genotypes at the time of death.

Model

The model of AL used was that described previously by Komen and co-workers²⁶ and subsequently adapted by Reisinger et al.²⁷ to obtain a model that resembles clinical practice. In this model, mice undergo anaesthesia with isoflurane and receive pain treatment with buprenorphine (0.1 mg/kg subcutaneously) before surgery in which a proximal colonic anastomosis is performed with seven interrupted extramucosal sutures (Prolene® 8/0; Ethicon, Johnson & Johnson, Somerville, New Jersey, USA), providing a leakage rate of 33.3 per cent. During surgery, the colon and caecum are kept moist with sterile saline. The body temperature of the mice is held constant at 37°C using a heat pad. The muscle layer and skin are closed using interrupted sutures (Vicryl® 4/0 and Monocryl® Plus 4/0 respectively; both from Ethicon). After surgery, the mice receive 0.5 ml saline subcutaneously as fluid therapy. All surgical procedures were performed by a single investigator who was blinded to genotype at the time of surgery. The main outcome after surgery was macroscopic evaluation of the anastomosis, as follows: no AL; small abscess, less than 0.5 cm³; large abscess, 0.5 cm³ or more; or faecal peritonitis.

Tissue and blood sampling

Histological assessment was performed on anastomotic tissue. To conserve the mucous layer, the anastomosis was dissected en bloc, leaving the sutures intact, and fixed in Carnoy solution (60 per cent methanol, 30 per cent chloroform, 10 per cent glacial acetic acid). Unaffected proximal and distal colonic tissues were obtained in the same manner, serving as control tissue. Mesenteric lymph nodes (MLNs) and spleen were snap-frozen in liquid nitrogen for further analysis. Blood samples were taken by intracardial puncture at time of animal killing, collected in EDTA vacuum tubes (BD Vacutainer®; BD, Stockholm, Sweden) and kept on ice. Blood samples were centrifuged at 4000 r.p.m., at 4°C for 15 minutes. Plasma was stored immediately in aliquots at -80° until analysis.

Measurements of mucous layer thickness

To evaluate whether mucus was present at the anastomotic site of control animals, measurements of mucus were performed as described previously²⁸, with some modifications. Briefly, unflushed proximal colon was opened longitudinally after removal of the mesenteric border. The tissue was pinned down on a silica gel plate with the

luminal side facing upwards. Pellet material was gently removed and the mucus was visualized by adding charcoal particles on top of the mucus. The thickness of the mucous layer was then determined by measuring the distance between the mucous surface and the epithelial surface using a micropipette viewed through a stereomicroscope (Leica MZ12; Leica Microsystems, Heerbrugg, Switzerland). Mucus was measured directly at the anastomotic site. Unaffected tissue was used for control measurements. The tissue was submerged in oxygenated Krebs's solution at all times. As Muc2^{-/-} mice do not have a functional mucous layer, this could not be measured in these animals²⁹.

Histological analysis

Tissue samples were embedded in paraffin and cut in 4-μm sections. To evaluate the morphology of cells and glycoproteins in the colonic mucous layer, standard haematoxylin and eosin, and periodic acid–Schiff–Alcian Blue (PAS-AB) staining was performed. Specimens were scored based on inflammation, granulocyte infiltration, fibroblast activity and neoangiogenesis, according to the Ehrlich and Hunt numerical scale (0-4) as modified by Phillips et al.³⁰.

Immunohistochemistry for Ki-67 (proliferation), cleaved caspase 3 (CC-3) (apoptosis) and Muc2 (to confirm the phenotype of the animals) was performed. In short, slides were deparaffinized in xylene and rehydrated in graded ethanol to distilled water. Antigen retrieval was performed using target retrieval solution (Dako, Glostrup, Denmark) at 95°C for 20min. Non-specific antibody binding was blocked using 5 per cent fetal calf serum in phosphate-buffered saline (PBS) (Ki-67) or 1 per cent bovine serum albumin (Asp175). Sections were then incubated with specific primary antibodies (Ki-67 clone Sp6, Thermo Fisher Scientific, Life Technologies, Bleiswijk, The Netherlands; CC-3 Asp175, Cell Signalling Technology, Leiden, The Netherlands; or anti-MUC2C3 (provided by the Mucin Biology Group)⁹). After washing, swine antirabbit IgG biotin-labelled antibody (Dako, Glostrup, Denmark) for Ki-67 and CC-3 staining, and fluorophore-conjugated CY3 antirabbit antibody (Life Technologies) for Muc2 staining were used as second antibodies. Nuclei were stained using (nickel) 3,3'-diaminobenzidine (DAB) or 4',6-diamidino-2-phenylindole (DAPI). All staining was evaluated by two independent observers using microscopy; they were blinded to the genotype of the mice. Ki-67 expression was analysed using ImmunoRatio software³¹, which calculates the percentage of positively stained nuclei (DAB) in relation to the other nuclei (haematoxylin). All CC-3 positive cells in one tissue sample were counted and comparisons were made between control and anastomotic tissue.

ELISA for intestinal fatty acid-binding protein

Intestinal fatty acid-binding protein (I-FABP) is a plasma marker of enterocyte damage, as these small cytosolic proteins are present in mature enterocytes at the tip of the villus and released into the circulation following loss of enterocyte membrane integrity³². Patients who developed AL after colorectal surgery were shown to have raised I-FABP plasma levels before the operation³³. I-FABP was measured in plasma using a commercially available sandwich enzyme immunoassay (lower detection limit 0.061 ng/ml; Cloud-Clone, Houston, Texas, USA) according to manufacturer's protocol.

Prostaglandin E2 supplementation

Inhibition of COX has been shown to increase the risk of AL, an effect suggested to be PGE2-mediated²⁷. To investigate whether PGE2 has a positive influence on anastomotic healing, mice received either vehicle (PBS) or 100 µg/g bodyweight of 16,16-dimethyl PGE2 (dmPGE2), a stable PGE2 analogue (Cayman Chemical, Ann Arbor, Michigan, USA), by twice-daily intraperitoneal injection. Both interventions started 1 day before surgery and continued until postoperative day (POD) 3, after which all mice were killed.

Quantitative PCR for bacterial translocation

Total DNA was extracted from snap-frozen MLN and spleen tissue using a combination of sodium dodecyl sulphate and proteolytic digestion with bead beating and phenol/chloroform DNA purification, as previously described³⁴. Bead beating was performed using Lysing Matrix E tubes in a FastPrep-24TM instrument (MP Biomedicals, Thermo Fisher Scientific, Gothenburg, Sweden). DNA was precipitated using 0.2 mol/l sodium chloride and isopropanol, then resuspended in 100 µl Tris-EDTA buffer and quantified using a NanoDropTM ND-1000 spectrophotometer (Thermo Fisher Scientific). DNA extractions from tubes containing no tissue served as contamination controls. Purified and quantified 16S DNA from *Escherichia coli* was used as a standard. Initially, bacterial ribosomal DNA (rDNA) was enriched from host DNA by limited-cycle (LC) PCR amplification of 16S rDNA using the universal 16S rDNA primer pair 27 F (AGAGTTTGATCMTGGCTCAG) and 1492R (CGGTTACCTTGTTACGACTT). Briefly, 50-µl PCR mixtures containing 480 ng DNA, 0.2 µmol/l primers and HotStarTaq Plus Mastermix (Qiagen, Sollentuna, Sweden) were subjected to 5 min at 95°C and 16 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1-5 min, followed by 10 min at 72°C. Sample, standard and control LC-PCRs were then compared by qPCR using the 16S rDNA primer pair 926 F (AAACTCAAAGGAATTGACGG) and 1062R (CTCACRRCACGAGCTGAC). In brief, 20-µl qPCR mixtures were prepared using 2 µl LC-PCR mixture, 0.3 µmol/l primers and SSoFastTM

EvaGreen® Supermix (Bio-Rad, Sundbyberg, Sweden) and subjected to 95°C for 5 min, then 30 cycles of 95°C for 15 s, 61·5°C for 15 s and 72°C for 20 s, with EvaGreen® fluorescence measured at the final step of each cycle. qPCR thermocycling and fluorescence measurement was performed on a CFX96™ instrument (Bio-Rad). Sample amplification curves were compared with contamination control curves, and samples were considered 16S-positive if they amplified before controls. Sample 16S was quantified using a standard curve generated from standard qPCR reactions.

Statistical analysis

Statistical analysis was performed using GraphPad Prism® 5.0 for Mac (GraphPad Software, San Diego, California, USA). Normality was tested using the Kolmogorov – Smirnov test. All continuous variables are presented as mean(s.d.) and compared using Student's t test, the Mann–Whitney U test, ANOVA or the Kruskal–Wallis test as appropriate. Dichotomous variables were compared using the χ^2 test.

Results

Anastomotic leakage rate is significantly higher in Muc2-deficient mice

All mice (n=44) completed follow-up of 3 days. After killing, the abdomen was reopened and the anastomotic site evaluated. Evaluation showed a wide range from normal healing/no AL to small and large abscesses, and even faecal peritonitis (Figure 8.1A–C). Muc2^{-/-} mice had AL more frequently (score greater than 2) compared with control animals (20 of 22 versus 7 of 22 respectively; $P<0.001$) (Figure 8.2A). Three animals in the Muc2^{-/-} group suffered from faecal peritonitis, whereas none of the control animals showed any sign of peritonitis. Further, both large (8 versus 1 in the control group) and small (9 versus 6 respectively) abscesses were found more frequently in Muc2^{-/-} mice. Bodyweight was similar at baseline in the two experimental groups, and remained so over time (Figure 8.2B). A significant decrease in welfare score was observed in Muc2^{-/-} mice from POD 1, with maximum effect at POD 3 ($P<0.001$) (Figure 8.2C).

Figure 8.1 Evaluation of the anastomosis: **A)** no anastomotic leakage, **B)** abscess (either small, less than 0.5 cm³, or large, 0.5 cm³ or more), **C)** faecal peritonitis.

Figure 8.2 **A)** Muc2^{-/-} mice showed more frequent anastomotic leakage (AL) (score greater than 2; 20 of 22 mice) compared with control animals (7 of 22). *P<0.001 (χ^2 test). **B)** Mean(s.d.) bodyweight was similar at baseline in the two experimental groups and did not differ over time. **C)** A significant decrease in mean(s.d.) Pommergaard welfare score was seen in Muc2^{-/-} mice from the first day after surgery, with maximum effect on day 3 (8.80(1.93) versus 10.76(1.30) in control mice; *P<0.001, 2 - way ANOVA with Bonferroni post hoc test (time)).

Mucus is present at the anastomotic site of control animals

The mucous layer was visualized by the addition of charcoal and its thickness was measured by means of a glass capillary attached to micrometer, observed via a stereomicroscope (Figure 8.3A). A mucous layer was observed in Muc2⁺ animals at the site of anastomosis as well as in the proximal and distal colonic tissues (Figure 8.3B). In the proximal colon, the mean mucous layer thickness was greatest between folds (238(17) μ m) and lowest on top of the folds (71(25) μ m). Mean thickness of the mucous layer at the anastomotic site was 119(65) μ m. In the control group, no significant difference was found in mucous layer thickness between heterozygous (Muc2^{+/-}) and WT (Muc2^{+/+}) mice, or between mice that had AL and those that did not. As Muc2⁻ deficient mice lack the structural component of intestinal mucus and therefore lack a

defined mucous layer, measurement of the mucous layer in $Muc2^{-/-}$ mice was not feasible.

Figure 8.3 **A)** Adding charcoal on the sample enables the thickness of the mucous layer to be measured. Mucous layer thickness is defined as the distance between the charcoal particles and the tissue, and was measured on top of colonic folds, between folds and at the anastomotic site. **B)** A mucous layer was observed in $Muc2^{+}$ animals, at the site of anastomosis as well as in the proximal and distal colonic tissues.

Histological analysis showed more inflammation in $Muc2$ -deficient mice

$Muc2^{-/-}$ mice showed a tendency for more leucocyte infiltration, and less collagen deposition and neoangiogenesis at killing on POD 3 compared with $Muc2^{+}$ animals (Figure 8.4A). In addition, continuation of the intestinal tissue was evident in the case of normal anastomotic healing, whereas AL caused disruption or dehiscence (Figure 8.4B). There was no difference in proliferation determined by Ki-67 staining between $Muc2^{-/-}$ and control mice at the serosal tissue of the anastomosis or in the enterocytes (Figure 8.4C,D). CC-3 staining showed no differences in apoptosis between $Muc2^{-/-}$ and control mice (Figure 8.4E,F).

A normal inner mucous layer, as shown by PAS-AB staining, was clearly observed in the $Muc2^{+}$ control mice, but was absent in $Muc2^{-/-}$ mice (Figure 8.5A,B). In addition, goblet cells in $Muc2^{-/-}$ mice showed only PAS-positive staining, whereas those from control animals also had positive AB staining. Immunohistochemistry revealed the presence of

Muc2-positive goblet cells in control animals, but in $Muc2^{-/-}$ mice there was no expression of Muc2, indicating no Muc2-positive goblet cells (Figure 8.5C,D).

Figure 8.4 **A)** Inflammatory parameters scored on day 3 after surgery did not differ significantly between experimental groups. Values are mean(s.d.). **B)** In $Muc2^{+}$ animals with normal anastomotic healing there was continuation of the intestinal tissue, whereas anastomotic leakage in $Muc2^{-/-}$ mice caused disruption or dehiscence (arrows) (haematoxylin and eosin stain). **C,D)** There was no difference in cell proliferation measured by Ki - 67 staining with 3,3' - diaminobenzidine (DAB) between $Muc2^{-/-}$ and control mice. Values are median, interquartile range and range. **E,F)** DAB staining for cleaved caspase (CC) 3 indicated no difference in apoptosis (arrows) between $Muc2^{-/-}$ and control mice. Values are mean(s.d.)

Figure 8.5 **A,B)** A normal inner mucous layer, as shown by periodic acid–Schiff–Alcian Blue (PAS - AB) staining, was clearly observed in the *Muc2*⁺ control mice (arrows), but was absent in *Muc2*^{-/-} animals. **C,D)** Immunohistochemistry revealed the presence of *Muc2* - positive goblet cells in control animals, but in *Muc2*^{-/-} mice there was no expression of *Muc2*, indicating no *Muc2* - positive goblet cells. The minimal staining found in *Muc2*^{-/-} is attributed to slight cross - reactivity of the anti - MUC2C3 antiserum with the mouse Fcgbp protein (Fc fragment of IgG - binding protein).

Epithelial cells were damaged in *Muc2*-deficient mice

When enterocytes are damaged they leak one of their main cytosolic proteins, I-FABP. Hence, measurement of I-FABP in plasma gives an estimate of intestinal tissue damage. *Muc2*^{-/-} mice showed significantly higher plasma levels of I-FABP compared with control

animals (3.88(2.63) versus 1.82(1.83) ng/ml respectively; $P=0.011$) (Figure 8.6A), indicating greater enterocyte damage in the $Muc2^{-/-}$ mice.

Figure 8.6 **A)** The concentration of intestinal fatty acid - binding protein (I - FABP) was significantly increased in $Muc2^{-/-}$ mice compared with that in control animals (mean(s.d.) 3.88(2.63) versus 1.82(1.83) ng/ml respectively), indicating more epithelial damage. * $P=0.011$ (Student's t test). **B)** Following prostaglandin (PG) E₂ supplementation, there was no reduction in the anastomotic leakage (AL) rate for $Muc2^{-/-}$ mice (10 of 11 animals for both vehicle and PGE₂), and the reduction in the AL rate in control animals was not significant ($P=0.362$, Student's t test per experiment as well as 2 - way ANOVA with post hoc testing). * $P<0.001$. **C)** Bacterial translocation to the mesenteric lymph node was present in both control and $Muc2^{-/-}$ animals that underwent surgery, but not in $Muc2^{-/-}$ mice that had no operation. * $P<0.050$ (Kruskal-Wallis test). **D)** No differences were found between experimental groups in bacterial translocation to the spleen. C,D) Box - and - whisker plots show values for median, interquartile range and range

PGE2 administration did not reduce anastomotic leakage in this model

dmPGE2 was administered 1 day before surgery and on every postoperative day until the animals were killed (day 3). With the protocol and doses used, no reduction in the rate of AL was observed in *Muc2*^{-/-} animals (10 of 11 animals for both vehicle and PGE2). The same was true for control animals (AL in 5 of 11 for vehicle versus 2 of 11 for PGE2; *P*=0.362) (Figure 8.6B). Within the control group, no difference was found in AL rate between heterozygous (*Muc2*^{+/-}) and WT mice.

Bacterial translocation in control and *Muc2*-deficient mice that had surgery

Bacterial 16S rDNA was quantified from MLN and spleen tissue from control and *Muc2*^{-/-} mice that underwent surgery, and from *Muc2*^{-/-} mice that had no surgical intervention. The MLN 16S load in both groups of mice having surgery was higher than that in the group that did not, indicating that the surgical procedure did cause increased bacterial translocation to the intestinal lymphatics (Figure 8.6C). No significant difference was detected between control and *Muc2*^{-/-} mice, although the median 16S load was 4-4 times lower in control mice compared with *Muc2*^{-/-} mice. Spleen load was similar in the three groups, with approximately 100 16S rDNA copies per mg tissue detected in most animals (Figure 8.6D). However, three of 11 mice in the *Muc2*^{-/-} surgery group had more than 104 16S copies/mg, indicating that more bacteria had penetrated the systemic tissue in a subgroup of these animals. Thus, the surgical procedure increased translocation of bacteria from the intestine, and *Muc2* deficiency resulted in a tendency towards higher systemic bacterial load.

Discussion

The incidence of AL has remained high over the years, despite extensive research, the introduction of new surgical techniques and the construction of a diverting stoma in high-risk patients³⁵. Many risk factors are being investigated, but the first line of defence in the gastrointestinal tract, the mucous layer, has been largely overlooked.

Mice deficient in the *Muc2* gene (*Muc2*^{-/-} mice) lack a functional mucous layer, and were more prone to develop AL than matched *Muc2*^{+/-} and *Muc2*^{+/+} controls. Control animals had a leakage rate of 32 per cent (7 of 22 animals), in line with the model of Reisinger and colleagues²⁷. In addition, *Muc2*^{-/-} mice had lower welfare scores than control animals despite co-housing, and matching for age and sex where possible. It should be noted that *Muc2*^{-/-} mice develop spontaneous colitis, although this often

remains asymptomatic in the animal facility³⁶. The effects observed in *Muc2*^{-/-} mice are therefore ascribed to the development of AL rather than to the colitis phenotype. Weight loss did not differ significantly between *Muc2*^{-/-} and control mice. However, the mice were killed only 3 days after surgery, and it is known that the difference in weight loss between animals with and those without leakage does not become evident until after 3 days²⁵. At this time animals without AL start to gain weight, whereas mice with AL start to gain weight only after POD 5²⁵. Not only did the *Muc2*^{-/-} mice show more clinical symptoms of AL than control mice, they also had significantly higher I-FABP levels, indicating enterocyte damage and more bacterial translocation. The damaged epithelia and increased translocation could indicate that the mucous layer is important for reducing the number of bacteria that reach the vulnerable anastomosis site, and hence is important in the healing process. In line with this, there is accumulating evidence^{37,38} that bacteria are involved in the pathogenesis of AL.

It was hypothesized that supplementation with dmPGE2 would reduce the AL rate by increasing mucous secretion. However, dmPGE2 did not reduce the AL rate in either *Muc2*^{-/-} or control mice. It has been suggested that dmPGE2 can prevent AL when COX-2 activity is decreased, induced either by drug treatment (COX inhibitors) or by knocking out the COX-2 gene²⁷. In the present study, no difference in AL rate was observed following PGE2 administration, despite using a stable analogue of PGE2 in the same dosage that had an effect in a Cox2-deficient mouse model. Perhaps PGE2 is effective only when there is a severe deficiency of PGE2, which is not the case in *Muc2*^{-/-} mice. A higher dose of dmPGE2 induces adverse reactions in mice, with dose- and time-dependent PGE2-induced diarrhoea³⁹. In the present study, no diarrhoea was observed, indicating that the PGE2 dose used was safe.

In the present study, only anastomoses in the proximal colon, and not in other parts of the gastrointestinal tract, were investigated. Interestingly, Yauw and co-workers⁴⁰ recently showed that diclofenac, a commonly used NSAID, caused AL in the ileum and proximal colon, but not in the distal colon. Furthermore, more recently, Rutegård et al.⁴¹ found no increased risk of AL with NSAID use in patients undergoing anterior resection for rectal cancer. Previous associations between AL and NSAIDs were derived from pooled data for colorectal surgery, not solely rectal resections. Additionally, it has been shown by ex vivo analysis of the mucous layer that PGE2 caused significantly increased mucous secretion in the small intestine and less in the proximal colon, and none in the distal colon¹⁹. Moreover, in humans, membrane protein profiling has demonstrated distinctive regional differences in the colon⁴². These different observations suggest that the healing-promoting effect of PGE2 might differ along the gastrointestinal tract, and that this might also be the case for the healing-promoting effect of a functional mucous layer suggested here for the proximal colon.

Potentially interesting factors that can influence the colonic mucous layer are certain bacteria or SCFAs. A frequent colonizer of the human colonic mucous layer is *Akkermansia muciniphila*, a mucin-degrading bacterium that is suggested to increase the thickness of the inner mucous layer¹⁸. The presence of *A. muciniphila* correlated with increased *Muc2* gene expression, suggesting that *A. muciniphila* can enhance its own nutrient-rich environment⁴³. Therefore, it can be hypothesized that supplementation with *A. muciniphila* or other bacteria before gastrointestinal surgery may boost mucous secretion, which can support anastomotic healing.

SCFAs from bacteria in the distal colon are important as the primary energy source for colonic epithelial cells, but also stimulate mucous secretion¹⁶. One of the most abundant SCFAs in the colon, butyrate, can strengthen distal anastomoses when given in enema form⁴⁴, perhaps through stimulated mucous secretion¹⁶. Thus, commensal bacteria in the outer mucous layer of the distal colon may help to prevent AL in animal models by producing SCFAs. Furthermore, mucins have been suggested to have a trophic effect, as they are not readily digested and can be considered an important nutrient source for many intestinal organisms. Colonic mucus also contains trefoil factor (TFF) 3, a molecule known to have a trophic effect in the colon⁴⁵. Mucus acts as a diffusion barrier, thereby hindering molecules such as TFF-3 from diffusing out quickly into the lumen, and increasing the local epithelial concentration. The colonic mucus and its main component MUC2 can thus be envisaged to have many effects that might be important for anastomotic healing.

When inflammation and cell proliferation were studied with immunohistochemistry, no differences were observed between the two experimental groups. This might be explained by the fact that mice were killed on day 3. It is known from other experiments^{26,27} that day 3 is early in the intestinal healing process and that potential differences become evident in later phases of healing. However, POD 3 was chosen for killing in the present study in order to prevent animal suffering.

Results from animal experiments cannot be translated directly to the human setting. Paradoxical to the observation that the thick mucous layer in the distal colon may be protective is that distal tumour location in patients is a known risk factor for the development of AL. For this reason, a diverting stoma is often constructed in clinical practice^{3,46}. Although animal findings should be interpreted with caution, they can lead to new insights and provide possible interventions for the human setting. One important lesson is that the controlled ischemia necessary in surgery can be maintained for some time (up to 1 h) and that the mucous layer recovers quickly after this time¹³. Owing to the slow biosynthesis of mucins, a second period of ischemia should be avoided⁴⁷.

The mucous layer of the gastrointestinal tract has been largely overlooked in research on AL, despite its function as a primary defence mechanism. In this study, mucus has been

shown to play an essential role in normal anastomotic healing, and is probably also crucial in other types of gastrointestinal healing. Further research on anastomotic healing should focus on positively influencing the mucous layer, to promote better postoperative recovery.

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Chapter 9

The shift in microbial composition and diversity in a rat model of NSAID induced anastomotic leak can be attributed to surgical stress

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Submitted

Abstract

Background

Growing evidence suggests a central role for the gut microbiome in the pathogenesis of anastomotic leakage. Nonsteroidal anti-inflammatory drugs (NSAIDs) have been recently identified as perioperative risk factor for AL and may also affect the microbiome.

Aim

The objective of this study was to explore the influence of anastomotic surgery and NSAID treatment on the microbiome composition of different bowel segments and the association with leak rates.

Methods

50 rats underwent intestinal resection and anastomosis construction in the ileum (IL), proximal colon (PC) or distal colon (DC). One PC group did not receive diclofenac, whereas all other groups did. Faecal samples collected from the anastomotic site on POD0 and POD3 were analyzed by 16S rRNA sequencing.

Results

More distally, the relative abundance of the Firmicutes phylum decreased, whereas the Bacteroidetes increased. Microbial diversity of the proximal and distal colon was significantly decreased on POD3 ($P=0.0025$ and $P=0.007$ respectively) compared to POD0. Clear differences in relative abundance of several species could be observed between preoperative and postoperative samples, in all investigated segments. Surgery was the main contributing factor to these observed changes: it explained 75% of the variance in microbiota between POD0 and POD3 in the ileum. Furthermore, analyses revealed no significant differences in alpha diversity metrics between leakage and non-leakage samples in the proximal and distal colon.

Conclusion

Surgical stress prompts a shift in microbial composition in all investigated segments. Microbial changes do not explain the increased risk for AL following NSAID treatment in this model.

Introduction

Anastomotic leak (AL) is the most feared complication following intestinal surgery. It is associated with high morbidity and mortality and occurs in 6-12% of patients after colorectal resection^{1,2}. Leak rates have failed to decline over the past decades despite intensive research in this field, emphasizing the need for a better understanding of leak pathogenesis.

Several recent studies have reinforced the hypothesis that gut bacteria play a central role in the pathogenesis of AL, by demonstrating a protective effect of antibiotics, and oral antibiotics in particular, against anastomotic leak^{3,4}. It has been shown that multiple surgery-related factors, such as fasting, bowel preparation, antibiotics, surgical trauma and analgesics, can influence the microbiome⁵, and in turn, that microbial changes and increased bacterial virulence can disturb anastomotic healing^{6,7}.

Since the past decade, nonsteroidal anti-inflammatory drug (NSAID) use has been associated with an increased risk of developing AL in both human and experimental studies^{8,9}. Although the main hypotheses are that this is caused by COX2-inhibition or topical toxicity of drug metabolites, several studies also support a role for the microbiome in NSAID-induced leakage¹⁰⁻¹³. Ueijima et al. demonstrated that the presence of specific microbes is a prerequisite for the induction of small intestinal ulcers in rats treated with NSAIDs¹⁰. Another study provided evidence that NSAIDs also have a direct influence on the microbiota composition. Following injection with indomethacin, an overgrowth of *E. faecalis* was observed in the rat small intestine, which resulted in the relative diminution of commensal microbes such as segmented filamentous bacteria¹⁴. Interestingly, high collagenase producing strains of *E. faecalis* have also been associated with AL in both experimental and clinical studies¹⁵. We have previously demonstrated that the use of the NSAID diclofenac causes leakage of anastomoses in the ileum and proximal colon in rats, but not in the distal colon¹⁶. These observed differences in AL may be explained by differences in microbiota, as this is an important discriminating factor between these segments^{17,18}.

The cumulative evidence suggests that the microbiome of the gut may play an important role in the pathogenesis of AL, and could also contribute to NSAID-induced intestinal complications. The aim of the present study is to capture the changes that occur in the composition of the rat microbiota in different intestinal segments as a result of anastomotic surgery and the administration of diclofenac, and to explore whether these changes can account for differences in diclofenac-induced leakage.

Materials and methods

Ethics

This experiment was conducted according to the Dutch “Experiments on Animals Act” and European Federation of Laboratory Animal Science Associations guidelines and was approved by the institutional Animal Ethics Committee of the Radboud University.

Animals

Faecal samples were collected from 50 Male Wistar rats (Harlan, Horst, The Netherlands), housed under standard laboratory conditions with free access to food and water. Rats were accustomed to laboratory conditions for one week prior to the start of the experiment. Animals were weighed daily and were inspected for signs of reduced wellbeing twice daily during the experiment. Signs that were monitored included red eyes, nose, and/or fur, piloerection, aberrant behaviour, distended abdomen, and diarrhoea. Humane endpoints were defined; animals were euthanized if they showed signs of severe discomfort (e.g. weight loss >20%, distended abdomen or severely reduced activity).

Study design

The experiment was conducted as previously described¹⁶. 50 rats underwent a 1 cm bowel resection followed by the construction of an anastomosis. Depending on the group randomization, resection and anastomosis construction occurred either in the ileum (IL), proximal colon (PC) or distal colon (DC), under strict aseptic conditions. No antibiotics were administered. Groups IL+ (n=10), PC+ (n=15) and DC+ (n=10) received diclofenac (3 mg/kg/day) until sacrifice on post-operative day 3 (POD3), with the first dose administered 15 minutes prior to the operation. Rats in group PC- (n=15) did not receive diclofenac.

On POD3 animals were euthanized by CO₂ asphyxiation. After euthanization, a relaparotomy was performed and the anastomosis and abdomen were inspected for macroscopic signs of anastomotic leakage. Two investigators independently assessed the presence and extent of leak by means of an anastomotic leak severity score. Any differences in scores were resolved by consensus. A score of ‘0’ constituted no signs of leakage, a score of ‘1’ constituted anastomotic abscesses, a score of ‘2’ represented free pus or large abscesses, and a score of ‘3’ stood for faecal peritonitis or visible dehiscence¹⁹.

Feces sample collection

Faeces samples were collected from the resected segment (i.e. IL or PC or DC) during the initial operation on POD 0 (samples 'D0') and again after sacrifice on POD 3 (samples 'D3'). Samples were snap frozen in liquid nitrogen and stored at -80°C .

DNA isolation

DNA isolation was performed using a combination of repeated bead-beating and column-based purification as described previously²⁰. Briefly, PSP lysis buffer (Strattec Molecular, Berlin, Germany) and approximately 50 mg of frozen stool aliquots were added to a sterile vial containing 0.5 g of 0.1 mm zirconia/silica beads and 4 3.0-3.5 mm glass beads (BioSpec, Bartlesville, USA). The samples were homogenized in a MagNA Lyser instrument (Roche, Basel, Switzerland) in three cycles of 1 minute at a speed of 5500 rpm, interspersed with cooling on ice for one minute in between cycles. DNA isolation was continued using the PSP Spin Stool Kit (Strattec Molecular, Berlin, Germany) according to the manufacturer's instructions. DNA was finally eluted in 200 μl elution buffer.

Sequencing

Amplicon libraries and sequencing were performed according to previously published protocols²¹. Briefly, the V4 region of the 16S rRNA gene was PCR amplified from each DNA sample in triplicate using the 515f/806r primer pair described previously²¹. Pooled amplicons from the triplicate reactions were purified using AMPure XP purification (Agencourt, Massachusetts, USA) according to the manufacturer's instructions and eluted in 25 μl 1 $\times$ low TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Quantification of amplicons was subsequently performed by the Quant-iT PicoGreen dsDNA reagent kit (Invitrogen, New York, USA) using a Victor3 Multilabel Counter (Perkin Elmer, Waltham, USA). Amplicons were mixed in equimolar concentrations to ensure equal representation of each sample, and sequenced on an Illumina MiSeq instrument.

Data analysis and statistics

Filtering, denoising, removing of chimeric sequences and clustering of sequences in Operational Taxonomic Units (OTUs) at 97% similarity was conducted using the LotuS pipeline (version 1.39)²². First, sequences with an average quality below 27, a read length below 170 bases, with one or more ambiguous bases, or containing homopolymer stretches of over 8 bases were discarded. Retained sequences were chimera filtered and clustered into OTUs using UPARSE23. Taxonomic annotation of

OTUs was derived from RDP naïve Bayes classifier annotations (minimum acceptance confidence set at 0.8). OTUs representing less than 0.01% of the total number of observations (sequences) and OTUs that were only observed in a single sample were discarded. Downstream analyses were conducted using QIIME version 1.8.1 and R version 3.1.324.

Taxonomy, alpha and beta diversity, microbial core

Differences in the relative abundance of bacterial phyla and genera were compared between day 0 (surgery) and day 3 (sacrifice), between IL, PC and DC, and between groups with and without diclofenac (applicable to proximal colon samples) using either the Wilcoxon signed-rank test (for the paired samples between day 0 and day 3) or the Mann-Whitney-U-test (for unpaired comparisons). The following metrics of species richness and diversity within communities (alpha-diversity) were determined at a rarefaction depth of 34,150 sequences/sample: observed OTUs (observed richness), Chao1 index (estimated richness), and Shannon index (diversity). Alpha diversity metrics between paired samples from day 0 and day 3 were compared by the Wilcoxon signed-rank test, whereas Mann Whitney U tests were used to compare alpha diversity metrics of group IL/PC+ with DC+, and group PC- with group PC+. The total number of pairs that were included in the analyses were: n=5 for PC-, n=14 for PC+ and n=10 for DC+. All P-values were adjusted for multiple comparisons using the False Discovery Rate [Benjamini-Hochberg]²⁵.

Beta-diversity, the diversity shared across samples, was determined by the Bray-Curtis dissimilarity (BC) at a rarefaction depth of 34,150 sequences/sample. Clustering of samples was visualized using Principal Coordinate analysis (PCoA).

Enterotyping

Enterotype analyses were performed as described previously by Arummougham and colleagues²⁶. We used the R27 package “vegan: Community ecology”, version 2.2-1 by Oksanen et al. from 2011 for calculating the Bray-Curtis distance on the genus-level relative abundance profiles. To cluster the samples, we used the Partitioning Around Medoids (PAM) clustering algorithm in the R27 package “Cluster analysis basics and extensions”, version 2.0.1 ed. by Maechler et al. from 2012. The optimal number of clusters was chosen based on Calinski-Harabasz (CH) index and validated by the silhouette index (57) using the R27 package ‘clusterSim’. The optimal number of 2 clusters was identified based on the CH index, which was confirmed by the silhouette index although clustering was moderate (SI 0.47).

Between-class analysis (BCA) was performed to plot the samples using the R27 package “Analysis of Ecological Data: Exploratory and Euclidean Methods in Environmental Sciences” version 1.7.2. by Dray et al. from 2015. The similarity percentage analysis (SIMPER)²⁸ was used to identify taxa contributing to similarity within- and dissimilarity between groups.

Results

Anastomotic leakage

Animal welfare, mortality and anastomotic healing assessment results were published previously. In summary, leak rates were 10 out of 10 (100%) in group IL+ and 22 out of 30 (73%) in group PC+ (22/30; 73%; $P=0.166$). Compared to group PC+, rates were significantly lower in group DC+ (1/10; 10%; $P=0.001$) and group PC- (0/15; 0%) ($P=0.001$). The mean leak severity score was significantly higher in the IL+ group compared to the PC+ group (2.8 ± 0.6 vs. 1.7 ± 0.5 , $P=0.004$) and significantly lower compared to the DC+ group (0.1 ± 0.3 , $P<0.001$).

Sequencing

In total 96 faecal samples were collected during the experiment. Because of insufficient material 17 samples could not be analyzed. Two samples failed during sequencing and were excluded from subsequent analysis. In total 77 samples remained, from which a total of 4,869,671 V4 reads were retained for downstream analysis after trimming, quality filtering, removal of potential chimeric reads and de-multiplexing. The number of sequences per sample ranged from 34,198 to 87,981 (median 63,354). Sequences were then clustered into 2,182 OTUs. After discarding OTUs that were only observed in a single sample and OTUs with a relative abundance of $<0.01\%$, 1,005 OTUs remained. The taxonomic composition of the samples on both phylum and genus level can be observed in Figures 9.1A and 9.1B, respectively.

Figure 9.1A Phylum abundance in different anatomical regions (IL=ileum, PC=proximal colon, DC=distal colon), pre- (D0) and post-surgery (D3) and with or without diclofenac (+ or – respectively).

Baseline microbiota composition and diversity in different segments of the intestinal tract

Taxonomy

Phylogenetic classification of the microbiota in the different anatomical locations identified 21 different families belonging to 9 bacterial phyla. The most abundant phyla in the fecal samples obtained from the ileum were Firmicutes, followed by Bacteroidetes and Actinobacteria. The most abundant genera were *Bacteroides*, *Lactobacillus*, *Escherichia-Shigella*, *Turicibacter* and *Bifidobacterium* (relative abundance of 23.91, 8.92, 7.17, 2.72 and 2.05% respectively). The genera that formed major constituents of the microbiota in the colon were *Lactobacillus*, *Bacteroides*, *Alloprevotella*, *Blautia* and *Alistipes*. More distally along the intestinal tract, the abundance of Firmicutes decreased, whereas Bacteroidetes showed an increase as compared to the ileum (see also supplementary material).

Figure 9.1B Genus abundance of 21 most abundant genera in different anatomical regions (IL=ileum, PC=proximal colon, DC=distal colon), pre- (D0) and post-surgery (D3) and with or without diclofenac (+ or – respectively).

Microbial diversity and community structure

Microbial richness and diversity of faeces samples collected before anastomosis construction was expressed as the number of observed species, Chao1 index and Shannon diversity index respectively. The analysis revealed baseline differences in diversity between the various segments of the intestine with a gradual increase in diversity from ileum towards the distal colon; see Figure 9.2 for a comparison of the different alpha diversity indexes. Preoperative faecal samples retrieved from the ileum demonstrated a significantly lower estimated microbial richness than samples retrieved from the proximal (PC- and PC+ combined) and distal colon ($P=0.002$ and $P=0.001$ respectively, as calculated from Chao1 data).

Figure 9.2 Comparison of alpha diversity indexes of pre-operative samples of the different anatomical locations: IL (ileum), PC (proximal colon), and DC (distal colon).

The microbial community structure, as expressed by the Bray-Curtis (BC) dissimilarity was also examined for each intestinal segment. Clustering of all samples based upon BC dissimilarities revealed a clear separation between ileal and colonic samples, represented by a separation along the first principal coordinate. Proximal and distal colonic samples did however not separate neither in this analysis nor when excluding ileal samples in a secondary analysis (results not shown).

The influence of surgery on microbial diversity and community structure

Surgery significantly decreased microbial richness and diversity in proximal (only PC+) and distal colon samples (Figure 9.3). A similar trend could not be observed in ileal samples, likely due to the limited number of paired pre- and post-surgery ileal samples.

Figure 9.3 Microbial richness (Chao 1 and Shannon index) for all groups measured pre- and post-surgery. Asterisk indicates significance <0.05.

To further examine the effect of surgery on the microbial community structure in specific intestinal segments, a PCoA plot was created for each segment individually. A clear separation between pre- and post-operative proximal colon samples was observed

along the first principal coordinate in PCoA plot based on the Bray-Curtis distance (Figure 9.4B). This separation was partially, but not completely, driven by a reduced microbial richness in post-operative samples (see supplementary data for a colored PCoA plot according to the Chao1 index). This surgery-effect was also observed for both ileum and distal colon samples (see Figure 9.4A and 9.4C respectively). In all intestinal segments, the same trend could be observed: all pre-operative samples clustered together, while the majority of the post-operative samples form a separate cluster with the exception of a few post-operative samples that remained clustered with the pre-operative samples.

Figure 9.4A Bray-Curtis: the first principal coordinate shows the effect of surgery on ileum samples: 75% of the variation could be attributed to PC1.

Figure 9.4B Bray-Curtis: the first principal coordinate shows the effect of surgery on PC+ and PC- proximal colon samples. No obvious effect of diclofenac can be observed in this separation.

Figure 9.4C Bray-Curtis: the first principal coordinate shows the effect of surgery on distal colon samples.

Enterotyping

In order to further elucidate the origin of the clustering observed in the Bray-Curtis analyses of proximal colon samples enterotyping was performed based on Partitioning Around Medoids. The Silhouette score yielded the strongest evidence for clustering in two groups (SH index=0.47), one group consisting of the pre-operative samples and one group consisting mainly of the post-operative samples. Bacterial taxa that contributed most to the observed clustering were the unclassified Lachnospiraceae, as well as *Bacteroides* and *Escherichia-Shigella*. Post-operatively, a marked decrease in the relative abundance of Lachnospiraceae could be observed and a sharp increase in the relative abundance of *Bacteroides* and *Escherichia-Shigella*.

Subsequently, paired analyses were performed to examine the differences in microbiome composition between pre- and post-operative proximal colon (PC+ and PC-) samples at phylum and genus levels (Figure 9.5A). These analyses revealed a significantly lower relative abundance of the Firmicutes phylum on D3 compared to D0 ($P<0.001$), whilst the decrease in the abundance of *Lactobacillus* (the most important genus within this phylum) was not significant ($P=0.26$). An overall increase in the phyla Bacteroidetes ($P=0.001$) and the genera *Bacteroides* ($P<0.001$) and *Alistipes* ($P<0.014$) was observed, as well as an increase in the Proteobacteria phylum ($P<0.001$), which confirmed the findings in the enterotyping analyses. The most important genera within the

Proteobacteria phylum showed the expected postoperative increase (Escherichia-Shigella $P<0.001$, Bilophila $P<0.001$ and Parasutterella $P<0.001$). A postoperative decrease in overall relative abundance was found for the phylum of Candidatus Saccharibacteria ($P=0.001$).

As was the case for the PC samples, paired analysis of the distal colon samples revealed a significantly higher abundance of several genera within the Firmicutes phylum, including the Blautia ($P=0.005$) and Clostridium sensu stricto ($P=0.022$) genera preoperatively, as well as a bloom of Proteobacteria genera such as Escherichia-Shigella ($P=0.007$) and Bilophila ($p=0.009$) post-operatively (Figure 9.5B).

The effect of diclofenac administration on microbial diversity and composition

Alpha diversity

As stated before, in the PC+ group a decrease in alpha diversity was observed in samples obtained at postoperative day 3 compared to the postoperative samples ($P=0,001$ for both alpha diversity measures). In the proximal colon group that did not receive diclofenac, microbial richness was also lower post-operatively, but this difference did not reach statistical significance (Chao1 index PC-D0: Mdn. 687,59, PC-D3: Mdn. 593,82, $z=-1,483$ $P=0,138$, Shannon index PC-D0: Mdn. 6,46, PC-D3: Mdn. 5,75, $z=-2,023$, $P>0,05$ after FDR correction). Surprisingly, significant baseline differences in microbial richness were found between PC-D0 and PC+D0 samples collected before anastomosis construction (Chao1 index $P=0,035$), but not in microbial diversity, (Shannon index $P=0,292$).

Despite these baseline differences, the diversity in the PC+D3 group and PC-D3 group was comparable (Chao 1 index PC+D3: Mdn. 652,97, PC-D3: Mdn. 593, 82, $P=0,694$, Shannon index PC+D3: Mdn. 5,93, PC-D3: Mdn. 5,75, $P=0,823$). The difference in diversity in the PC+ group between D0 and D3 was not significantly greater than that in the PC- group ($P=0,123$ and $P=0,956$ for Chao1 and Shannon index respectively). See Figure 9.6 for the associations between the different time-points for the paired samples.

Figure 9.5A Cladogram representation of microbiota composition of proximal colon samples on different taxonomical levels. Green indicates significant higher relative abundance in pre-operative samples, higher relative abundance of post-operative samples is depicted in red.

Akk:Akkermansia
 Ali:Alistipes
 Allo:Alloprevotella
 Ba:Bacteroides
 Barn:Barnesiella
 Bif:Bifidobacterium
 Blt:Blautia
 Blau:Blautia
 But:Butyrivibrio
 E/S:Escherichia/Shigella
 Fl:Flavonifractor
 I:Clostridium sensu stricto
 Lab:Lactobacillus
 Osc:Oscillibacter
 Pb:Parabacteroides
 Ps:Parasutterella
 Ros:Roseburia
 Rum:Ruminococcus
 Sac:Saccharibacteria incertae sedis
 Tur:Turicibacter
 XIVa:Clostridium XIVa
 XIVb:Clostridium XIVb

Figure 9.5B Cladogram representation of microbiota composition of distal colon samples on different taxonomical levels. Green indicates significant higher relative abundance in pre-operative samples, higher relative abundance of post-operative samples is depicted in red.

Figure 9.6 Difference in Chao1 index for paired samples on different time points between groups PC- and PC+.

Beta diversity

To examine whether the microbial community structure within the PC+D3 and PC-D3 groups was more similar than the microbial community structure between the groups, the average within- and between-group Bray Curtis dissimilarity was examined. Samples within the PC+D3 group showed the strongest homogeneity, as indicated by significantly lower within-group Bray Curtis dissimilarity compared to the PC-D3 group ($p = <0.001$) (Figure 9.7). When comparing OTUs between PC+D3 and PC-D3 group, only one out of >1000 OTU's was found to be significantly different: the OTU belonging to *Anaerostipes* from the family of Lachnospiraceae ($p = 0.046$ after FDR correction). Together with the similar post-operative decrease in alpha-diversity in the PC+D3 and PC-D3, these results provide little evidence for an effect of diclofenac administration on microbiota composition.

Relation between microbial composition and diversity and the occurrence of AL in different intestinal segments

Anastomotic leakage was observed in ileal and proximal colon (PC+) samples, but only sporadically in PC- or distal colon samples. For the analysis of the association between AL and microbial composition and diversity, only paired samples were taken into account: in the PC+ group, 10/14 rats (71,4%) showed signs of AL, whereas only 1 of 10 rats in the distal colon (10%) leaked. In order to investigate the relation between the microbial composition and the occurrence of leakage in the proximal colon and distal colon

samples, we compared alpha diversity scores between leakage and non-leakage samples. Alpha diversity indices were not significantly different between postoperative leakage and non-leakage samples from the proximal colon (Chao1 index: Mdn. 642,40 vs. 682,91 respectively, $z=-0.990$, $P=0.37$, Shannon index: Mdn. 5,66 vs. 6,07 respectively, $z=-1,414$, $P=0.19$), or the distal colon (Chao1 index: Mdn. 606,00 vs. 665,53 respectively, $z=-1,219$, $P=0.40$, Shannon index: Mdn. 6.01 vs. 6.20, respectively, $z=-0,522$, $p=0.80$).

Figure 7.7 Within-group Bray-Curtis dissimilarity of proximal colon samples. Samples within the PC+D3 group showed the strongest homogeneity.

We further examined the relative abundance between the leakage and non-leakage samples in the PC+ group, which showed a significant lower relative abundance for the phylum Actinobacteria in the preoperative samples (Mdn. 0.0027 vs. 0.0219, $z=-2.121$, $P=0.035$) as well as a lower relative abundance of Bacteroidetes in the postoperative samples of the leakage group (Mdn. 0.457 vs. 0.567, $z=-2.263$, $P=0.024$). The other most abundant genera and phyla showed no difference in relative abundance between leakage and non-leakage samples.

Discussion

In the present study, the effect of anastomotic surgery and perioperative NSAIDs on the microbiome of different gut segments was explored. In the proximal and distal colon, a clear reduction of microbial diversity was observed three days after surgery. Changes were most apparent in the proximal colon and involved a diminution of the relative abundance of the Firmicutes (mainly attributed to a decrease in Lachnospiraceae) and an increase in the relative abundance of the Bacteroidetes phylum and Proteobacteria genera such as *Escherichia-Shigella*. The species richness did not change as evidently in the ileum as a result of the surgical intervention, but this can be explained by the relatively low richness at baseline. The PCoA on the other hand revealed a strong influence of surgery in the ileum: 75% of the variance between D0 and D3 samples could be explained by the intervention. Contrary to our hypothesis and available literature, no significant difference was observed in taxonomic composition and diversity following diclofenac treatment.

A possible explanation is that the current study was not able to detect such influence, as surgery alone has proven to be a strong factor to alter the microbiota, and may have subsequently camouflaged influences exerted by the administration of diclofenac⁷. Other studies have shown that NSAID treatment lead to an obvious decrease in the number of gram-positive bacteria^{13,29}. Another study found increased numbers of Enterobacteriaceae and Acidominococcaceae in the faeces of NSAID-users compared to controls³⁰.

The trauma of undergoing surgery alone may suffice in explaining the alteration of the normal microbial composition⁷. Lactobacilli and short chain fatty acids, both considered to have a cytoprotective effect, decrease by more than 90% within 6 hours after major trauma³¹. Our results are also in concordance with a study by Shogan et al. in which a decrease in the relative abundance of the Firmicutes phylum and an increase in Proteobacteria genera such as *Escherichia-Shigella* was observed post-operatively⁵. Shogan et al., however, found a more dramatic increase in the relative abundance of *Enterococcus*. Because of the short time frame in which this alteration takes place, it is considered to be a result from chemosignalling rather than a depletion of nutrients in the gut³². Another possible explanation is the exposure of the gut lumen to atmospheric air, which will affect the obligate anaerobic species^{33,34}.

Several features of the luminal content as well as the structure of the intestine have been suggested to play a role in the observed segmental differences^{17,18}. The small intestinal content is known to have a lower pH, higher oxygen and bile acid levels as compared to the colon, which facilitates colonization by facultative anaerobes, mainly Lactobacillaceae and Enterobacteriaceae (belonging to the Firmicutes and

Proteobacteria phyla respectively). The colon on the other hand, characterized by low oxygen levels, slow transit and high nutrient levels, is mainly inhabited by Bacteroidetes or Firmicutes phyla. The microbiome changes observed in this study were greater in the proximal colon compared to the distal colon. The anastomotic leak rate was also higher in the proximal colon.

It is possible that the microbial shift in the proximal colon increased its susceptibility to damage caused by diclofenac. Other studies have shown that certain antibiotics and probiotics reduce the severity of NSAID-induced enteropathy, especially when they target gram-negative bacteria^{35,36}. Another important clue for the interaction between NSAIDs and bacteria is the finding that NSAID enteropathy does not occur in germ free mice, but, when *Eubacterium limosum* or *E. coli* is introduced, intestinal ulceration is observed^{10,37}.

However, there may be other explanations than an altered microbiota composition for the increased leakage rates in the ileum and proximal colon in this study. Inhibition of cyclo-oxygenase (COX) consequently leads to inhibition of its products: the prostaglandins. By inhibiting the prostaglandins E2 (PGE2) and I2 (PGI2), two important inflammatory mediators in the gut, several hallmarks of inflammation are reduced as well³⁸. As they also play an important role as mediators of mucosal defence and repair, NSAID administration leaves the gut lining more susceptible to cytotoxic components of the luminal content³⁹.

Another property of this group of drugs that may explain the observed alterations is their metabolism. Many NSAIDs (including diclofenac) are excreted through bile after being absorbed in the distal intestine and being glucuronidated in the liver. After their excretion together with the bile (free or glucuronidated), they exert secondary effects on the intestinal wall, contributing to epithelial damage⁴⁰.

The decrease in the relative abundance of Lachnospiraceae observed in the colonic segments may have detrimental consequences for epithelial permeability as many of the intestinal butyrate producers belong to the Lachnospiraceae family⁴¹. Aside from facilitating tight junction assembly, butyrate is associated with many other beneficial properties in the colon. Butyrate acts as an energy source for intestinal epithelial cells, reduces oxidative stress in the mucosa and by extension inflammation, and is an anti-carcinogenic⁴². Interestingly, in inflammatory bowel disease the most consistent change that is observed is also a marked decrease in the representation of the Firmicutes phylum (including Lachnospiraceae)⁴³.

No clear association between microbial composition and the occurrence of anastomotic leakage was found in this study. This could be explained by the relatively low numbers of leakage, as well as the strong influence of the surgical intervention on the microbiota. A recently conducted clinical study did find a positive correlation between the occurrence of CAL and low microbial diversity, as well as with a high abundance of Bacteroidaceae and Lachnospiraceae families⁴⁴. It has been further suggested that aside from microbial composition and diversity, microbial virulence also plays an important role in leakage pathogenesis. Bacteria have been shown to demonstrate an upregulation of virulence factors such as collagenase activity, swarming motility and epithelial adhesion in response to changes in their direct environment such as phosphate depletion⁴⁵, release of inflammatory mediators⁴⁶, and the release of endogenous opioids⁴⁷. Even symbiotic microbes can express virulence as a reaction to cues coming from their host. This increase in bacterial virulence can promote the emergence of a pathobiome capable of penetrating the intestinal barrier and causing anastomotic leak^{32,48}. In order to investigate this hypothesis, analyses cannot remain limited to taxonomic composition and diversity quantification; analysis of the functional context of microbial species is vital in order to determine the clinical relevance of the presence of specific bacterial species.

The strength of this study is the simplicity of the model used; results are not tainted by the administration of antibiotics, bowel preparation or radiotherapy – interventions that are commonly used in other animal experiments and, most importantly, in clinical practice. This allowed us to isolate and identify changes on the microbiota composition that were induced by surgery or NSAIDs alone. We included the proximal colon anastomosis in our experiments, a type of anastomosis that is often neglected in experimental CAL research, despite the fact that right-sided (ileocolic) anastomosis are common and show significant leak rates in recent series⁴⁹. The most important limitation of this study is the low leak rate of the distal colon anastomoses, which can largely be attributed to the type of model used. Several additional interventions can be employed in order to increase the leakage rate of distal anastomoses (e.g. ischemia, radiotherapy, morphine), but this would increase the complexity of this specific model and would hamper interpretation of single intervention effects.

In summary, surgical stress, and not NSAID treatment, prompts a shift in microbial composition and decrease in diversity in a rat model of NSAID induced anastomotic leak. The increased risk for AL that is induced by the administration of NSAIDs is therefore likely to be attributable to other properties of this drug class. However, as microbial changes have been previously described to occur with the use of NSAIDs, more research is needed to clarify if these changes make the gut more susceptible to diclofenac-induced leakage and anastomotic leak in general.

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Supplementary data

Bubblechart depicting the relative abundance of the ten most common genera in the ileum (IL), proximal colon (PC) and distal colon (DC).

Chapter 10

General discussion

General discussion

Colorectal cancer is the most common oncological disease in the Netherlands, with over 15.000 newly diagnosed cases every year. This number is thought to further increase due to the recently initiated national colorectal cancer-screening program and the ageing population. The large majority of patients that undergo treatment in a curative setting will eventually undergo colorectal surgery. Although several techniques can be used, depending on tumour location, the majority of these include resection of the diseased bowel part, which is usually followed by the construction of an anastomosis in order to restore bowel continuity. One of the most feared complications after this type of surgery remains colorectal anastomotic leakage (CAL), in which a failure of the anastomosis leads to spill of faecal content into the normally sterile abdominal cavity. The presence of faecal material and bacteria can induce the formation of abscesses, peritonitis, and in severe cases sepsis, and may eventually lead to death. Despite the fact that important improvements have been made in the perioperative care, such as the implementation of fast-track protocols, advances in anaesthesia and minimally invasive techniques, the numbers of leakage have remained stable over the past decades – around 7-10%¹. The occurrence of CAL has been associated with increased rates of morbidity and mortality, negatively affects health-related quality of life due the increased risk of reoperation and the construction of a permanent ileo- or colostomy, increases health care costs by a threefold and, finally, is associated with an increased risk of cancer recurrence and cancer-related death²⁻⁵.

In this thesis, we have focused on several important aspects of colorectal anastomotic leakage in different phases of care for the colorectal patient.

PART I: increasing international consensus on the definition of colorectal anastomotic leakage

In the first part of the thesis, we have aimed at increasing insight in the use of different definitions for CAL. The definition of what exactly should be considered a leakage after colorectal anastomotic surgery has remained a topic of discussion for many years. Our literature study in **Chapter 3** showed that no common definition exists, despite the fact that the number of papers published on this topic has increased tremendously over the years, and despite the fact that several new definitions have been proposed⁶. The heterogeneity in study outcome hampers the execution of statistically strong meta-analyses, that are needed to provide level A evidence for recommendations and

guidelines. Furthermore, we believe it is important to strive for uniformity on the reporting of clinical data as well, as this would increase understanding of certain findings and further aid the comparison of studies. Uniformity in data reporting has gained increased attention over the years and has led to the formulation of the CONSORT statement for RCTs. We believe that adherence to such guidelines greatly improves data reporting and we are in favour of the use of similar formats for studies conducted in the field of CAL.

The survey study (**Chapter 2**) increased our insight in the differences of what surgeons consider an anastomotic leak in different countries (in this case, the Netherlands and China), and partly explained an important variation between reported leakage rates in Western and Eastern clinical studies⁷⁻¹⁰. Such differences can of course partially be explained by variables such as operation technique, tumour location and patient characteristics. We investigated whether these reported differences could also be explained by differences in definitions used in separate countries.

Both studies stress the need for a new and generally accepted definition, and indicate the importance of guidelines with regards to what topics and data should be addressed in new studies considering colorectal anastomotic leakage. In collaboration with the Dutch Taskforce for Anastomotic Leakage, we drafted a set of 11 points that we advise to report in newly published papers.

Future perspectives and recommendations

In our literature study, we show that authors tend to formulate their own definitions rather than using an existing definition. This can be caused by the existence of broad or rather vague definitions such as ‘a defect in the bowel wall at the anastomotic site, leading to communication of intra- and extraluminal compartments’¹¹, which can be difficult to use in clinical practice. Furthermore, we feel that it is important to increase support by the scientific community in order to improve implementation of new definitions. In the first part of this thesis, we laid the foundation of the discussion for the proper definition of colorectal anastomotic leakage, which is currently being formulated by the use of a worldwide Delphi analysis. This analysis will be done with the help of renowned international researchers and colorectal surgeons in order to increase the final implementation of a newly formed definition. The co-authors and myself strongly believe that the use of a graded system with treatment recommendations for the different severity grades will enhance uniformity of patient care and may lead to decreased delay in treatment.

PART II: Developing a predictive algorithm for anastomotic leakage

Many studies have been conducted focusing on risk factors for leakage, which can be subdivided into patient and surgery related risk factors. Patient related risk factors include genetic predisposition, drug use such as NSAIDs or steroids, smoking, male gender and old age and preoperative radiotherapy¹²⁻¹⁶. Surgery-related risk factors include the height of the anastomosis (the closer to the anal verge, the higher the risk of CAL), duration and amount of blood loss during surgery, the oxygenation of the tissue and the microbiota composition of the gut^{1,17}. Despite the knowledge of these risk factors, it is impossible to make an adequate risk assessment for the risk of CAL for every individual patient. The results of the REVEAL study (**Chapter 4**), in which we aim to develop a predictive algorithm for the risk of anastomotic leakage, will aid the physicians involved in colorectal care and their patients to make tailored decisions to the construction of a deviating ostomy and an anastomosis prior to undergoing surgery. The results of the study will further lead to earlier detection of anastomotic leakage. In the early postoperative phase, physicians rely on nonspecific markers such as fever, abdominal pain and C-Reactive Protein (CRP) levels in blood to distinguish a patient with a normal postoperative course from a patient that is suffering from a small or fulminant anastomotic leak^{18,19}. It has been shown that early recognition of the problem leads to early intervention, and this in turn helps to keep the clinical consequences to a minimum²⁰. As many of the patients with CAL display nonspecific signs and symptoms, CT scans are commonly used to exclude the presence of an anastomotic leak in case an abnormal postoperative course is suspected. Unfortunately, the false-negative rates of CT scans are relatively high, which often leads to a significant delay in diagnosis and initiation of treatment^{21,22}.

As part of the on-going REVEAL study, we studied the composition of volatile organic compounds (VOCs) in exhaled air and examined whether this composition shifts in the event of a leak. Volatile organic compounds in breath are a reflection of metabolic processes in our body both from exogenous (bacteria, viruses) and endogenous sources, of which the breakdown products are released into the blood stream and, via the alveoli, into the exhaled air²³. We investigated whether specific metabolic profiles can be used as an early predictor of colorectal anastomotic leakage. We have sampled a large number of patients prior to surgery and on every postoperative day, and are currently analysing the samples by means of mass spectrometry.

Apart from the preoperative phase, a lot of information can be gained from the period of surgery itself. As mentioned before, attention should be taken regarding several perioperative factors such as the mean blood pressure, body temperature and the

amount of blood loss, as they have been shown to influence the postoperative course after major surgery. With regards to colorectal surgery, another parameter is of interest: the microperfusion of the intestine, as this has been suggested to be predictive for colorectal anastomotic leakage^{24,25}. In **Chapter 5**, we have focused on the use of near infrared imaging (NIRF) to assess the quality of perfusion of the intestine, and the influence of fluorescence intensity on the incidence of colorectal anastomotic leakage in a small group of patients who were also included in the REVEAL study (**Chapter 4**). In this pilot study, we found that fluorescence intensity was significantly lower in patients who eventually developed CAL. We however did not find a relationship between signal intensity or anastomotic leakage and several markers for intestinal healing or cell damage, which can partly be attributed to the lack of specificity of these markers and the relatively small sample size²⁶. We believe that larger patient numbers are needed to further investigate these relationships and to formulate cut-off values for the fluorescent signal.

Future perspectives and recommendations

Many generations of both clinical and experimental researchers have focused on the problem of colorectal anastomotic leakage, and despite many important revelations that have been done over the past decades, CAL remains an important postoperative problem in a relatively large portion of patients. Clearly, with the finishing of this thesis, we have been unable to get rid of this feared complication. However, important steps have been taken in the right direction. For example, the REVEAL study is nearly coming to an end, which means that very soon we will be able to implement a reliable risk assessment tool in the preoperative work up of every individual patient diagnosed with colorectal cancer. With the use of computer learning algorithms, we will be able to find the most reliable combination of markers in for example blood, stool and exhaled air. Eventually, we will be able to allocate every individual patient to a risk classification before the surgery has even taken place. This would be very useful in the discussion between health care professional and patient with regards to construction of an anastomosis and/or a deviating ostomy. Furthermore, as the study also aims to decrease the treatment delay that is now often seen because of the lack of reliable postoperative markers, we believe that fewer casualties will be caused by the complication due to early recognition of the problem.

We believe that colorectal anastomotic leakage is a multifactorial disease, with numerous known and possibly unknown risk factors involved. This has made preoperative risk assessment and even postoperative diagnosis unspeakably difficult. We however feel that with the use of large patient populations and datasets (for example

from existing databases and innovative computational techniques), it is possible to overcome these difficulties. We therefore strongly support the collaborative trend we see in all fields of medicine and also in research focused on colorectal anastomotic leakage, of which the Dutch Taskforce is a very good example.

As mentioned above, in Chapter 5 we focused on the use of NIRF imaging to assess microperfusion of the intestine. In this pilot study, we tried to establish an objective cut-off value for the fluorescent signal to predict CAL, but failed to do so due to the small sample size. As we believe that NIRF imaging can be used as a safe and effective method to assess the perfusion of the intestine at the site of the future anastomosis²⁷, a larger study with a larger sample size is recommended in order to determine the ideal cut-off for signal intensity.

PART III: Considerations regarding perioperative care of the colorectal surgical patient

In the third part of this thesis we have focused on important aspects and questions regarding the perioperative care of the colorectal patient. During the past decade, enhanced recovery after surgery protocols have been implemented in order to shorten time of hospitalization, decrease the number of adverse events after surgery and fasten the general recovery of our patients after invasive surgery. For every individual patient, important decisions have to be made before, during and after surgery. In this thesis, several aspects of the perioperative care have been studied.

In many cases, it is decided during surgery whether a diverting ostomy is made to protect the anastomosis from the passing faecal stream. The practice of whether or not to construct a temporary (ileo)stomy also differs heavily between surgeons and institutions, and like many subjects in this surgical field, no clear guidelines exist on this topic. In case a temporary ostomy is indeed constructed, it is also unclear which type of stoma (ileo or colon) and which interval between the primary surgery and the stoma reversal is preferable. Another question is which diagnostic tool is most accurate in the assessment of the integrity of the anastomosis before temporary stoma reversal. The latter was investigated in **Chapter 6**, where we used a large national cohort of patients operated because of rectal carcinoma in 2011, and in which we investigated the accuracy of colonoscopy and contrast enema to assess the patency of the rectal anastomosis. Very little literature exists on the routine use of colonoscopy in order to evaluate the quality of the anastomosis, but it is generally considered safe in use and is associated with low costs. The contrast enema is also known to be safe in use^{28,29}, but is associated with the risk of false positive test, where contrast is seen in the ‘dog ears’ of

the staple line and confused for extraluminal leakage. In up to 41%, contrast enema detects radiological leaks that may not require intervention¹⁸. In those cases, the temporary ostomy could have been safely reversed. Despite the lack of a statistical significant difference in the odds of the diagnosis of anastomotic leakage between the two modalities, several advantages for the colonoscopy can be described, as it can also detect intraluminal cancer recurrence (although rare), advanced adenomas and anastomotic stricture, which occurs in up to 30% of cases, and can then be immediately used to resolve the stenosis by means of balloon dilation^{30,31}. However, before we are able to make strong recommendations on this matter, it is desirable to perform an RCT with a large sample size.

Future perspectives and recommendations

The large Snapshot cohort from patients who underwent rectal surgery in 2011 as is a good example as to what collaboration on a national scale can do³². From this and other national databases, we will be able to gain more insight in several research questions, because we can access large pools or retrospective data. The ultimate scientific method however remains the randomized controlled trial. In the case of colorectal anastomotic leakage, large patient numbers are needed to gain sufficient strength because of the relatively low incidence of the complication. Together with other members of the Taskforce Colorectal Anastomotic Leakage, we are finally able to initiate large RCTs in multiple centres and to answer burning questions in the field of colorectal surgery.

PART IV: Novel insights in the (patho)physiology of anastomotic healing and leakage

In the final part of this thesis, we have aimed to increase the understanding of the processes and structures of the intestine involved in colorectal anastomotic healing and leakage. We have focused on possible novel preventive techniques that aim directly to these structures and mechanisms of interest. In **Chapter 7** we investigated the use of butyrate to support the healing processes of the intestinal wall after colonic resection in a rat model. This study is the first that aims to improve the method of butyrate supplementation in animal models. Butyrate is known to restore epithelial integrity, decrease apoptosis, and mediates the colonic inflammatory response^{33,34}. In this chapter, we investigated whether enemas and/or intraluminal patches are suitable to deliver butyrate directly to the cells of interest: the colonocytes. Unfortunately, in this study, a single injection of butyrate in the colonic lumen right before construction of the

anastomosis was insufficient to ameliorate healing in the colon, whilst an intraluminal patch caused severe stenosis. Despite the disappointing results from this study with regards to the methods to deliver the drug of interest to the site of the anastomosis, we believe that treatment with butyrate with the right vector can enhance the patency of the colonic anastomosis, making it less susceptible to anastomotic leakage. Several new approaches are currently being investigated, such as nano-based drug delivery systems that allow to encapsulate and slowly release drugs at the site of interest^{35,36}.

Another important factor that we believe plays a major role in the protection against CAL is the mucus layer. Despite the fact that it is the first line of defence in the gastrointestinal tract, it has remained largely overlooked in colorectal surgery and anastomotic leakage. In **Chapter 8** we investigated the protective role of the mucus layer in heterozygous and muc2 knockout mice, and studied whether the influence of prostaglandin E2 (PGE2), a product of the two isoforms of cyclo-oxygenase (COX) 1 and 2, could improve anastomotic healing. As expected, the leakage rates for Muc2 knockout were much higher than those of heterozygous animals, but I was unable to establish a relationship between the supplementation of PGE2 and healing in this model. This can be explained by the fact that in these knockout mice, there was no mucus layer present at all, and it could therefore not be enhanced. It remains interesting to investigate whether enhancement of mucus layer thickness in humans by means of PGE2 supplementation is possible and leads to increased protection of the bowel wall against pathogens and surgical stress. Furthermore, this mucus layer is a habitat of a wide range of bacterial species, which are known to have important functions and interactions with the bowel itself. Indeed, butyrate (investigated in **Chapter 7**) is produced by some of these bacteria, as they break down dietary fibres into short-chain fatty acids (SCFA's). As mentioned before, these SCFA's comprise an important nutritional source for the enterocytes.

Over the recent years, important new insights have been created regarding the influence of the microbiome of the gut on bowel wall physiology, and – more specifically – on the incidence of colorectal anastomotic leakage. It has been shown for example that gut bacteria influence the vascularisation of the intestinal mucosa and the processes of wound healing³⁷. While some bacteria can obviously hamper healing and induce inflammation, compelling evidence suggests that the presence of other species of bacteria can lead to improved healing³⁸. Germ-free versus conventional studies have shown that the presence of bacteria increases the tensile strength of the wound³⁹. The group of Alverdy et al. has done important work on the influence of certain pathogenic species such as collagenase-producing *E. faecalis* on the healing anastomosis⁴⁰⁻⁴².

During the past few years, compelling evidence regarding the negative influence of NSAIDs on the healing of the colorectal anastomosis has led to the ban of these drugs during the perioperative period. However, the precise mechanisms behind this have been not completely unravelled. Several authors have suggested interplay between NSAIDs and the composition of the microbiome to be the cause of these detrimental effects of this drug type. In **Chapter 9**, we have looked in depth in the relationship between non-steroidal anti-inflammatory drugs and the luminal microbiome composition in a rat model for anastomotic leakage, and found that not NSAIDs, but the surgical damage itself was the most important contributing factor for the postoperative shift in microbiota.

Future perspectives and recommendations

The use of mechanical bowel preparation and oral and/or systemic antibiotics have a major impact on the composition of the microbiome. Whilst mechanical bowel preparation leads to relatively short-term alteration of the microbiome (due to loss of the mucosal mucus, which is an important habitat for bacteria⁴³), antibiotics affect the composition in a more severe and long lasting manner⁴⁴⁻⁴⁶. The microbial imbalance that is caused by any form of bowel preparation may negatively affect the processes involved in intestinal healing⁴⁷. We believe that future therapies should be directed at specific pathological bacteria and that we should aim for preservation of the 'normal' microbiome of the gut.

We believe that fundamental understanding of the processes involved in normal healing of the intestine is crucial to the development of new preventive techniques for CAL. We are currently working with an innovative method to clarify the processes of intestinal healing and leakage: mass spectrometry imaging (MSI). MSI allows us to assess the spatial distribution of a wide range of molecules, possibly detecting prognostic markers and identifying pathways as a target for effective therapies. We have recently set up a longitudinal, discovery based experiment that combines high-resolution mass-spectrometry imaging data with results from immunohistochemistry staining on tissue of the healing and leaking anastomoses of the rat colon. In this study, we aim to unravel the mechanistic processes behind normal anastomotic healing and those behind colorectal anastomotic leakage. We have constructed a lipid profile for both conditions, which may serve as the basis of additional research into the pathways of healing and leakage. Further analyses on human intestinal tissue will show us whether these processes are also applicable to the human setting, and may eventually lead to the development of methods that may finally ban this dreaded complication.

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Part V

Addendum

English summary

English summary

In Europe, colorectal cancer (CRC) is the second most common cause of cancer related deaths in both men and women. It is the second most common form of cancer in women preceded only by breast cancer, and the third most common in men, after lung and prostate cancer. The incidence rates are strongly influenced by behavioural risk factors such as smoking, poor diet (low consumption of fruits and vegetables, and high consumption of red meats), obesity, physical inactivity, and alcohol consumption. Over the past years, a rapid increase of the overall incidence of colorectal cancer has been observed, which can be contributed to the adaptation of a more western lifestyle and diet in medium to high human development index (HDI) countries, and the implementation of national screening programmes in the highest indexed HDI countries. It is thought that these incidence rates will continue to rise even further during the next few decades; some even estimate a 60% increase by 2030.

An important corner stone for treatment of CRC remains surgery, which is often combined with (neo)adjuvant chemo(radio)therapy, depending on tumour location and stadium. Important improvements have been made over the past decades with regards to the perioperative care of the colorectal patient, including the implementation of 'fast-track' protocols and, for example, minimal invasive surgery. Despite these improvements, complications are not uncommon after this type of surgery, and colorectal anastomotic leakage in particular is still the most feared one. Anastomotic leakage occurs when the anastomosis does not heal properly, leading to the leakage of luminal content (faeces and bacteria), into the normally sterile abdominal cavity, which can result in abscess formation, peritonitis and, in severe cases, sepsis.

In around 7-12% of cases of colorectal surgery, leakage of the anastomosis occurs, which is associated with a prolonged hospital admission, an increased risk of reoperation and ICU admission, and mortality. Several large studies have also shown an association between anastomotic leakage and an impaired oncological outcome, for example a decreased disease-specific survival and an increased risk of recurrence.

Despite the intensive research that has been done on this topic during the past decades by research groups worldwide, we have been unable to bring down the numbers of leakages. This can largely be contributed to the lack of understanding of the processes involved in the normal healing of the gut and the pathways involved in leakage.

Formulating a definition for colorectal anastomotic leakage

What is considered to be the definition of colorectal anastomotic leakage varies considerably from physician to physician. The existing definitions are rather vague and,

therefore, not used in clinical practice. This makes comparison of large randomized studies and their outcomes difficult, and hampers the formulation of guidelines. The first part of this thesis focuses in more detail on this problem. We used a survey (Chapter 2) and a large literature study (Chapter 3) to specify what exactly clinicians think anastomotic leakage is. The survey was conducted both in the Netherlands and in China, and showed that there are large regional differences as to what clinicians consider leakage. This can explain the large variation in reported incidence rates between studies conducted in the East and the West. With the literature study, we looked closer into the definitions used and what surgery-related parameters are described in the studies. It appeared that most authors prefer to use their own wording and definitions rather than definitions that were formulated by others. Also, the information provided on the cases that were included in the study with regards to the surgery and the postoperative course varied strongly between studies. Together with the Dutch Taskforce for Anastomotic Leakage, we drafted a set of 11 points that are advised to be reported in future papers. The results from these two studies have formed the basis of a consensus study which is currently being conducted by our research group. In this Delphi study (not included in this thesis), we asked a number of highly specialised surgeons around the world to give their input regarding this matter, and through several rounds of questions, we aim to formulate a new definition of CAL. As this new definition will be approved by important authors worldwide, it is very likely to be accepted and used by the rest of the scientific community.

Developing a risk assessment tool for leakage

In the second part of the thesis, the study protocol of the REVEAL study (Chapter 4) is discussed. The REVEAL study is an on-going prospective observational study that aims to develop an algorithm to assess the risk of developing anastomotic leakage. Before the start of this study, it was not possible for clinicians to assess the risk of leakage adequately, despite our knowledge on the risk factors that were described above. Based on a large number of different parameters such as plasma levels of several inflammatory markers, pre-operative CT scans, breath samples and the composition of the microbiome in stool samples, we aim at developing a risk score that can predict the risk of leakage before patients actually undergo surgery. This would make it easier for the clinician to discuss important aspects of the surgery with the patient, for example whether or not to construct a bowel anastomosis in case of a very high pre-operative risk score. In cases like these, the surgeon and patient could decide to not make an anastomosis, but rather to construct a permanent stoma. Based on the assessed risk, recommendations regarding postoperative monitoring of the patient could also be

given, such as routine blood tests or CT scans. Another important goal of the REVEAL study is to decrease the time between development of the complication and diagnosis. Studies have shown that early diagnosis and subsequent treatment with antibiotics, drainage or surgery decreases the consequences of a leak significantly.

Chapter 5 presents the results of a subset of patients included in the REVEAL study, in which we used an imaging technique called near infrared fluorescence (NIRF) imaging to assess the perfusion of the intestine. These patients were administered a substance intravenously, that can be visualised in the tissues with the use of a certain light. The better the vascularisation of the intestines, the more blood is delivered to the tissues, which leads to a more intense fluorescent signal. In this so-called pilot study, we found that the intensity of the imaging signal was lower in patients who eventually developed a leak. The study shows that NIRF imaging is a safe and effective method to assess the perfusion of the intestine at the site of the future anastomosis.

Different imaging modalities to assess the quality of the anastomosis

During the past decade or so, important improvements have been made regarding the in-hospital care of the patient. Enhanced recovery protocols have been drafted to shorten recovery and hospital admission time, and to reduce postoperative complications such as ileus or pneumonia. Whereas certain aspects have been captured in these protocols, other important decisions have to be made for every individual patient, such as whether or not to construct a temporary ostomy. The presence of a temporary ostomy also leads to new discussion points, for example what is the best interval between the index surgery and temporary stoma reversal. In Chapter 6, we used a large national cohort of patients to investigate which of two imaging modalities (colonoscopy or CT scan with contrast) was most suitable to assess the integrity of the anastomosis before patients underwent temporary stoma reversal. In this study, no statistically significant differences were observed between the two tests, but they both have pros and cons. The CT scan with contrast has the risk of being 'false positive', in which it may look as if there is a leak, when in reality there is none. Some advantages for the colonoscopy can be described on the other hand, as it can simultaneously detect new polyps or stenosis of the anastomosis, which could then immediately be treated. However, new randomized studies with larger patient numbers are needed before we can make recommendations for either technique.

Understanding the processes involved in leakage

In the final part of the thesis, we aimed at increasing the understanding of the processes that are involved in normal intestinal healing and leakage. Three different studies were

discussed in this section. In Chapter 7 we investigated whether supplementation of a specific substance called butyrate would reduce the risk of colorectal anastomotic leakage in rats. Butyrate is a so-called short-chain fatty acid (SCFA), and forms the main source of energy for the cells of the large intestine. The bacteria that reside in the intestine produce butyrate by the breakdown of dietary fibres. Studies have shown that butyrate has positive effects on cell renewal and that it decreases inflammation in the intestine. In Chapter 7, we looked at three different application routes to deliver butyrate to the intestinal cells: 1) by single injection right before construction of the anastomosis, 2) by continuous effusion from a patch that was placed in the lumen, or 3) by intermittent doses of butyrate by means of an enema. The first and second techniques seemed either insufficient or dangerous (the patches caused obstruction of the intestine), but some positive results were observed in the animals that received enemas on several consecutive days. However, this cannot be translated into clinical practice directly, as clinicians are usually weary of giving enemas in the early postoperative phase in case of low rectal anastomoses. During the days after the surgery the anastomosis is still vulnerable, and the risk of rupture of the anastomosis is high due to the increased intraluminal pressure of the enema.

Another study that was discussed in the final part of the thesis was on the mucous layer of the intestine. This study was conducted in collaboration with a research group in Gothenburg, Sweden. The entire intestine is lined by a layer of mucous, which forms the first line of defence against passing stool and harmful bacteria. It is produced by a certain cell type of the intestinal wall and provides a natural habitat for certain types of 'beneficial' intestinal bacteria. In the study presented in Chapter 8, a specific strain of mice was used that completely lack this mucous layer, as well as normal 'wild-type' mice. The results from this study showed that this mucous layer is indeed necessary for the normal processes of intestinal healing. Newer studies are needed to investigate methods to support the normal function of the mucous layer.

In several chapters of this thesis, the importance of the composition of the microbiome and 'beneficial' bacteria was mentioned. In other studies it has also been shown that there are certain bacterial strains that pose serious risks for the newly constructed anastomosis. Some bacteria develop harmful characteristics when exposed to certain triggers (for example certain drugs such as opioids, or simply the surgery itself), and produce enzymes that break down the intestinal wall. Another issue that has received a lot of attention in the past decades are the group of drugs of the NSAIDs (non-steroidal anti-inflammatory drugs). These are very commonly used analgesics that are believed to have a negative effect on intestinal healing, by mechanisms that are not yet completely understood. Some have suggested a relation between NSAIDs and the composition of

the microbiome to be the cause of these detrimental effects. In the final chapter of this thesis (Chapter 9) we have looked in depth into this, and found that not the use of these drugs, but the surgical damage itself was the most important influencing factor for the difference in microbiome composition after surgery.

Nederlandse samenvatting

Nederlandse samenvatting

Darmkanker is de tweede meest voorkomende oorzaak van kanker-gerelateerde sterfte voor zowel mannen als vrouwen. Het is de tweede meest voorkomende vorm van kanker bij vrouwen na borstkanker, en wordt bij mannen alleen voorafgegaan door long- en prostaatkanker. De incidentie wordt voor een belangrijk deel beïnvloed door de levensstijl van patiënten, waarbij men moet denken aan factoren als roken, slechte eetgewoonten (lage consumptie van groenten en fruit, hoge consumptie van rood vlees), obesitas, weinig beweging en het gebruik van alcohol. De afgelopen paar jaar werd er een sterke toename van het aantal gevallen van darmkanker gezien, welk kan worden toegeschreven aan het ontwikkelen van een meer Westerse levensstijl en dieet in landen met een middel tot hoge ontwikkelingsindex (Engels: human development index (HDI)), en door de start van nationale screeningprogramma's in de landen met de hoogste ontwikkelingsindex. Men verwacht dat deze incidentie nog alleen maar verder zal stijgen de komende decennia; experts spreken zelfs over een toename van 40% in 2030.

De belangrijkste hoeksteen voor de behandeling van colorectaal carcinoom blijft nog altijd chirurgie, welk vaak wordt gecombineerd met (neo)adjuvante chemo(radio)therapie, afhankelijk van de tumor locatie en het stadium waarin de ziekte verkeert. De afgelopen decennia werden er belangrijke verbeteringen doorgevoerd in de perioperatieve zorg van de colorectale patiënt, zoals ook de implementatie van 'snel-herstel' protocollen en bijvoorbeeld de introductie van minimaal invasieve chirurgie. Ondanks deze ontwikkelingen zijn complicaties na dit type chirurgie niet ongewoon, en blijft colorectale naadlekkage een erg gevreesde. Colorectale naadlekkage treedt op wanneer de anastomose (naad) niet goed geneest, en leidt tot de lekkage van darminhoud (ontlasting en bacteriën), naar de normaal steriele buikholte. Dit leidt tot de vorming van abscessen, buikvliesontsteking (peritonitis) en in ernstige gevallen, bloedvergiftiging (sepsis).

In ongeveer 7-12% van de operaties treedt er naadlekkage op, welk geassocieerd is met een verlengde opnameduur, een verhoogd risico op re-operatie, opname op de Intensive Care, en op overlijden. Tevens tonen verschillende grote studies aan dat er ook een relatie bestaat tussen het optreden van naadlekkage en een verhoogd risico op het ontwikkelen van een recidief.

Ondanks de grote aantallen studies die er wereldwijd naar deze complicatie zijn verricht door verschillende onderzoeksgroepen wereldwijd, is het nog niet mogelijk gebleken om het risico op naadlekkage te verminderen. We denken dat dit grotendeels verklaard kan worden omdat er nog weinig inzicht bestaat in de processen die betrokken zijn bij de normale darmgenezing en de pathways betrokken bij naadlekkage.

Het formuleren van een definitie van colorectale naadlekkage

Wat precies wordt verstaan onder naadlekkage verschilt sterk van arts tot arts. De bestaande definities zijn erg abstract en worden derhalve weinig toegepast in de dagelijkse praktijk. Dit maakt het vergelijken van uitkomsten van grote gerandomiseerde studies erg moeilijk, en het formuleren van richtlijnen vrijwel onmogelijk. Het eerste deel van dit proefschrift focust daarom op dit probleem. Er werd gebruik gemaakt van een vragenlijst (Hoofdstuk 2) en een uitgebreide literatuurstudie (Hoofdstuk 3) om uit te zoeken wat door artsen precies wordt verstaan onder naadlekkage. The vragenlijst werd zowel in Nederland als in China afgenomen, en toonde grote regionale verschillen tussen de gehanteerde definities van naadlekkage. Dit kan de grote variatie in de gerapporteerde incidentie van naadlekkage verklaren tussen studies die in het Westen en het Oosten werden uitgevoerd.

Met behulp van de literatuurstudie konden we in meer detail kijken naar de definities die gebruikt worden en welke chirurgie-gerelateerde gegevens werden gepubliceerd in de studies. Het bleek dat de meeste auteurs het liefst gebruik maakten van een eigen geformuleerde definitie, en niet van een reeds bestaande definitie. Daarnaast waren er grote verschillen aan te wijzen in de informatie die de auteurs publiceerden over het type chirurgie en het postoperatieve beloop. Samen met de Nederlandse Taskforce Naadlekkage stelden we 11 punten als advies voor toekomstige publicaties.

De resultaten van deze twee studies vormden de basis van een consensus studie die op dit moment wordt uitgevoerd door onze onderzoeksgroep. In deze Delphi studie (die niet in deze thesis werd opgenomen), vroegen we een aantal zeer gespecialiseerde chirurgen wereldwijd om hun mening te geven over deze kwestie. In een aantal rondes waarin de chirurgen hun feedback kunnen geven, zullen we een nieuwe definitie van naadlekkage formuleren. Omdat deze nieuwe definitie wordt goedgekeurd door belangrijke auteurs op het gebied van naadlekkage, is het waarschijnlijk dat deze beter wordt geaccepteerd door anderen die zich bezighouden met colorectale naadlekkage.

Het ontwikkelen van een risicoanalyse voor naadlekkage

In het tweede deel van dit proefschrift wordt het studie protocol van de REVEAL studie (Hoofdstuk 4) beschreven. Deze REVEAL studie is een nog lopende prospectieve observationele studie gericht op het ontwikkelen van een risicoanalyse voor naadlekkage. Voor de start van deze studie was het voor artsen vrijwel onmogelijk om een goede inschatting te maken van dit risico, ondanks onze brede kennis over risicofactoren zoals hierboven beschreven. Op basis van een groot aantal verschillende parameters zoals bijvoorbeeld plasma concentraties van ontstekingsmarkers, preoperatieve CT scans, uitademingslucht en de samenstelling van de darmbacteriën in

ontlastingssamples, richten we ons middels deze studie op het ontwikkelen van een risico-score die het risico op naadlekkage al vóór de operatie kan inschatten. Een adequate inschatting van dit risico zou het voor de behandelend arts makkelijker maken om belangrijke aspecten van de operatie met de patiënt te bespreken, bijvoorbeeld de keuze om wel of geen anastomose aan te leggen in het geval van een zeer hoog risico. In deze gevallen zouden de chirurg en de patiënt samen kunnen besluiten om geen darm anastomose, maar liever een permanent stoma aan te leggen. Gebaseerd op het vooraf bepaalde risico zouden er ook aanbevelingen kunnen worden gedaan over het postoperatieve traject, bijvoorbeeld over het routinematig afnemen van bloedsamples of vervaardigen van CT scans.

Een belangrijk neven doel van de REVEAL studie is het verminderen van de tijd tussen het ontwikkelen van de complicatie en de daadwerkelijke diagnose ervan. Studies hebben namelijk aangetoond dat vroege diagnose en behandeling met bijvoorbeeld drainage, antibiotica of een nieuwe operatie, het risico op ernstig nadeel van de complicatie fors verminderen.

In Hoofdstuk 5 presenteren we de resultaten van een kleinere studie binnen de REVEAL studie, bij wie er gebruik werd gemaakt van een beeldvormende techniek genaamd nabij-infrarood fluorescentie (NIRF) om de doorbloeding van de darm te kunnen visualiseren. Er werd tijdens de operatie bij deze patiënten een stofje via de bloedbaan toegediend die in weefsels oplicht wanneer er met licht van een bepaalde golflengte op wordt geschonden. Een betere bloedvoorziening van de darmen betekent een grotere intensiteit van het fluorescente signaal. In deze zogenaamde pilotstudie, vonden we dat de intensiteit van het geobserveerde signaal lager was bij patiënten die uiteindelijk naadlekkage zouden ontwikkelen. De studie toonde aan dat NIRF een veilige en effectieve methode is om de perfusie van de darm ter plaatse van de toekomstige anastomose in te schatten.

Verschillende beeldvormende technieken om de kwaliteit van de anastomose te testen

Tijdens het laatste decennium zijn er belangrijke verbeteringen doorgevoerd in de zorg die wordt geleverd in de dagen rondom de chirurgie. Zogenaamde versneld-herstel protocollen (Engels: Enhanced recovery protocols) werden opgesteld om het herstel en de opnameduur te verkorten, en om postoperatieve complicaties zoals ileus of longontsteking te verminderen. Bepaalde aspecten van de zorg konden reeds in deze protocollen worden gevangen, terwijl andere belangrijke beslissingen per individuele patiënt moeten worden genomen, zoals het wel of niet aanleggen van een tijdelijk dunne darm of dikke darm stoma. De aanwezigheid van een dergelijk ontlastend stoma

brengt ook weer discussiepunten met zich mee, zoals wat het optimale tijdsinterval is tussen de initiële chirurgie en het opheffen van het tijdelijk stoma. In Hoofdstuk 6 maakten we gebruik van de gegevens die werden verzameld in een groot nationaal cohort van patiënten om te onderzoeken welke van twee diagnostische modaliteiten (colonoscopie of CT scan met contrast) het beste kan worden ingezet om de integriteit van de darmnaad te controleren, voordat het stoma kan worden opgeheven. In deze studie werden geen statistisch significante verschillen gevonden tussen de twee testen, maar er zijn voor beide voor- en nadelen te beschrijven. Bij gebruik van een CT scan met contrast bestaat er het risico dat de test vals-positief is, wat wil zeggen dat het lijkt alsof er een lekkage is, maar dat dit in werkelijkheid niet zo is. Een voordeel van de colonoscopie is dat er tegelijkertijd ook nieuwe poliepen of een stenose van de anastomose kan worden gezien, welk dan direct kan worden behandeld. Echter, op basis van de resultaten van onze studie kunnen hier geen conclusies over worden getrokken, en er zijn grotere gerandomiseerde studies nodig met meer patiënten voordat er aanbevelingen kunnen worden gedaan voor een van de twee modaliteiten.

Meer inzicht in de processen die een rol spelen bij naadlekkage

In het laatste deel van dit proefschrift hebben we ons gericht op het vergroten van het inzicht in de processen die betrokken zijn bij normale darmgenezing en bij lekkage. In het vierde en laatste deel worden drie verschillende studies beschreven. In Hoofdstuk 7 hebben we onderzocht of de suppletie van een specifieke stof genaamd butyraat (of boterzuur) het risico op naadlekkage in ratten verminderd. Butyraat is een zogenaamde korte-keten vetzuur (SCFA), en vormt de belangrijkste bron van energie voor de cellen van de dikke darm. De bacteriën die in huizen in de darm produceren butyraat door middel van de afbraak van voedingsvezels. Studies hebben aangetoond dat butyraat een positief effect heeft op celvernieuwing en dat het de inflammatie in de darm doet verminderen. In Hoofdstuk 7 hebben we gekeken naar drie verschillende toedieningsmanieren om butyraat aan te bieden aan de intestinale cellen: door een eenmalige injectie direct voordat de naad wordt aangelegd, bij continue afgifte door een patch die in het lumen van de darm werd geplaatst, en door intermitterende doses van butyraat door middel van klysma's. De eerste twee technieken bleken echter onvoldoende effect te hebben of gevaarlijk (de patches zorgden voor een obstructie van de darm), maar er werden wel positieve resultaten gevonden in de groep die klysma's ontvingen. De translatie naar de dagelijkse praktijk is echter niet eenvoudig, omdat artsen vaak terughoudend zijn in het geven van klysma's gedurende vroege postoperatieve fase in het geval van lage darmnaden. Tijdens de eerste dagen na

chirurgie is de anastomose nog erg kwetsbaar en het risico op ruptuur van de anastomose door de verhoogde intraluminaire druk is daardoor hoog.

Een andere studie die werd beschreven in het laatste deel van dit proefschrift is het onderzoek naar de mucuslaag van de darm. Deze studie werd uitgevoerd in samenwerking met een onderzoeksgroep uit Gotenburg, Zweden. De gehele darm is van de binnenzijde bekleed door een mucuslaag die de eerste bescherming vormt tegen passerende ontlasting en schadelijke bacteriën. De mucus wordt geproduceerd door een specifiek celtype in de darmwand en vormt een natuurlijke omgeving voor bepaalde typen goede darmbacteriën. In de studie beschreven in Hoofdstuk 8, werd er een bepaald type muis gebruikt waarbij de mucuslaag in zijn geheel ontbreekt, maar ook normale zogenaamde 'wild-type' muizen. De resultaten van deze studie laten zien dat de mucuslaag inderdaad nodig is voor de normale processen van darmgenezing. Nieuwe studies zijn nodig om methoden te onderzoeken die deze normale functie verder kunnen ondersteunen.

In verschillende hoofdstukken van dit proefschrift werd het belang van de samenstelling van het microbioom en de goede darmbacteriën genoemd. In andere studies werd ook getoond dat er bepaalde bacteriesoorten zijn die een groot risico vormen voor de pas aangelegde anastomose. Sommige bacteriën ontwikkelen schadelijke eigenschappen wanneer ze worden blootgesteld aan bepaalde invloeden (zoals opioïden, of zelfs de chirurgie zelf), en produceren dan enzymen die de darmwand afbreken. Een ander veel onderzocht onderwerp is de invloed van bepaalde medicijnen, de zogenaamde NSAIDs (non-steroidale anti-inflammatoire drugs, bijvoorbeeld diclofenac of ibuprofen). Dit zijn zeer veelgebruikte pijnstillers waarvan wordt gedacht dat ze een negatief effect hebben op de darmgenezing. Het mechanisme hierachter is echter nog niet geheel opgehelderd. Sommige auteurs suggereerden dat er een verband zou bestaan tussen het gebruik van NSAIDs en de samenstelling van het microbioom die zou zorgen voor deze negatieve effecten. In het laatste hoofdstuk van dit proefschrift (Hoofdstuk 9) werd er in detail gekeken naar deze relatie, en werd er gevonden dat niet zozeer deze medicijnen, als wel de chirurgische schade zelf de meest belangrijke factor was voor de verandering in samenstelling van het microbioom na chirurgie.

Valorisation

Valorisation

The societal and economic impact of scientific research has become increasingly important during the last decade. This chapter therefore focuses on the impact that the knowledge acquired from the different projects described in this thesis may have on the society and economy, and discusses the influence these results have on the current scientific focus in the field of colorectal anastomotic leakage.

Economical and societal impact

10,000 oncological colorectal resections are performed on a yearly basis in the Netherlands. Of these, 10% (1000 patients) will be complicated by colorectal anastomotic leakage (CAL). It is predicted that the incidence rates of colorectal carcinoma will increase substantially over the next decades due to the implementation of the national screening programme for colorectal cancer and the ageing population. This would also implicate a strong increase in patients suffering the consequences of CAL. Patients who develop CAL experience significant morbidity, and the mortality rates associated with the occurrence of CAL are also still high: around 15% of the patients who develop a leak will eventually die of the consequences. These numbers/percentages have remained equally high the last decades.

The occurrence of the complication after colorectal surgery causes a three- to fivefold increase in healthcare costs compared to a similar surgery with a normal postoperative course. The exact additional costs associated with CAL have not been intensively studied in the Netherlands, but American studies have calculated the extra financial burden associated with leakage per 1000 operated patients to lay around 25.7 million euros. These costs are mainly generated due to the prolonged hospital stay (7-10 days), the increased employment of diagnostic tests, reoperations, and/or when the patient is temporarily admitted to the intensive care unit. The costs that are indirectly involved with the complication are generated in rehabilitation, physiotherapy and prolonged absence from work.

The burden of the complication goes beyond its economical impact, as CAL is also associated with a substantial impact on the Health-Related Quality of Life (HRQoL) of our patients. It is believed that quality of life is affected by the same factors that cause the increase in costs associated with CAL (prolonged hospital stay, absence from work), but also by the need of the construction of a stoma. If a reoperation is needed when CAL occurs, the anastomosis is usually taken down and a permanent colostomy is

constructed. Studies have shown that these stomas (either temporary or permanent) severely affect patients' body image and quality of life and lead to depression and anxiety. Lastly, the occurrence of CAL has been associated with an impaired oncological outcome, leading to an increase in local recurrence and a decrease in disease-specific survival.

Reducing the incidence of CAL

We believe that it is possible to achieve a significant reduction in the incidence of leakage rates and the associated morbidity and mortality by a combination of several techniques. We feel that with the REVEAL study, that currently is still on-going in the MUMC+ in Maastricht, Zuyderland Medical Centre in Sittard-Geleen/Heerlen and in VieCuri Medical Centre in Veldhoven, an important step in the right direction can be made. We believe that a combination of patient-derived factors such as a compromised immune response, sarcopenia, genetic predisposition, an aberrant intestinal microbiome composition, and surgery-related factors such as surgical stress, ischemia and a derailed systemic response, are responsible for the occurrence of CAL. Based on data from our own previous studies, in which we found that certain plasma markers and genetic factors are predictive of the risk of CAL, the REVEAL study is set up to collect a large set of patient data and patient samples (plasma, exhaled air, faeces, buccal swabs, tissue). With these data and with the use of machine-learning models, we will be able to assess the risk of the development of CAL in every individual patient. Until now, it has been extremely difficult to predict which patient will leak following surgery; no specific lab test exists that gives an adequate indication for the risk, and also the findings during surgery (e.g. aspect of the bowel and the quality of the anastomosis) have been shown to be of insufficient predictive value for CAL. With the results of the REVEAL-study, we will be able to make an adequate estimation of the risk of CAL for every individual patient, even before they have been subjected to surgery. A clear risk classification – and the associated guidelines for each risk class - will ameliorate communication between clinicians and patients. Moreover, with these results, one can also identify the patients with low risk of leakage, possibly saving them from an unnecessary deviating stoma and the associated impact on their quality of life. With the results from the REVEAL-study, the decision to construct an anastomosis or to opt for a permanent colostomy in the frail patient will no longer be based on the 'gut feeling' of the surgeon, but supported by evidence from a large multicentre observational study. As we have passed the 80% inclusion of in total 558 patients, we expect to be able to present the first results shortly.

Beside the preoperative risk assessment, the REVEAL study has a second important goal: to shorten time to diagnosis of CAL following surgery. This would also lead to a decrease in mortality and health care costs, as research has shown that early diagnosis leads to improved outcome. Lastly, a combination of metabolic and microbial signatures derived from the REVEAL data, more insight is generated in the processes of colorectal anastomotic healing and leakage. This could also lead to the development of new preventive and treatment strategies, such as nutritional interventions or influencing the microbiome by anti- or probiotics etcetera - strategies that are desperately needed, as our current best preventive method of severe CAL is a deviating stoma.

New research has stressed the importance of the surgery itself as a period that can be influenced in order to improve outcome. We participated in the nationwide Leak Check study, which investigated several factors that can be improved during surgery. This study showed that high peri-operative glucose-levels, low preoperative haemoglobin levels, low body temperature are all modifiable risk factors that can be improved and possibly reduce the rates of leakage. Another important tool that can be used during surgery to aid the battle against CAL is the use of fluorescence imaging to assess the perfusion of the intestine at the future anastomotic site during surgery. This is in line with our own work on intestinal fatty acid binding protein (I-FABP), which is a marker for intestinal damage and shows a significant increase in case of intestinal hypoperfusion.

We believe that in order to further reduce the leakage rates, new and innovative techniques are needed to further reduce the incidence of CAL. In the general introduction of this thesis, we have elaborated on why increased insight in the healing processes of the intestine and the pathophysiology behind CAL is relevant for current and future research. Several studies presented in this thesis have aimed at gaining knowledge on certain pathways, and possible fields of interest for such preventive methods have been investigated. In combination with the results from the clinical study (REVEAL), we will be able to select patients that will benefit most from new therapies, hopefully reducing the risk of a leakage to acceptable levels.

Importance of collaboration

During the past four years, fruitful collaborations have been established with different research groups in the Netherlands and abroad. Our research group has been part of the Taskforce Colorectal Anastomotic leakage, which is a platform for clinicians and researchers in the Netherlands to exchange ideas on this topic. Collaboration on a national level allows for initiation of new projects in multiple centres simultaneously, of

which the Leak Check is a good example. The consensus survey and definition review (Chapter 2 and 3, respectively) have already showed what combined (international) forces could bring about. The final part of the quest to a generally accepted definition for CAL is currently being conducted across all continents in the form of a Delphi analysis. In this analysis, many renowned researchers are asked for their opinion and are given the opportunity to elaborate on their thoughts together, eventually coming to consensus about a certain topic. We are positive that the new definition to be presented soon will be adopted by researchers and clinicians worldwide.

Dankwoord

Dankwoord

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List of publications

List of publications

Bosmans JW, **Jongen AC**, Bouvy ND, Derikx JP. Colorectal Anastomotic Healing: Why unravelling the biological process that leads to anastomotic leakage should be the main priority prior to conducting intervention studies - *BMC Gastroenterol.* 2015 Dec 21;15:180

Jongen AC, Bosmans JW, Kartal S, Lubbers T, Sosef M, Roumen RM, Stoot JH, Derikx JP, Bouvy ND Predictive factors for anastomotic leakage after colorectal surgery: The REVEAL-study. A study protocol for a prospective observational study - *JMIR Res Protoc.* 2016 Jun 9;5(2):e90

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Mommers E, Hong L, **Jongen AC**, Bouvy ND – Baseline performance of the ischemic button model for induction of adhesions in male and female laboratory rats – *Submitted*

Bosmans JW, **Jongen AC**, Scognamiglio F, Boonen BT, Hoebers N, Gijbels MJ, Jocken JW, Bouvy ND - Caution should be taken with glycerol as plasticizer in biomaterials designed for intra-abdominal use; evidence from in vitro assays as well as an experimental rat model – *Submitted*

Plat VD, Derikx JP, **Jongen AC**, Nielsen K, Sonneveld DJ, Tersteeg JJ, Crolla RM, van Dam DA, Cense HA, de Meij TG, Tuynman JB, de Boer NK, Daams F - Diagnostic accuracy of urinary intestinal fatty acid binding protein in detecting colorectal anastomotic leakage

Jongen AC, Meyer YM, Stassen LP, Derikx JP, Bouvy ND, Dutch Snapshot Research Group - Contrast enema vs. colonoscopy prior to temporary stoma reversal in rectal surgery; results of a national snapshot study - *In preparation*

Book chapter:

Jongen AC, van Woerden V, van Vugt JL, de Hoogt PA, de Wijkerslooth de Weerdesteijn EM, Tegels JJ, Stoot JH - Improving outcome in gastrointestinal and hepatopancreaticobiliary surgical oncology by preoperative risk assessment and optimization of perioperative care. Book chapter – *Oncology Critical Care*

Curriculum vitae

Curriculum vitae

Audrey Jongen was born on the 20th of July in Sittard, the Netherlands. After graduating from grammar school at the Trevianum in Sittard in 2007, she started her medical training at the University of Maastricht. Her interest in the scientific background of the medical decisions made in clinical practice was aroused during the Bachelor phase of Medicine and she worked several weeks on a clinical database involving patients with cervical cancer at the Maastricht Clinic. During the years that followed, she developed a strong preference for the surgical specialties and she decided to do a clinical rotation at the surgery department in Ferrara, Italy. Her final year of Medicine stood also completely in the light of surgery, when she did a combined clinical and scientific rotation under the supervision of Prof. dr. Nicole Bouvy. Directly following this final year, she started a PhD project under the supervision of Prof. dr. Nicole Bouvy, Dr. Joep Derikx, and Dr. Kaatje Lenaerts, which finally resulted in this thesis. After a year of working as ANIOS at the department of general surgery at the Amsterdam University Medical Centres, location AMC, she started her surgical residency at the Maastricht University Medical Centre+ in July 2019 under the supervision of Prof. dr. Laurents Stassen.