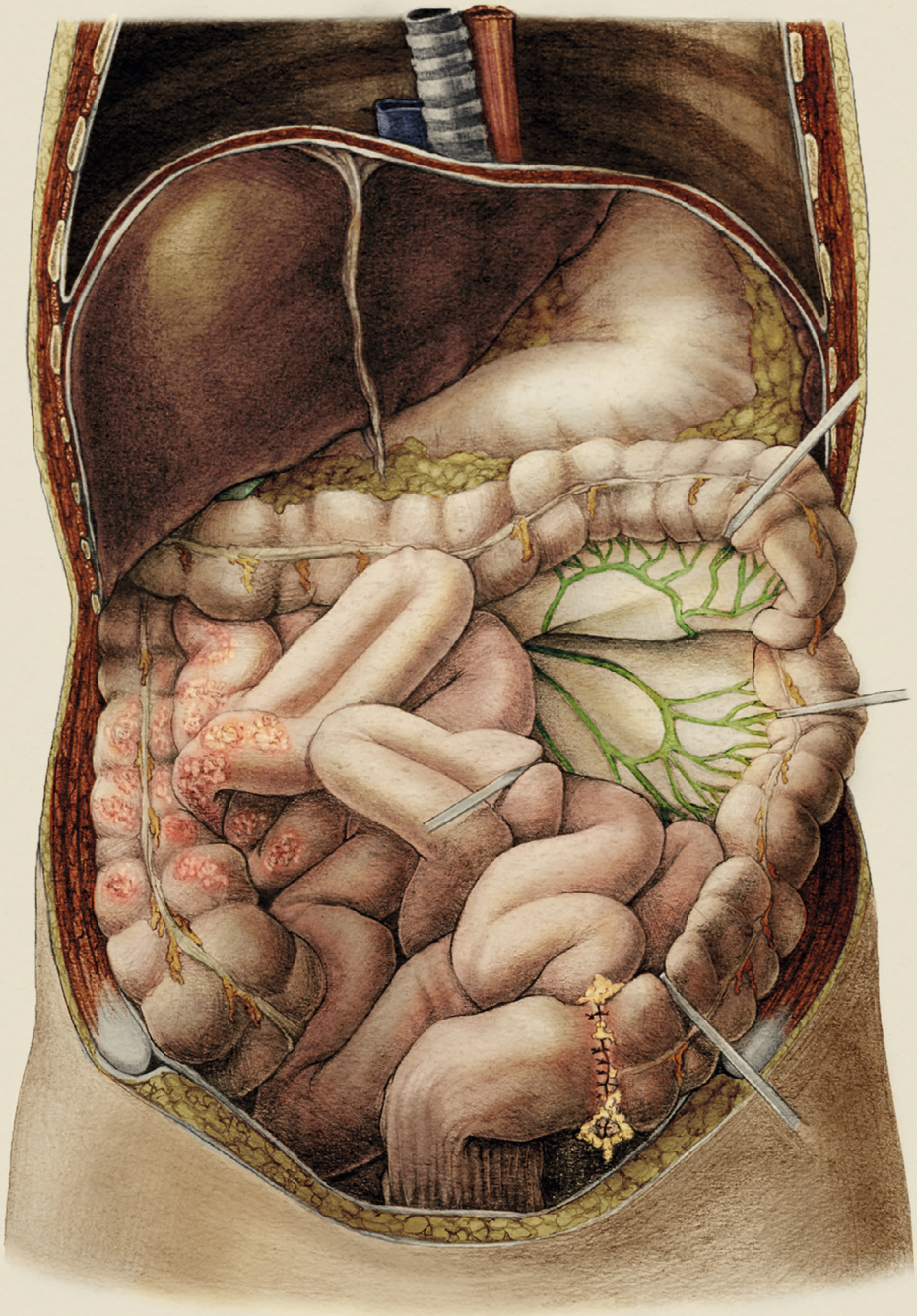
Transforming Outcomes in Colorectal Surgery



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Transforming Outcomes in Colorectal Surgery

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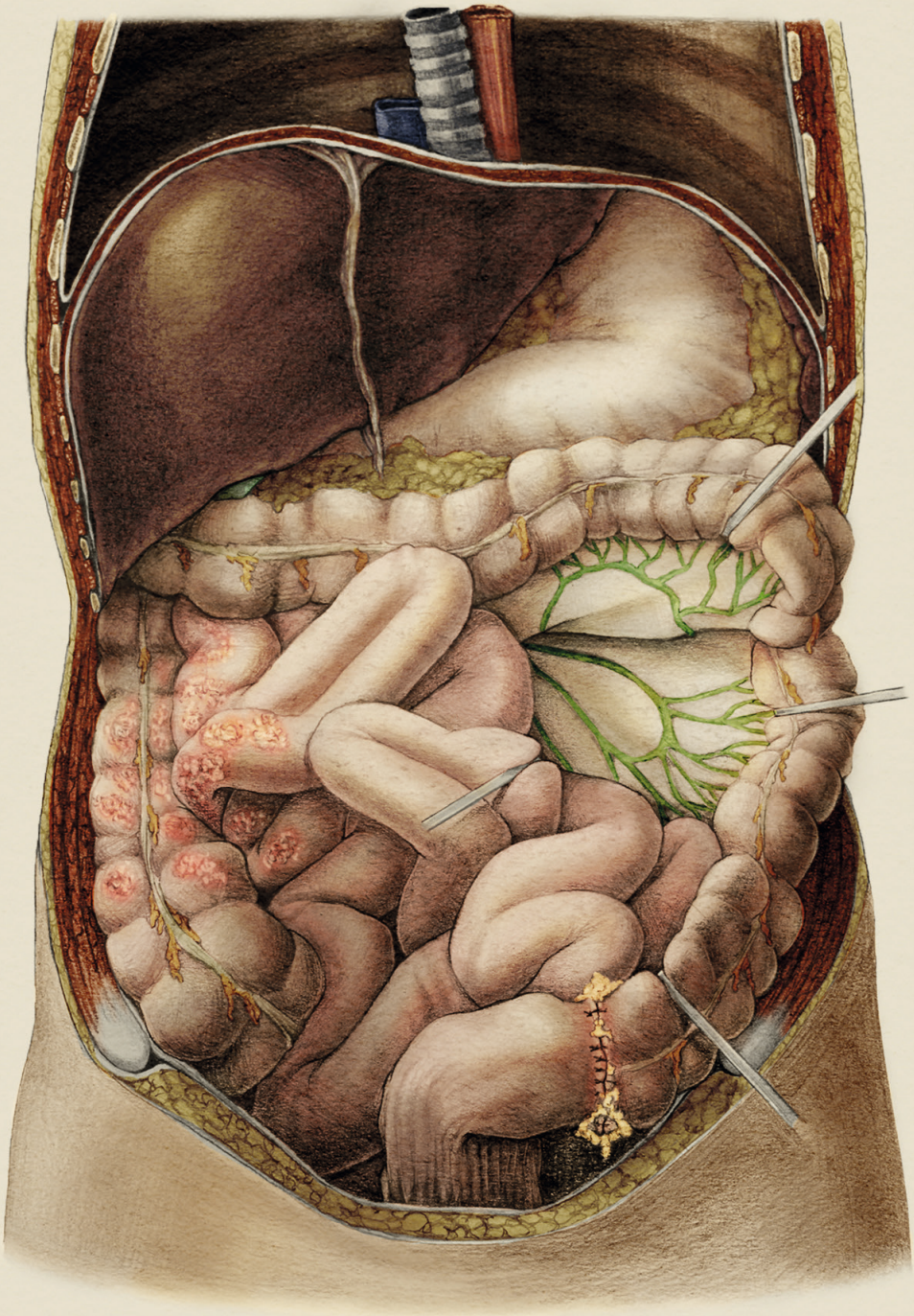
TABLE OF CONTENTS



CHAPTER 1

GENERAL INTRODUCTION

COLORECTAL CANCER

Colorectal cancer (CRC) ranks as the third most prevalent cancer globally, comprising around 10% of all cancer cases. Moreover, it stands as the second primary cause of cancer-related deaths worldwide [1]. The primary approach for curative treatment of CRC involves surgical removal of the tumor and adjacent lymph nodes. Choice of the best surgical procedure depends on the tumors' location and patients' condition, but often involves the creation of a primary anastomosis. The emphasis on improving patient outcomes in colorectal surgery has emerged as a central focus in medical research and clinical care. It is crucial to implement strategies to prevent associated complications to mitigate the impact of CRC surgery and improve patients' outcomes. The most feared complication after colorectal surgery is anastomotic leakage (AL). It is known that AL patients have a worse overall survival and poorer oncological outcomes, especially after rectal cancer surgery [2-4]. Besides, the most important and ultimately life-threatening feature of CRC is the ability to still metastasize after curative surgery. These so-called metachronous metastases have the poorest outcomes when they spread to the peritoneum, as treatment options are limited [5]. Minimizing the risk to prevent both AL and PM after colorectal surgery is essential as their development can lead to cancer progression, decreased quality of life, and poorer prognosis. As prevention is better than cure, taking proactive measures to minimize risks through meticulous surgical techniques, appropriate perioperative care, and early detection strategies can significantly improve patient outcomes and reduce the need for more complex and aggressive treatments later on.

ANASTOMOTIC LEAKAGE

Background

AL represents the most common major complication following colorectal resections. Severity of AL spans from minor defects with no evident extravasation of air or fluid to significant dehiscence, with or without localized abscess, phlegmon, and diffuse purulent and/or fecal peritonitis [6, 7]. These leaks can manifest early or late postoperatively, taking the form of fistulae, anastomotic strictures, chronic sinuses, or abscess cavities [7, 8]. The clinical impact of AL varies from minimal or no symptoms, particularly in diverted patients, to substantial morbidity and mortality arising from abdominal and/or pelvic sepsis [9]. AL also exerts a detrimental influence on oncological outcomes, functional results, and quality of life due to the necessity for reoperation, permanent diversion, or delayed ostomy reversal [9-12]. Difficult etiology and heterogeneous presentation of AL is reflected by its wide reported incidence rates, ranging from 2% to 25% [10-12].

Reporting of AL

Despite the growing number of literature that delves into the occurrence, origins, risk factors, treatment methods, and short/long-term consequences of AL after colorectal

surgery, interpreting the findings remains challenging due to significant heterogeneity in AL reporting [13]. This variation not only impacts reported incidence rates in clinical registries but also undermines the reliability of reported outcomes among colorectal surgery patients. Examination of an extensive colorectal dataset in the Netherlands has suggested a potential underestimation of AL rates following colorectal cancer resections in international literature [9]. The audit suggests that AL may intricately be involved in up to 23% of low anterior resections, especially when considering both acute and delayed leaks, as well as leaks that may manifest asymptomatically in patients with fecal diversion. This underscores the imperative need for a standardized and widely acknowledged definition of AL in colorectal surgery, as the absence of such a consensus limits the meaningfulness of comparing outcomes across wordwide medical centers. The lack of uniformity in AL definitions also hampers efforts to identify and categorize risk factors, standardize treatment protocols, and implement quality improvement initiatives with the objective to reduce AL occurrences. Furthermore, the lack of consensus also influences surgical trials investigating AL rates, particularly when AL is specified as the primary endpoint.

In 2010, the International Study Group of Rectal Cancer (ISREC) published a consensus on definition and grading AL, particularly in the context of anterior resection for rectal cancer, which stands out as the most frequently referenced and has garnered support through validation [14]. However, despite its acknowledgment, this definition has not garnered widespread endorsement from surgical societies or widespread adoption among practicing surgeons. Although several consensus guidelines and position statements aiming to establish standardized definitions of AL [8, 13-15], a universally accepted definition remains elusive. Yet, consensus on the radiologic definition or standardized assessment of CT-scans is also still lacking [13, 15].

Risk factors for AL

Although the creation of an anastomosis is a surgical technique, numerous patient related risk factors linked to a higher risk of the development of AL have been identified, offering opportunities for improved prevention and early detection of this significant complication. Nonmodifiable factors, including male gender, comorbidities, and the tumor's proximity to the anal verge, are among these contributors that can be assessed before surgery. Modifiable risk factors encompass smoking, alcohol consumption, obesity, neoadjuvant treatment, and the use of certain drugs [16]. Beside preoperative patients factors, intraoperative factors may also play a rol. The most important one is adequate blood perfusion, which is widely recognized as a crucial factor for the successful healing of the anastomosis and thereby reducing the risk of AL [17].

Bowel perfusion assessment

Adequate anastomotic perfusion is important for anastomotic healing, as good blood flow promotes tissue viability, cellular metabolism, and collagen synthesis, all of which are critical

for the formation of a strong and durable anastomosis. Insufficient perfusion can lead to tissue ischemia, delayed wound healing, and ultimately an increased risk of anastomotic breakdown and leakage [18]. Hence, there is a rising interest in employing real-time perfusion assessment techniques to aid in surgical decision-making and enhance outcomes. Through the identification of tissue areas exhibiting compromised perfusion, surgeons can potentially steer clear of establishing an anastomosis in those regions, opting instead for tissues with more favorable perfusion.

The most common intraoperative adjunct to assess bowel perfusion is by using *near-infrared fluorescence angiography* (NIRF). In short, a fluorophore is intravenously injected and, upon excitation at a specific wavelength, emits light at another specified wavelength (typically infrared) immediately following vessel division and/or completion of the anastomosis [17]. Using an optic dye like indocyanine green (ICG) have proved to be effective for bowel perfusion assessment and AL reduction after colorectal surgery [19-22]. As this technology enhances intraoperative decision-making by guiding surgeons to optimize perfusion and minimize the risk of AL, optimalisation of fluorescence imaging with new and better camera systems, development new optical dyes, quantification methods and assessing outcomes in large trials, is very popular in fluorescence-guided surgery research. Besides, near infrared fluorescence imaging can be used to visualize other structures too, like lymph nodes and the ureter, and might be helpful for multiple purposes during surgery.

Another technique that has emerged as promising modality for real-time assessment of bowel perfusion is *laser speckle contrast imaging* (LSCI). It is a non-invasive imaging technique that assesses blood flow dynamics by exploiting the speckle pattern created when coherent light interacts with moving objects, in particular red blood cells [23]. Previous research indicates that LSCI can achieve real-time intraoperative visualization of intestinal micro perfusion deficits, allowing for accurate prediction postoperative ischemic complications [24, 25]. Therefore, LSCI can be a useful tool to mitigate ischemia-related complications such as AL and improve patients' outcomes after CRC surgery. With this revealing capacity, it is important to perform additional preclinical validation, quantification, and feasibility assessment of LSCI to facilitate its potential in surgical decision-making when constructing colorectal anastomoses.

Long-term oncological outcomes

Already 15 years ago, an analysis of patients who did develop AL after lower anterior resections (LAR) for rectal cancer, showed that overall survival was reduced, but oncologic outcomes were not significantly influences by AL [26]. Later, a meta-analysis on this topic concluded that AL was associated with high local recurrence and poor survival (both overall and cancer-specific), but not with distant recurrence after anterior resections [27]. More recent studies showed that rectal cancer patients who developed AL after anterior resection or laparoscopic total mesorectal excisions (TME) had an increased risk of local recurrence, and even a decrease in overall survival, cancer-specific survival, and disease-free survival [2-4]. In

contrast, some studies did not find these significantly higher recurrence rates [28, 29]. For colonic resections, a systematic review including 69,047 patients in which 2,555 patients developed AL, found that AL was significantly associated with impaired overall survival, disease free survival and cancer specific survival, but not with higher recurrence rates [30]. These similar findings were also published later on by a study including both colon and rectal cancer patients [31]. They showed that long-term oncological outcomes were negatively influenced by the occurrence of AL after rectal cancer surgery, but not for colon cancer; although the authors stated this was probably due to low power of this study. A recent large retrospective Dutch population-based study including 65,299 colon cancer patients and 22,855 rectal cancer patients stated that AL had a stage-dependent negative impact on survival, but no independent association with disease recurrence after CRC resection [32].

Although oncological outcomes are not always significantly influenced by the occurrence of a leak, we know that survival rates are impaired for CRC patients after AL. Yet, it is necessary to avoid any further risk of poorer oncological and survival outcomes, which can be achieved by risk reduction of recurrence/metastatic spread after curative CRC surgery.

PERITONEAL METASTASES

Background

Peritoneal metastases (PM), commonly referred to as peritoneal carcinomatosis, signify the dissemination of metastatic lesions across the peritoneal surface within the abdominal cavity. These deposits possess the capability to infiltrate abdominal organs and structures, frequently leading to complications such as bowel obstruction, ureteral obstruction, and malignant ascites [33]. They may be identified either during the initial treatment of the primary tumor (referred to as synchronous PM) but also through follow-up assessments after primary surgery (referred to as metachronous PM) [34]. The incidence of metachronous PM is estimated in 4–12% of patients who undergo curative resection for colon cancer and in 2–19% of patients who undergo curative resection for rectal cancer [35], with an estimated average of 5% in all colorectal patients [33]. Although recurrence as PM seems to be a rare event in CRC patients after curative resection, consequences are notably significant. It is generally considered as a palliative situation when extensive spread is present as the typical life expectancy following diagnosis of PM spans from six to twelve months if no intervention takes place [36-38]. The limited efficacy of routine imaging techniques often leads to a failure in detecting PM, attributed to their small size and the inherently low contrast resolution of soft tissue in which they manifest. Consequently, their true incidence is probably underestimated, which is also reflected by autopsy reports [5, 33, 35, 39].

Treatment options

Only a selection of physically fit patients with limited colorectal PM (based on a low peritoneal cancer index (PCI) score) are considered eligible for current available treatment options [33]. The most common applied treatment is the surgical removal of all visible tumor deposits which is called cytoreductive surgery (CRS), followed by the application of heated chemotherapy in the abdominal cavity, known as hyperthermic intraperitoneal chemotherapy (HIPEC). Pressurized intraperitoneal aerosol chemotherapy (PIPAC) is also increasingly investigated as a palliative treatment option for patients [40]. Nonetheless, the exposure of tumor cells to the cytostatic drug remains restricted in both HIPEC and PIPAC, diminishing the therapeutic effectiveness [41]. Subsequently, minimally invasive alternatives that are applicable to a larger selection of patients with prolonged exposure of the cytostatic to the cancer cells are currently investigated. Recently, new research has been done to develop such a new minimally invasive treatment for PM, namely intraperitoneal administration of a cytostatic-loaded supramolecular hydrogel [41-43]. These previous investigations demonstrated enhanced survival among animals subjected to mitomycin C (MMC)-loaded hydrogels in a PM model. Given the promising nature of these findings, there arises not only potential interest in employing this approach as a therapeutic intervention but also in considering its application as a prophylactic intervention during primary surgery to mitigate the likelihood of metachronous PM in at-risk patients. It is therefore important to further investigate this hydrogel in colorectal surgery setting.

Risk factors and prevention

Given that the prospects for preventing metachronous peritoneal metastases (PM) are more encouraging than for synchronous PM, previous research on risk factors for metachronous PM is more comprehensive [33]. Various studies indicate that independent risk factors for metachronous PM include advanced tumor stages, infiltration of the radial margin, elevated preoperative tumor markers, emergency surgery, a primary tumor in the colon rather than the rectum, and the presence of free intraperitoneal cancer cells both before and/or after the resection of the primary tumor [33, 44, 45]. Currently, these clinical risk factors do not function as a landmark to apply certain prophylactic treatments. In recent years, there has been a growing recognition of the potential role of biomarkers in disease course prediction and not only offering a promising avenue for early detection, but also for potential preventive interventions [46]. The role of biomarkers may play an interesting role in the prevention of metachronous colorectal PM. If specific biomarkers, based on DNA/RNA alterations identified in the primary colorectal tumor during curative surgery, could characterize colorectal PM patients that have a higher risk of developing metachronous PM, these patients may benefit from preventive treatments regimes.

AIMS AND OUTLINE OF THIS THESIS

By examining innovative approaches and intercontinental expert opinions, this thesis aims to shed light on promising avenues that contribute to improved patient outcome after CRC surgery. While this thesis may not offer unequivocal solutions to the aforementioned discussion points, it does present novel perspectives within four distinct areas; evidence overview and reporting of colorectal AL, improvement of bowel perfusion assessment, impact of colorectal AL on patients, and prevention of metachronous PM.

PART I: Increasing international consensus on current evidence and reporting of anastomotic leaks after colorectal cancer surgery

Aims

- To increase insight in how AL is currently reported in high level evidence literature;
- To provide an overview of evidence-based statements regarding AL and a subsequent reporting framework that can be used to standardize AL reporting in the future;
- To create a radiological scoring system that can be used to standardize the assessment of AL on computerized tomography (CT) scans in the future.

The first part of this thesis focuses on the reporting of AL after CRC surgery. **Chapter 2** is a systematic review that focuses on the use of different AL definitions in high-level evidence literature (randomized controlled trials, systematic reviews, and meta-analyses) and additional reported elements that are related to AL. In this chapter, we also highlight the importance of standardized reporting of AL. Subsequently, **Chapter 3** reflects an international consensus project, in which the overview of current evidence regarding colorectal AL is presented, followed by a reporting framework to standardize reporting of colorectal AL after oncological surgery. As radiological assessment of AL plays a key role in the diagnostic phase, **Chapter 4** displays a study protocol for the development of a radiological scoring system that can be used to assess AL on CT-scans and to radiologically report its assessment in a standardized way.

Part II: Improving bowel perfusion assessment to reduce the risk of anastomotic leaks

Aims

- To study the feasibility and quantification of intestinal perfusion and ureter visualization with indocyanine green (ICG) and methylene blue (MB) using a new near-infrared fluorescence imaging system;
- To study the feasibility and quantification of intestinal perfusion during surgery with laser speckle contrast imaging (LSCI).

Part II of this thesis provides insights into bowel perfusion assessment to reduce the risk of AL. **Chapter 5** is a feasibility study in a porcine model which evaluates a new imaging system that is able to visualize both MB and ICG. This study illustrates the use of MB for both ureter visualization and bowel perfusion assessment. Subsequently, **Chapter 6** is a quantification analysis in which MB and ICG are compared in a porcine model using ischemic bowel loops. **Chapter 7** highlights the clinical implications of LSCI by performing a porcine experiment on anastomotic site selection. In this study, we demonstrate how LSCI can provide valuable real-time feedback on intestinal tissue perfusion during surgery. **Chapter 8** is a preclinical validation of LSCI for bowel perfusion assessment in a porcine model. In this quantification study, we perform a correlation analysis between laser speckle units and local lactate levels in ischemic bowel loops and assess inter-observer variability.

PART III: Patients' perspectives on colorectal anastomotic leaks

Aims

- To create an overview of the current knowledge of the impact on Quality of Life (QoL) of patients after colorectal AL;
- To obtain a more in-depth understanding of patients' experiences after AL.

The third part of this thesis gives insights into the patients' perspective on AL after CRC surgery. **Chapter 9** is a systematic review that provides an overview of current literature on the impact on the QoL of patients after AL. In this chapter, we also give additional recommendations on how to improve future AL research in relation to QoL. In **Chapter 10**, a qualitative interview study provides an insight into patients' experiences after developing an AL. In addition to summarizing the identified interview themes, we emphasize the key factors highlighted by patients that can directly enhance clinical practice and improve patient outcomes.

PART IV: Prevention of metachronous peritoneal metastases after colorectal cancer surgery

Aims

- To create an overview of the current knowledge on predictive biomarkers in primary colorectal tumors for PM;
- To identify predictive biomarkers in primary colorectal tumors for metachronous PM;
- To evaluate the safety of intraperitoneal cytostatic-loaded supramolecular hydrogel administration after the creation of a colon anastomosis.

Part IV of this thesis describes potentials to reduce the risk of developing PM by considering prophylactic interventions in patients who are at risk. **Chapter 11** provides an overview of current knowledge on specific biomarkers in the primary colorectal tumor that could

serve as a prediction tool to estimate the risk of distant peritoneal spread. **Chapter 12** is an explorative study in which primary colorectal tumor samples are analyzed to identify specific DNA and/or RNA that may predict metachronous PM after curative resection. As intraperitoneal administration of cytostatic loaded hydrogels is a promising preventive strategy for patients who have a high risk of developing metachronous PM, we evaluate the safety of a certain intervention in **Chapter 13**. This evaluation describes the effect of an intraperitoneal mitomycin-loaded hydrogel on anastomotic healing in a rodent model, based AL scores, adhesion scores and microscopic evaluation.

PART V: Summary, discussion and impact

This thesis is completed by a summary, general discussion and additional future perspectives in **Chapter 14**. **Chapter 15** provides an impact paragraph, followed by the Dutch summary of this thesis in **Chapter 16**.

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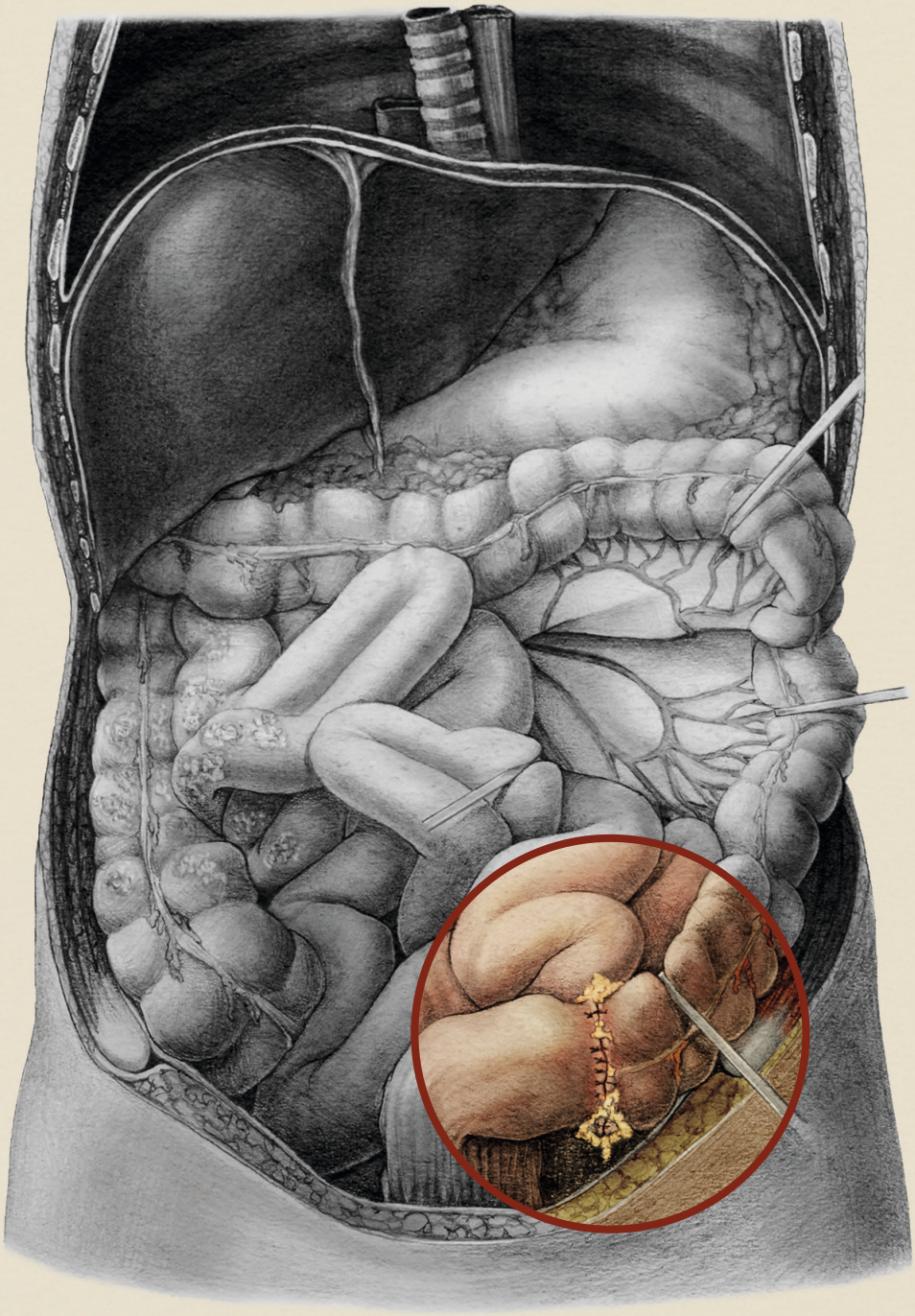
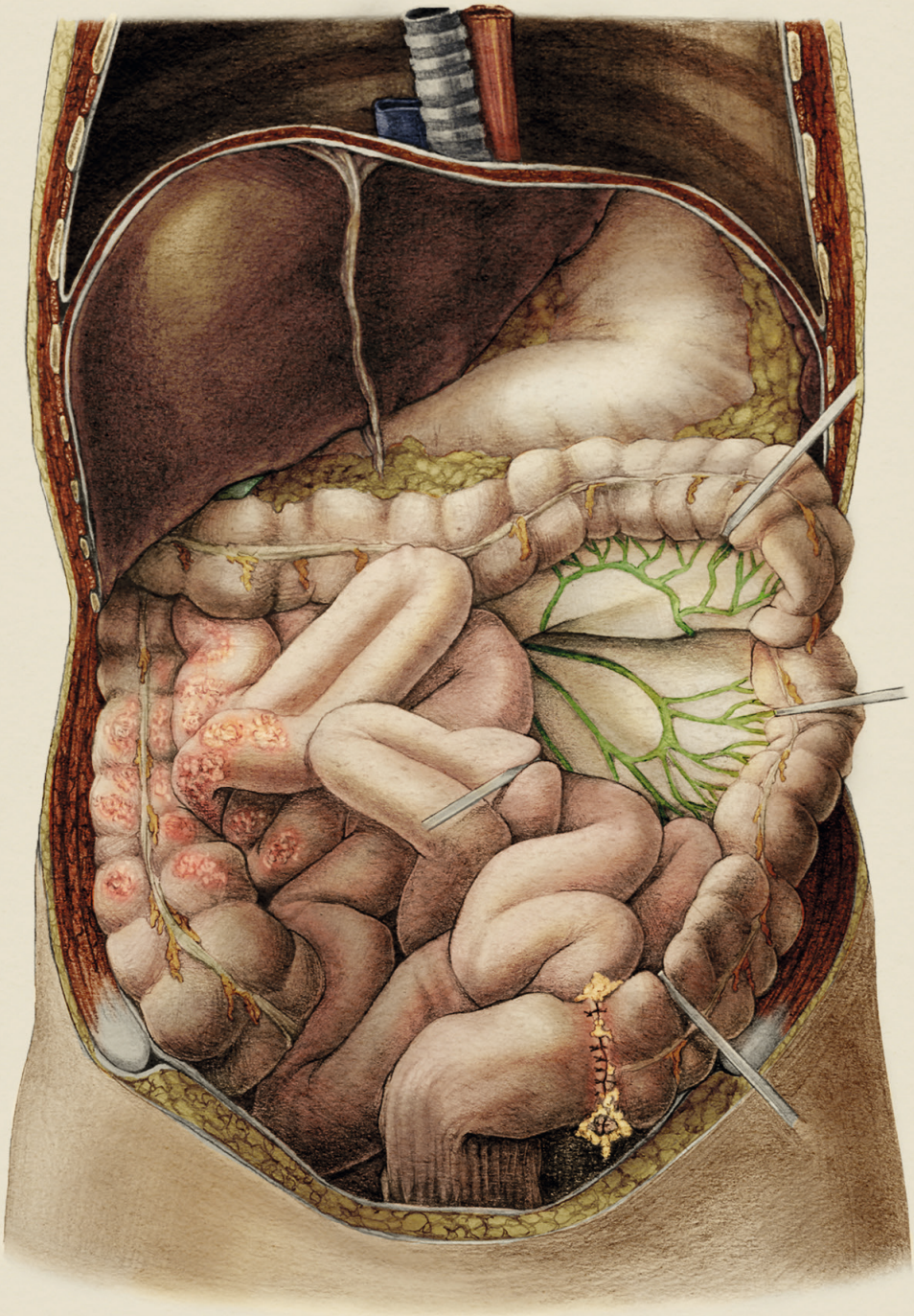


PART I

INCREASING INTERNATIONAL CONSENSUS ON CURRENT EVIDENCE AND REPORTING OF ANASTOMOTIC LEAKS AFTER COLORECTAL CANCER SURGERY



CHAPTER 2

QUALITY OF REPORTING ON ANASTOMOTIC LEAKS IN COLORECTAL CANCER TRIALS: A SYSTEMATIC REVIEW

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ABSTRACT

Background. Although attempts have been made in the past to establish consensus regarding the definitions and grading of the severity of colorectal anastomotic leakage, widespread adoption has remained limited.

Objective. A systematic review of the literature was conducted with the objective of examining the various elements used to report and define anastomotic leakage in colorectal cancer resections.

Data sources and study selection. A systematic review, using the PubMed, Embase, and Cochrane Library Database, of all published randomized controlled trials, systematic reviews, and meta-analyses containing data related to adult patients undergoing colorectal cancer surgery and reporting anastomotic leakage as a primary or secondary outcome, with a definition of anastomotic leakage included.

Outcomes. Definitions of AL, clinical symptoms, radiological modalities and findings, findings at reoperation, as well as grading terminology or classifications for AL.

Results. Of the 471 articles reporting anastomotic leakage as a primary or secondary outcome, a definition was reported in 95 studies (45 randomized controlled trials, 13 systematic reviews, and 37 meta-analyses), involving a total of 346,140 patients. Of these 95 articles, 68% reported clinical signs and symptoms of AL, 26% biochemical criteria, 63% radiological modalities, 62% radiological findings, and 13% findings at re-intervention. Only 45% (n=43) of included studies reported grading of anastomotic leakage severity or leak classification, and 41% (n=39) included a timeframe for reporting.

Limitations. There was a high heterogeneity between the included studies.

Conclusion. This evidence synthesis confirmed incomplete and inconsistent reporting of anastomotic leakage across the published colorectal cancer literature. There is a great need for the development and implementation of a consensus framework for defining, grading, and reporting anastomotic leakage.

Keywords. Anastomotic leakage; consensus; colorectal surgery; systematic review; definitions, severity grading, reporting.

INTRODUCTION

Despite advances in preoperative risk assessment, operative techniques and strategies, and postoperative care, the incidence of anastomotic leakage (AL) after colorectal cancer (CRC) surgery has not improved over the recent decades; with an incidence of 1.5 to 23% and with mortality rates as high as 16%-29% [1-5]. AL negatively impacts oncological outcomes, functional outcomes, and quality of life due to reoperation, permanent diversion, or delayed ostomy reversal [2, 3, 5]. In addition, AL leads to increased hospital costs adding to the overall economic burden associated with CRC surgery [6]. AL can present as small defects without air or fluid extravasation or large defects with or without localized abscess, phlegmon, and/or peritonitis [7, 8]. The clinical impact of AL varies from minimal or no symptoms to substantial morbidity and mortality from abdominal and/or pelvic sepsis [9]. Clinical studies where AL serves as a primary endpoint are often difficult to compare given considerable heterogeneity in the definition, severity grading, and diagnostic modalities used to assess AL.

Despite efforts to create a validated consensus definition and severity grading system by the International Study Group of Rectal Cancer (ISREC) in 2010; this has not been widely adopted in clinical practice [10-12]. A survey study among Dutch and Chinese colorectal surgeons highlighted ongoing lack of national and international agreement on definitions of AL [13]. Hence, several definitions of AL continue to be used in studies, with most controversy surrounding the radiological criteria considered diagnostic of AL. A panel of eight senior US surgeons attempted to reach consensus on the definition of AL, specifically evaluating clinical and radiological criteria [14]. Consensus could only be achieved in a few specific cases for both a radiological and clinical description, and only for specific types of interventions.

The development of an internationally accepted standardized framework for defining, reporting and, grading colorectal AL is needed to facilitate earlier identification, reporting and treatment of AL in order to reduce short and long-term sequelae. A widely implemented standardized framework could serve as a template for clinical trials where the incidence of AL is used as a clinical end point. This systematic review aimed to gain insight into the different elements contributing to the general definition and reporting of AL in the literature. The findings of this study will serve as the basis of an ongoing project to develop a framework for reporting and grading AL after CRC surgery (Consensus Reporting of colorectal Anastomotic Leaks; CoReAL).

METHODS

This systematic review was reported according to the guidelines of the “Preferred Reporting Items for Systematic Reviews and Meta-Analyses” (PRISMA) [15]. The protocol has been prospectively registered at PROSPERO ID 454660.

Search and Information Sources

A literature search was performed on November 4, 2022 in the PubMed, Embase, and the Cochrane Library Database using MeSH-, Emtree-, and free terms (Supplementary 1). Reference lists of all publications were searched for additional studies. The cross-referencing method was continued until no further relevant publications were identified.

Selection Process

Inclusion and exclusion criteria

Randomized controlled trials (RCTs), systematic reviews (SRs), and meta-analyses (Mas) containing data related to adult (>18 years) patients with CRC and in which AL was a primary or secondary outcome, were considered eligible. Studies published before 2000 (date of the first systematic review concerning AL definitions), other publication types and articles not in English or Dutch were excluded. Articles were excluded if AL was not a primary or secondary outcome as stated in the methods section, no AL definitions were stated in the study, or patients were not undergoing oncological procedures.

Study selection

All search results were imported into a web tool designed for SRs (Rayyan) [16]. Firstly, all duplicates were removed. Secondly, the screening of studies for eligibility was independently performed by 2 reviewers (DH, OM), using the predefined in- and exclusion criteria in two phases. In the first phase, articles were screened based on title and abstract. Disagreements between reviewers were resolved by initial discussion to create consensus and/or by one of the senior authors (NB). As part of the second phase, full texts were assessed. If the eligibility criteria were met after full-text screening by both reviewers, article inclusion followed. All references were stored in the Endnote Reference Management Tool.

Data Items and Collection Process

Two reviewers (DH, OM) independently extracted data from text, tables, and figures in a standardized, predefined datasheet. Data extraction for each article included first author, year of publication, country, study design, number of patients, number of studies in case of a SR or MA, study aims, surgical details, definitions or criteria used for AL assessment (clinical, biochemical, radiologic criteria and/or finding during reoperation), all definitions of AL, clinical symptoms associated with definitions of AL, radiological modalities and findings used in the diagnosis of AL, findings at reoperation for AL, as well as grading terminology or classifications for AL. We ensured definitions and reporting elements were not double-counted by cross-referencing RCTs included in systematic reviews. When systematic reviews provided their own AL definitions without detailing those from included studies, we treated these as separate entries. This method maintained data integrity. Data acquired through the outlined search strategy was summarized in tables.

Study Risk of Bias Assessment

To assess the methodological quality of the included studies, the risk of bias was independently assessed by 2 reviewers (DH, OM). RCTs were assessed using the RoB2 tool, while (systematic) reviews and meta-analyses were assessed using the ROBIS tool [17, 18]. All types of bias were evaluated and judged as low-, moderate-, or high risk resulting in an overall bias judgement. The bias was visualized using the Risk-of-bias visualization (Robvis) tool [19].

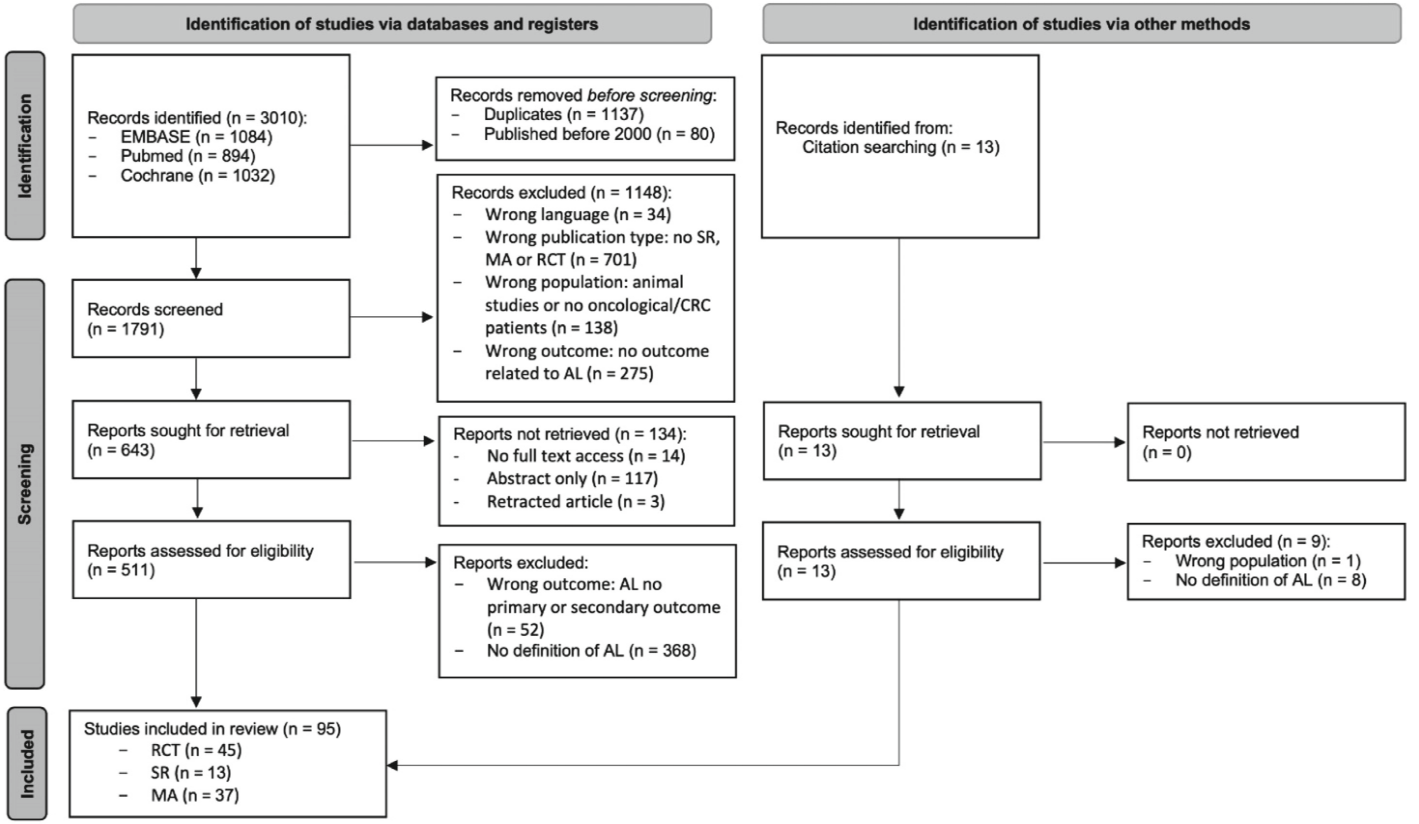
RESULTS

Study Selection

The electronic search yielded 1,792 studies after removing duplicates and publications before 2000. After screening abstracts, 644 potentially eligible studies remained, based on the predefined inclusion and exclusion criteria. Full-text assessment from 134 studies was not possible (i.e., no full-texts available, or retracted articles), whereafter 511 articles remained eligible. Reference checking resulted in 13 additional studies, resulting in 524 studies for full-text assessment. Fifty-three studies did not meet inclusion criteria; the remaining 471 studies reported AL as a primary or secondary outcome. Of these, 376 did not report a definition of AL, which resulted in the inclusion of 95 studies. The study selection process is summarized in Figure 1.

Study characteristics

The 95 studies included 45 RCTs, 13 SRs, and 37 meta-analyses (MAs) published between 2000 and 2022. The main characteristics of the included studies are summarized in Table 1.



AL, anastomotic leak; CRC, colorectal cancer; MA, meta-analysis; RCT, randomized controlled trial; SR, systematic review.

Figure 1. Flow diagram of selections process based on the PRISMA 2020 guidelines.

Table 1. Characteristics of included studies

Author	Year	Country	Study design	Number of patients*	Number of studies	Aim of the study	Type of resections included
Alekseev et al. [20]	2020	Russia	RCT	380	N/A	To evaluate the usefulness of ICG FA in reducing AL in patients undergoing a stapled colorectal anastomosis.	(L)AR with TME, left colectomy
Altomare et al. [21]	2021	Italy	RCT	54	N/A	To compare incidence of AL and severity of postop complications in patients undergoing LAR with diverting stoma or LAR with reinforcement of the anastomosis without diverting stoma.	LAR with TME
Ansari et al. [22]	2017	Australia	RCT	326	N/A	To compare acute adverse events and postoperative complication rates in a randomized trial of short-course versus long-course preoperative radiotherapy.	APR, (L)AR, Hartmann procedures
Badawi et al. [23]	2015	Saudi Arabia	SR	6921	31	To review risk factors for and protective strategies against AL following minimally access surgery for CRC.	(L)AR
Bakker et al. [24]	2017	The Netherlands	RCT	402	N/A	To evaluate the efficacy of the C-seal device in reducing AL following stapled colorectal anastomoses.	All types of colorectal resections with stapled anastomoses
Balciscueta et al. [25]	2020	Switzerland	SR and MA	1267	4	To evaluate the incidence of AL rate following laparoscopic rectal surgery following one vs two stapler firings for rectal transection.	AR
Bao et al. [26]	2022	Italy	RCT follow-up	311	N/A	To evaluate overall survival, disease-free survival, and local and distant recurrence in patients with AL following LAR.	LAR

Table 1. Continued

Author	Year	Country	Study design	Number of patients*	Number of studies	Aim of the study	Type of resections included
Blanco-Colino et al. [27]	2018	Spain	SR and MA	1302	5	To evaluate AL rates using ICG fluorescence imaging vs standard surgical care in CRC surgery.	LAR with TME, right colectomy, left colectomy, sphincter-saving resection
Boelens et al. [28]	2014	The Netherlands	RCT	123	N/A	To investigate whether early enteral nutrition (EEN), as a bridge to a normal diet, can reduce postoperative ileus.	LAR, APR, Hartmann procedure
Bretagnol et al. [29]	2010	France	RCT	178	N/A	To assess postoperative outcomes in patients undergoing sphincter-saving rectal resection for cancer without preoperative MBP.	Mesorectal excision, sphincter-saving resection
Brisinda et al. [30]	2009	Italy	RCT	77	N/A	To compare surgical outcomes of end-to-end and end-to-side anastomosis after AR for T1 – T2 rectal cancer.	AR with TME or PME
Brown et al. [31]	2001	Singapore	RCT	59	N/A	To assess the effect of prophylactic drainage after LAR when anastomoses are located below the peritoneal reflection.	LAR with total- or wide mesorectal excision
Bülow et al. [32]	2006	Denmark	RCT	194	N/A	To compare AL rates after AR with a loop ileostomy vs transanal stenting vs both vs neither.	Anterior resection
Cong et al. [33]	2015	China	SR	16178	37	To evaluate AL requiring laparotomy and the associated rate of diverting stoma in initial AR for rectal cancer.	(ultra)LAR, sphincter-saving resection
Cong et al. [34]	2014	China	SR and MA	24232	39	To evaluate AL requiring reoperation and compare mortality in patients with AL relative to overall postoperative mortality after AR for rectal carcinoma.	AR

Table 1. Continued

Author	Year	Country	Study design	Number of patients*	Number of studies	Aim of the study	Type of resections included
Cong et al. [35]	2013	China	SR	24288	70	To evaluate the pooled incidence and severity of AL and determine the average rate of AL for each grade after AR for rectal cancer.	AR, (ultra)LAR, sphincter-saving resection
Maggiore et al. [36]	2018	Egypt	RCT	57	N/A	To compare the short-term operative as well as oncologic outcomes of robotic-assisted and laparoscopic rectal cancer resections	AR, (ultra)LAR, APR
Debakey et al. [37]	2022	China	SR and MA	1556	7	To evaluate the TDT effect on AL prevention.	Laparoscopic rectal resections
Deng et al. [38]	2020	Italy	RCT	252	N/A	To evaluate the usefulness of intraoperative assessment of anastomotic perfusion using ICG angiography in patients undergoing left-sided colon or rectal resection with colorectal anastomosis.	LAR, left colectomy
Emile et al. [39]	2022	Egypt	SR and MA	8786	27	To assess changes in surgical plan based on ICG fluorescence angiography on the rates of AL.	All types of colorectal procedures
Finochi et al. [40]	2020	France	MA	5115	12	To compare postoperative outcomes between patients undergoing rectal cancer resection performed by totally laparoscopic approach compared to those who underwent intraoperative conversion.	APR, sphincter-saving resection
Floodeen et al. [41]	2013	Sweden	RCT	45	N/A	To compare patients with symptomatic AL following LAR for cancer diagnosed during the initial hospital stay with those in whom leakage was diagnosed after hospital discharge.	LAR
Fujii et al. [42]	2018	Japan	RCT	331	N/A	To clarify whether the IMA should be tied at the origin (high tie) or distal to the left colic artery (low tie) in relation to AL.	AR

Table 1. Continued

Author	Year	Country	Study design	Number of patients*	Number of studies	Aim of the study	Type of resections included
Fujii et al. [43]	2019	Japan	RCT subanalysis	331	N/A	To determine if the IMA should be tied at the origin (high tie) or distal to the left colic artery (low tie) in relation to AL.	AR
Gadan et al. [44]	2020	Sweden	RCT	232	N/A	To investigate the incidence of and risk factors for permanent stoma beyond 5 years following LAR.	LAR
Guenaga et al. [45]	2003	Brazil	SR	5805	18	To assess the safety and effectiveness of MBP based on morbidity and mortality following colorectal surgery.	LAR
Ha et al. [46]	2015	Korea	SR and MA	1118	6	To evaluate the effectiveness of transanal tube placement to prevent AL after LAR for rectal cancer using a stapling technique.	LAR
Ha et al. [47]	2017	South Korea	SR and MA	78434	34	To assess the oncologic outcomes of AL following restorative surgery for CRC	All types of colorectal procedures
Habeeb et al. [48]	2023	Egypt	RCT	74	N/A	To compare outcomes of open colorectal anastomosis with side-to-end vs end-to-end configuration in non-emergent sigmoid and rectal cancer surgery in adults.	(Ultra)LAR
Hajibandeh et al. [49]	2019	UK	MA	436	4	To compare outcomes of temporary loop ileostomy closure during or after adjuvant chemotherapy following rectal cancer resection.	LAR
He et al. [50]	2022	China	RCT follow-up	203	N/A	To analyze long-term impact of radiation on major LARS and permanent stoma rates.	LAR
Hüser et al. [51]	2008	Germany	SR and MA	2729	27	To evaluate the benefit of a defunctioning ileostomy or colostomy after LAR for CRC.	LAR

Table 1. Continued

Author	Year	Country	Study design	Number of patients*	Number of studies	Aim of the study	Type of resections included
Ivanov et al. [52]	2011	Serbia	RCT	71	N/A	To establish if intraoperative air testing may reduce the dehiscence rate of stapled colorectal anastomoses.	Sigmoid resection, LAR, Sigmoidostomy diversion, Hartmann procedure
Jafari et al. [53]	2021	USA	RCT	347	N/A	To evaluate whether the use of fluorescence angiography to ensure anastomotic perfusion decreases AL after LAR.	LAR
Karim et al. [54]	2020	Switzerland	SR and MA	18039	18	To evaluate cancer-specific outcomes after curative rectal cancer surgery comparing AL with no leak.	All types of colorectal procedures
Kastora et al. [55]	2021	UK	SR and MA	25395	15	To assess whether NSAIDs, and their sub-categories, increase AL in colonic anastomoses and to identify whether this affects specific anastomotic sites.	Right hemicolectomy, left hemicolectomy, AR
Kelly et al. [56]	2014	UK	SR and MA	14344	19	To compare short-term and oncological outcomes following CRC resection performed by surgical trainees and expert surgeons.	All types of colorectal procedures
Kim et al. [57]	2022	Korea	SR and MA	1431	12	To compare the effects of high versus low IMA ligation in CRC surgery.	(L)AR
Koedam et al. [58]	2022	The Netherlands	RCT	1832	N/A	To evaluate oncological outcomes with and without AL after CRC surgery.	All types of colorectal procedures
Lee et al. [59]	2018	Australia	SR and MA	1418	7	To evaluate the predictive value of cardiopulmonary exercise testing and field walk tests in surgical outcomes after CRC surgery.	All types of colorectal procedures

Table 1. Continued

Author	Year	Country	Study design	Number of patients*	Number of studies	Aim of the study	Type of resections included
Lin et al. [60]	2021	China	SR and MA	3137	11	To investigate whether intraoperative ICG angiography can reduce the incidence of AL.	LAR
Lindgren et al. [61]	2011	Sweden	RCT follow-up	233	N/A	To assess the risk for permanent stoma after LAR for rectal cancer.	LAR
Lu et al. [62]	2016	Australia	MA	13655	11	To evaluate the best current evidence assessing AL in rectal cancer resections with curative intent and its impact on survival and cancer recurrence.	All types of rectal procedures
Ma et al. [63]	2020	China	SR and MA	3480	18	To assess the relationship between AL and long-term oncological outcomes after curative AR for rectal cancer.	AR
Ma et al. [64]	2019	China	RCT secondary analysis	125	N/A	To quantify the changes in pelvic anatomic features caused by preoperative radiotherapy for CRC on pelvic MRI and evaluate the ability to predict AL.	TME
Machado et al. [65]	2003	Sweden	RCT	100	N/A	To investigate functional outcomes of pouch vs non-pouch side-to-end anastomosis after standard TME surgery.	LAR with TME
Mari et al. [66]	2019	Italy	RCT	214	N/A	To compare the incidence of GU dysfunction and evaluate the incidence of AL and oncological outcomes in patients undergoing elective lap LAR + TME with either high or low ligation of the IMA.	LAR with TME
Matsuda et al. [67]	2015	Japan	RCT	100	N/A	To clarify whether the level of ligation of the IMA in patients with rectal cancer affects defecatory function.	AR

Table 1. Continued

Author	Year	Country	Study design	Number of patients*	Number of studies	Aim of the study	Type of resections included
Matthiessen et al. [68]	2007	Sweden	RCT	234	N/A	To assess if there is a difference in the rate of symptomatic AL in patients randomized to fecal deviation.	LAR
Mcdermott et al. [69]	2015	UK/Ireland	SR	-	451	To evaluate the role of preoperative, intraoperative, and postoperative factors in the development of colorectal AL.	All types of colorectal procedures
Menahem et al. [70]	2017	Germany	MA	660	3	To evaluate if drainage of the extraperitoneal anastomosis after rectal surgery impacts the postoperative complication rate.	Rectal resections
Mhatre et al. [71]	2016		SR	20441	-	To identify risk factors for AL and identify a standardized diagnostic protocol to reduce delay in diagnosis of AL.	All types of colorectal procedures
Mrak et al. [72]	2016	Austria	RCT	166	N/A	To determine whether a protective diverting ileostomy reduces the AL rate.	LAR
Neutzling et al. [73]	2012	Brazil	SR	1233	9	To compare the safety and effectiveness of stapled and handsewn colorectal anastomosis. The following primary hypothesis was tested: the stapled technique is more effective because it decreases complications.	All types of colorectal procedures
Oguz et al. [74]	2007	Turkey	RCT	109	N/A	To investigate the effect of L-alanine-L-glutamine on postoperative complication rate and duration of hospitalization in patients operated for CRC.	All types of colorectal procedures
Okkabaz et al. [75]	2017	Turkey	RCT	74	N/A	To analyze the outcomes of j-pouch and side-to-end anastomosis in rectal cancer patients treated with lap hand-assisted LAR.	LAR

Table 1. Continued

Author	Year	Country	Study design	Number of patients*	Number of studies	Aim of the study	Type of resections included
Pata et al. [76]	2009	Belgium	SR with a MA and sensitivity analysis	4417	45	To determine whether a defunctioning stoma should be constructed routinely after TME or whether it could be used selectively to ensure patient safety.	TME
Peeters et al. [77]	2005	The Netherlands	Retrospective analysis of RCT	924	N/A	To identify risk factors for symptomatic AL in patients undergoing TME for rectal cancer.	TME
Peters et al. [78]	2017	The Netherlands	RCT post hoc analysis	112	N/A	To investigate the relationship between POI and inflammation and AL after CRC resection.	All open colorectal resections
Podda et al. [79]	2020	Italy	SR and MA	1120	4	To determine whether prophylactic drainage after colorectal anastomoses confers any advantage in the prevention and management of AL.	All types of colorectal procedures
Pucciarelli et al. [80]	2019	Italy	RCT	379	N/A	To assess whether colonic J pouch reconstruction after LAR reduces the incidence of AL compared to standard straight colorectal anastomosis.	LAR
Qi et al. [81]	2022	China	SR and MA	580	8	To evaluate the predictive value of peritoneal fluid cytokines in the detection of AL following colorectal surgery.	All types of colorectal procedures
Qu et al. [82]	2015	China	SR and MA	4580	14	To quantify the clinicopathologic factors predictive for AL in patients undergoing laparoscopic AR for rectal cancer.	Laparoscopic AR
Ren et al. [83]	2021	China	RCT	64	N/A	To provide a basis for evaluating the safety and effectiveness of laparoscopic TME.	Laparoscopic TME
Rojas-Machado et al. [84]	2016	Spain	SR and MA	-	68	To develop a new prognostic index to predict the risk of developing AL after CRC surgery.	All types of colorectal procedures

Table 1. Continued

Author	Year	Country	Study design	Number of patients*	Number of studies	Aim of the study	Type of resections included
Rolph et al. [85]	2004	UK	Intervention review	903	3	To assess the effectiveness and safety of a prophylactic drain after elective colorectal anastomosis.	All types of colorectal procedures
Rutkowski et al. [86]	2014	Poland	RCT	177	N/A	To evaluate the rate of local recurrence and distant recurrence in patients after R0 resection.	TME
Saber et al. [87]	2013	Egypt	RCT	156	N/A	To evaluate the efficacy of tube cecostomy as an alternative to colostomy in the managing patients with left-sided colonic carcinoma and rectal cancer with respect to postoperative morbidity and mortality and functional outcomes.	All left colon or rectal cancer resections
Sangiorgio et al.	2021	Italy	Intervention review with MA	252	6	To systematically assess the efficacy of parenteral and oral antibiotic prophylaxis compared to parenteral-only prophylaxis for the prevention of SSI in patients undergoing laparoscopic surgery for CRC resection.	All types of laparoscopic colorectal resections
[88]**Schardey et al.** [89]	2020	Germany	RCT	80	N/A	To study the efficacy of topical antibiotic treatment on the incidence of AL in rectal cancer surgery.	(L)AR
Selvamani et al. [90]	2022	USA	SR	3451	12	To examine the need for blood markers that assist in the early diagnosis of AL after surgery.	All types of colorectal procedures
Senagore et al. [91]	2014	US	RCT	258	N/A	To assess whether the use of a synthetic, bioabsorbable staple line reinforcement material with circular staplers would reduce postoperative AL in patients with a colorectal, coloanal, or ileoanal anastomosis.	All types of colorectal procedures

Table 1. Continued

Author	Year	Country	Study design	Number of patients*	Number of studies	Aim of the study	Type of resections included
Shigeta et al. [92]	2016	Japan	SR and MA	909	4	To evaluate the usefulness of a TDT for the prevention of AL after an AR for rectal cancer.	AR
Singh et al. [93]	2014	New Zealand	SR and MA	2483	7	To evaluate the predictive value of CRP in this setting.	All types of colorectal procedures
Škrabec et al. [94]	2022	Spain	SR	N/A	9	To review and to assess the quality of the scientific articles regarding early and late AL after CRC surgery and their risk factors.	All types of colorectal procedures
Snijders et al. [95]	2012	The Netherlands	MA	10343	22	To compare AL-related mortality in comparison to overall postoperative mortality after LAR for rectal cancer.	LAR
Su'a et al. [96]	2017	New Zealand	SR	8988	36	To assess biomarkers as potential diagnostic tests for preclinical detection of AL.	All types of colorectal procedures
Su'a et al. [97]	2020	New Zealand	MA	1639	8	To evaluate the accuracy of procalcitonin in the early diagnosis of AL following CRC surgery.	All types of colorectal procedures
Tamura et al. [98]	2021	Japan	RCT	161	N/A	To assess the incidence of AL in patients with rectal cancer after laparoscopic AR with or without TDT on the hypothesis that it could contribute to prevent AL without reference to diverting stoma.	LAR
Tan et al. [99]	2009	Singapore	MA	11429	25	To evaluate the need for routine stoma formation.	LAR
Tocchi et al. [100]	2000	Italy	RCT	112	N/A	To investigate the role of omentoplasty, by means of intact omentum, in preventing AL after rectal resection.	AR

Table 1. Continued

Author	Year	Country	Study design	Number of patients*	Number of studies	Aim of the study	Type of resections included
Ulrich et al. [101]	2009	Germany	RCT	34	N/A	To evaluate the need for diverting ileostomy in patients undergoing LAR.	LAR
Van't Sand et al. [102]	2011	The Netherlands	RCT subgroup analysis	63	N/A	To evaluate the effects of MBP on morbidity and mortality after AL in elective colorectal surgery.	All types of colorectal procedures
Wang et al. [103]	2017	China	SR and MA	11535	14	To evaluate the impact of AL on disease recurrence and survival.	AR
Wang et al. [104]	2016	China	SR and MA	909	4	To evaluate the efficacy of TDT placement after AR.	AR
Whistance et al. [105]	2013	UK	SR	N/A	194	To summarize and undertake an in-depth analysis of outcome reporting in CRC surgery.	All types of colorectal procedures
Wiggins et al. [106]	2015	UK	SR and MA	2296	6	To compare the outcomes of gastrointestinal anastomosis with and without the use of omentoplasty.	All types of colorectal procedures
Wright et al. [107]	2017	UK	SR	N/A	13	To appraise the current evidence base into local biomarkers of AL allowing the identification of the most promising emerging biomarkers and discussion of their limitations and future potential clinical role	All types of colorectal procedures
Wu et al. [108]	2014	China	MA	5612	11	To provide a comprehensive evaluation of the role of a protective stoma in LAR for rectal cancer.	LAR
Xiao et al. [109]	2011	China	RCT	398	N/A	To investigate whether the use of a TDT as an alternative endoluminal diversion technique for rectal carcinoma can reduce the 30-day leakage rate after LAR.	LAR

Table 1. Continued

Author	Year	Country	Study design	Number of patients*	Number of studies	Aim of the study	Type of resections included
Yang et al. [110]	2016	China	RCT	79	N/A	To evaluate the anti-infectious effects of perioperative probiotics treatment in patients undergoing CRC resection.	All types of colorectal procedures
Yang et al. [111]	2019	China	SR and MA	8456	24	To evaluate the current scientific evidence of LCA non-preservation versus LCA preservation in CRC surgery.	All left colon or rectal cancer resections
Yeung et al. [112]	2021	MA	MA	6647	23	To perform a MA of current CRP data in AL after colorectal surgery.	All types of colorectal procedures
Zhang et al. [113]	2016	China	MA	1803	11	To determine whether prophylactic placement of a drain in colorectal anastomosis can reduce postoperative complications.	LAR
Zhao et al. [114]	2021	China	RCT	560	N/A	To assess the effect of TDT in AL prevention after laparoscopic LAR for rectal cancer.	Laparoscopic LAR

*AR, anterior resection; CRC, colorectal cancer; CRP, c-reactive protein; ICG, indocyanine green; IMA, inferior mesenteric artery; LAR, low anterior resection; LCA, left colic artery; MA, meta-analyses; MBP, mechanical bowel preparation; POI, postoperative ileus; RCT, randomized controlled trial; SR, systematic review; SSI, surgical site infection; TDT, transanal drainage tubes, TME, total mesorectal excision. *only malignant cases/patients after oncological resections; - authors did not report a total amount of included patients or it was not clear to separate benign from malignant cases.*

Risk of bias in studies

Forty-five RCTs (47%) were assessed for risk of bias (Figure 2A and 2C). The judgment was based on the categories of bias arising from the randomization process, bias due to deviations from intended interventions, bias due to missing outcome data, bias in the measurement of the outcome, and bias in the selection of the reported results. Upon evaluation, the highest risk of bias was attributed to the randomization process and deviations from intended interventions. Overall, nearly half of the studies (44%) were determined to have a high risk of bias.

Fifty (53%) SRs with or without meta-analysis were assessed for risk of bias (Figure 2B and 2D) Risk assessment was based on study eligibility criteria, identification and selection of studies, data collection and study appraisal, and synthesis and findings. In general, these studies had a lower risk of bias than RCTs, with just a quarter of studies (24%) judged as having a high risk of bias.

Terminology, definitions, and timeframe for AL reporting

The term most frequently used to describe the complication of failure of the integrity of the anastomosis was anastomotic leakage. Other terms used less commonly included anastomotic dehiscence, insufficiency, failure, breakdown, defect, or separation. Nearly half of included studies (n = 44. Table 2 and Supplement 2) used a more extensive definition to describe AL[20, 23, 24, 26, 28, 30, 33, 35, 36, 38, 39, 42, 48, 50, 55, 57, 58, 60, 62-65, 69, 70, 73, 79, 84, 85, 88-90, 94-96, 98-100, 103-105, 107, 108, 111, 112]. The most commonly described definition was the ISREC definition (n = 25), which describes an AL as a defect of the intestinal wall at the anastomotic site (including suture and staple lines of neo-rectal reservoirs) leading to a communication between the intra- and extra-luminal compartments. The timeframe during which AL was diagnosed was reported in 39 studies, of which most (n = 24, 62%) reported AL only within 30 days after index surgery.

Other reporting elements

An overview of all reporting elements is displayed in Table 3.

Clinical and biochemical elements

A total of 65 studies (68%) reported clinical signs and symptoms associated with AL, either as part of the formulated definition or in the description of the method of diagnosis [20-22, 25-27, 29, 31, 32, 34, 36-46, 49-53, 58, 61-64, 68-79, 81-83, 85-87, 92-96, 98-100, 102, 104, 105, 107, 109, 111, 113, 114]. The most frequently described clinical signs/symptoms were purulent or feculent discharge from a drain, peritonitis, fever, and fistula formation. Additionally, 26% (n = 25) of publications reported biochemical elements in the description of the method of AL diagnosis [25, 31, 34, 36, 40, 51, 52, 64, 69-72, 78, 79, 82, 94, 96, 100, 102, 105, 109, 112-115]. The most described biochemical markers were leukocytosis and C-reactive protein (CRP).

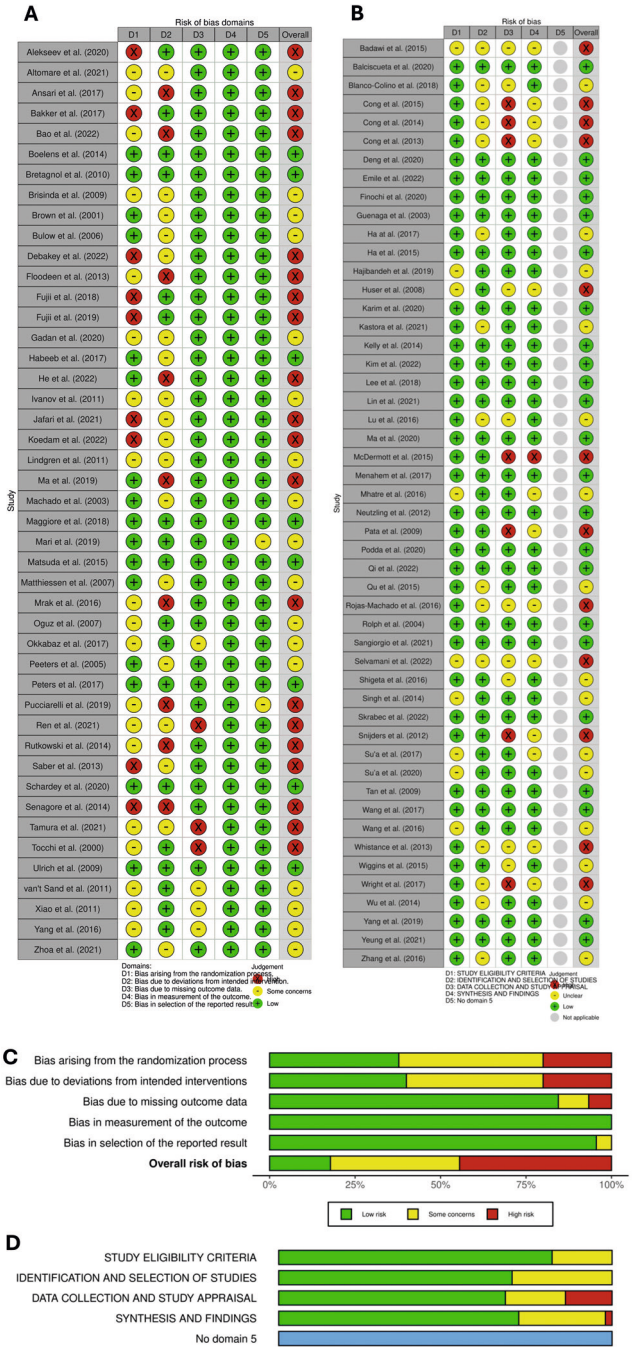


Figure 2. Risk-of-Bias **(A)** based on the RoB2 tool for RCTs: Summary of the domain-level judgements for each study; **(B)** based on the ROBIN tool for systematic reviews and meta-analyses: Summary of the domain-level judgements for each study. **(C)** within each bias domain for RCTs; **(D)** within each bias domain for systematic reviews and meta-analyses.

Table 2. Overview of definitions and timeframes used in the included studies.

Definitions	**N = 44/95, 46%**
A defect of the intestinal wall at the anastomotic site (including suture and staple lines of neo-rectal reservoirs) leading to a communication between the intra- and extra-luminal compartments.*	25 (57%)
Leak originating from staple/suture line	6 (14%)
Incontinuity at the anastomotic site detected clinically or radiologically within 30-60 days after surgery	3 (7%)
Anything other than a regular, uniform caliber at the level of the anastomosis	2 (5%)
Other definitions**	12 (27%)
Timeframe of AL diagnosis (after surgery)	**N = 39/95, 41%**
Within 7 days	1
Within 14 days	1
Within 30 days	24
> 30 days	1
Within 90 days	2
Within 12 weeks	1
Within 6 months	1
Within hospital stay	2
No time limit reported (> 6 months)	4
Systematic review reports different times for all included articles	2

*Percentages are calculated based on number of publications reporting an element. *Definition according to ISREC, the International Study Group of Rectal Cancer; **See supplementary 2.*

Radiological modalities and elements

Radiological modalities were specified in 63% (n = 60) of publications [20-22, 25-31, 36, 38-44, 46, 50, 53, 58, 61, 63, 64, 66, 68-72, 74-82, 85-87, 89, 91-93, 95-97, 99, 100, 102, 105-107, 109, 111, 113, 114]. Most authors confirmed the suspicion of AL by computed tomography (CT) scan. In more than half of studies, the authors did not specify if the CT scan was performed with or without oral or rectal contrast. If specified, most of them used contrast enemas. Besides CT scans, endoscopic studies (*e.g.,* sigmoidoscopy and rectoscopy) were used to assess AL. Other modalities used included X-ray with or without contrast, gastrografin enema, ultrasound, magnetic resonance imaging (MRI), and positron emission tomography (PET). An abdominal or pelvic collection and/or abscess in the proximity of the anastomosis was the most frequently described imaging finding when diagnosing a leak. Extravasation of contrast, the presence of air or fluid around the anastomosis, and descriptions of anastomotic dehiscence, breakdown of any staple line, and an anastomotic defect were also used.

Reoperations

Findings at reoperation were described in 13% (n = 12) of the included publications [22, 27, 28, 53, 66, 76, 78, 93, 95, 97, 102, 105]. The most frequently reported finding was visualization of anastomotic dehiscence and/or anastomotic defect at the time of reoperation. Other findings at reoperation were fistula formation and postoperative peritonitis.

AL Severity Grading systems

Grading or classification of AL severity was reported in 45% of included studies (n = 43) [12, 20, 21, 23, 26, 28, 29, 33-36, 38, 39, 41-43, 47, 49, 50, 56, 57, 60, 64, 66, 69, 70, 73, 74, 78, 80, 84, 85, 89, 93-96, 98, 100, 102, 113-115]. Nearly half of publications used the ISREC grading system. This classification ranks AL into three grades (grade A, B, or C) based clinical management [10]. The Clavien-Dindo grading was used in 19% (n = 8) of publications [28, 29, 36, 42, 43, 57, 78, 95]. Leaks were classified as major vs minor leaks in 14% of the papers (n = 6), while radiological vs clinical and clinical vs subclinical leaks were reported in four papers [21, 49, 64, 66, 70, 73, 74, 80, 93, 95, 97, 100, 105, 113].

Table 3. Overview of reported elements subdivided in clinical, biochemical, imaging, re-interventions and grading terms.

Reporting Element	**Number of publications**
Clinical signs and/or symptoms	**N = 65/95, 68%**
Discharge from the drain	51 (78%)
Peritonitis	42 (65%)
Fever	25 (38%)
Fistula formation (*e.g.*, rectovaginal fistula etc.)	23 (35%)
Discharge from the wound	17 (26%)
Local physical examination (*e.g.*, bowel obstruction, gastric retention, facial dehiscence and/or abdominal pain)	14 (22%)
Anastomotic dehiscence/defect	11 (17%)
Discharge of pus per rectum	8 (12%)
Sepsis	8 (12%)
Cardiac complications (*e.g.*, atrial fibrillation and/or tachycardia)	5 (8%)
Deterioration of clinical condition	3 (5%)
Tachypnea	3 (5%)
Decreased urine production	3 (5%)
Mental status change (*e.g.*, agitation or lethargy)	3 (5%)
Nutritional status (*e.g.*, tube feeding or total parental nutrition)	3 (5%)
Diarrhea	1 (2%)
Organ failure	1 (2%)
Abdominal distension	1 (2%)
Biochemical elements	**N = 25/95, 26%**
Leukocytosis / White cell count	22 (88%)
CRP elevation	7 (28%)
Worsening of renal function (*e.g.*, creatinine or urea)	3 (12%)
Increase of pro-calcitonin	2 (8%)
Leukopenia	1 (4%)
pH changes	1 (4%)
Lactate (increase)	1 (4%)
Pyruvate (increase)	1 (4%)
Cytokines (increase)	1 (4%)
Lysozymes (increase)	1 (4%)
Matrix metalloproteinases (increase)	1 (4%)
Culture of intra-abdominal bacteria	1 (4%)
Other postoperative inflammatory markers (*i.e.*, I-FABP, TNFRSF1A, IL-6, IL-8, CCL2)	1 (4%)

Table 3. Continued

Reporting Element	Number of publications
Modality	**N = 60/95, 63%**
CT scan	
Not specified	36 (60%)
With contrast (not specified)	6 (10%)
With contrast enema	6 (10%)
With intravenous contrast	1 (2%)
With oral contrast	1 (2%)
Endoscopy	
Not specified	13 (22%)
Sigmoidoscopy	11 (18%)
Rectoscopy	5 (8%)
Proctoscopy	2 (3%)
Colonoscopy	1 (2%)
Enteroscopy	1 (2%)
Unspecified contrast studies	
Contrast enema	20 (33%)
Water soluble contrast enema	7 (12%)
Radiological contrast study	3 (5%)
Water soluble contrast study	2 (3%)
X-ray	
With contrast (*e.g.*, not specified or water soluble)	5 (8%)
With contrast enema (*e.g.*, not specified or water-soluble)	4 (7%)
Not specified	1 (2%)
Fluoroscopy	
Gastrografin enema	4 (7%)
Ultrasound	3 (5%)
MRI	2 (3%)
PET	1 (2%)
Imaging findings	**N = 59/95, 62%**
Abdominal or pelvic collection / abscess in the proximity of the anastomosis	54 (92%)
Extravasation of contrast	16 (27%)
Presence of fluid/ air around the anastomosis	9 (15%)
Anastomotic dehiscence / Breakdown of any staple line / Anastomotic defect	10 (17%)
Fistula formation (*e.g.*, rectovaginal fistula etc.)	9 (15%)
Fecal peritonitis	1 (2%)
Abscess with a communication to the anastomosis	1 (2%)
Re-intervention findings	**N = 12/95, 13%**
Evidence of an anastomotic defect or dehiscence	9 (75%)
Fistula formation	3 (25%)
Postoperative peritonitis	2 (17%)
Air, fluid, GI contents, or contrast material	1 (8%)
Pericolic abscess or phlegmon	1 (8%)
Pelvic, intraabdominal or retroperitoneal abscess	1 (8%)
Generalized purulent peritonitis	1 (8%)
Generalized fecal peritonitis	1 (8%)

Table 3. Continued

Reporting Element	Number of publications
Grading terms	**N = 43/95, 45%**
ISREC classification	21 (49%)
Other classifications:	
Clavien-Dindo	8 (19%)
Hinchey	1 (2%)
Major vs minor leaks	6 (14%)
Radiological vs clinical leaks	4 (9%)
Clinical vs subclinical leaks	4 (9%)
Generalized vs localized leaks	1 (2%)
Early vs late leaks	1 (2%)
Significant vs non-significant leaks	1 (2%)
Complete vs partial leaks	1 (2%)

CRP, C-reactive protein; I-FABP, intestinal fatty acid binding protein; TNFRSF1A, TNF receptor superfamily member 1A; IL-6, interleukine-6; IL-8, interleukine-8; CCl2, C-C motif chemokine ligand 2; CT, Computerized tomography scan; MRI, Magnetic resonance imaging; PET, Positron emission tomography.

DISCUSSION

This systematic review aimed to evaluate the various elements and criteria used to report on the definition and grading colorectal AL following CRC resections. This current review of the literature reveals the lack of a widely accepted and applied definition of colorectal AL. Despite the increase in the number of high level of evidence publications (RCTs, SRs and MA's) on this topic in recent years, 72% (n = 376) of publications screened for eligibility did not include a specific definition to assess the presence of AL, even though the incidence of AL served as a primary or secondary outcome. Based on our literature search, only 18% (n = 95) of eligible studies specified how AL was defined. In order to gain knowledge of general definitions of AL across eligible publications, specific elements contributing to the definition and grading of the severity of leaks were compared across studies when applicable (*i.e.*, clinical-, biochemical-, radiological-, findings at reoperation, and severity grades). The latter led to another noteworthy finding; the extensive range of elements utilized, led to vast variations in the reported colorectal AL rates (based on the various categories or domains used), and ultimately resulted in difficulty comparing findings across studies.

Overall, to support the diagnosis of an AL, clinical signs and symptoms were used in 68% of included studies, radiological modalities and radiological findings in 63% and 62% respectively, biochemical elements in 26% and findings at reoperation only in 13% of studies. In addition, 45% of studies reported grading the severity of AL, with 46% reporting a more detailed definition and 41% including a timeframe for AL reporting.

A consensus study by Helsdingen et al. (2020) already reported recommendations for a definition and category elements of AL based on experts' opinions[13] By comparing the results of our review to the recommendations formulated in this consensus, we confirm a lack of reporting the categories suggested (clinical parameters, laboratory tests, radiological findings, findings during reoperation, grading systems, timing, location of the tumor). The most common element used for AL reporting was clinical symptoms and signs associated with AL. Compared to the ISREC definitions, our results for clinical elements showed many similarities. However, several clinical elements from our search were not included in the original ISREC classification [10]. The most frequently used biochemical result was leukocytosis. In contrast, while CRP was also included in the ISREC classification, its use was only mentioned in 7 studies [36, 64, 69, 71, 78, 96, 112]. There is no uniformity in recommendations regarding a preferred imaging modality when suspecting an AL. The most often used modality to support the diagnosis of a leak in our analysis was by CT. However, it was often unclear whether these were CTs performed with rectal, intravenous or oral contrast. While a previous SR and MA by Kornmann et al. reported the scarce and poor quality of evidence regarding the predictive value of CT in diagnosing AL, Matsuda et al. and Lim et al. specifically used CT for confirmation when there was suspicion of AL [8, 12, 116]. For now, it is unclear how much additional information rectal contrast provides over clinical assessment for low anastomoses.[117] Notably, the role of endoscopic assessment in the assessment of AL is poorly investigated despite low procedural risk and rapid detection of AL [118]. Besides the type of imaging modality used, the detailed findings are important too. The most frequently described finding was an abdominal or pelvic collection and/or abscess in the proximity of the anastomosis on CT scan although radiological criteria considered diagnostic of AL remain controversial [14]. Upon diagnosis of AL, the type of re-intervention and findings at reintervention were underreported in the summarized evidence. It is important to report the type of re-intervention(s) as this may correlate with time to resolution of AL, return to function and long term outcomes and quality of life. Only 13% of included studies reported type of re-intervention(s) which highlights a significant gap in reporting.

The lack of standardized definitions and agreement on the specific elements of an AL contributed to significant variations in the reported rates, making it challenging to identify risk factors for leaks and evaluate the effectiveness of specific therapeutic and prophylactic interventions. Most studies considered AL to involve a breach in the integrity of the intestinal wall at the site of colorectal or colonanal anastomosis, with severity ranging from incidental findings to life-threatening sepsis requiring further surgery. However, substantial variability was uncovered regarding the minimum criteria for reporting AL.

Grading of the severity of AL may have major implications with respect to timing and type of required intervention, prognosis, short- and long-term outcomes. However, less than half of included studies reported grading or classification of AL. The most common grading system reported was the ISREC classification, followed by the Clavien-Dindo classification, although this is not specific to AL [119]. Furthermore, our results also showed that there was some effort

towards classifying leaks based on degree of clinical severity (*i.e.,* significant vs. non-significant leaks, clinical vs. radiological leaks, etc.), however the specific terminology used was ill-defined and non-standardized. One important attribute that may play an important role in reporting and managing ALs is the timeframe in which AL is identified, with clear distinction between early vs late or delayed leaks. Our review found that the timeframe of leak diagnosis, i.e. early and late or delayed, was only reported in one article,[41] and most other studies described a 30 day postoperative timeframe for reporting. Including early and late timeframes as an element in the standardized reporting of AL may prevent under-reporting of late/delayed leaks and their sequelae, facilitate earlier management and improve long-term outcomes.

The stigma associated with leaks and the use of institutional AL rates as a measure of surgical quality may contribute to the generalized reluctance to investigate leaks early and consistently, as reflected in the wide range of reported diagnostic elements in our review. This stigma must be balanced against the potential benefits of adopting a standardized reporting framework that facilitates earlier diagnosis, management, and resolution of leaks. Also within current reporting systems like The National Surgical Quality Improvement Program (NSQIP), the reporting of an AL is presently contingent upon the specific intervention undertaken and does lack background information (this encompasses a spectrum of scenarios: instances where no documented treatment intervention is recorded, cases managed through interventional methods, situations addressed with non-interventional or nonoperative approaches, instances necessitating reoperation, situations where there is no definitive diagnosis of a leak or a leak-related abscess, and cases categorized as unknown). The need for standardized, well-accepted terminology for reporting of AL remains an important issue especially when evaluating the effectiveness of targeted interventions and/or comparing procedural outcomes. Before formulating a novel framework for reporting and grading colorectal AL, that will gain wide acceptance, several issues need to be addressed. Consensus agreement needs to be reached with respect to which clinical and/or radiologic or endoscopic, and/or biochemical elements are most suggestive of AL, as reporting rates of these elements vary widely. Secondly, agreement is also needed with respect to grading the severity of leaks, that may not only take into account the type of intervention(s) required, but also short and long-term sequelae and impact on patients. Thirdly, additional elements relevant to the timeframe of diagnosis and management of leaks should be routinely incorporated in reporting, with clear distinction between early vs late/delayed AL diagnosis. Lastly, additional features of AL with potential implications on outcomes and interventions, may need to be included such as anastomotic height and protective fecal diversion.

There are some limitations of the current work. The heterogeneity between the included studies and varying presentations of data prohibited a more detailed analysis. Also, not all papers solely reported on oncological cases. Furthermore, a deliberate choice was made to only include high-level evidence publications (*i.e.*, RCTs and SRs with or without MAs). However, based on the findings of these studies, the urgency of achieving uniformity in the

reporting and grading of colorectal AL is highlighted. This uniform process would facilitate quality assurance in the reporting of diagnostic elements, enable transparant of study results and reliable interpretation of meta-analyses. The development of a general outcome AL set may be helpful to tackle further reporting gaps. Consequently, the findings of this study may inform the development of consensus framework for the reporting and grading of AL after CRC surgery.

CONCLUSION

This systematic review highlights substantial heterogeneity in the elements used to define colorectal AL across high level evidence literature, reflecting the need for a widely accepted framework that can guide definition, grading, and reporting of AL. Standardized reporting of AL is essential for mitigating delays in diagnosis and treatment, promoting the development of treatment guidelines, and addressing existing shortcomings.

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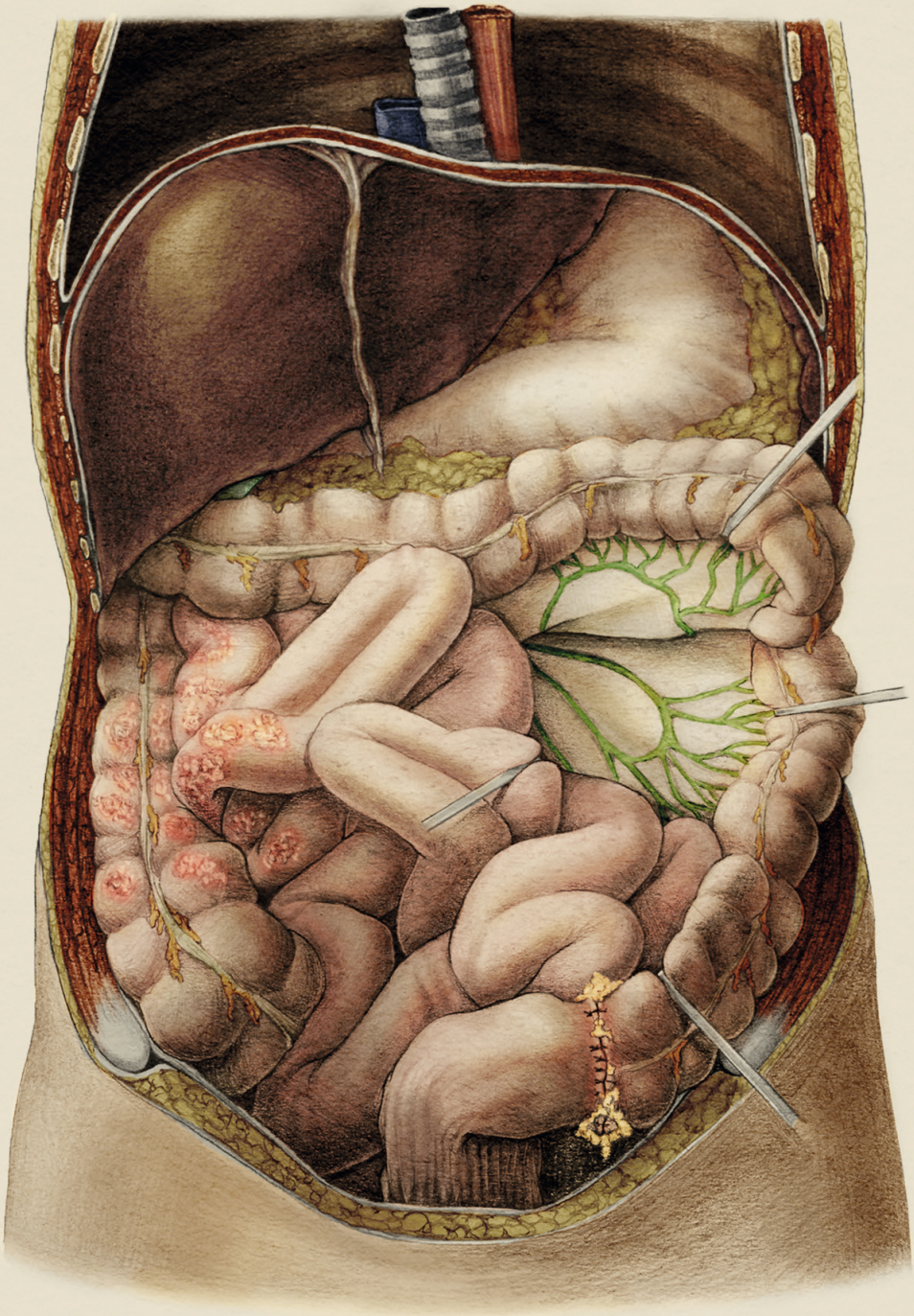
SUPPLEMENTARY

The following supplementary material can be downloaded from:

- *Table S1. Search strategy*
- *Table S2. All theoretical definitions formulated by included studies*



CHAPTER 3

INTERNATIONAL **CO**NSENSUS ON **RE**PORTING **A**NASTOMOTIC **L**EAKS AFTER COLORECTAL CANCER SURGERY: THE COREAL REPORTING FRAMEWORK

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Submitted

ABSTRACT

Background. Anastomotic leak frequently complicates colorectal anastomoses with persistently high morbidity and mortality. The significant variability in published leak rates reflects the lack of consistency in reporting variables that may impact the occurrence, management, short and long-term outcomes of patients.

Objective. The Consensus for Reporting of colorectal Anastomotic Leaks (CoReAL) is an international collaborative that developed a standardized evidence-based framework for reporting key variables related to the entire episode of colorectal anastomotic leak in cancer patients.

Methods. Along the preoperative, intraoperative, short- and long-term postoperative phases of a left-sided colorectal anastomotic leak, key questions regarding all potentially relevant variables were formulated. A literature review was conducted to generate evidence-based statements in response to these questions. Statements that reached consensus, together with input from patients' experience and experts' opinion, were incorporated into the framework as reporting elements. Modified Delphi methodology, including online voting and an in-person consensus meeting, was used to generate consensus statements based on the literature review, and to develop the reporting framework. An international panel of 32 colorectal surgeons with expertise in the field of colorectal anastomotic leaks, representing 6 surgical societies, along with radiologists, research collaborators, patients, healthcare economists and surgical trial methodologists. Evidence-based statements and reporting elements with >70% agreement were included.

Results. Consensus among experts was achieved on 33 evidence-based statements and 43 reporting elements for the CoReAL framework. The reporting elements encompassed evidence-based statements (27), patient perspectives (7), as well as expert opinion (9)..

Conclusions. This international consensus provides an evidence-based standardized framework for reporting of key variables related to a colorectal anastomotic leak following oncologic resection.

Keywords. Anastomotic leakage; consensus; colorectal surgery; reporting; patient outcomes.

INTRODUCTION

Anastomotic leak (AL) represents a critical and challenging complication of colorectal cancer (CRC) resections with substantial impact on short and long-term outcomes. Despite advances in preoperative risk assessment, surgical technique and postoperative care, the prevalence of colorectal AL ranges from 1.5% to 23% with sequelae that range from minimal to severe morbidity, and mortality rates as high as 16%-29% [1-5]. The lack of consensus on how AL is defined, graded and reported complicates our understanding of the true prevalence of AL and our ability to compare risk factors, interventions and outcomes of AL across studies [6,7]. In recent years, there has been a growing recognition of the importance of standardizing the reporting of colorectal ALs. While previous endeavors have been undertaken to achieve consensus on definitions and severity grading of colorectal AL, widespread adoption and reporting have remained limited [8-12].

A recent systematic review of the literature to assess the quality of reporting of AL in CRC trials, highlighted significant heterogeneity across trials. Among studies where colorectal AL following CRC resection was a primary or secondary endpoint, only 20% provided clear reporting of how AL was defined, with even fewer describing diagnostic modalities and/or re-interventions for AL in the short and long-term study follow-up [13]. This lack of reporting undermines the validity of clinical trials, complicates the comparison of any given interventions on outcomes of AL across studies, ultimately hindering the assessment of the effectiveness of strategies to mitigate AL [14-17]. We hypothesized that the lack of an acceptable standardized reporting system leads to widespread underreporting of colorectal AL and subsequent anastomotic complications. This inconsistency in reporting of AL represents a clinical and scientific gap that prompted the development of a consensus framework aimed at enhancing the quality of reporting of AL in both clinical practice and clinical trials.

The aim of this Consensus on Reporting colorectal Anastomotic Leaks (CoReAL) project was to create a framework to standardize the reporting of AL following left-sided colorectal cancer resections with a colorectal anastomosis based on expert consensus, informed by high-level published evidence and patient perspectives.

MATERIALS AND METHODS

The CoReAL project consisted of two phases (Figure 1). First, all available evidence regarding key questions related to factors that may or may not contribute to the development, severity and short and long-term outcomes after AL, was analyzed and used to develop evidence-based statements. Second, the evidence statements were complemented with expert opinions and patients' perspectives to develop a reporting framework. The topic of colorectal AL was divided into four phases along the AL episode of care, including preoperative,

intraoperative, postoperative short-term, and postoperative long-term phases. A working group (WG) was created for each phase.

Research team

An expert panel of surgeons functioned as the team leads (PS, NF, MB, NB) together with a surgical research fellow (DH). The coordinating team extended invitations to a diverse group of colorectal expert surgeons to join the expert panel. Criteria for invitation included previously leading or contributing to surgical trials in CRC, research on colorectal AL, or participated in the development of AL guidelines. This research group comprised of 32 expert surgeons and 12 research collaborators representing six international surgical societies; the American Society of Colon and Rectal Surgeons (ASCRS), the Society of American Gastrointestinal and Endoscopic Surgeons (SAGES), the European Association for Endoscopic Surgery (EAES), the European Society of Coloproctology (ESCP), the Endoscopic and Laparoscopic Surgeons of Asia (ELSA) and the Colorectal Surgical Society of Australia and New Zealand (CSSANZ). The research team was distributed across the four working groups, with balanced representation from the various surgical societies (Appendix A1). Experts from related specialties were consulted over the course of the project, including 3 radiologists, 4 industry representatives as well as 10 patient advocates who developed an AL following colorectal resection. MK and SvK served as health outcomes and trial methodologist providing guidance throughout all phases of the project.

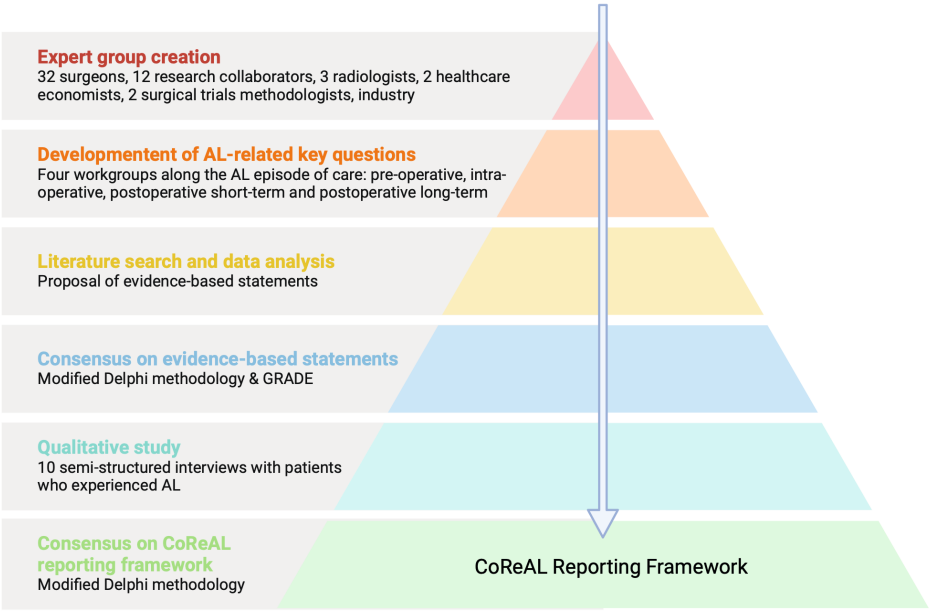

Figure 1. Overview of the steps that were taken to create the CoReAL reporting framework

Research questions and search strategy

Along the four phases of the AL episode of care, the coordinating team developed a comprehensive list of key questions related to AL (Supplementary S1). Following expert input, the final list was divided among the corresponding WGs for further investigation. A literature search was conducted to assess the evidence related to each question, led by DH and a librarian. The search was performed in PubMed, Embase, and Cochrane electronic libraries on November 3, 2022 (Supplementary S2). Only high-level evidence articles were selected, including randomized controlled trials (RCTs) and systematic reviews with or without meta-analyses, where AL after CRC surgery was a primary or secondary outcome. Eligible articles were required to be published in English after 2000. Articles that did not report on oncological left-sided colorectal resections were excluded. All search results were imported into Rayyan [18] to allocate manuscripts to a given topic and WG, with initial eligibility determined based on title and abstract review. DH and research collaborators performed the screening for each question. In cases of disagreement, the team leads acted as referees. Eligible full-text articles were reviewed and summarized. The search was updated on July 26, 2023.

CoReAL definitions

As previously demonstrated in our recent systematic review on the quality of AL reporting in CRC trials, significant heterogeneity exists in AL reporting and definitions, a potential source of flawed comparisons [13]. In order to overcome this limitation, the team agreed to define AL in the broadest way possible rather than to follow any specific criteria, including the International Study Group of Rectal Cancer (ISREC) definition [8]. Thus, for this consensus, AL was defined as any breach or failure in the integrity of the anastomosis, including dehiscence, insufficiency, failure, breakdown, defect, or separation, regardless of the diagnostic modality (radiologic, endoscopic, intraoperative) and irrespective of clinical or biochemical manifestations. Defining the timing of AL diagnosis was considered important due to its different implications on healthcare resource utilization and outcomes. Based on consensus, AL was considered “early” when diagnosed 90 days or less from the index surgery, and “late” or “delayed” when diagnosed after 90 days.

Data extraction and Evidence-based statements

Data extraction was conducted using RevMan Web (Review Manager Web, Computer program, Version 4.12.0. The Cochrane Collaboration, 2022). General information regarding oncologic colorectal AL, including definitions, severity assessment, diagnostic timeframe, clinical symptoms, biochemical tests, imaging modalities, type of re-interventions, and long-term outcomes, were collected, using standardized forms. Key outcome measures related to AL (e.g. relative risk, odds ratio, hazard ratios) were extracted for every research question. If systematic reviews showed overlapping data, the lowest quality study was excluded, or a new overview was created which only included mutually exclusive studies. If no overlap was found, data was pooled using RevMan Web tools. Methodological quality and risk of bias for included studies were assessed by two research collaborators using the RoB2 tool for RCTs and the

ROBIS tool for systematic reviews and meta-analyses [19,20]. Bias was visualized with risk-of-bias visualization tool in RevMan Web. After summarizing and presenting all the evidence related to each question, evidence-based statements were formulated by each WG to address all AL-related questions. The formulation of the statements was based on the level of evidence (LoE) according to the Grading of Recommendations Assessment, Development and Evaluation (GRADE) guidelines, rated as 'high', 'moderate', 'low', or 'very low' [21]. Standardized wording used to phrase statements is shown in Table 1. When the LoE was very low, the research team did not formulate a statement but flagged the topic as lacking evidence.

Table 1. Phrasing of the statements based on the Level of Evidence (LoE)

LoE according to GRADE	Wording statement
High	Does (not)
Moderate	Probably does (not)
Low	May do (not)
Very low / expert opinion	*No statement formulated*

Consensus on drafted statements

A two-phase modified Delphi method, consisting of an online survey and an in-person consensus meeting, was used to achieve consensus on all evidence-based statements. In the first phase, the statements from each WG with LoE, were presented to all 32 team experts who subsequently voted online on each of these statements using a 9-point Likert scale, with consensus defined as >70% agreement (a score of ≥7 on the Likert scale). In the second phase, statements that did not reach consensus were discussed on Day 1 of a 2-day in-person consensus meeting held in Boston in October 2023. Research collaborators presented the data analysis for all statements that did not reach consensus and facilitated discussions and rephrasing of the statements among experts. Another round of voting was carried out with consensus achieved with >70% agreement.

Patient engagement

Patients' personal experience with AL were captured across all the phases of patient journey through semi-structured interviews and a qualitative synthesis [22]. In-depth semi-structured patient interviews were conducted among 10 patients who experienced an AL. To obtain a wide range of perspectives, a maximum variation sampling strategy was employed, ensuring diversity in age, sex, and the severity of AL. The results of this qualitative analysis were presented during the in-person consensus meeting and incorporated into the reporting framework.

Development of the reporting framework and consensus process

The reporting framework encompassing reporting elements along all four phases of the AL episode, was constructed from the statements that achieved consensus, with input derived from qualitative analysis of patients' interviews as well as from experts. The framework was

developed using a modified Delphi method conducted in two phases. On day 2 of the in-person consensus meeting, each WG formulated a list of reporting elements derived from the consensus statements with additional elements derived from patients and experts. The reporting elements were then presented to the wider group for discussion followed by voting. The results were consolidated to create an initial draft of the reporting framework. In the 2nd phase, all experts were asked to rate on a 9-point Likert scale, their agreement with the inclusion of each element in the final reporting framework in an online survey. Consensus was achieved with >70% agreement (a score of ≥7 on the Likert scale). All the reporting elements that achieved consensus were incorporated into the final CoReAL framework.

RESULTS

The literature search yielded 2,989 abstracts, of which 453 were included and analyzed. The search was updated on July 26, 2023, and yielded an additional 24 articles, for a total of 477 included articles.

Evidence-based statements

The first online Delphi round was completed by 30 experts and the second round by 26 experts who attended the in-person consensus meeting (23 in-person and 3 connected remotely). By the end of Day 1, 33 evidence-based statements reached consensus (Table 2). For 13 topics, the evidence was insufficient to formulate a statement (Table 3). Experts' commentary on the consensus statements can be found in Supplementary S3.

Table 2. Overview of evidence-based statement with corresponding Level of Evidence (LoE) and percentage of agreement.

LoE	Statement	No. of Delphi round: % of agreement
Preoperative topics		
M	1. Modifiable preoperative risk factors that probably do increase AL rates after CRC surgery include obesity, alcohol, smoking, and low preoperative serum albumin level.	1: 100%
M	2. Not-modifiable preoperative risk factors that probably do increase AL rates after CRC surgery include male gender, high ASA (>2), diabetes, cardiovascular disease, renal disease, chronic steroid use, advanced TMN stage (T3-4), tumor size > 5cm, tumor location < 12cm from the anal verge, complicated tumor (perforation or obstruction), and neoadjuvant therapy (preoperative chemotherapy, radiotherapy, long course chemoradiotherapy, short course radiotherapy).	1: 96.67%
M	3. Adding pre- and postoperative probiotics to standard nutrition probably does not reduce AL rates after CRC surgery.	1: 80%
M	4. The use of preoperative oral mechanical bowel preparation alone probably does not influence AL rates after CRC surgery.	1: 80%
L	5. The addition of mechanical bowel preparation to single phosphate enema may not influence the AL rates after CRC surgery.	1: 83.33%
M	6. The addition of preoperative oral antibiotics (to mechanical bowel preparation and perioperative IV antibiotics) probably reduces AL rates after CRC surgery.	1: 96.67%
L	7. Sarcopenia, diagnosed with L3 skeletal muscle index, may not influence AL rates after CRC surgery but the evidence is very uncertain.	2: 83.87%
Intraoperative topics		
L	8. High IMA ligation may be associated with higher AL rates compared to low IMA.	2: 73.03%
M	9. Routine splenic flexure mobilization when performing anterior rectal resections probably does not affect AL rates.	1: 76,67%
M	10. Choice of either performing a laparoscopic or open CRC resection probably does not affect AL rates.	1: 86,67%
M	11. Conversion (from laparoscopic to open surgery) for patients undergoing rectal cancer resection may increase AL rates.	1: 76,67%
L	12. The choice of either performing open TME, robotic TME, laparoscopic TME or transanal TME may not affect AL.	1: 86,67%
L	13. Firing more than one stapler during laparoscopic rectal resection may be associated with higher AL rates.	1: 90%
M	14. The use of ICG (fluorescence angiography) for bowel perfusion assessment during CRC surgery is probably associated with a decrease in AL rates.	2: 76,92%
L	15. Prophylactic fecal diversion may reduce the severity of AL after rectal cancer surgery.	2: 88,46%
L	16. Intraoperative anastomotic integrity assessments (air leak test and/or endoscopy) may be associated with lower AL rates.	1: 100%
L	17. The choice of either performing intraoperative endoscopy or air leak test alone, may not influence AL rates after CRC surgery.	1: 80%
L	18. Staple line reinforcement may not affect AL rates after CRC surgery.	1: 83,33%

Table 2. Continued

LoE	Statement	No. of Delphi round: % of agreement
L	19. Anastomotic reinforcement with omentoplasty may not affect AL rates after CRC surgery.	1: 90%
M	20. Using a prophylactic pelvic drain for rectal surgery probably does not affect overall AL rates after CRC surgery.	2: 96,15%
Postoperative short-term topics		
M	21. In addition to the confirmed overall benefits of ERAS, the use of an ERAS protocol probably does not impact AL rates after CRC surgery.	2: 73.08%
L	22. Within the context of ERAS protocols, short-term use of NSAIDs in the postoperative phase may not influence the prevalence of AL after CRC surgery.	2: 84.62%
M	23. Serial CRP in the early postoperative phase probably has strong negative predictive value for AL after CRC surgery.	2: 100%
M	24. While serial CRP on itself is not diagnostic for AL, increased values in the early postoperative phase probably are a predictor of an adverse postoperative event after CRC surgery.	2: 84.62%
M	25. Endoscopy or CT scan (with at least IV-contrast) may be more accurate than water-soluble contrast enema for the diagnosis of AL after CRC surgery.	2: 96.61%
L	26. Following a minimally invasive CRC operation, a laparoscopic approach as re-intervention may be safe and feasible for the management of AL.	2: 96.15%
L	27. In a hemodynamically stable patient with AL after CRC surgery, transanal or endoscopic management, either alone or in combination with other modalities, may be safe.	2: 96.15%
L	28. The development of AL after CRC surgery may result in higher mortality rates compared to patients without AL.	2: 92.30%
Postoperative long-term topics		
L	29. The development of AL after CRC surgery may result in higher overall complications compared to patients without AL.	1: 100%
L	30. The development of AL may be associated with increased healthcare costs due to increased hospital stay after rectal cancer surgery.	1: 100%
L	31. The development of AL after CRC surgery may be a risk factor for permanent stoma compared to patients without AL.	2: 96.15%
M	32. The development of AL is probably associated with decreased overall survival and disease-free survival after CRC surgery.	1: 96.67%
M	33. The development of AL is probably associated with an increased local recurrence after rectal cancer surgery.	1: 90%

AL, anastomotic leak; IV, intravenous; CRC, colorectal cancer; CRP, C-reactive protein; ERAS, Enhanced Recovery After Surgery; IMA, inferior mesenteric artery; NSAID, nonsteroidal anti-inflammatory drugs; TME, total mesorectal excision. LoE; Level of Evidence, green = moderate (M), orange = low (L).

Table 3. Overview of topics that require further investigation

Topic	Reason to not formulate a statement
Preoperative	
• Preoperative selective decontamination compared to broad-spectrum antibiotics	Too low level of evidence
• Anemia correction	Too low level of evidence
• Oral nutritional supplement/ support	Too much heterogeneity in the duration of administration, different types of oral nutritional support and no clear consensus on what the definition is of immunonutrition.
Intraoperative	
• Human factors*	Too low level of evidence
• Anastomotic configuration**	The reported data in the analysis is very scarce and heterogeneous, overall evidence was too low.
• Anesthesia factors or intraoperative risk scoring systems	Too low level of evidence and lack of worldwide validation.
Postoperative short-term	
• Clinical predictions scores	Not described in high level evidence literature.
• Peritoneal fluid markers	Not described in high level evidence literature.
• Low fiber diet	Not described in high level evidence literature.
• Laxatives	Not described in high level evidence literature.
Postoperative long-term	
• Impact on QoL	Too low level of evidence
• Financial impact	Too low level of evidence. The expert team felt like additional intervention, imaging modalities and paramedical care were contributors within the statement.
• Chronic sequalae of AL	Evidence too low, not well described in high level evidence literature.

**hospital volume, surgeon volume, surgeon specialization; **side-to-side versus side-to-end versus end-to-end versus J-pouch, anti-peristaltic versus isoperistaltic, intracorporeal versus extracorporeal, handsewn versus stapled, immediate versus delayed (Turnbull-Cutait), compression versus handsewn versus stapled, and single vs double layered anastomosis. AL; anastomotic leak, QoL; Quality of Life.*

CoReAL reporting framework

By the end of day 2 of the in-person consensus meeting, 46 reporting elements were included in the reporting framework including 7 preoperative, 14 intraoperative, 7 postoperative -index admission, 8 postoperative -30 to 90 day period, and 10 postoperative -long-term elements. Following the 2nd online Delphi round, three elements did not reach consensus. Of the 43 reporting elements that reached consensus, 27 reporting elements were derived from evidence-based consensus statements, 7 were based on patient perspectives and 9 from expert opinion (Table 4). Patient-centered elements were informed by the results of our qualitative analysis and included preoperative discussion regarding the potential need for a stoma after surgery, preparation and planning for possible stoma creation, postoperative assessment and management of potential sequelae of AL, and the impact of AL on QoL

and functional outcomes [22]. Consensus was reached to report these outcomes for at least one year after surgery, with the aspirational goal of reporting on oncologic, survival, and functional status at 2 and 5 years postoperatively. Although the evidence suggested that mechanical bowel preparation alone and routine splenic flexure mobilization did not significantly impact AL rates, both were included as elements based on expert opinion that they reflected standard practice during left-sided restorative proctectomy for cancer. In addition, 6 intraoperative elements reflecting the intraoperative difficulty (3) and surgical pitfalls (3) were based on expert opinion. Although these elements are not based on evidence, they were felt to serve as surrogates for the technical and human factors that likely contribute to AL. Lastly, given the importance of documenting resolution (or lack thereof) of AL and its potential sequelae, status of the anastomosis was included as a postoperative element to be captured beyond 90 days and up to 1 year postoperatively.

Table 4. Reporting elements included in the CoReAL reporting framework

Reporting elements	Agreement (%)	Background
Preoperative elements		
1. Modifiable risk factors	92.31	Evidence based
2. Preoperative oral antibiotics	92.31	Evidence based
3. Mechanical bowel preparation	88.45	Expert opinion
4. Other risk factors	84.62	Evidence based
5. Was the potential need of a postoperative/permanent stoma discussed?	84.62	Patient centered
6. Was the patient referred to a stoma therapist preoperatively?	76.92	Patient centered
Intraoperative elements		
7. Diverting stoma creation	100	Evidence based
8. Intraoperative difficulty: Distance of the anastomosis (cm) from AV	100	Expert opinion
9. Anastomotic integrity testing	96.15	Evidence based
10. Number of stapler loads for rectal transection	92.31	Evidence based
11. Intraoperative difficulty: Redo pelvic surgery	92.31	Expert opinion
12. Conversion MIS to open	88.46	Evidence based
13. Pitfalls: pelvic stapler failures	88.46	Expert opinion
14. Pitfalls: Unplanned multivisceral resection or repair (of organ injury)	84.62	Expert opinion
15. Splenic flexure mobilization	84.62	Expert opinion
16. Intraoperative difficulty: acute blood loss requiring blood transfusion	80.77	Expert opinion
17. Location of inferior mesenteric artery ligation	76.92	Evidence based
18. Pitfalls: other device failures	76.92	Expert opinion
19. Perfusion assessment of conduit with fluorescence angiography	73.08	Evidence based

Table 4. Continued

Reporting elements	Agreement (%)	Background
Reporting elements before discharge (index admission)		
20. Mortality	100	Evidence based
21. Re-interventions until discharge	100	Evidence based
22. Stoma creation	100	Evidence based
23. Diagnostic modality for AL	96.15	Evidence based
24. Length of hospital stay	92.31	Evidence based
25. Length of stay in the ICU	84.62	Evidence based
26. Serial CRP measurement	84.62	Evidence based
Reporting elements after discharge – 30 days AND up to 90 days		
27. Mortality	100	Evidence based
28. Readmission	100	Evidence based
29. Re-interventions after initial discharge	100	Evidence based
30. Stoma creation and closure	100	Evidence based
31. Diagnostic modality for AL	96.15	Evidence based
32. Anastomotic complication	96.15	Patient centered
33. Length of hospital stay	88.46	Evidence based
34. Length of stay in the ICU	84.61	Evidence based
Reporting elements after 90 days (long term)		
35. Re-interventions after 90 days *	100	Evidence based
36. Stoma information *	96.15	Evidence based
37. Anastomotic complications *	96.15	Patient centered
38. Oncological outcomes **	96.15	Evidence based
39. Mortality **	96.15	Evidence based
40. Anastomotic status *	92.31	Expert opinion
41. Functional outcomes: LARS (LARS score) **	88.46	Patient centered
42. Quality of life assessment (EQ-5D score) **	84.62	Patient centered
43. Functional outcomes: Incontinence (Wexner FI score) **	80.76	Patient centered

AV, anal verge; FI, fecal incontinence; ICU, intensive care unit; LARS, low anterior resection syndrome.
** Up to 1 year; ** At 1, 2 (when possible) and 5 years (when possible).*

DISCUSSION

Prior attempts to achieve consensus on definitions of colorectal AL have had limited success. As demonstrated in a recent systematic review of the quality of reporting of AL across CRC trials, substantial variability in the reporting of contributing factors, diagnostic modalities, interventions and impact of colorectal AL on functional and oncologic outcomes persists. These variables are of important value not only to patients, but also administrators, quality officers, payers, and industry [13,23-28].

The CoReAL project aimed to bridge this gap by developing a standardized reporting framework for patients undergoing left-sided colorectal cancer resections. Drawing upon the most robust evidence available, along with insights from patients and experts, the CoReAL framework encompasses key variables related to the development, severity and postoperative outcomes of AL to create a comprehensive, data-driven and patient-centered approach for the clinical reporting of AL. We believe that integration of this framework into the clinical workflow will promote risk stratification for AL, adoption of evidence-based preventive and mitigation strategies, and demonstrate that time to diagnosis and corrective intervention correlates with persistence of long-term sequelae and patient reported outcomes.

The strength of the manuscript derives from the rigorous methodology for consensus development among a large group of international experts with a wide range of practices. Representation of several surgical societies was critical for endorsement, dissemination, and subsequent adoption by members. The core of the project's achievement lies in its evidence synthesis, which is encapsulated in 33 evidence-based statements derived from the highest level of evidence. The framework differs from prior consensus efforts in that it is largely built on these evidence-based statements and enriched by patients' experiences and experts' opinions to ensure it achieved its objectives whilst remaining relevant and meaningful to all relevant stakeholders [8-10].

The CoReAL reporting framework consists of 43 elements, organized along the four phases of the AL episode. This structured approach was intended to standardize reporting practices rather than replace existing AL classification systems. We envisage the framework to become integrated into the clinical workflow, ensuring that implementation does not disrupt but complements current documentation. Preoperative elements can be included in standard assessments or informed consent discussions. Intraoperative elements can be added to operative report templates for CRC resections, while discharge summaries should incorporate short-term postoperative elements from the index admission and any readmissions, with follow-up reports including data up to 30, 90 days, and beyond.

To date, institutions have only been required to report 30-day leak, re-intervention, re-operation, and readmission rates, which have been used as colectomy-specific quality benchmarking. This has reinforced the stigma associated with the reporting of AL and deterred clinical teams from interrogating anastomoses early, particularly when subclinical leaks are suspected. Another shortcoming of traditional quality reporting is that it does not consider whether steps were taken to mitigate the risk of leaks, identify and manage them early, effectively shortening the time to resolution. Extending the reporting period beyond 90 days is also critical to document resolution of AL, assess the true impact on healthcare resource utilization, and capture oncologic and functional sequelae, which are often omitted in shorter follow-up periods [29,30]. The proposed extended reporting timeframe for AL, which

reached consensus among experts, was aligned with patients' feedback regarding the need for better supporting patients affected by long-term sequelae of AL.

Widespread adoption of the CoReAL framework holds the potential to standardize and de-stigmatize the reporting of AL. By providing a clear and consistent methodology, the framework can enhance the quality of AL-related research and clinical care. This standardization will help ensure that data collected across different studies and clinical settings are comparable and reliable, facilitating better comparison of trials and meta-analyses. Additionally, standardized reporting can help demystify AL for patients, providing them with clearer information about their prognosis and long-term impact of their condition. The framework could also serve as a model for clinical trials employing AL as a clinical endpoint, as well as for benchmarking surgical outcomes and postoperative complications at the institutional level.

This consensus project and its outcomes should still be considered in the context of certain limitations. Although we included an international cohort of experts, our sampling did not encompass all regions of the world, notably South America, and most of Asia and Africa were not represented. This geographic gap may limit the generalizability of our findings. To address this shortcoming, we plan to include these regions in the upcoming implementation phase. Our patient cohort consisted of 10 individuals, and while their diversity was ensured by using a maximum variation sampling strategy, this sample size may not encompass the full spectrum of factors meaningful to patients. Because of the paucity of evidence for some topics, evidence-based statements were primarily based on a moderate-to-low level of evidence. A substantial challenge lies in the large number of reporting elements proposed and their integration into the clinical workflow. Questions arise regarding who will input data, at which timepoints, and the resources required for this reporting. Specifically, capturing long-term oncologic and QoL outcomes is difficult to operationalize, even at the 1-year timepoint, given loss to follow-up and resource constraints. It is also crucial to acknowledge that some reporting elements included in the framework lacked evidence, which may hinder broad acceptance and implementation.

The next phase of the CoReAL project will solicit stakeholder feedback and address perceived challenges to implementation to improve the feasibility and utility of the reporting framework in clinical practice. Currently, the framework is undergoing evaluation by the ASCRS membership and other collaborating societies to gauge agreement with the reporting elements and potential adoption in the clinical setting. The research team is conducting semi-structured interviews with surgeons, residents and nurses in focus groups to evaluate for the likelihood of adoption and compliance with the current version of the CoReAL framework. Factors that may contribute to poor adoption will be explored. Further work is needed to evaluate the utility of the reporting framework using real-world clinical datasets, as well as the feasibility of data collection through electronic health records.

CONCLUSION

The CoReAL project's international collaborative consensus reporting framework represents an important advancement towards standardization of reporting colorectal AL. By building on the highest level of evidence and incorporating diverse expert and patient perspectives, this framework may help to enhance the quality of reporting of anastomotic leak, de-stigmatizing leak, and moving our field towards a patient-centered approach that can lead to improving patient outcomes and future research.

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APPENDIX

A1. Working groups and topic allocations

Topics	Experts		Surgical research collaborators
Preoperative	Nader Francis* Deborah Keller Neil Hyman Patricia Tejedor	Jasper Stijns Benjamin Shogan Chelliah Selvasekar Ian Paquette	Anse De Sadeleer Marta Botti
Intraoperative	Patricia Sylla* Abe Fingerhut Mahdi Al-Taher Simon NG Siu Man Sherief Shawki	Freek Daams Michel Adamina Elizabeth Wick Mehraneh Dorna Jafari Marina Yiasemidou	Danique Heuvelings Saba Balvardi Samuel Lai Zoe Garoufalia
Postoperative short-term	Marylise Boutros* Tina van Loon Jennifer Davids Ian Jenkins	William Tzu-Liang Chen Jeremie Lefevre David Clark	Audrey Jongen Nariaki Okamoto Himani Bhatt Gianluca Pellino
Postoperative long-term	Nicole Bouvy* Stephanie Breukink Justin Maykel Alberto Arrezzo	Tan Arulampalam Roel Hompes Steven Wexner	Anke Gielen Jenny Moon

**Team leads*

A2. Detailed reporting framework

a. Preoperative reporting elements

Modifiable risk factors	**Other risk factors**	**Mechanical bowel preparation**
☐ Alcohol: ……/ week ☐ Active smoker: …… cigarettes / day ☐ Serum albumin level < 30 days before the surgery: ……	☐ Drop down menu - BMI - Gender: male / female - ASA score - Diabetes: yes (Hba1c:….) / no - Cardiovascular disease: yes / no - Chronic renal failure or insufficiency: yes / no - Chronic steroid use: yes / no - Clinical T-stage: T1 or T2 (not advanced) / T3 or T4 (advanced) - Tumor size: > 5cm / < 5 cm - Tumor location: right / transverse / left / rectal - Complicated tumor: yes (perforation / obstruction) / no - Neoadjuvant therapy: preop chemotherapy / radiotherapy / long course chemotherapy / short course chemotherapy / immunotherapy	☐ Yes ☐ No
Was the patient referred to a stoma therapist preoperatively?	**Was the potential need of a postoperative/ permanent stoma discussed?**	**Preoperative oral antibiotics**
☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes, Specify: …… ☐ No

b. Intraoperative reporting elements

Conversion MIS to open	**Number of staple loads for rectal transection**	**Perfusion assessment of conduit with fluorescence angiography**	**Anastomotic integrity testing**	**Location of inferior mesenteric artery ligation**
☐ Yes ☐ No ☐ N/A = open	☐ N/A = handsewn ☐ 0 ☐ 1 ☐ 2 ☐ >2	☐ Yes ☐ No	☐ Yes ☐ Consequence: … ☐ No	☐ Distal to the left colic artery ☐ Proximal to the left colic artery
Pitfalls	**Diverting stoma creation**	**Splenic flexure mobilization**	**Intraoperative difficulty**	
Critical events ☐ Pelvic stapler failure ☐ Other device failures ☐ Unplanned multivisceral* resection or repair	☐ No ☐ Yes ☐ unplanned ☐ planned - ileostomy - colostomy	☐ Yes ☐ No	☐ Distance of the anastomosis (cm) from AV ☐ Acute blood loss requiring intraop blood transfusion ☐ Redo pelvic surgery	

c. Postoperative early (surgery – 90 days) reporting elements

Before discharge (index admission)
POD of AL diagnosis: ..... days
LOS: ..... days
ICU LOS: ..... days

Mortality:
☐ Yes: POD .....
☐ No

Serial CRP measurement	**Mode of diagnosis**	**Re-interventions until discharge**	**Stoma creation**
☐ Yes ☐ No	☐ No ☐ Yes ➔ *Drop down menu:* ☐ CT scan - POD: ..... Contrast: PO / IV / rectal / NA ☐ Endoscopy - POD: ..... ☐ Contrast enema - POD: ..... ☐ Surgical - POD: ..... - EUA - Transabdominal: minimal invasive / open	☐ None ☐ Yes ➔ *Drop down menu:* ☐ Antibiotics ☐ Radiological drainage - POD: ..... - Trans-abdominal - Trans-gluteal ☐ Transanal/endoscopic repair - POD: ..... - Endosponge - Repair - Clipping - Drainage - Dilatation ☐ Abdominal: minimal invasive / open - POD: ..... - Takedown - Repair - Redo - Drainage - Tissue flap - Stoma creation ☐ Other:	☐ NA ☐ Yes - Ileostomy • Loop • End - Colostomy • Loop • End

c. continued

<u>After discharge – 30 days</u> AND <u>90 days</u>
☐ Not applicable
POD of AL diagnosis: ..... days

Mortality:
☐ Yes: POD .....
☐ No

Readmission	**Diagnostic modality**	**Re-interventions after initial discharge**	**Stoma**	**Anastomotic complication**
☐ Yes LOS: ..... days ICU LOS: ..... days ☐ No	☐ N/A ☐ CT scan - POD: ..... Contrast: PO / IV / rectal / NA ☐ Endoscopy - POD: ..... ☐ Contrast enema - POD: ..... ☐ Surgical - POD: ..... ☐ EUA - Transabdominal: minimal invasive / open	☐ None ☐ Antibiotics ☐ Radiological drainage - POD: ..... - Trans-abdominal - Trans-gluteal ☐ Transanal/endoscopic repair - POD: ..... - Endosponge - Repair - Clipping - Drainage - Dilatation ☐ Abdominal: minimal invasive / open - POD: ..... - Takedown - Repair - Redo - Drainage - Tissue flap - Stoma creation ☐ Other:	**Creation** ☐ NA ☐ Yes - Ileostomy • Loop • End - Colostomy • Loop • End **Stoma closure** ☐ N/A ☐ Yes: POD ..... ☐ Reversal planned ☐ No: - Ongoing leak - Medically ineligible - Ongoing cancer tx - Patient declined - Other:	☐ Resolved/None ☐ Leak - Defect, dehiscence or sinus - Stricture or stenosis - Fistula - Osteomyelitis

d. Postoperative late (90+ days) reporting elements

Re-interventions after 90 days *Until 1 year*	**Anastomotic status** *Until 1 year*	**Stoma** *Until 1 year*	**Anastomotic complications** *Until 1 year*
☐ No ☐ Yes ➔ *Drop down menu:* ☐ Antibiotics ☐ Radiological drainage - POD: ..... - Trans-abdominal - Trans-gluteal ☐ Transanal/endoscopic repair - POD: ..... - Endosponge - Repair - Clipping - Drainage - Dilatation ☐ Abdominal: minimal invasive / open - POD: ..... - Takedown - Repair - Redo - Drainage - Tissue flap - Stoma creation ☐ Other:	☐ Primary ☐ Secondary ☐ Healed ☐ Not healed	☐ No stoma ☐ Reversal planned ☐ Stoma ➔ *Drop down menu:* - Ileostomy • Loop • End - Colostomy • Loop • End Reason: • Ongoing leak • Medically ineligible • Ongoing cancer tx • Patient declined • Other: ..... • Reversal planned	☐ Resolved/None ☐ Leak - Defect, dehiscence or sinus - Stricture or stenosis - Fistula - Osteomyelitis

Oncological outcomes *1 year – 2 years – 5 years*	**Functional outcomes** *1 year – 2 years – 5 years*	**Quality of Life** *1 year – 2 years – 5 years*	**Mortality** *1 year – 2 years – 5 years*
Local recurrence ☐ Yes ☐ No Distant recurrence ☐ Yes ☐ No	☐ LARS - LARS score ..... ☐ Incontinence - Wexner FI score	☐ EQ-5D score	☐ No ☐ Yes: POD .....

SUPPLEMENTARY COREAL

S1. All research questions formulated by the coordinating team that were assessed during the literature search.

1. Preoperative topics

- What patient characteristics are preoperative risk factors for AL in colorectal cancer surgery?
- What is the effectiveness of prehabilitation versus no prehabilitation prior to elective colorectal cancer surgery on the percentage of AL?
- What is the effectiveness of mechanical bowel preparation versus no preparation prior to elective colorectal cancer surgery on the percentage of AL?
- What is the effectiveness of prophylactic oral antibiotics versus no ABX prior to colorectal cancer surgery on the percentage of AL?
- What is the effectiveness of anemia correction versus no correction prior to colorectal cancer surgery on the percentage of AL?
- What is the effectiveness of sarcopenia assessment versus no assessment prior to colorectal cancer surgery on the percentage of AL?

2. Intraoperative topics

- What is the comparative effectiveness of alternative operative technique (operations & anastomosis techniques) on the percentage of AL?
- What is the effectiveness of perfusion assessment versus no perfusion assessment during colorectal cancer surgery on the postoperative percentage of AL?
- What is the effectiveness of prophylactic diversion versus no prophylactic diversion during colorectal cancer surgery on the postoperative percentage of AL?
- What is the effectiveness of performing an integrity test versus no test during colorectal cancer surgery on the postoperative percentage of AL?
- What is the effectiveness of anastomotic reinforcement versus no reinforcement during colorectal cancer surgery on the postoperative percentage of AL?
- What is the effectiveness of placing a prophylactic drain versus no drain in the abdominal or pelvic cavity after colorectal cancer surgery on the percentage of AL?
- What is the effectiveness of an intraoperative ERAS protocol versus no protocol during colorectal cancer surgery on the percentage of AL?
- What is the effectiveness of transanal decompression tube versus no tube during low anterior colorectal cancer surgery on the percentage of AL?
- What is the effectiveness of using a validated intra-operative AL risk scoring system versus no scoring system during colorectal cancer surgery on the percentage of AL?
- What is the effectiveness of bypass devices versus no during colorectal cancer surgery on the postoperative percentage of AL?
- What is the influence of human factors during colorectal cancer surgery on the postoperative percentage of AL?

3. Postoperative short-term topics

- What is the diagnostic accuracy of validated clinical predictions scores for early identification of AL after colorectal cancer surgery?
- What is the diagnostic accuracy of biochemical markers on the percentage early detected ALs after colorectal surgery?
- What is the positive predictive value of postoperative imaging on the percentage of AL diagnoses after colorectal cancer surgery?
- What is the positive predictive value of endoscopic examination on the percentage of AL diagnoses after colorectal cancer surgery?
- What is the effectiveness of a low fiber diet versus regular diet postoperatively on the percentage of AL after colorectal cancer surgery?

- What is the effectiveness of postoperative prophylactic ABX versus no ABX on the percentage of AL after colorectal cancer surgery?
- What is the effectiveness of NSAID use versus no NSAID use on the percentage of AL after colorectal cancer surgery?
- Does the use of laxatives (e.g. movicolon or magnesiumhydroxide) versus no use of laxatives impact the percentage of AL?
- What is the effectiveness of an ERAS protocol versus no ERAS on the percentage early detected AL after colorectal cancer surgery? Does early discharge impact severity of AL after colorectal cancer surgery?
- What is the safety/feasibility of a minimal invasive approach versus open reintervention for AL after colorectal cancer surgery?
- What other approaches/techniques are present in the literature regarding reintervention for AL after colorectal cancer surgery?

4. Postoperative long term topics

- What is the oncologic impact of AL versus no AL following colorectal cancer surgery?
- What is the impact of AL versus no AL on quality of life after colorectal cancer surgery?
- What is the impact of AL versus no AL on the frequency of additional interventions for sequelae after colorectal cancer surgery?
- What is the impact of AL versus no AL on healthcare costs after colorectal cancer surgery?
- Are early and late leaks different identities?
- How should the impact of AL be measured after colorectal cancer surgery?
- When should we measure the impact of AL after colorectal cancer surgery?

S2. Search strategy

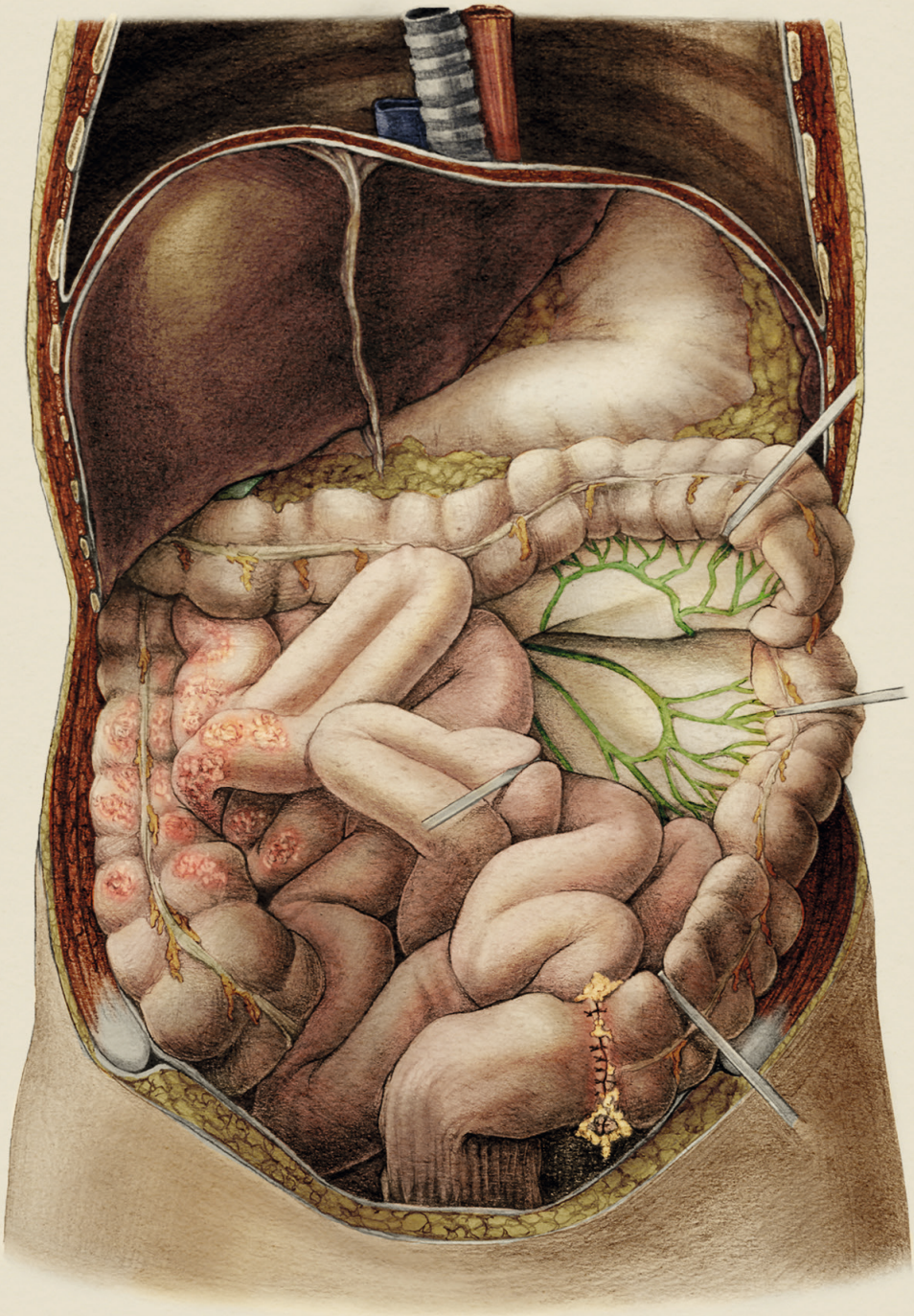
- **Pubmed:** ((((“Colorectal Neoplasms”[MeSH] OR ((“Neoplasms”[MeSH] OR carcinoma*[tiab] OR adenocarcinoma*[tiab] OR neoplas*[tiab] OR tumour*[tiab] OR tumor*[tiab] OR oncolog*[tiab] OR malignan*[tiab] OR cancer*[tiab]) AND (colorectal*[tiab] OR colon[tiab] OR colonic[tiab] OR rectal[tiab] OR rectum[tiab] OR sigmoid*[tiab]))) AND (“Colectomy”[MeSH] OR “Colorectal Surgery”[MeSH] OR “Rectum/surgery”[MeSH] OR “Colon/surgery”[MeSH] OR ((large bowel[tiab] OR colorectal*[tiab] OR colon[tiab] OR rectum[tiab] OR rectal[tiab] OR ileocaecal[tiab] OR caecum[tiab] OR low anterior[tiab]) AND (resection*[tiab] OR surg*[tiab] OR anastomo*[tiab] OR “Anastomosis, Surgical”[MeSH])) OR (colectom*[tiab] OR hemicolectom*[tiab] OR “total mesorectal excision*”[tiab] OR proctocolectom*[tiab] OR “abdominal perineal resection*”[tiab]))) AND (“Anastomotic Leak”[MeSH] OR (anastomo*[tiab] AND (“adverse effects”[Subheading] OR “complications”[Subheading] OR leak*[tiab] OR complication*[tiab] OR defect*[tiab] OR separation*[tiab] OR dehiscence*[tiab] OR breakdown*[tiab] OR abscess*[tiab])))) AND (((systematic review[pt] OR (((systematic review[ti] OR systematic literature review[ti] OR systematic scoping review[ti] OR systematic narrative review[ti] OR systematic qualitative review[ti] OR systematic evidence review[ti] OR systematic quantitative review[ti] OR systematic meta-review[ti] OR systematic critical review[ti] OR systematic mixed studies review[ti] OR systematic mapping review[ti] OR systematic cochrane review[ti] OR systematic search and review[ti] OR systematic integrative review[ti]) NOT comment[pt] NOT (protocol[ti] OR protocols[ti])) NOT MEDLINE [subset]) OR (Cochrane Database Syst Rev[ta] AND review[pt])) OR (“Meta-Analysis”[pt] OR meta analysis[ti])) OR (“Randomized Controlled Trial”[pt] OR “Controlled Clinical Trial”[pt] OR ((random*[tiab]

AND (controlled[tiab] OR control[tiab] OR placebo[tiab] OR versus[tiab] OR vs[tiab] OR group[tiab] OR groups[tiab] OR comparison[tiab] OR compared[tiab] OR crossover[tiab] OR cross-over[tiab]) AND (trial[tiab] OR study[tiab])) OR ((single[tiab] OR double[tiab] OR triple[tiab]) AND (masked[tiab] OR blind*[tiab])))))) NOT (((“Animals”[MeSH]) OR “Models, Animal”[MeSH] NOT “Humans”[MeSH]) NOT (letter[pt] OR comment[pt] OR editorial[pt]))

- **Embase:** (exp colorectal tumor/ or exp colorectal cancer/ or ((neoplasm/ or (carcinoma* or adenocarcinoma* or neoplas* or tumour* or tumor* or oncolog* or malignan* or cancer*).ti,ab,kw.) adj3 (colorectal* or colon or colonic or rectal or rectum or sigmoid*).ti,ab,kw.)) and (exp colorectal surgery/ or exp rectum surgery/ or exp colon surgery/ or ileoanal anastomosis/ or ileorectal anastomosis/ or ((large bowel or colorectal* or colon or rectum or rectal or ileocaecal or caecum or low anterior).ti,ab,kw. adj3 (resection* or surg* or anastomo*).ti,ab,kw. or (colectom* or hemicolectom* or “total mesorectal excision*” or proctocolectom* or “abdominal perineal resection*”).ti,ab,kw.)) and (postoperative complication/su or exp anastomosis leakage/ or anastomosis/co or (anastomo* adj3 (leak* or complication*)).ti,ab,kw.) and ((((“systematic review”/ or (systematic review.ti. or systematic literature review.ti. or systematic scoping review.ti. or systematic narrative review.ti. or systematic qualitative review.ti. or systematic evidence review.ti. or systematic quantitative review.ti. or systematic meta-review.ti. or systematic critical review.ti. or systematic mixed studies review.ti. or systematic mapping review.ti. or systematic cochrane review.ti. or “systematic search and review”.ti. or systematic integrative review.ti.)) or (meta analysis/ or meta analysis.ti.) or (randomized controlled trial/ or ((random*.ti,ab. and (controlled.ti,ab. or control.ti,ab. or placebo.ti,ab. or versus.ti,ab. or vs.ti,ab. or group.ti,ab. or groups.ti,ab. or comparison.ti,ab. or compared.ti,ab. or crossover.ti,ab. or cross-over.ti,ab.) and (trial.ti,ab. or study.ti,ab.)) or ((single.ti,ab. or double.ti,ab. or triple.ti,ab.) and (masked.ti,ab. or blind*.ti,ab.))))) NOT ((exp animal/ or nonhuman/) NOT exp human/) NOT (letter or editorial).pt.)
- **Cochrane:** (“Colorectal Neoplasms”[MeSH] OR ((“Neoplasms”[MeSH] OR carcinoma*:ti,ab,kw OR adenocarcinoma*:ti,ab,kw OR neoplas*:ti,ab,kw OR tumour*:ti,ab,kw OR tumor*:ti,ab,kw OR oncolog*:ti,ab,kw OR malignan*:ti,ab,kw OR cancer*:ti,ab,kw) AND (colorectal*:ti,ab,kw OR colon:ti,ab,kw OR colonic:ti,ab,kw OR rectal:ti,ab,kw OR rectum:ti,ab,kw OR sigmoid*:ti,ab,kw))) AND (“Colectomy”[MeSH] OR “Colorectal Surgery”[MeSH] OR “Rectum/surgery”[MeSH] OR “Colon/surgery”[MeSH] OR ((large bowel:ti,ab,kw OR colorectal*:ti,ab,kw OR colon:ti,ab,kw OR rectum:ti,ab,kw OR rectal:ti,ab,kw OR ileocaecal:ti,ab,kw OR caecum:ti,ab,kw OR low anterior:ti,ab,kw) AND (resection*:ti,ab,kw OR surg*:ti,ab,kw OR anastomo*:ti,ab,kw OR “Anastomosis, Surgical”[MeSH])) OR (colectom*:ti,ab,kw OR hemicolectom*:ti,ab,kw OR “total mesorectal excision*”:ti,ab,kw OR proctocolectom*:ti,ab,kw OR “abdominal perineal resection*”:ti,ab,kw)) AND (“Anastomotic Leak”[MeSH] OR (anastomo*:ti,ab,kw AND (“adverse effects”[Subheading] OR “complications”[Subheading] OR leak*:ti,ab,kw OR complication*:ti,ab,kw OR defect*:ti,ab,kw OR separation*:ti,ab,kw OR dehiscence*:ti,ab,kw OR breakdown*:ti,ab,kw OR abscess*:ti,ab,kw))

S3. Expert commentary on the statements

- Statement 7: Although the statement on sarcopenia, experts believe sarcopenia may be associated with AL and future research needs to be performed to investigate the impact of sarcopenia and frailty to AL outcomes.
- Statement 9: Splenic flexure mobilization represents a way to create a tension free anastomosis. The experts asked themselves 'What is tension free?' and concluded this cannot be measured objectively. They therefore decided to formulate the statement regarding flexure mobilization, with the expert note that if there is no tension free anastomosis, the risk of AL increases and flexure mobilization is a way to reduce this risk, but, as stated here, this is not necessary to perform routinely.
- Statement 11: As conversion reflects intraoperative difficulty, it is not the conversion itself that increases AL rates, but reflects the fact that the operation was difficult, which is a risk factor for AL development.
- Statement 15: The 'severity' of leaks is something difficult to measure, but the experts decided to use this phrasing as it's more about the consequences of leaks instead of the rates.
- Statement 27: The experts state that transanal or endoscopic management is possible when appropriate expertise is available. Besides, some experts stated that this should never be performed alone but always in combination with a lavage, although this opinion was not supported by everyone, nor was this specified in the evidence.



CHAPTER 4

PROPOSAL OF A REPORTING AND DATA SYSTEM FOR COLORECTAL ANASTOMOTIC LEAKAGE (CAL-RADS): A STUDY PROTOCOL FOR A CATEGORICAL CT ASSESSMENT SCHEME

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Study protocol

ABSTRACT

Introduction. Surgery is the primary curative option for colorectal cancer (CRC), but it can lead to significant post-operative complications, including anastomotic leakage (AL). AL occurs in 8-15% of cases and is associated with high morbidity and mortality. Diagnosing AL can be challenging due to ambiguous clinical presentations and high false-negative rates in imaging. The Colorectal Anastomotic Leakage Reporting and Data System (CAL-RADS) project aims to standardize the radiological (computed tomography; CT) assessment of AL.

Study Objectives. This study protocol outlines the first definitions of CAL-RADS. The primary objective is to validate the CAL-RADS score by assessing interobserver variability. The secondary objective is to correlate CAL-RADS scores with clinical re-interventions.

Methods. This multicenter, retrospective observational study involves collaboration among several Dutch medical centers. It includes 150 patients who underwent abdominal CT scanning within 90 days after colorectal surgery. Six categories were included in the CAL-RADS. Categories 1 to 5 follow an increasing risk for AL, from unlikely risk to a known leak. Category 0 indicates an inadequate imaging. Six radiologists will assess the CT scans using the CAL-RADS score. Initial test cases will be reviewed and discussed to ensure consistency. Interobserver agreement will be evaluated using Fleiss' kappa on the final CAL-RADS scores.

Results. Patient inclusion and data extraction were completed in April 2024. The test cases were finalized in May 2024 and showed good results. Radiologists are currently assessing the CT scans, with final results expected in early 2025.

Discussion. Standardizing CT scan reporting for AL through CAL-RADS is expected to improve early detection, reduce diagnostic errors, and enhance patient outcomes. The system aims to provide a clear framework for assessing AL, facilitating better communication among healthcare providers in a consistent manner, providing recommendations for subsequent management. The established standardization in reporting offers advantages for different stakeholders with the potential to ultimately enhance the overall quality of care.

Keywords: Anastomotic leakage, Reporting and Data System, abdominal computed tomography scans, colorectal surgery.

INTRODUCTION

Surgery remains the primary curative option for individuals with colorectal cancer (CRC), yet it can result in significant post-operative complications, with anastomotic leakage (AL) being particularly concerning. AL arises in 8-15% of colorectal surgery cases and is linked to elevated morbidity rates and short-term mortality rates reaching up to 39% [1]. The manifestation of AL can range from abdominal discomfort and mild fever to peritonitis and severe sepsis. This ambiguous progression often hampers prompt radiological assessment, and even when conducted, diagnoses frequently remain uncertain. Prior studies have reported false-negative rates ranging from 17-52% for both contrast enemas and computerized tomography (CT) scans, resulting in significant delays in re-intervention [2, 3].

Given that delayed detection of AL is linked to unfavorable outcomes and premature re-intervention may result in numerous negative re-explorations, it's crucial to critically look at radiological examinations and additionally weight the risks of delayed intervention against the morbidity of re-intervention. Physicians have a limited set of parameters to assess the likelihood of AL development on CT scans, which may help improve earlier recognition and re-interventions of AL after colorectal surgery. As the sensitivity of abdominal CT scanning after colonic surgery is considered low, we must make efforts to recognize, interpret, and communicate the imaging findings pertaining to the abdomen.

In early 2022, the American Society for Colorectal Surgery (ASCRS) initiated the Consensus on Reporting and Defining Colorectal Anastomotic Leaks (CoReAL) project to facilitate development and nationwide dissemination of AL information and tools together with the Society of American Gastrointestinal and Endoscopic Surgeons (SAGES), the European Association for Endoscopic Surgery (EAES), the European Society of Coloproctology (ESCP), the Endoscopic and Laparoscopic Surgeons of Asia (ELSA) and the Colorectal Surgical Society of Australia and New Zealand (CSSANZ). Within this network, a standardized reporting framework for AL after colorectal surgery was developed. Besides, the research team proposed a standardized CT assessment scheme for AL. Developing this classification system would make it possible to compare data across institutions and populations and, thus, provide a basis for gathering scientific evidence and improved communication with referring physicians when assessing CT scans for the suspicion of a leak. Building on the standardization efforts seen in systems like Breast Imaging Reporting and Data System (BI-RADS), the Lung Imaging Reporting and Data System (Lung-RADS), and Prostate Imaging Reporting and Data System (PI-RADS), the authors opted for the term Colorectal Anastomotic Leakage Reporting and Data System (CAL-RADS) [4].

STUDY OBJECTIVES

This protocol presents the first definitions of CAL-RADS, along with the expected next steps. The objectives of the CAL-RADS study are twofold. The primary aim is to validate and assess clinical feasibility of the CAL-RADs score by assessing interobserver variability of the proposed system. Secondary, we want to estimate a correlation between the performed clinical re-interventions and the given CAL-RADS score.

METHODS

This is a study protocol for a multicenter, retrospective observational study (non-WMO research). This study is approved by the Medical Ethical Committee of the Maastricht University Medical Centre (no. 2023-0348), and additional participating centers that contribute to patient inclusion.

Research group and participating centers

The CAL-RADS study will be a collaboration between Maastricht Universitair Medisch Centrum (MUMC+), Amsterdam Universitair Medisch Centrum (Amsterdam UMC), Catharina Ziekenhuis Eindhoven (CZE) and Antoni van Leeuwenhoek (AVL). Besides radiologists, there will be a surgeon from every hospital involved as well.

Study population and definitions

An observer study will be conducted on a set of randomly selected abdominal CT scans from a group of consecutive patients who presented with a possible AL after oncological colorectal surgery between 2018 and 2024. Specifically, all patients underwent CT scanning within 90 days after their primary surgery due to clinical deterioration, raising the question of a possible leak for the radiologist to assess. This group will include 100 patients who were officially diagnosed with an AL, and 50 patients who did not have an official AL diagnosis or any registered complications. An AL was defined as any defect of the intestinal wall at the anastomotic site (including suture and staple lines of neorectal reservoirs) leading to a communication between the intra- and extraluminal compartments, as proposed by the ISREC group [5]. All patients will be retrospectively included from MUMC+, Amsterdam UMC, and CZE. Data collected will include patient characteristics (age, sex, comorbidities), type of surgical procedure, CT scan details (postoperative day, use of contrast, technical details, and report), clinical signs, biochemical markers, and re-interventions. These data will be stored in an online database in Castor.

CAL-RADS proposal

The CAL-RADS assessment scheme allows for the categorization of a given non-enhanced abdominal CT scan into groups related to the likelihood of a patient having AL after colorectal

surgery. This CT classification proposal, presented in Table 1, was developed based on findings from 30 patients who suffered from an AL after colorectal surgery, together with an extensive literature search that gathered all reported elements on imaging and radiological findings [6]. The system underwent iterative refinement based on feedback from the included radiological experts.

Table 1. Overview of CAL-RADS categories and the corresponding level of suspicion for AL after colorectal surgery

	Likelihood of AL	Findings	Management
0	(Technical) Inadequate CT		Consider repeating the CT
1	Leakage unlikely	• Expected amount of postoperative air and no localized peritoneal fluid	No recommended intervention
2	Probable no leak	• Ileus with an expected amount of post-operative air and no localized peritoneal fluid	Follow-up based on clinical parameters
3	Possible a leak	• Excessive and/or increasing post operative extraluminal gas; • With/without anastomotic abnormal bowel wall thickening; • Without peri-anastomotic fluid	Suggest close observation and a low threshold for additional examination
4	Highly suggestive of a leak	• Excessive and/or increasing postoperative extraluminal gas, with peri-anastomotic fluid and/or; • Presence of a peri-anastomotic abscess; • Disruption of the anastomotic integrity	Appropriate intervention suggested
5	Known leak		

The designation of the CAL-RADS 0 category indicates that the scan lacks the diagnostic quality necessary for the reporting radiologist to definitively attribute or exclude one of the other CAL-RADS categories. This deficiency may arise from severe artifacts or incomplete abdominal coverage. It is important not to interpret this as a conclusive assessment, and if feasible, a repeat scan should be considered. The CAL-RADS 1 category encompasses cases with an abdominal CT scan that is either normal or exhibits expected amount of postoperative air. There are no signs of localized peritoneal fluid. The CAL-RADS 2 category comprises cases featuring radiological findings consistent with an ileus and an expected amount of post-operative air. Again, there are no signs of localized peritoneal fluid. The third category includes findings that, while some may be typical for AL, have still some overlaps with a normal postoperative image. Therefore, CAL-RADS 3 indicates a possible leak. Inclusion in this category is warranted by findings such as excessive and/or increasing post operative extraluminal gas with or without anastomotic abnormal bowel wall thickening, but still without peri-anastomotic fluid. This category implies suspicion for AL and therefore low threshold for additional examination. The fourth category reflects the image is highly suggestive of a leak. Features of the CAL-RADS 4 are excessive and/or increasing postoperative extraluminal gas

with peri-anastomotic fluid and/or presence of a peri-anastomotic abscess. Also, any signs of disrupted integrity of the anastomosis will lead to this score. This category implies a very high level of suspicion for AL and a subsequent intervention is suggested. The final CO-RADS 5 category indicated proven AL after endoscopic examination or a surgical intervention.

CT scoring procedure

CT images will be anonymously extracted from the picture archive and communication system. The CAL-RADS study will involve observers (radiologists) with varying levels of experience in interpreting abdominal CT scans for suspected AL after colorectal surgery using the CAL-RADS score. A total of six observers will participate in scoring the CT scans with the proposed CAL-RADS score. These observers will be blinded for all extracted patient data regarding AL outcomes. First, five test cases will be assessed by each radiologist and then discussed in a plenary session. These test cases will validate the accuracy of the radiologists' scores and identify any discrepancies or biases in their interpretations before the official assessment. The plenary discussion will ensure that everyone is well-versed in the criteria and protocols, leading to more consistent and accurate readings. All 150 included CT scans will be assessed using a standardized excel sheet to score the criteria and add comments if necessary. Afterwards, the final CAL-RADS scores will be added to the Castor Database for every patient.

Statistical analysis

Statistical analysis will be performed using SPSS (IBM SPSS Statistics for Apple, Version 27, Armonk, New York, NY, USA) and GraphPad Prism (GraphPad software for Apple, version 8.0.0, San Diego, CA, USA). Data will be presented as the mean ± standard deviation or median and interquartile range based on normality of data. A 4 x 4 confusion matrix will be made separately per observer, in which the CAL-RADS score of the observer will be compared with the median CAL-RADS score of the remaining observers. Subsequently, a similar matrix will be computed by aggregating all individual 4 x 4 tables. To assess interobserver agreement, the Fleiss' kappa (κ) value will be calculated among observers. The κ values are derived by comparing the CAL-RADS scores of each observer to the median score of the remaining observers. Interobserver agreement is categorized as slight (κ = 0.01 – 0.20), fair (κ = 0.21 – 0.40), moderate (κ = 0.41 – 0.60), substantial (κ = 0.61 – 0.80) or almost perfect (κ = 0.81 – 1.00) [5].

RESULTS AND DISCUSSION

Patient inclusion and data extraction were finalized in April 2024. The test cases, which yielded good results, have been completed. Currently, the radiologists are assessing the CT scans. Results are expected in early 2025.

As the use for CT scanning has gained validation in numerous clinical trials for evaluating patients with suspected AL after colorectal surgery, standardizing reporting is a key element to foster broader adoption in clinical practice, reduce errors, and ultimately enhance patient outcomes. The primary objective of the current study is to develop a CAL-RADS classification system that offers uniform categories for final assessment, accompanied by recommendations for subsequent management. It is crucial to emphasize that the CAL-RADS classification is intended to complement the final impression of the report, especially since the report will furnish detailed information regarding the timing, type of surgery and other relevant findings. We advise to use the CAL-RADS classification on a per-patient basis for clinically suspected AL cases after colorectal surgery.

The CAL-RADS score will be developed by the CoReAL collaborative group and supportive radiological societies, providing a framework that builds on other reporting schemes for surgical complications but expands the concept in a way similar to systems like BI-RADS. Categories 1–4 provide increasing suspicion for AL after colorectal surgery at unenhanced abdominal CT, thus allowing for task-specific cutoff points for clinical decision making. Before clinical use, this score must show substantial interobserver agreement. If it does, the system may fulfill the need for a structured and fast reporting system that decreases ambiguity in communications with referring physicians and facilitates collection of CT performance data for further research of this worldwide colorectal surgery problem. Additionally, the effectiveness in clinical practice and flexibility in selecting optimal cutoff points for diverse clinical decisions should be investigated to make this type of system even more valuable.

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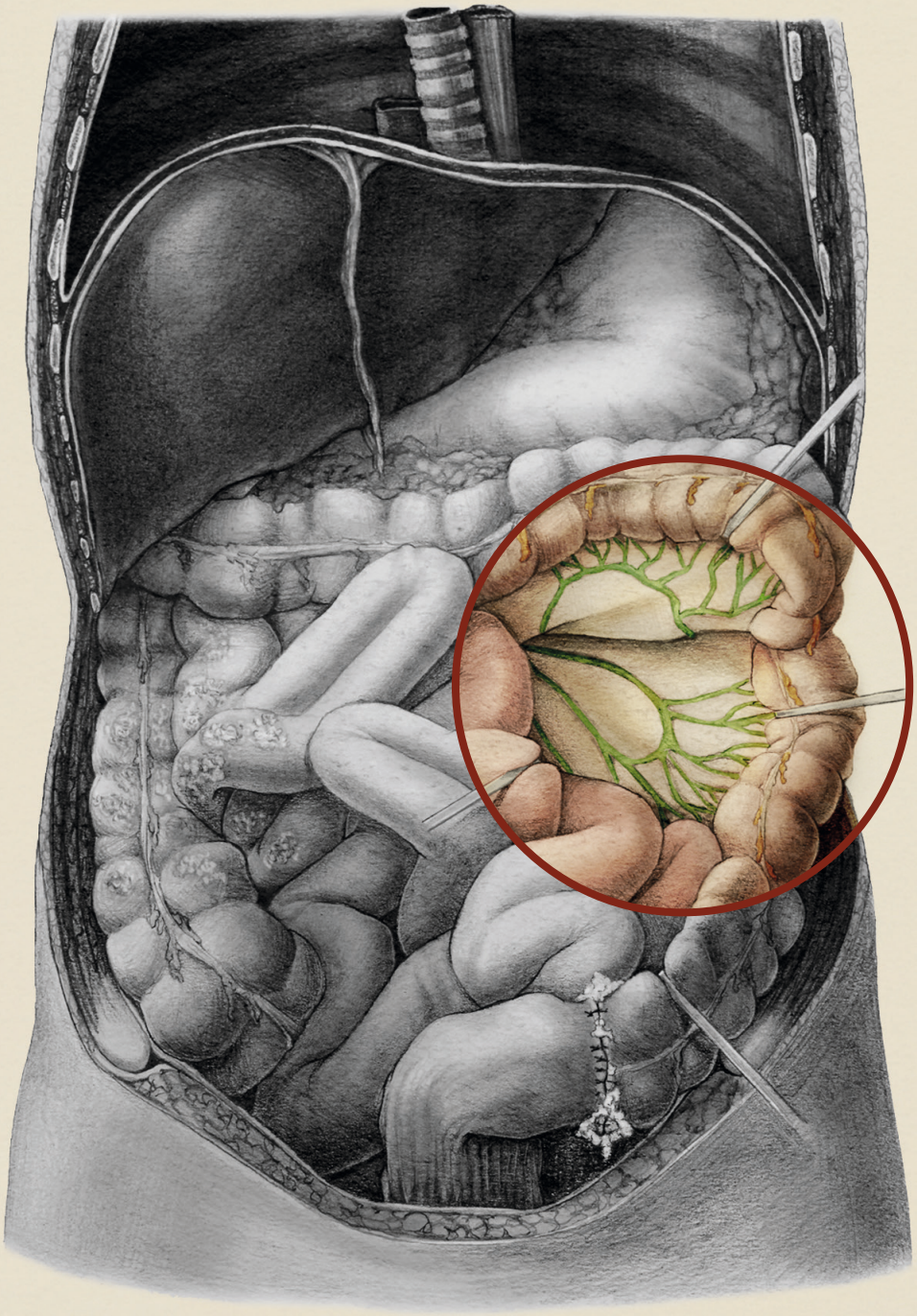
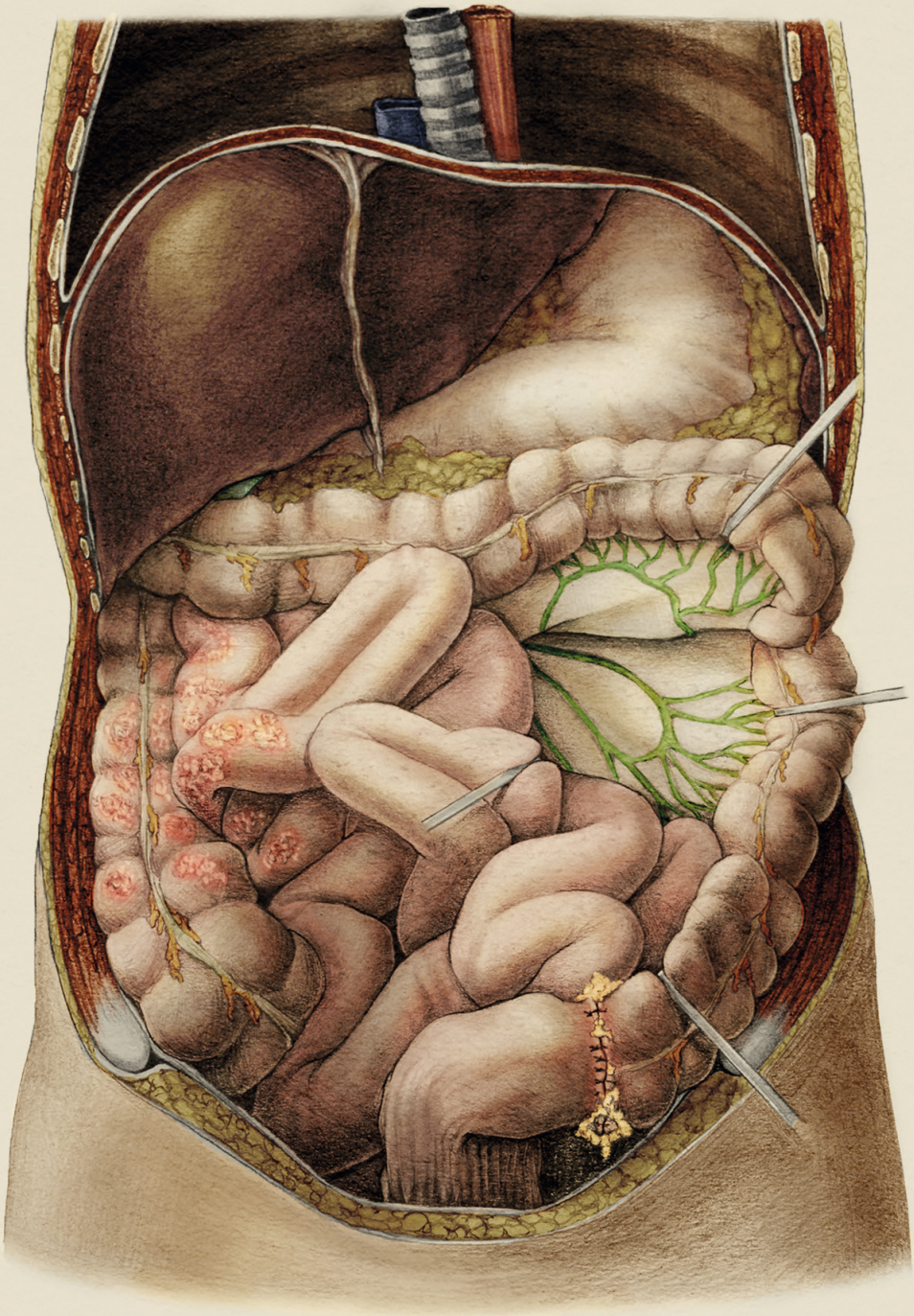


PART II

IMPROVING BOWEL PERFUSION ASSESSMENT TO REDUCE THE RISK OF ANASTOMOTIC LEAKS



CHAPTER 5

SIMULTANEOUS FLUORESCENCE IMAGING OF BOWEL PERFUSION AND URETER DELINEATION USING METHYLENE BLUE: A DEMONSTRATION IN A PORCINE MODEL

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ABSTRACT

Background. Intraoperative near-infrared fluorescence imaging (NIRF) with preoperative optical dye administration is a promising technique for quick and easy intraoperative visualization of the ureter and for an improved, real-time assessment of intestinal perfusion. During colorectal surgery, there is a need for simultaneous non-invasive ureteral imaging and bowel perfusion assessment, using one single camera system.

Aims. The purpose of this study is to investigate the feasibility of simultaneous intestinal perfusion and ureteral imaging using a single commercially available NIRF imaging system.

Methods. Six Landrace pigs underwent laparotomy under general anesthesia in this experiment. An intravenous (IV) dose of 0.2 mg/kg indocyanine green (ICG) was given to assess bowel perfusion. Two pairs received a methylene blue (MB) iv injection of 0.75 mg/kg, 0.50 mg/kg or 0.25 mg/kg respectively to investigate ureteral visualization. Quest Spectrum Fluorescence Camera (Quest Medical Imaging, Middenmeer, The Netherlands) was used for NIRF imaging.

Results. Ureter visualization and bowel perfusion under NIRF imaging was achieved in all animals. All ureters were visible after five to ten minutes and remained clearly visible until the end of every experiment (120 – 420 minutes). A mixed model analysis did not show any significant differences neither between the three groups nor over time. Importantly, we demonstrated that bowel perfusion could be visualized with methylene blue (MB) as well. We observed no interference between ICG and MB and a faster washout of MB.

Conclusion. We successfully demonstrated simultaneous fluorescence angiography with ICG and ureteral imaging with MB in the same surgical procedure, with the same commercially available NIRF imaging equipment. More importantly, we showed that the use MB is adequate for bowel perfusion assessment and ureter visualization with this NIRF imaging system. Besides, MB showed an earlier washout time, which can be clinical beneficial as a repeated dye injection may be necessary during a surgical procedure.

Keywords. Perfusion assessment; ureteral delineation; methylene blue; indocyanine green; intraoperative near-infrared fluorescence imaging; anastomotic leakage.

INTRODUCTION

Anastomotic leakage (AL) is one of the most dreaded complications after colorectal surgery. Probably the most important cause of AL is impaired perfusion of the bowel. Assessment of bowel perfusion is therefore one of the crucial strategies in reducing the incidence of AL [1, 2]. Another feared complication during colorectal surgery is ureteral injury. In order to prevent iatrogenic damage, the surgeon must be aware of the exact location of the ureter.

Intraoperative near-infrared fluorescence imaging (NIRF) with preoperative optical dye administration is a technique for quick and easy intraoperative visualization of the ureter [3-5] and for an improved assessment of anastomotic perfusion [2, 6-11]. However, to date there is no clinical study which evaluates simultaneous fluorescence-enhanced ureteral delineation and intestinal perfusion in the same surgical procedure over time and the possibility of using one single dye. Over the last decade, (pre-)clinical studies have been performed to visualize the ureter. Due to the exclusive clearance of indocyanine green (ICG) by the liver, it is not suitable for ureteral imaging since it is not cleared in the urine. Methylene blue (MB) on the other hand, a clinically approved and widely used dye, is excreted by the kidneys and can consequently be administered for non-invasive ureteral imaging. However, results of clinical and pre-clinical experiments investigating the feasibility of MB for ureteral imaging have shown conflicting results regarding its added clinical value [3, 4]. This may be due to the characteristics of the dye itself, having only a weak fluorescent signal, or to the laparoscopic equipment used. The latter refers to a disadvantage of MB, which is excited at ~670nm, in contrast to other dyes such as ICG which is excited at ~800nm. As a result, the use of MB requires specifically developed equipment. The vast majority of imaging systems used in the studies with MB thus far were experimental and not commercially available for clinical use [5].

In colorectal surgery, there is a need for simultaneous non-invasive ureteral imaging and bowel perfusion assessment. The latter can be achieved by finding a single dye that can simultaneously identify these structures, or an adequate NIRF imaging system that can simultaneously identify these structures with two different dyes. Our group has already successfully studied and reported on the first approach [12]. However, this was a pre-clinical study that is not yet ready for clinical implementation. The use of two dyes simultaneously for ureteral imaging and bowel perfusion imaging has become potentially feasible now that a commercial imaging system is available for such an approach.

The aim of this study was to investigate the feasibility of simultaneous intestinal perfusion and ureteral imaging using a single commercially available NIRF imaging system.

MATERIALS AND METHODS

This feasibility study was performed at the central animal facilities of Maastricht University (Maastricht, The Netherlands). Animals were used in compliance with Dutch regulations and legislation concerning animal research, and the study was performed according to a protocol approved by the Experimental Animal Committee of Maastricht University (DEC-UM) (approval number: 2017-021-001). Informed written consent was not applicable.

Animals

A total of six mature (35-45 kg) female Landrace pigs were used for this study. A pig model was chosen because of the anatomical similarities between humans and pigs, and previous successful application of NIRF imaging in pigs [13]. Animals were used in compliance with the regulations of Dutch legislation concerning animal research, and the study was performed according to an approved protocol by the local animal ethics committee.

Preparation of the dyes

MB (Proveblue, Provepharm Life Solutions, Marseille, France) was diluted in a sterile phosphate-buffered saline (PBS) solution to a concentration of 1 mg/ml. In a previous review, MB doses ranging from 0.25 to 1 mg/kg were studied [14]. A dose of 0.75 mg/kg resulted in the highest target-to-background ratio of the fluorescence image of the ureter [14]. However, in another review by van Manen et al. [15], a dose of 0.25 mg/kg was recommended. In our own experience in a clinical pilot study using this dye, we found that NIRF imaging was strongly influenced by the dose/concentration of the dye [4]. Consequently, in this study, we investigated three different MB doses: two pigs received a bolus IV injection of 0.75 mg/kg (group 1), two other pigs of 0.50 mg/kg (group 2), and the final two pigs 0.25 mg/kg (group 3). Additionally, ICG (Verdye, Diagnostic Green GmbH, Aschheim, Germany) was diluted in a sterile H_2O solution to a concentration of 2.5 mg/ml. A dose of 0.2 mg/kg was given as a bolus IV injection. This dose is based on the current frequently clinically used dose range in patients as was previously found in our analysis of 1,240 patients registered in the EURO-FIGS registry on fluorescence angiography [16].

Fluorescence imaging system

The commercially available Quest Spectrum Fluorescence Camera (QUEST SPECTRUM®, Quest Medical Imaging, Middenmeer, The Netherlands) was used for NIRF imaging. To ensure standardized measurements and prevent potential movement of the camera, the camera was fixed with a custom-made mechanic, articulated arm, which was connected to the surgical table. The distance of the camera tip to the target organ was measured with a sterile paper ruler and was 15 cm in all procedures. During NIRF imaging, environmental lights were dimmed preventing ambient light interference. Because of this standardization, and prevention of motion of the camera and animal, we ensured to have high quality of images through the surgical procedure. The 800 nm channel was used to capture ICG fluorescence

(ICG mode) while the 700 nm channel was used for MB fluorescence imaging (MB mode). The information captured during recording was visualized within several different fluorescence formats and displayed onto a screen while performing the procedures. First, a color image of the surgical field was presented together with the NIRF image to allow surgical guidance. Additionally, two overlay modes, including a fluorescence intensity map and a NIRF image that is projected over the colored image, were projected onto the same screen.

Anesthesia

All surgical procedures were performed under general anesthesia. Standard medication used to ensure proper sedation and analgesia was as follows: intramuscular injection of Zolazepam/Tiletamine (6 mg/kg, Virbac, Barneveld, The Netherlands) and Thiopental (10 mg/kg, Panpharma SA, Trittau, Germany), a combination of sufentanyl (0.01 mg/kg/h, Hameln Pharma GmbH, Hameln, Germany), Propofol (9 mg/kg/h, B. Braun Melsungen AG, Melsungen, Germany), and Midazolam (1 mg/kg/h, Aurobindo, Baarn, The Netherlands) intravenously. All alterations in vital parameters were monitored by the animal anesthesiologist.

Surgical procedure and measurements

After general anesthesia, a midline laparotomy was performed by an experienced surgeon. First, a loop of the small bowel with a length of approx. 15 cm, at 250 cm measured from the gastric pylorus, was selected as a region of interest. The camera system was switched to ICG mode followed by ICG injection. Bowel perfusion imaging was performed for at least 120 seconds. The same procedure was repeated under MB mode whereafter MB was administered. Consequently, the area where the left ureter would be expected was identified after 120 seconds and continuous left ureteral imaging in MB mode was performed until 5 minutes after dye administration. The latter was repeated every 10 minutes for a total of minimum 120 minutes (T120) after MB dye injection. Bowel perfusion imaging in both ICG and MB modes was performed in parallel for every 10 minutes in two pigs to investigate the washout pattern. In the other four pigs, only T0 measurements were taken. The identification of the right ureter occurred in the meantime, without any further recordings. An overview of the surgical procedure and measurements is shown in Figure 1. At the end of the protocol, animals were euthanized with a lethal dose of pentobarbital (200 mg/kg).

Statistical analysis and quantification of the fluorescence imaging

All NIRF videos were post-analyzed with Quest Spectrum software (ResearchTool v4.3 and TBR tool v1.0). The ureteral fluorescence imaging was assessed and quantified by calculating the fluorescence intensity (FI) and the target-to-background ratio (TBR; FI of target/FI background) [13, 17]. Background values were calculated approximately 1 cm on either side of the ureter, with solely retroperitoneal tissue. The FI of target was calculated based on the fluorescence signal during peristaltic contractions of the ureter by drawing a circle of interest in the corresponding region. Numerical variables were presented as means and standard deviation (SD) or median and interquartile range (IQR) where appropriate. To evaluate the

statistical significance of numerical variable differences observed between groups and estimate group effect, a mixed model analysis was performed. Differences were considered significant when the *p* value was < 0.05. All the statistical analyses were performed with the SPSS© software (version 27).

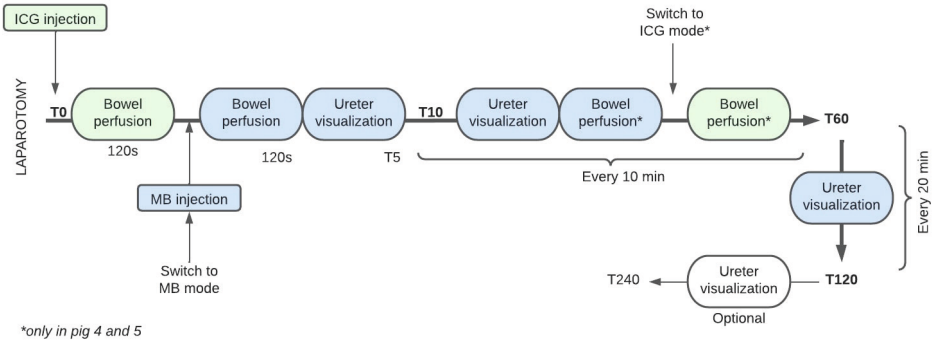

Figure 1. Overview of surgical procedure and measurements.

RESULTS

A total of six pigs were included in this study. Median weight was 39.25 ± 3.13 kg (IQR 36.00 – 42.25). All animals were followed for at least 120 minutes after dye administration. The maximum observation time took 420 minutes. Animal characteristics and clinical data are summarized in Table 1.

Table 1. Pig characteristics and clinical data

Group	Pig 1 1	Pig 2 1	Pig 3 2	Pig 4 2	Pig 5 3	Pig 6 3
Weight (kg)	41.00	43.00	42.00	37.50	36.00	36.00
MB dose (mg/kg) ***Total dose (mg)***	0.75 *30.75*	0.75 *32.25*	0.50 *21.00*	0.50 *18.75*	0.25 *9.00*	0.25 *9.00*
ICG dose (mg/kg) ***Total dose (mg)***	0.20 *8.20*	0.20 *8.60*	0.20 *8.40*	0.20 *7.50*	0.20 *7.20*	0.20 *7.20*
Length of observation (min)	420	240	360	240	120	360
Number of ureters visualized *(n)*	2	2	2	1*	2	2

**due to renal agenesis, only right kidney in situ*

Ureter visualization analysis

A total of 11 ureters in six pigs were identified. The reason why one ureter could not be identified was due to renal agenesis (only right kidney in situ). All ureters were clearly distinguishable from their surroundings. In all six experiments (based on three different doses of MB), the ureters were visible within five to ten minutes after dye administration

and remained clearly visible until the end of every experiment (Figure 2). For the surgical team, no visual differences between the higher, middle, or lower doses were seen during the experiments. All evaluations showed a persistent clear delineation of the ureters in NIRF mode. The fluorescence signal was maximal during peristaltic contractions of the ureter. TBR values of all left ureters (except for pig no. 4, right ureter) at different time points are summarized in Table 2. The highest measured TBR was 8.89. All details about the mixed model analysis are presented in Table 3. Univariate tests did not show a significant difference when comparing the three dose groups (p = 0.345). The mean TBR value was the highest in group 2 (5.21 ± 0.37). The latter was confirmed in pairwise comparisons, which showed group 2 differed the most in mean TBR value compared to both other groups. The mean difference was 0.745 (p = 0.250) and 0.833 (p = 0.209) compared to group 1 and group 3, respectively. The relation between group and time was not significant (p = 0.855), indicating that the group effect did not significantly differ at different time points. As a consequence, the group effect was computed over all time points and showed non-significant effect either.

No adverse reactions were observed in any of the animals after MB injection. In the first pig, the left ureter was purposely transected at the end of the experiment. The leakage of urine due to ureteral damage could be clearly visualized with the NIRF imaging system (Supplementary, Figure A).

Table 2. TBR values for different time points

Ureter **MB dose**	**Pig 1** **Left** **0.75mg/kg**	**Pig 2** **Left** **0.75mg/kg**	**Pig 3** **Left** **0.50mg/kg**	**Pig 4** **Right** **0.50mg/kg**	**Pig 5** **Left** **0.25mg/kg**	**Pig 6** **Left** **0.25mg/kg**
TBR 5	1.32 ± 0.12	1.18 ± 0.14	0.87 ± 0.25	0.96 ± 0.25	0.91 ± 0.23	2.98 ± 0.22
TBR 10	4.61 ± 0.35	5.70 ± 0.45	3.00 ± 0.29	5.61 ± 0.52	1.00 ± 0.16	5.89 ± 0.28
TBR 20	4.96 ± 0.41	3.95 ± 0.45	5.02 ± 0.37	7.88 ± 0.62	2.42 ± 0.28	5.98 ± 0.90
TBR 30	6.00 ± 0.15	4.08 ± 0.62	7.00 ± 0.34	5.35 ± 0.61	1.08 ± 0.43	4.76 ± 0.44
TBR 40	5.45 ± 0.31	3.51 ± 0.33	7.94 ± 0.35	4.34 ± 0.57	6.39 ± 0.44	-
TBR 50	4.29 ± 0.27	3.92 ± 0.36	5.75 ± 0.39	7.04 ± 0.59	4.47 ± 0.58	5.06 ± 0.26
TBR 60	4.46 ± 0.21	4.27 ± 0.50	6.42 ± 0.31	6.11 ± 0.51	6.41 ± 0.73	7.10 ± 0.57
TBR 80	6.58 ± 0.37	3.94 ± 0.41	8.89 ± 0.89	3.20 ± 0.38	3.69 ± 0.35	4.67 ± 0.33
TBR 100	3.97 ± 0.25	4.21 ± 0.49	4.63 ± 0.50	5.59 ± 0.46	7.51 ± 0.54	2.61 ± 0.31
TBR 120	7.02 ± 0.68	4.17 ± 0.50	3.86 ± 0.45	4.71 ± 0.34	8.84 ± 0.65	4.09 ± 0.32
TBR 180	-	2.60 ± 0.41	-	4.18 ± 0.37	-	-
TBR 240	5.51 ± 0.42	2.66 ± 0.24	5.46 ± 0.52	6.69 ± 0.68	-	-
TBR 360	-	-	3.86 ± 0.28	-	-	2.48 ± 0.20
TBR 420	4.36 ± 0.36	-	-	-	-	-

TBR, Target to Background ratio.

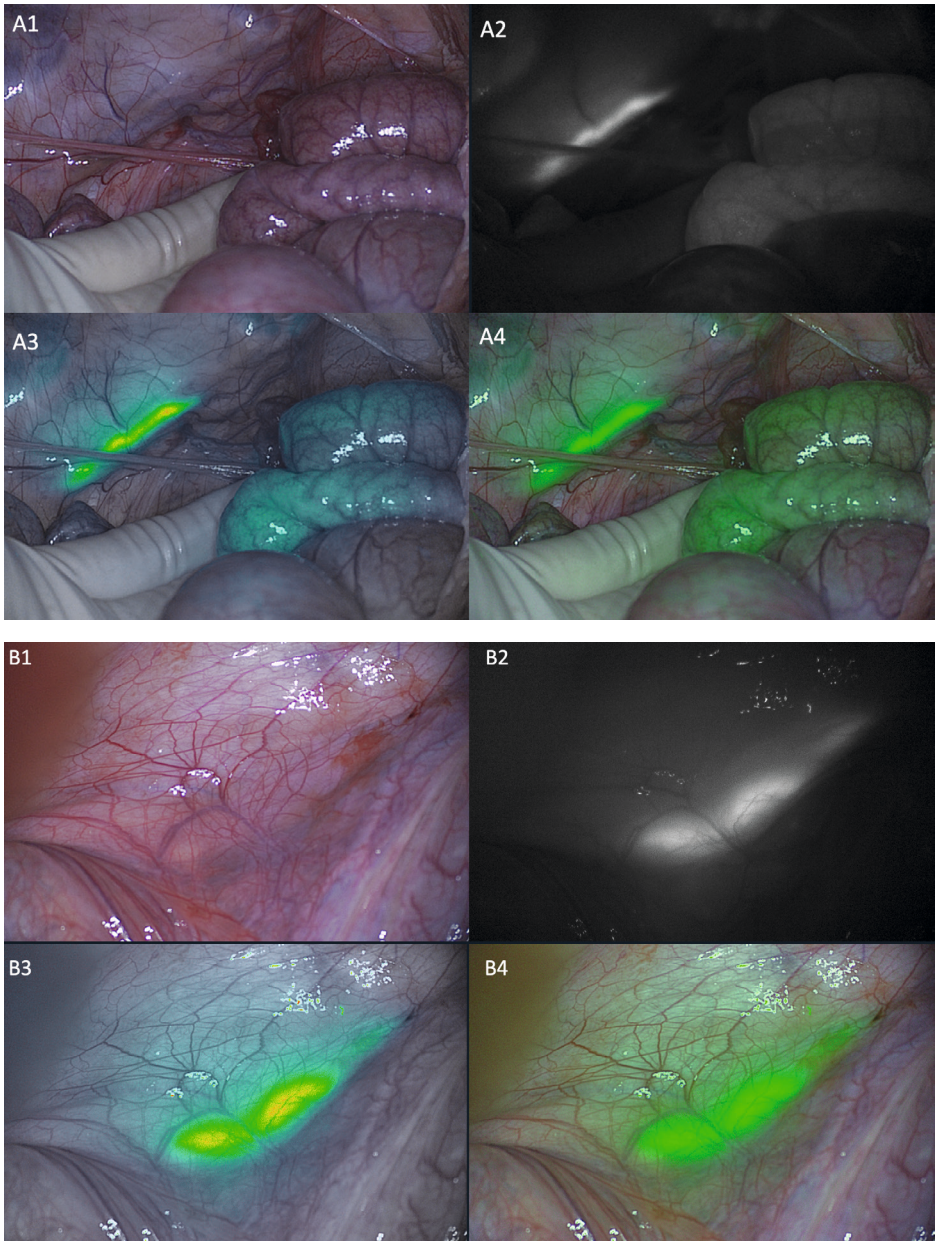

Figure 2. Examples of left ureter visualization with MB during peristaltic contraction. **(A)** (1) Color image of the surgical field, (2) NIRF image, (3) gradient overlay image, and (4) green overlay image of the left ureter in MB mode 10 minutes after MB injection (pig 2). **(B)** (1) Color image of the surgical field, (2) NIRF image, (3) gradient overlay image, and (4) green overlay image of the left ureter in MB mode 360 minutes after MB injection (pig 6).

Table 3. Mean TBR values for different time points among different MB dose groups

TBR ± SD	**Group 1 (0.75mg/kg)**	**Group 2 (0.50mg/kg)**	**Group 3 (0.25mg/kg)**	***p* value***
TBR 5	1.95 ± 1.46	0.92 ± 0.06	1.21 ± 0.04	0.826
TBR 10	3.45 ± 3.46	4.31 ± 1.84	5.16 ± 0.77	0.617
TBR 20	4.20 ± 2.52	6.45 ± 2.02	4.46 ± 0.71	0.374
TBR 30	4.76 ± 2.92	6.18 ± 1.17	5.04 ± 1.36	0.178
TBR 40	6.39 ± N/A	6.14 ± 2.55	4.48 ± 1.37	0.544
TBR 50	4.66 ± 0.42	6.40 ± 0.19	4.11 ± 0.26	0.405
TBR 60	6.76 ± 0.49	6.27 ± 0.22	4.37 ± 0.13	0.356
TBR 80	4.77 ± 0.14	6.04 ± 4.02	5.46 ± 1.87	0.760
TBR 100	5.00 ± 3.38	5.11 ± 0.67	4.09 ± 0.17	0.812
TBR 120	5.00 ± 1.28	4.29 ± 0.60	5.60 ± 2.02	0.751
Mean (95% CI)	4.46 ± 0.376 (3.31 – 5.62)	5.21 ± 0.37 (4.01 – 6.40)	4.38 ± 0.37 (3.18 – 5.57)	0.345

*TBR, Target to Background ratio; *Univariate tests within mixed model analysis*

Bowel perfusion analysis

In all pigs, a clear macroscopic NIRF visualization of the perfusion in ICG and MB mode was achieved in all pigs within a few seconds after dye administration (Figure 3). After 20 seconds, maximal intensities were reached (Figure 4). No interference between ICG and MB was observed when switching to either mode. In pigs 4 and 5, bowel perfusion was assessed every 10 minutes for at least 60 minutes to investigate washout and fluorescence intensities over time. After 50 minutes of dye administration, ICG was still clearly visible while MB was almost no longer visible in pig 4 (MB dose of 0.5 mg/kg) (Figure 5). After 60 minutes, only a few spots of MB dye were still visible. Pig 5 (MB dose of 0.25 mg/kg) also showed an earlier washout time of MB compared to ICG, after 40 minutes of MB injection. No adverse reactions were observed in any of the animals after ICG administration.

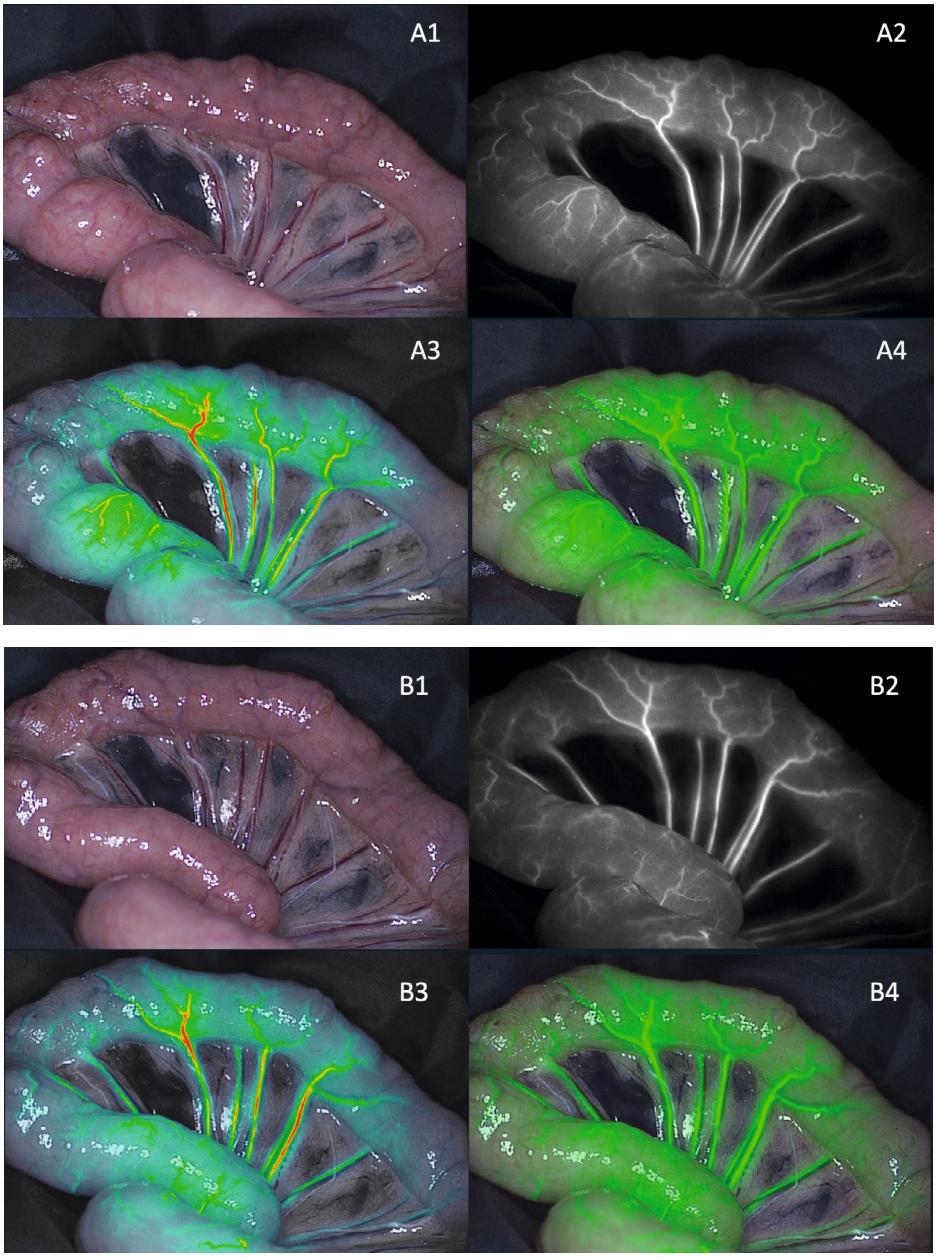

Figure 3. Bowel perfusion assessment in ICG and MB mode directly after dye injection (pig 4). **(A)** (1) Color image of the surgical field, (2) NIRF image, (3) gradient overlay image, and (4) green overlay image in ICG mode straight after ICG injection. **(B)** (1) Color image of the surgical field, (2) NIRF image, (3) gradient overlay image, and (4) green overlay image in MB mode immediately after MB injection (dose of 0.5 mg/kg).

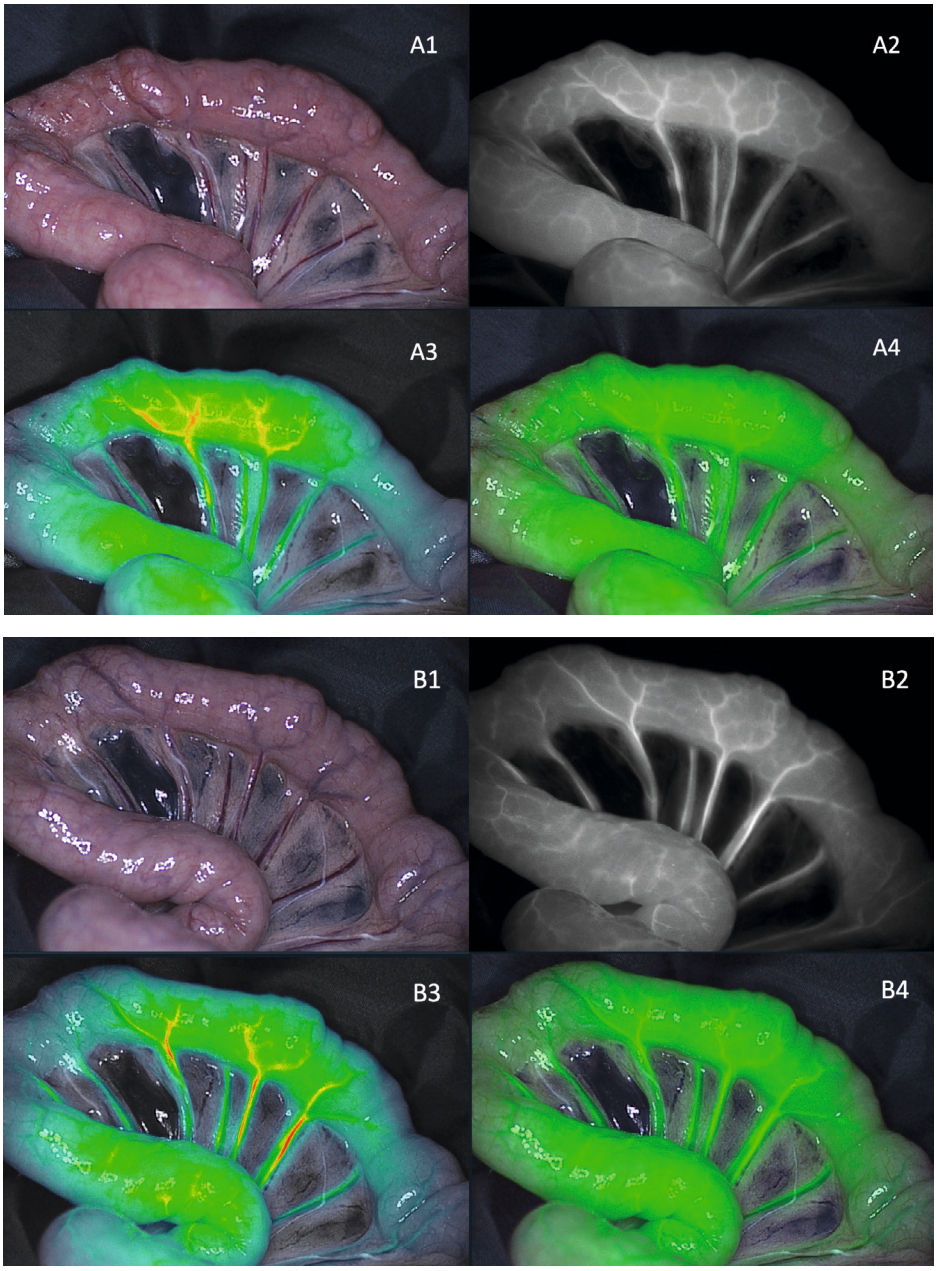

Figure 4. Bowel perfusion assessment in ICG and MB mode 20 seconds after dye injection (pig 4). **(A)** (1) Color image of the surgical field, (2) NIRF image, (3) gradient overlay image, and (4) green overlay image in ICG mode 20 seconds after ICG injection. **(B)** (1) Color image of the surgical field, (2) NIRF image, (3) gradient overlay image, and (4) green overlay image in MB mode 20 seconds after MB injection (dose of 0.5 mg/kg).

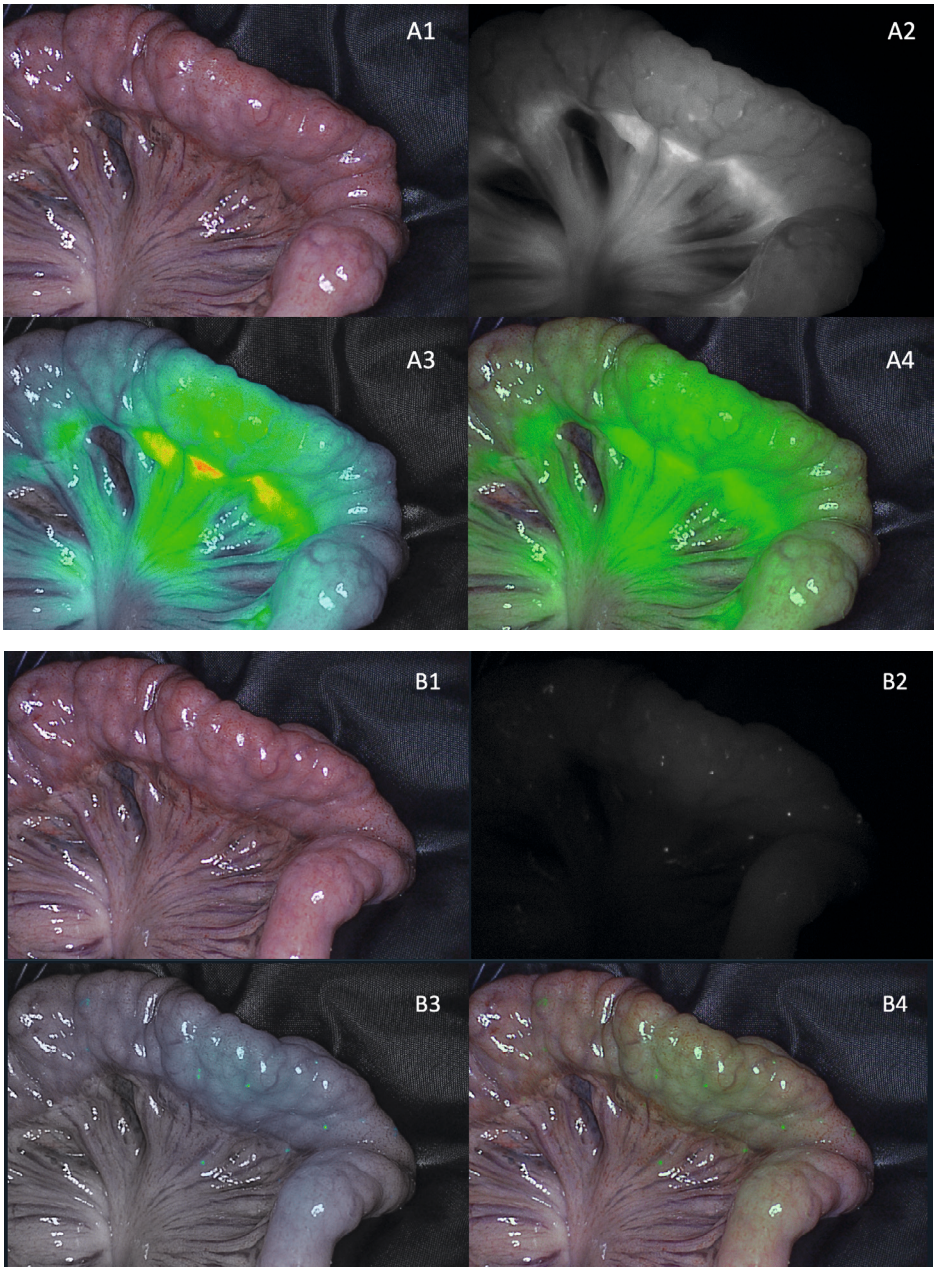

Figure 5. Bowel perfusion assessment in ICG and MB mode 50 minutes after of dye injection (pig 4). **(A)** (1) Color image of the surgical field, (2) NIRF image, (3) gradient overlay image, and (4) green overlay image in ICG mode 50 minutes after ICG injection. **(B)** (1) Color image of the surgical field, (2) NIRF image, (3) gradient overlay image, and (4) green overlay image in MB mode 50 minutes after MB injection (dose of 0.5 mg/kg).

DISCUSSION

This study demonstrated that simultaneous ureteral and bowel perfusion imaging in the same surgical procedure using a single commercially available NIRF imaging system and with the use of FDA-approved fluorescent dyes is feasible. The current findings provide surgeons with a potentially powerful tool to enhance the visibility of the ureter and assess bowel perfusion during colorectal procedures.

With MB, clear identification of the ureters was achieved under NIRF imaging in all animals, as well as assessment of intestinal perfusion. The pigs were allocated to 3 different dose groups to determine the optimal dose of MB. Although no significant differences in TBR values were found among groups, the dose of 0.50 mg/kg MB appeared to be the most optimal with the highest TBR values in this animal study. A previously described optimal dose in the first human study using MB to identify the ureter was 0.25 mg/kg [18]. Additionally, we found the ideal time to administer MB to be 10 minutes prior to requiring ureter delineation and that ureteral imaging remains possible at least until 240 minutes after a single bolus of MB dye administration.

Bowel perfusion assessment was successful in all pigs with ICG. A key finding is that we have demonstrated that bowel perfusion can be visualized with MB as well. Cwalinski et al. created an overview of the role of MB as a fluorophore in a surgical setting [19]; however, bowel perfusion assessment was not mentioned. The latter suggests that MB may represent a versatile substitute for ICG in intestinal perfusion imaging, especially in cases where there is also a need for intraoperative ureteral identification or when ICG is contraindicated. Another clinically relevant finding of this study is that there was no interference between ICG and MB in bowel perfusion assessment. As a result, MB alone may enable us to clearly assess intestinal perfusion in combination with ureteral imaging without the need for ICG.

In several studies [5, 17, 20], our groups have thoroughly explored the potential of novel dyes for the purpose of intraoperative ureteral imaging. Although showing promising results, such novel dyes are still in an experimental phase and it is expected that it will take several years before they will be available for clinical use. A previous pre-clinical study by our group has demonstrated the simultaneous assessment of bowel perfusion and ureteral delineation with a single dye [12]. However, the dye used in that study (IRDye® 800BK) is not yet approved for clinical use. In contrast, MB has been widely used in humans with a good safety profile. It is cheap and clinically available. In addition, MB is approved by the US Food and Drug Administration (FDA) for many indications [21].

We believe that the use of a single dye for bowel perfusion assessment and ureteral imaging has several advantages. Most importantly, it reduces the potential risk of adverse reactions as only one dye is administered and contributes to the efficiency of the procedure. One point

of attention is the fact that MB can only be used in patients with adequate renal function and the intensity of the ureteral signal is influenced by the peristaltic movement of urine through the ureter [18]. There are some known adverse effects after MB administration such as hypertension, dyspnea, hemolysis, methemoglobinemia, nausea and vomiting, and pain in the chest when administering doses above 2 to 7 mg/kg. Refractory hypotension and skin discoloration are known upon administration of 20 to 80 mg/kg [21]. As the previous mentioned doses are much higher than needed for ureter delineation and bowel perfusion assessment as demonstrated in this study (even visible with the lowest dose of 0.25mg/kg), such adverse events are not expected for this indication. MB is currently safely used for visualization of thyroid and parathyroid glands, pancreatic neuroendocrine tumors, and breast cancer tumors and sentinel nodes within therapeutic doses of <2 mg/kg [19].

The current study also demonstrated a faster washout of MB compared to ICG during bowel perfusion assessment, which is known to remain fluorescent for long periods after dye administration. This finding may allow for repeated MB bolus administrations within the same procedure for the purpose of perfusion assessment. We hypothesize that three MB dye characteristics could play a role in the faster washout. First, the molecular weight of both dyes is different, which may result in a difference of diffusion of the dyes into the capillaries; MB has a molecular weight of 319.85 Da as compared to 774.963 Da for ICG [22]. Secondly, MB is more hydrophobic than ICG, which has two hydrophobic and two hydrophilic molecule groups [23-25]. Thirdly, the binding properties of both dyes are probably different: ICG tends to bind to plasma proteins [26], whilst this is not well described for MB. An earlier MB washout time can be beneficial, as a repeated dye injection for bowel perfusion assessment may be necessary during a surgical procedure. When MB is completely washed out, a second dose can be given without the interference of previous signals. We also observed a difference between the washout time of an MB dose of 0.5 and 0.25 mg/kg (50 and 40 minutes respectively). The differences observed in both pigs are probably due to the MB dose administered.

A recent clinical pilot study successfully demonstrated that it is feasible to delineate the ureters with MB and assess the perfusion with ICG using the same camera system [27]. The authors included 12 patients who underwent complex open or laparoscopic colorectal surgeries and demonstrated successful ureteral delineation with MB in 91.6% of cases, and successful bowel perfusion assessment with ICG in all cases. In this pilot study, all measurements were only taken immediately after dye injection, without a follow-up in time. Besides, bowel perfusion was not visualized with MB. We believe, as demonstrated in our study, that the next step would be to focus only on MB fluorescence imaging for ureteral and perfusion imaging. The relatively fast washout of the MB dye may allow for repeated MB administration; one prior to the surgical procedure for ureteral imaging and one during the procedure for perfusion imaging. Based on the results of this study and previous articles in the literature, we have designed a further animal study in which intestinal perfusion quantification for MB compared to ICG will be explored in more detail.

One of the main limitations of this animal study is the small sample size. However, for the feasibility of our hypothesis and to respect the 3R principle (replace, reduce, refine) in animal research as described by Russell and Burch [28], the current number of animals was deemed sufficient. The current results must be interpreted with caution and human studies are necessary to evaluate the reproducibility of our finding in a clinical setting. In addition, while most of the elective abdominal clinical procedures are performed laparoscopically, in this study due to logistical reasons the camera system used was an open camera system (for use after laparotomy). Fortunately, a laparoscopic variant of the camera used in this study is commercially available.

The duration of the study per animal was not exactly the same. The study setup was to have a follow-up time of 120 minutes (most related to a clinical setting) of observation in all animals. This requirement was met in all animals. As an interesting additional finding, we were also interested in the fluorescence signal of the ureter over time. The difference in the timings in the various animals after 120 minutes can be simply explained due to logistical reasons. As we performed more than one operation per day, the timing of the start of the procedure was the most important factor in the maximum time of follow-up after dye injection.

This feasibility animal study has provided the basis for further, larger human studies evaluating dual-imaging camera systems, using only one single dye (MB) for ureteral imaging and bowel perfusion assessment. In line with ongoing animal intestinal perfusion quantification research by our team, we believe a next step should be to further investigate the use of MB in assessing bowel perfusion, as this is not well described [19] and take this research to the human setting in colectomy procedures.

CONCLUSION

Our study shows the feasibility of simultaneous fluorescence angiography with ICG and ureteral imaging with MB in the same surgical procedure, using the same commercially available NIRF imaging equipment. As both dyes can be used in humans, we believe that there is high potential for clinical translational. Additionally, this dual camera system allows for the simultaneous assessment of bowel perfusion and ureteral visualization, using a single dye (MB). Further human studies are necessary to translate our findings to clinical application.

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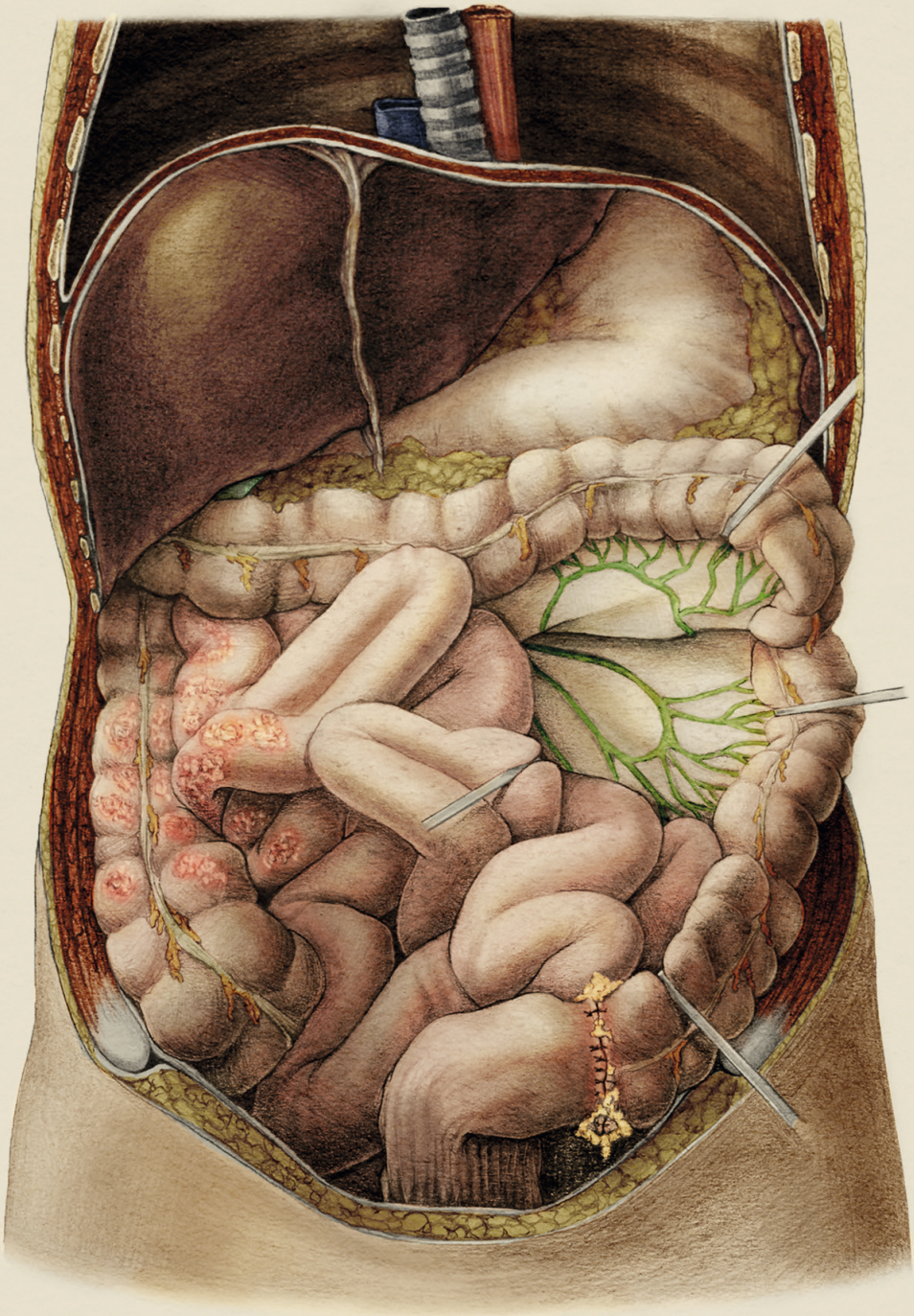
SUPPLEMENTARY

The following supplementary material can be downloaded from:

- *Figure A. Left transected ureter visualization in pig 1 with MB*



CHAPTER 6

QUANTITATIVE ANALYSIS OF INTESTINAL PERFUSION WITH INDOCYANINE GREEN (ICG) AND METHYLENE BLUE (MB) USING A SINGLE CLINICALLY APPROVED FLUORESCENCE IMAGING SYSTEM: A DEMONSTRATION IN A PORCINE MODEL

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ABSTRACT

Background. Near-infrared fluorescence (NIRF) angiography with intraoperative administration of indocyanine green (ICG) has rapidly disseminated in clinical practice. Another clinically approved, and widely available dye, methylene blue (MB), has up to now not been used for this purpose. Recently, we demonstrated promising results for the real-time evaluation of intestinal perfusion using this dye. The primary aim of this study was to perform a quantitative analysis of bowel perfusion assessment for both ICG and MB.

Methods. Four mature female Landrace pigs underwent laparotomy under general anesthesia. An ischemic bowel loop with five regions of interest (ROIs) with varying levels of perfusion was created in each animal. An intravenous (IV) injection of 0.25 mg/kg - 0.50 mg/kg MB was administered after 10 minutes, followed by NIRF imaging in MB mode and measurement of local lactate levels in all corresponding ROIs. This procedure was repeated in ICG mode (IV dose of 0.2 mg/kg) after 60 minutes. The Quest Spectrum Fluorescence Camera (Quest Medical Imaging, Middenmeer, The Netherlands) was used for NIRF imaging of both MB and ICG.

Results. Intraoperative NIRF imaging of bowel perfusion assessment with MB and ICG was successful in all studied animals. Ingress (i/s) levels were calculated and correlated with local lactate levels. Both MB and ICG ingress values showed a significant negative correlation ($r = -0.7709$; $p = <0.001$; $r = -0.5367$, $p = 0.015$ respectively) with local lactate levels. This correlation was stronger for MB compared to ICG, although ICG analysis showed higher absolute ingress values.

Conclusion. Our fluorescence quantification analysis validates the potential to use MB for bowel perfusion assessment besides the well-known and widely used ICG. Further human studies are necessary to translate our findings to clinical applications.

Keywords. Bowel perfusion assessment; methylene blue; indocyanine green; intraoperative near-infrared fluorescence imaging; anastomotic leakage; bile duct imaging.

INTRODUCTION

Anastomotic leakage (AL) is a highly concerning complication, which can occur after colorectal surgery with anastomosis formation. Impaired blood flow to the bowel is considered the primary factor contributing to AL. Consequently, bowel perfusion assessment is a critical approach to reduce the occurrence of AL [1, 2]. Intraoperative near-infrared fluorescence imaging (NIRF), using administration of an optical dye, offers a convenient and versatile method to improve the assessment of anastomotic perfusion. Indocyanine green (ICG) is the dye that is most commonly used for this purpose as it provides favorable results [3-7]. Recently, our research team demonstrated in an experimental study that methylene blue (MB), another widely available dye, showed promising results too [8]. As it is partly cleared by the kidneys, it was previously demonstrated that it could successfully visualize the ureters intraoperatively. Less expected was the demonstration that it could also be used for intraoperative perfusion imaging, reflecting a potential benefit in comparison to ICG that is exclusively cleared by the liver and subsequently cannot visualize the ureters non-invasively.

Our promising results with MB were gathered using a commercially available NIRF imaging system, which can visualize both dyes due to its bimodal properties (QUEST SPECTRUM®, Quest Medical Imaging, Middenmeer, The Netherlands). Since this NIRF imaging system uses two different wavelength modes, it solves one of the drawbacks related to the use of MB, namely its excitation characteristics. MB has an excitation peak of about 700 nm, an excitation wavelength of 668 nm, and an emission of 688 nm which can be seen with the naked eye [9]. These characteristics are different from ICG that is excited at around 800nm, until recently requiring a different imaging system. So far, the majority of imaging systems used in MB studies were experimental and not commercially accessible for clinical purposes conversely to the system used in the present study. As the Federal Drug Administration (FDA) and the European Medicines Agency (EMA), have approved the clinical use of MB as well [9], this dye may be promising to use for multipurpose NIRF imaging. Our research team therefore considered it clinically relevant to investigate the use of MB for bowel perfusion assessment.

The objective of the current study was to conduct a quantitative analysis of bowel perfusion assessment using a commercially available NIRF imaging system, comparing the visualization obtained with MB and ICG.

MATERIALS AND METHODS

This study was performed at the central animal facilities of Maastricht University (Maastricht, The Netherlands) and was approved by the local Experimental Animal Committee (DEC) (2017-021-001). All animals were used in compliance with Dutch regulations and legislation concerning animal research.

Animals and anesthesia

Four mature female Landrace pigs (35-45kg) underwent general anesthesia ensured with appropriate analgesia using the following medication: intramuscular injection of Zolazepam/ Tiletamine (6 mg/kg, Virbac, Barneveld, The Netherlands) and Thiopental (10 mg/kg, Panpharma SA, Trittau, Germany), a combination of sufentanyl (0.01 mg/kg/h, Hameln Pharma GmbH, Hameln, Germany), Propofol (9 mg/kg/h, B. Braun Melsungen AG, Melsungen, Germany), and Midazolam (1 mg/kg/h, Aurobindo, Baarn, The Netherlands) intravenously. All pigs were intubated and mechanically ventilated. Alterations in vital parameters were monitored by an animal anesthesiologist, and whenever necessary, anesthesia and analgesia were intensified. At the end of the procedure, all animals were euthanized with a lethal dose of 200 mg/kg pentobarbital.

Preparation of dyes

The preparation of dyes was carried out as previously described [8]. In short, MB was diluted in a sterile phosphate-buffered saline (PBS) solution to a concentration of 1 mg/mL and ICG in a sterile H_2O solution to a concentration of 2.5 mg/mL. An IV dose of 0.25 or 0.50 mg/kg of MB (doses based on a previously published dose finding study [8]) and 0.2 mg/kg of ICG was administered. This ICG dose is the current frequently clinically used dose range in patients, based on the analysis of 1,240 patients registered in the EURO-FIGS registry on fluorescence angiography [10].

Surgical procedure and measurements

After appropriate sedation and analgesia, a midline laparotomy was performed by an experienced surgeon. A small bowel loop with a length of approximately 15 cm, was measured at 250 cm from the gastric pylorus. To ensure optimal exposure, the loop was placed on a gauze. The mesenteric side with at least 8 vessels was transected to create a gradual ischemic loop, as described in an earlier study [11]. After compromising the intestinal tissue perfusion (T = 0), 5 ROIs (Figure 1) were marked and defined as follows: 2 on the lateral sides of the loop (well-perfused), 1 in the exact middle of the loop (not perfused), and 2 between the lateral and middle ROIs (partly perfused = watershed area). This method has been previously explained by our group [12]. Subsequently, a systemic lactate measurement was taken from the central ear vein. MB was injected after 10 minutes (T = 10) and bowel perfusion imaging in MB mode was performed for at least 60 seconds. Sixty minutes after ischemic loop creation, the camera system was switched to ICG mode, followed by ICG injection (T = 60) and fluorescence quantification analysis for 60 seconds. This procedure was directly followed by local capillary lactate sampling by puncturing the serosa at each of the 5 ROIs. The latter was done using a 23 Gauge needle and an EDGE lactate analyzer (ApexBio, Taipei, Taiwan, People's Republic of China), which only requires a small drop of blood (3 µl) [12]. As lactate is a marker of ischemia [13], it was used as the gold standard to correlate the fluorescence signal. All animals were followed for a minimum of 120 minutes (T = 120). A schematic overview of the surgical procedure is displayed in Figure 1.

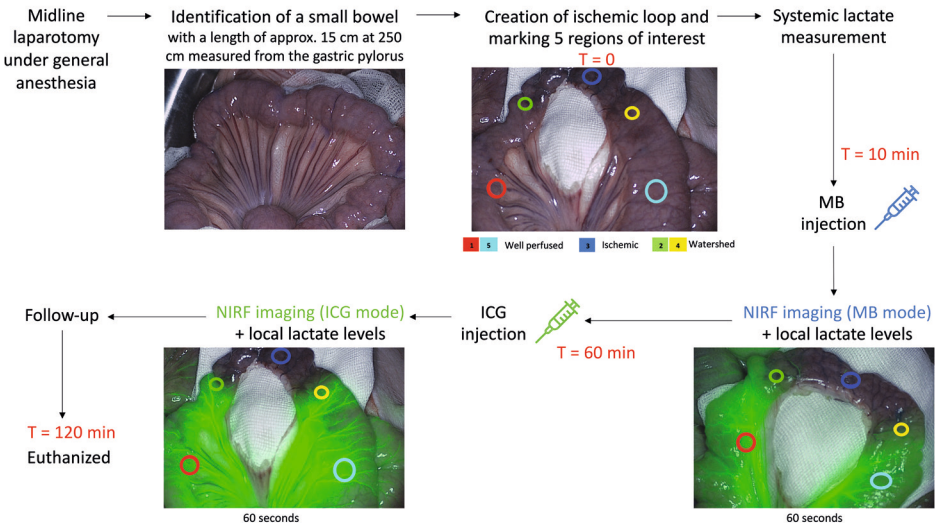

Figure 1. Schematic overview of the surgical procedure and measurements. T = 0 represents the timepoint at which the ischemic loop was created. After 10 minutes (T = 10) MB was injected. One hour after the creation of the ischemic bowel loop (T = 60), ICG was injected. NIRF imaging continued until at least 120 minutes.

Fluorescence imaging system

The commercially available Quest Spectrum Fluorescence Camera (QUEST SPECTRUM®, Quest Medical Imaging, Middenmeer, The Netherlands) was used for NIRF imaging. The camera was fixed with a custom-made mechanical arm, which was connected to the surgical table to ensure a stable vision and distance to the ROI. The camera tip was fixed at 15cm from the target organ in all operations. To avoid ambient light interference, all lights of the operating room were dimmed. As introduced, this camera system has bimodal properties and both channels were used in the current study; an 800 nm channel to capture ICG fluorescence (ICG mode) and a 700 nm channel for MB fluorescence imaging (MB mode). During the surgical procedure, four images were displayed on the screen (Supplementary S1). First, a standard color image of the surgical field was displayed alongside a grayscale NIRF image to aid surgical guidance. Moreover, two overlay modes were utilized, comprising a fluorescence intensity map and a NIRF image projected onto the colored image, all presented on the same screen.

Statistical analysis and quantification

All NIRF images and videos were post-analyzed with Quest Spectrum software (ResearchTool v4.7, Quest Medical Imaging, Middenmeer, The Netherlands). As all ROIs were intraoperatively marked with a surgical marker, this was visible on all recordings and the exact same ROIs could be used during the analysis. A tracker synchronized the ROI with movement, and afterwards the software created a time-intensity curve of the measured intensity of the specific ROIs. The measured fluorescence intensity is displayed in arbitrary units (a.u.). Baseline subtraction was

applied to all time-intensity curves. The ingress was the parameter used for the quantitative analysis of bowel perfusion assessment. The ingress quantifies the inflow in terms of increase in fluorescence intensity per second in the ROI (increase in a.u. per second: i/s). The ingress was calculated over a timeframe of 20 seconds after the end of the baseline.

Numerical variables were presented as medians with interquartile range (IQR). A Spearman's rho was calculated to correlate local lactates with the fluorescence parameter. A $p < 0.05$ was considered significant. All statistical analyses were performed with the GraphPad Prism (GraphPad software for Apple, version 8.0.0, San Diego, California, United States).

RESULTS

A total of 4 pigs were included in this experiment (Table 1). Systemic lactate levels confirmed that there was no systemic ischemia during the experiment. No intraoperative dye-related complications occurred.

Table 1. Animal characteristics

	Pig 1	**Pig 2**	**Pig 3**	**Pig 4**
Weight (kg)	42.00	37.50	36.00	36.00
MB dose (mg/kg)	0.50	0.50	0.25	0.25
ICG dose (mg/kg)	0.20	0.20	0.20	0.20
Systemic lactate (mg/dL) ***T10 / T60***	19 / 23	15 / 23	18 / 21	12 / 14
Lactate ROI 1 ***T10 / T60***	21 /19	15 / 8	19 / 17	12 / 12
Lactate ROI 2 ***T10 / T60***	28 / 57	40 / 41	16 / 16	59 / 53
Lactate ROI 3 ***T10 / T60***	102 / 73	36 / 68	50 / 84	43 / 69
Lactate ROI 4 ***T10 / T60***	34 / 91	42 / 46	15 / 8	43 / 51
Lactate ROI 5 ***T10 / T60***	24 / 22	17 / 26	15 / 17	12 / 26

T = time in minutes.

Bowel perfusion quantification

Time-intensity curves

In all included pigs, a clear macroscopic NIRF visualization of perfusion was achieved. For the well-perfused ROIs (1 and 5), the majority of curves displayed a steep ingress. In the time-intensity curves in the second ROI, marked as a watershed region, an inflow pattern comparable to ROI 4, also marked as a watershed region, was most often seen. For the ROIs

with low perfusion (ROI 3), a clearly non-steep ingress and lower maximum fluorescence intensity is demonstrated compared to watershed and normal perfusion ROIs. An example of both MB and ICG time-intensity curves is displayed in Figure 2.

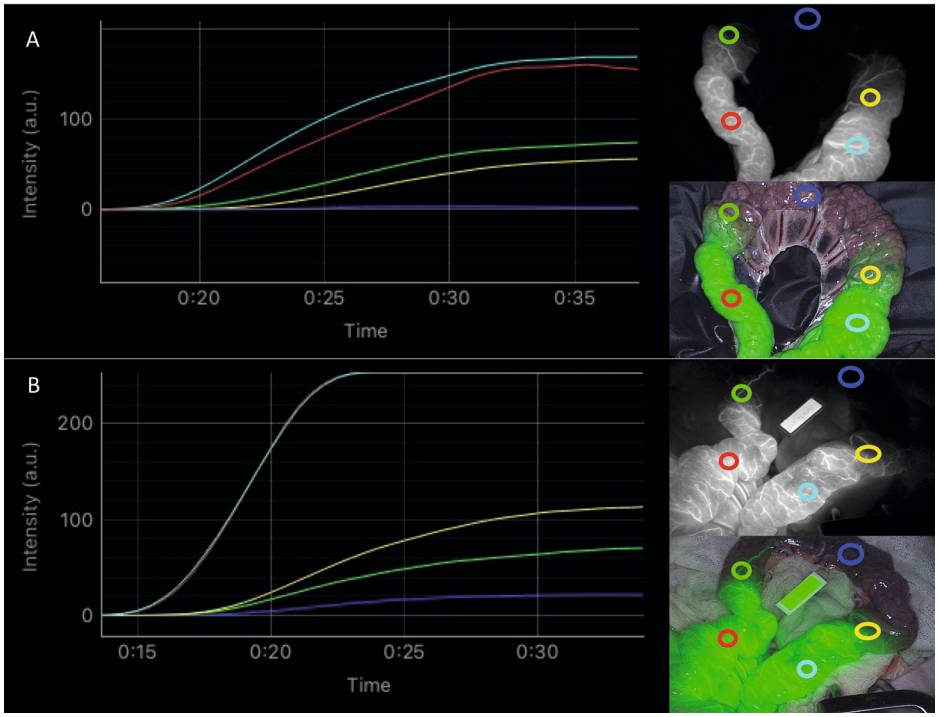

Figure 2. Time-intensity curve examples (Pig 3). ROI 1 = red (well-perfused), ROI2 = green (watershed), RO3 = blue (ischemic), ROI4 = yellow (watershed), ROI 5 = cyan (well-perfused), with corresponding NIRF images at 12 seconds. **(A)** MB mode (T = 10 min). **(B)** ICG mode (T = 60 min), red and cyanin line partly overlap.

Fluorescence quantification analysis: ingress correlation to lactate levels

First, all images of the ischemic loop during MB administration were analyzed (at T = 10 min). Ingress (i/s) values were calculated in all ROIs (Figure 3A). ROIs 1 and 5 had a faster development of brightness as compared to ROIs 2, 3, and 4. It is also objectively proven with the fluorescence quantification analysis of the ingress in which ROI 3 had the lowest ingress. Compared to local lactate levels, the opposite patterns were seen; ROI 3 had lower levels of lactate compared to watershed and well-perfused areas (Figure 3B). A Spearman's correlation test showed a significant negative correlation between the ingress levels in the ischemic bowel loop and the corresponding local lactate levels (r = -0.7709, 95% CI: -0.9073 to -0.4878; *p* = <0.001) for MB fluorescence quantification analysis (Figure 3C).

Secondly, the fluorescence quantification analysis of all images of the ischemic loop during ICG administration was performed (T = 60 min). All ROIs showed a similar development of increase in fluorescence intensity as during MB analysis (Figure 3D). Compared to MB, absolute values were higher. Local lactate levels of ROI 3 also had lower levels compared to all other areas (Figure 3E) and most of the values were higher than at T = 10 min. A second Spearman's correlation test showed a significant negative correlation between ingress levels in the ischemic bowel loop and corresponding local lactate levels (r = -0.5367, 95% CI: -0.7965 to -0.1096; p = 0.015) for ICG fluorescence quantification analysis (Figure 3F).

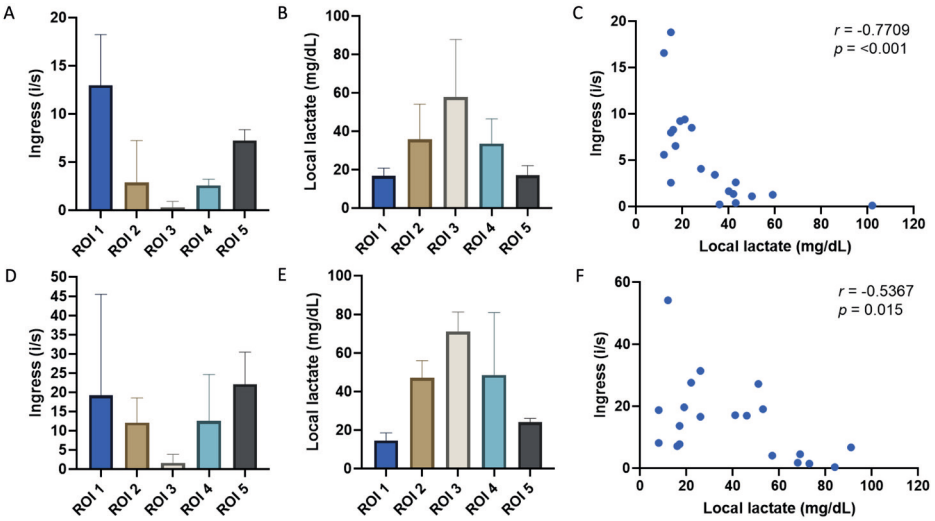

Figure 3. Results of bowel perfusion analysis. A-B-D-E values present medians, whiskers indicate the 75th percentile. **(A)** Ingress values of the 5 ROIs during MB administration (T = 10 min). **(B)** Local lactate levels of the 5 ROIs during MB administration (T = 10 min). **(C)** Scatterplot of ingress values and local lactate during MB administration showing a significant negative correlation (Spearman's rho = -0.7709, 95% CI: -0.9073 to -0.4878; p = <0.001). **(D)** Ingress values for the 5 ROIs during ICG administration (T = 60 min). **(E)** Local lactate levels of the 5 ROIs during ICG administration (T = 60 min). **(F)** Scatterplot of ingress values and local lactate during ICG administration showing a significant negative correlation (Spearman's rho = -0.5367, 95% CI: -0.7965 to -0.1096; p = 0.015). All detailed information is provided in Supplementary S2.

In one animal, NIRF imaging was performed of a non-ischemic bowel loop after injecting MB and ICG to compare absolute ingress values with the same method of administration, at the same timing. The ingress values were 7.02 i/s and 11.89 i/s for MB and ICG, respectively. No adverse reactions were observed in any of the animals after MB and ICG administration.

DISCUSSION

In this preclinical animal study, we have successfully performed a quantitative analysis of NIRF imaging for bowel perfusion using MB with a commercially available fluorescence imaging system. This analysis showed a significant negative correlation between local lactate levels (as a marker for ischemia) and MB ingress values. This correlation was stronger than the correlation for ICG quantification values, although the absolute ingress values of ICG were higher compared to MB. This camera system solved two significant drawbacks of MB as discussed in the previous literature [14]: (1) its absorption and emission in the vicinity of 700 nm, which is susceptible to increased background auto-fluorescence, and (2) the need for distinct equipment settings.

To imitate the clinical scenario involving bowel ischemia and/or inadequate anastomotic perfusion, we generated ischemic bowel loops in our experiment. As our goal was to validate fluorescence signals, we used lactate levels as an indicator of the perfusion state of the different ROIs [11, 13, 15]. It should be noted that some lactate levels decreased after one hour. A potential explanation could be linked to the existence of small overlapping vessels on the serosa, emanating from a neighboring intestinal segment with better perfusion. This setup might contribute to a slight reperfusion effect, a phenomenon recognized previously by Diana et al. [13]. For both MB and ICG imaging results, we found a significant negative Spearman's correlation for the local lactate and corresponding ingress values in the same ROI. The results indicate that both dyes are suitable to assess bowel perfusion. Interestingly, the correlation of MB was stronger compared to the one of ICG (Spearman's rho of -7709 and -0.5367, respectively). The latter may suggest that the use of MB for bowel perfusion assessment with this camera system may be more accurate. In contrast, we observed higher absolute ingress values for ICG imaging compared to MB. The finding that MB has a stronger correlation as opposed to ICG which shows higher absolute values may be attributed to the fact that ingress values were calculated at different time points (T = 10min for MB and T = 60min for ICG). To compare absolute ingress values, we performed NIRF imaging in one animal immediately after injecting MB and ICG into a non-ischemic bowel loop. The ingress values were 7.02 i/s for MB and 11.89 i/s for ICG, still indicating a slight difference. However, our research team considers the differences in the absolute values and correlation negligible as the real-time images obtained during surgery were very clear and informative for both dyes. We therefore do not state that one dye is better than the other, but we can conclude that MB may be as good as ICG for bowel perfusion assessment based on our quantitative analysis.

Adverse effects are important to consider when performing NIRF imaging with an optical dye. MB is a safe drug at a therapeutic dose below 2 mg/kg [9, 16], which is eight to four times higher than used in the current study (0.25 mg/kg and 0.5mg/kg). There are some known adverse effects when administering doses above 2 mg/kg, such as hypertension, dyspnea, hemolysis, methemoglobinemia, nausea and vomiting, and pain in the chest [17], and it may

precipitate serotonin toxicity if combined with other serotonergic drugs at doses above 5 mg/kg [18]. When levels are >7 mg/kg, many of the adverse effects occur [16, 17, 19]. Refractory hypotension and skin discoloration are only known upon administration of 20 to 80 mg/kg [17], and anaphylactic reaction is extremely rare [9]. As the previous mentioned doses are much higher than needed for bowel perfusion assessment as demonstrated in this study, such adverse events are not expected for this indication. It is important to know that MB is contra-indicated together with serotonergic drugs, in glucose-6-phosphate dehydrogenase deficient patients, in patients with renal failure, and in pregnant women [9, 16]. Compared to ICG, MB has some more adverse reactions when administered in higher doses, but is currently completely safely used for visualization of thyroid and parathyroid glands, pancreatic neuroendocrine tumors, and breast cancer tumors and sentinel nodes within doses of <2 mg/kg [9].

Although the use of ICG fluorescence is recommended in colorectal surgery to assess tissue perfusion [4], there is still no consensus on how to quantify fluorescence angiography. Previous studies were conducted to establish and gather validity evidence for a method of quantifying fluorescence angiography [20-24]. This revealed that bowel perfusion quantification is a feasible method to differentiate between different perfusion patterns, highlighting the possibility of using standardized imaging protocols [21]. According to a recent consensus paper on ICG fluorescence angiography, we concur with the authors' standpoint that additional investigation into quantitatively evaluating fluorescence is imperative. This will help to reduce the subjective variability associated with perfusion assessment [4], and make it easier to compare study outcomes with different dyes, and will improve the validity and reproducibility of such data in daily practice.

The unique aspect of the present study is that, to our knowledge, no previous study has demonstrated the use of MB for bowel perfusion imaging in addition to the well-known and widely used ICG, within a single operative procedure and with a single commercially available NIRF imaging system. Based on the findings presented in this study and our previous investigations [8, 25], we can conclude that MB, when used in a dedicated imaging system, offers a range of simultaneous and multipurpose functionalities, all achieved solely through the administration of a single dose of MB. Based on several studies and recent consensus papers, the incorporation of ICG fluorescence for perfusion assessment during colorectal surgery has been shown to substantially decrease the risk of AL [2, 4, 14, 26]. It can even result in modifications to the resection line and/or adjustment of the anastomosis, and leads to shorter hospital stay and reduced overall morbidity [4]. ICG fluorescence highlights the added value of performing NIRF for bowel perfusion assessment. Considering that fluorescence imaging is not currently used in all medical facilities routinely, we anticipate that the findings from this study, along with our previous research, will encourage clinicians to explore the use of MB fluorescence. The advantage of using a single dye for multiple purposes makes it an appealing option for clinical practice.

Limitations

There are some limitations in this animal study. The small sample size is a notable limitation. However, to ensure the feasibility of our hypothesis and adhere to the 3R principle (i.e., replace, reduce, refine) in animal research [27], the number of animals used in the study was considered adequate. Despite this small sample size, we believe that the correlations we present are sufficiently illustrative. Each pig underwent 5 measurements, resulting in a total of 20 measurements for each correlation analysis, which is sufficient for a Spearman correlation. Another limitation is that, although laparoscopic procedures are the norm for most elective abdominal clinical procedures, we used an open camera system in this study due to logistical constraints. Fortunately, there is a commercially available laparoscopic variant of the camera system used in our study. Additionally, while anastomotic perfusion is commonly required during colorectal resection and anastomosis creation, we used small bowel loops in our experiment. This decision was based on the challenges posed by the fixed and spiral orientation of a pig's colon, making the small bowel a more suitable choice for illustrative purposes. It is essential to acknowledge that it differs from human colonic tissue, underscoring the need for human studies to provide crucial insights. Consequently, it is critical to interpret the current results cautiously, and human studies are necessary to assess the reproducibility of our findings in a clinical context.

CONCLUSION

To conclude, we successfully performed a quantification analysis of a commercially available NIRF imaging system in this study. We demonstrated a significant negative correlation of ingress values of MB and ICG fluorescence quantification analysis with local lactate levels. This validates the potential to use MB for bowel perfusion assessment besides the well-known and widely used ICG. Further human studies are necessary to translate our findings to clinical applications.

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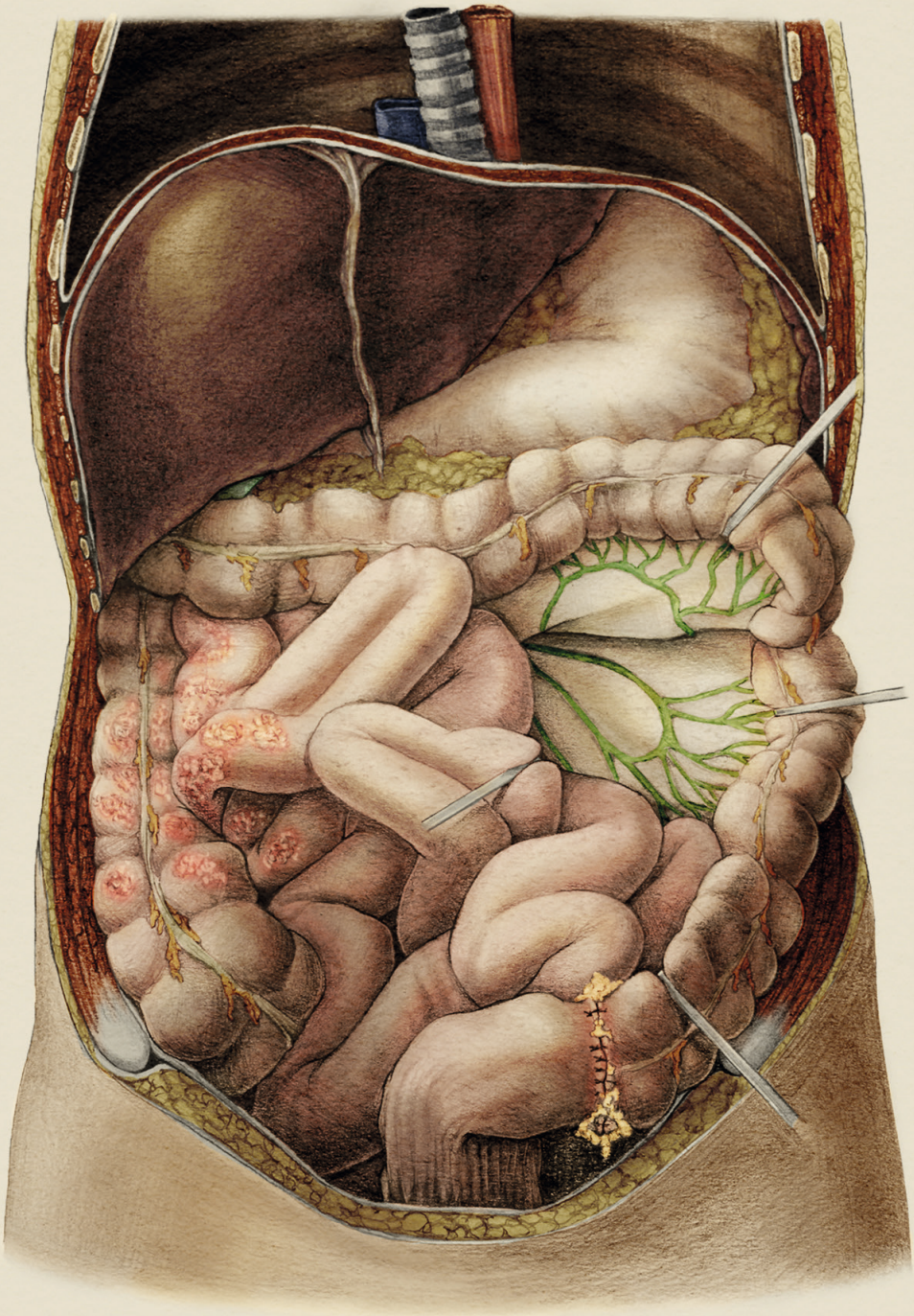
SUPPLEMENTARY

The following supplementary material can be downloaded from:

- *Figure S1. Example of four displayed images/modes during surgery*
- *Table S2. Overview raw datapoints as presented*



CHAPTER 7

REAL-TIME INTESTINAL PERFUSION ASSESSMENT FOR ANASTOMOTIC SITE SELECTION USING LASER SPECKLE CONTRAST IMAGING: VERIFICATION IN A PORCINE MODEL

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ABSTRACT

Introduction. Adequate blood perfusion is widely recognized as a crucial factor for successful healing of an anastomosis and avoid anastomotic leakage. This study aimed to determine if laparoscopic laser speckle contrast imaging, can provide valuable feedback for identifying the state of tissue perfusion. Therefore, we explored the efficacy and feasibility of a new laser speckle contrast imaging system to assess real-time intestinal perfusion.

Methods. Three gradually perfused porcine small bowel loops were created, and five senior surgeons were asked to assess the perfusion differences based on laser speckle contrast images using PerfusiX-Imaging®. Subsequently, the study evaluated the impact of laser speckle contrast imaging on decision-making for anastomosis creation. Afterwards, a questionnaire was completed by all surgeons to assess the usability of the device.

Results. Results demonstrated a high accuracy (100%) in identifying compromised perfusion and detecting perfusion differences between loops using the imaging system. In case of compromised perfusion, all surgeons recommended against creating an anastomosis based on the visual feedback. The questionnaire revealed that the senior surgeons were satisfied with the perfusion imager, particularly in terms of minimal latency, ease of use and set up, and ability to accurately represent blood flow patterns as these questions showed a (very) strong agreement in 80%.

Conclusion. Laser speckle contrast imaging can provide valuable real-time feedback on intestinal tissue perfusion during surgery, enabling surgeons to select optimal tissue segments for a well-perfused anastomosis. However, further research is required to validate the efficacy in clinical settings and its potential impact on surgical outcomes in patients.

Keywords. Anastomotic leakage; image-guided surgery; laparoscopic surgery; laser speckle contrast imaging; perfusion assessment.

INTRODUCTION

Anastomotic leakage (AL) is a major complication following gastrointestinal surgery and remains the foremost concern for gastrointestinal surgeons. The occurrence rate of AL varies between 1-19% depending on the anatomic location of the anastomosis [1, 2, 3, 4]. The AL etiology is influenced by various factors, including patient characteristics, peroperative factors, and tissue perfusion [2, 5, 6, 7]. Adequate blood perfusion is widely recognized as a crucial factor for successful healing of an anastomosis [1, 8, 9, 10]. Insufficient perfusion can impair the natural healing of the body, compromising the repair process and increasing the risk of AL. In recent years, there has been growing interest in utilizing real-time perfusion assessment techniques to guide surgical decision-making to improve outcomes [11]. By identifying tissue areas with compromised perfusion, surgeons can potentially avoid creating an anastomosis in those regions and opt for better-perfused tissue, thereby minimizing the risk of AL [12].

Real-time identification of intestinal perfusion to guide surgeons towards creating a bowel anastomosis using tissue with optimal perfusion, can be achieved using laser speckle contrast imaging (LSCI) [13, 14, 15] with instantaneous and continuous 2D-perfusion maps [16, 17, 18]. This imaging technique allows visualization of tissue perfusion in real-time, without the need for contrast agents [19]. By integrating the LSCI system into current laparoscopic video systems and surgical workflow, surgeons can have immediate access to visual information on tissue perfusion during the procedure. This additional feedback may serve as a valuable tool to identify regions with compromised perfusion, prompting surgeons to select alternative tissue segments for anastomoses that exhibit better perfusion [20]. If real-time identification of intestinal perfusion proves feasible and effective, it could serve as a valuable adjunct in surgical practice, providing surgeons with additional information used in better substantiated clinical decision making and optimize patient outcomes.

We hypothesized that the use of LSCI will enable surgeons to make better informed decisions regarding anastomotic site selection prompting surgeons to select alternative tissue segments for anastomosis that exhibit better perfusion, and therefore potentially reduce AL rates in future patients. Therefore, the current study aimed to assess real-time identification of intestinal perfusion using laparoscopic LSCI, subsequent decision making based on this assessment, and the efficacy and feasibility of the used LSCI device.

MATERIALS AND METHODS

This study was performed at the animal facility of Maastricht University Medical Center, Maastricht, The Netherlands. The animal was treated in compliance with the regulations of the Dutch legislation concerning animal research and ARRIVE guidelines, and a protocol approved by the Local Experimental Animal Committee (DEC) (number 2017-021-001).

Animal

A female Dutch Landrace pig weighting approximately 35 kg was used in this study. The pig underwent an acclimatization period of one week in the animal keeping facility prior to the experiment. During this period, the pig had free access to water but was fasted for 24 hours. For anesthesia induction, a combination of medications was administered intravenously. This included sufentanyl at a dosage of 0.01 mg/kg/h (Hameln Pharma GmbH, Hameln, Germany), Propofol at a dosage of 9 mg/kg/h (B. Braun Melsungen AG, Melsungen, Germany), and Midazolam at a dosage of 1 mg/kg/h (Aurobindo, Baarn, The Netherlands). These medications were used to induce a state of anesthesia in the pig. The pig was mechanically ventilated to ensure adequate respiration throughout the procedure. The ventilation was adjusted as needed to maintain appropriate oxygenation and ventilation. During the procedure, anesthesia was maintained using a continuous infusion of sufentanyl and propofol and additional doses were given whenever necessary. At the end of the experimental procedure, the pig was euthanized using a lethal dose of 200 mg/kg Pentobarbital (AST Farma, Oudewater, The Netherlands).

Laser Speckle Contrast Imaging

The PerfusiX-Imaging® device, developed by LIMIS Development BV (Leeuwarden, The Netherlands), was used for acquiring LSCI images. The system is designed to work in conjunction with standard laparoscopic equipment (Figure 1). For this study an Olympus laparoscopic video system (OTV-S190, Olympus, Hamburg, Germany) and a 30-degree laparoscope (EndoEye, Olympus, Hamburg, Germany) were used. LSCI is a non-invasive imaging technique that offers high spatial and temporal resolution for subsurface perfusion measurements 21. It can capture large surface areas without the need of a contrast agent. The technique leverages the coherent properties of laser light to provide real-time perfusion information. LSCI is a real-time 2D-perfusion imaging technique that relies on low power laser light to illuminate the tissue of interest. The laser light produces a random interference pattern, known as the speckle pattern, on the camera sensor. This pattern undergoes changes when underlying red blood cells move, corresponding to the rate of blood flow. Consequently, the blurring of the image or loss in contrast within the speckle pattern represents blood flow. Notably, the laparoscope was used without modification allowing the device to integrate into surgical practice. The device houses a red laser and allows for a fast, instant switching between conventional white light and laser light. As a result, 2D perfusion maps were generated in real-time and made directly available in the operating room. These maps provided visual representations of tissue perfusion, enabling immediate perfusion assessment and analysis during the surgical procedure.

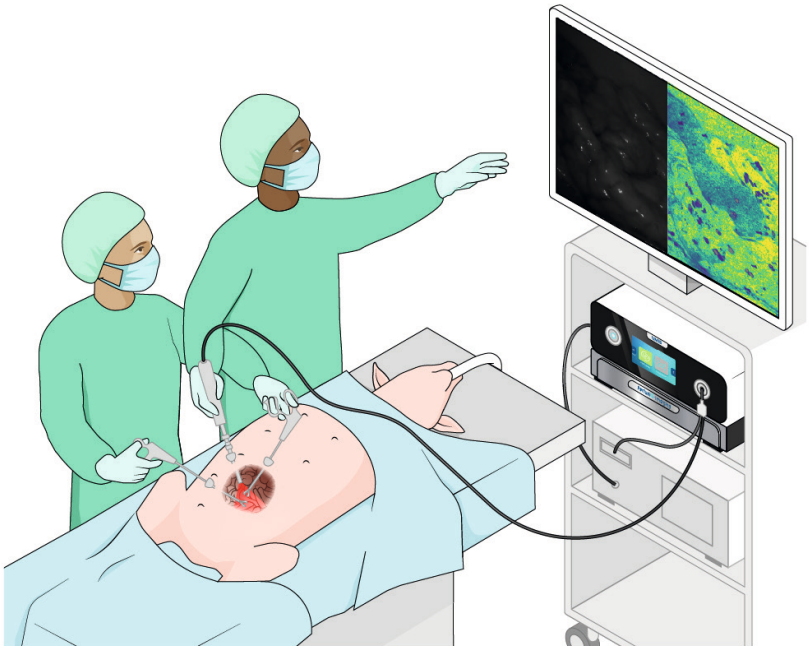

Figure 1. Graphic representation of the experimental setup.
Illustration made by Sieben Medical Art, © 2023 Sieben Medical Art.

Surgical procedure and identification of three differently perfused intestinal loops

After proper sedation and analgesia, laparoscopic instruments were introduced by an experienced colorectal surgeon (M.A-T.). During the experiment, a small bowel ischemic loop model was used that was previously described by Diana et al.[22]. In short, small bowel loops with a length of approximately 15 centimeters were selected and arteries at the mesenteric side of the small bowel loop were transected to impair perfusion. To evaluate the ability of surgeons to identify and differentiate ischemic intestinal loops with varying levels of perfusion using both LSCI derived visual feedback and conventional white light images, three differently perfused ischemic intestinal loops were created, each measuring approximately 15 centimeters in length. The first loop underwent tissue perfusion compromise through the dissection of 15 arteries 90 minutes prior to surgeon evaluation. The second loop underwent a lesser perfusion alteration with fewer dissected arteries, with the dissection of eight arteries occurring just five minutes before questioning. The third loop had unaltered perfusion. To assess the state of perfusion, three sections of the small bowel loops were selected: the middle section of the first loop (with compromised perfusion), the end section of the second loop (with more recent perfusion alteration and less compromised perfusion), and a section from a healthy loop (with normal perfusion). The state of perfusion was confirmed by three specialists who examined white light images of the tissue. Discoloration of the tissue, previously shown to be indicative of ischemic intestinal tissue using LSCI, was considered the

gold standard [18]. To mitigate potential bias from the white light images, the LSCI perfusion mode was activated when the surgeons entered the operating theatre and the (dissected) mesentery of the bowel loops was covered by a gauze for blinding. All five senior surgeons participating in the study entered the operating room individually and were asked to answer three specific questions based on the LSCI derived visual feedback. The questions posed to the surgeons were as follows: (1) Could you identify an ischemic intestinal loop? (2) Could you detect a perfusion difference in the other two loops? (3) Could you identify the best perfused loop? After responding to the questions, the surgeons were shown the corresponding white light images for further evaluation and comparison.

Identification of anastomotic perfusion

To evaluate the ability of senior surgeons to make decisions regarding anastomosis creation based on additional visual feedback, a hand-sewn anastomosis was created using a healthy and an ischemic small bowel loop. The ischemic loop was created 30 minutes prior to questioning by dissecting eight peripheral arteries and veins. The state of perfusion was confirmed by three specialists based on white light images. All five senior surgeons participating in the study entered the operating room individually and were asked three specific questions based on the perfusion images. The questions posed to the surgeons were as follows: (1) Would you advise creating an anastomosis based on this additional visual feedback? (2) Can you identify a perfusion difference? and (3) What is the worst perfused tissue?

Usability of PerfusiX-Imaging for intestinal perfusion assessment

A questionnaire was designed to assess the usability of the device. The questionnaire consisted of six items, each addressing a specific aspect of usability. The items were answered using the Likert scale from one to five, with the one representing the least favorable response and five indicating the most favorable response. The questionnaire can be found in Supplemental material S1.

RESULTS

The surgical procedure was performed without any complications nor adverse events.

Identification of three differently perfused intestinal loops

The results indicated that surgeons demonstrated a good ability to identify ischemic intestinal loops using LSCI derived visual feedback (Figure 2). Specifically, all five senior surgeons correctly identified the ischemic loop, achieving a 100% accuracy rate when relying solely on this feedback. After the identification of the ischemic bowel loop using only LSCI, the white light images were shown. All surgeons still agreed with the identified ischemic region and no one doubted his/hers decision based on this additional information. Regarding the ability to detect perfusion differences in the other two loops, again LSCI derived visual feedback

enabled all five surgeons to accurately identify the differences, resulting in a 100% accuracy rate. When asked to identify the best perfused loop, four out of five (80%) surgeons provided correct answers based solely on the laser speckle images.

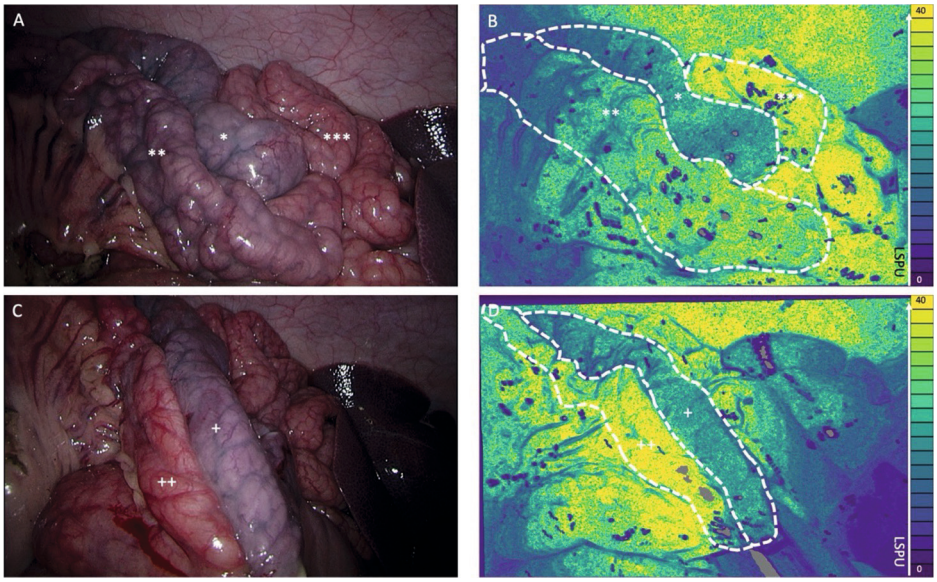

Figure 2. (A) The white light image of the three differently perfused loops. * Indicates the ischemic loop, ** indicates the compromised loop and *** indicates the healthy loop. **(B)** The PerfusiX-Imaging® perfusion image with the three differently perfused loops. Blue indicates low perfusion and yellow indicates high perfusion. * Indicates the ischemic loop, ** indicates the compromised loop and *** indicates the healthy loop. **(C)** The white light image of the anastomosis with a bad perfused segment indicated by + and an uncompromised segment indicated with ++. **(D)** The PerfusiX-Imaging perfusion image of the anastomosis. Blue indicates low perfusion and yellow indicates high perfusion. The compromised segment indicated by + and an uncompromised segment indicated with ++.

Identification of anastomotic perfusion

The study's findings demonstrated that the LSCI perfusion images had an impact on the surgeons' decision-making concerning anastomosis creation. All five senior surgeons, when presented with LSCI feedback (Figure 2B and D), recommended against creating an anastomosis, resulting in a recommendation rate of 100%. In terms of identifying perfusion differences, the LSCI feedback alone proved to be highly effective, as all surgeons correctly identified the differences, leading to a 100% accuracy rate. Similarly, when asked to identify the worst perfused tissue, all surgeons provided correct answers based solely on the LSCI feedback. The inclusion of white light images did not alter the accuracy in this regard.

Usability for intestinal perfusion assessment

The collected data from the questionnaire were analyzed to assess the usability of PerfusiX-Imaging®. The questionnaire was filled in by five senior surgeons. No one disagreed on any of the questions. All surgeons (strongly) agreed on the minimal latency during the surgical procedure. Besides, 80% of the surgeons (n = 4) (strongly) agreed that the system was easy to use, easy to set up, able to visualize perfusion and able to visualize watershed areas. Two surgeons agreed and one strongly agreed (total of 60%) on the statement that the LSCI information reflected the expected pattern of blood flow. An additional 60% agreed on the good quality of the displayed data. The results from the survey are displayed in percentages in Figure 3.

DISCUSSION

In the current animal study, we successfully acquired laser speckle contrast images during intestinal surgery using laparoscopic LSCI setup, demonstrated the capability of indicating ischemic bowel regions with this technique, and demonstrated the usability of LSCI system for intestinal perfusion assessment.

The use of LSCI feedback allowed us to visualize and detect differences of intestinal perfusion, which can serve as a critical indicator of tissue perfusion. The noticeable attributes of LSCI appear especially captivating when applied to the creation of intestinal anastomoses. In this context, it becomes imperative to conduct an intraoperative evaluation of intestinal microperfusion to confirm the vitality of the recently established anastomosis, aiming to avert complications stemming from insufficient blood supply, such as AL. The conventional approach of the surgeon relying on visual examination has demonstrated marked subjectivity and offers minimal predictive efficacy [12]. The latter stimulated the advancement of perfusion imaging methods, especially near-infrared fluorescence imaging [22, 23]. Fluorescence angiography has some distinct disadvantages compared to LSCI. These include the need for a fluorescence dye and the inability to repetitively and continuously assess bowel perfusion due to wash-out effects [24, 25]. In contrast, we were able to detect the location of the intestinal watershed area in real time without the need to administer an exogenous dye with LSCI. Identifying the location of the intestinal watershed area with LSCI could serve as a so-called red flag technique in guiding surgeons towards an anastomosis created with better perfused tissue. The ability to assess anastomotic perfusion in real-time provides surgeons with important information that complements their conventional assessment methods. This additional feedback empowers surgeons to detect perfusion differences between tissue segments and identify the worst and best perfused tissue more accurately [16, 17, 18, 20].

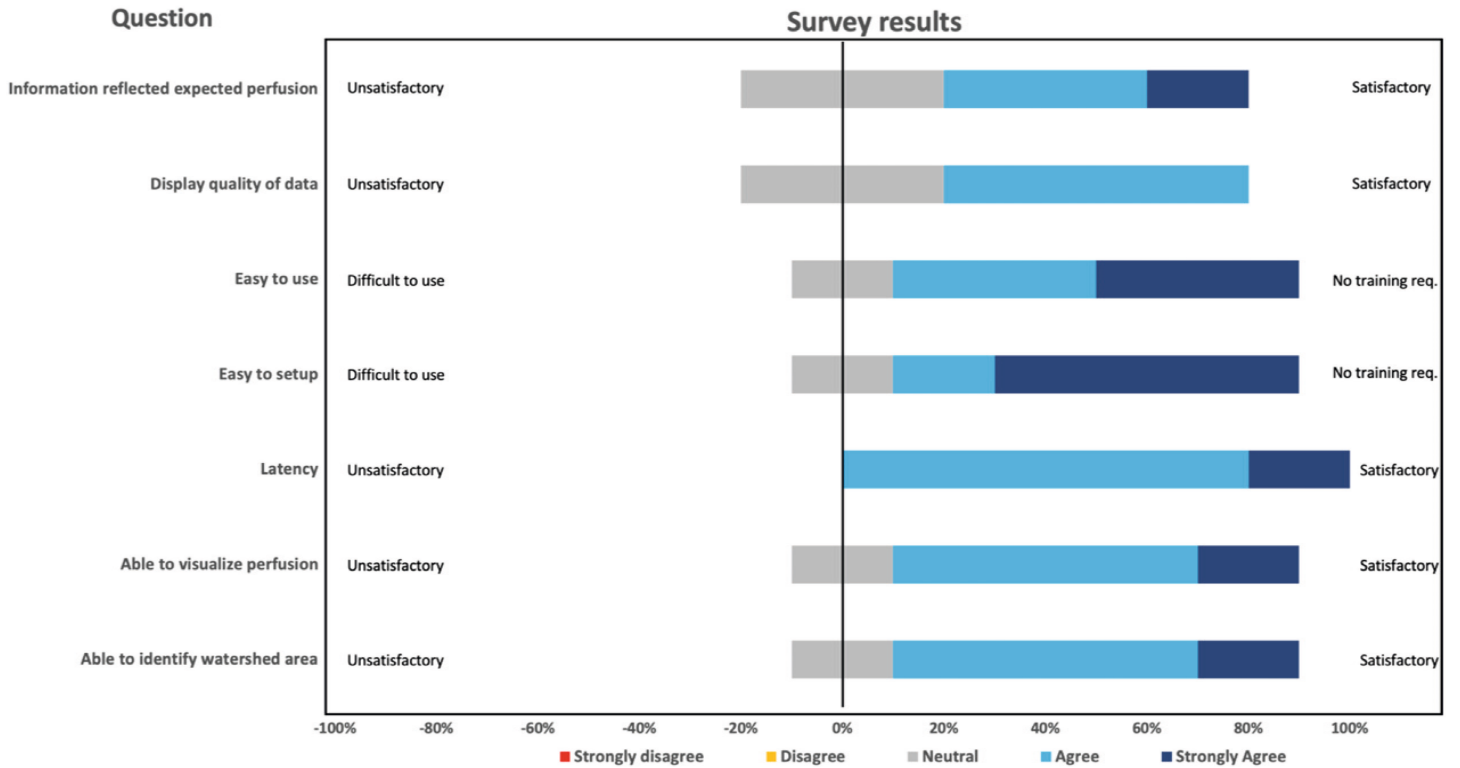

Figure 3. The results from the survey filled in by five surgeons represented in percentages in a Likert profile graph. The answers ranged from strongly disagree/bad to strongly agree/good. The questions were (1) The PerfusiX-Imaging perfusion information reflected the expected pattern of blood flow? (2) How was the display quality of PerfusiX-Imaging on displaying the blood flow? (3) PerfusiX-Imaging was easy to use during surgery? (4) How easy was it to setup PerfusiX-Imaging? (5) Was there latency during the perfusion imaging? (6) PerfusiX-Imaging was able to visualize tissue perfusion intraoperatively? And (7) I was able to identify the intestinal watershed area using PerfusiX-Imaging.

LSCI is still seen as a less commonly used perfusion imaging technique, although its clinical applications seem promising. In contrast to other imaging techniques such as near-infrared fluorescence imaging (NIRF), there is no need for any pharmaceuticals or dyes. Additionally, LSCI captures the possibility for continued perfusion evaluation, without difficulties such as residual signals or wash-out effects [26]. Future validation and exploration are necessary to assess the exact value of this current red flag technique in colorectal surgery. In the current study the surgeons were asked to draw conclusions based on LSCI images (verification). Although, for clinical setting it may be interesting to perform the current study in opposite direction and investigate if LSCI provides additional information compared to the white light images (validation). Additionally, it is interesting to compare these outcomes with other imaging technique such as fluorescence angiography as previous research has already shown the complemental role of LSCI compared to NIRF in parenchymal perfusion assessment [27]. Yet, it may also be interesting to deeper evaluate inter-observer variability of experienced surgeons as used in this study, compared to less experienced residents. LSCI could emerge as a more direct, real-time, and repeatable approach in providing quantitative information on tissue perfusion [27, 28, 29]. Developing additional methods to quantify the LSCI output can extra enhance the accuracy and reliability of LSCI. Given that this research exclusively focusses on establishing the practicability of measuring perfusion and identifying perfusion discrepancies at the anastomosis, future studies should focus on evaluating the device's performance in clinical trials and investigate surgical clinical consequences such as AL rates and overall patient recovery.

The results from the survey indicated that the senior surgeons were overall very satisfied with PerfusiX-Imaging® as a perfusion imager and its use during the surgical procedure. The system's ability to accurately represent blood flow patterns, high display quality of data, ease of use, efficient setup, minimal latency, and real-time visualization of tissue perfusion were positively acknowledged. However, it is important to note that this study was conducted in an animal model, and further research is needed to validate the efficacy and feasibility of the LSCI device in clinical settings. Besides, while anastomotic perfusion is commonly required during colorectal anastomotic creation, we used small bowel loops in our experiment. This decision was based on the difficult curly nature of a pig's colon, making the small bowel a more suitable choice for illustrative and surgical technical purposes. Consequently, the generalizability of our findings to human patients should be further investigated. Yet, previous research has shown good results using the same device for colonic perfusion assessment in a human population20. Also, for this experiment we conducted numerous experiments with separate bowel loops, however, it is essential to note that these observations were derived from a single animal. Consequently, it is imperative to be cautious when interpreting the data presented in this study, given its limited sample size. For our animal studies, we consider it of paramount importance to adhere to the principles of the 3R's: replacement, reduction, and refinement 30. As such, the current experimental design was considered adequate for assessing the hypothesis.

CONCLUSION

The PerfusiX-Imaging® device provided visual feedback for assessing reduced intestine perfusion in ischemic loops, detecting perfusion differences between loops, and identifying the best perfused loop in a porcine model. The real-time 2D-perfusion maps offered immediate and continuous information on tissue perfusion, which may help to select optimal sites for anastomosis creation. Although surgeons were overall very satisfied with using the system, further research is required to validate the efficacy in clinical settings and its potential impact on surgical outcomes in patients.

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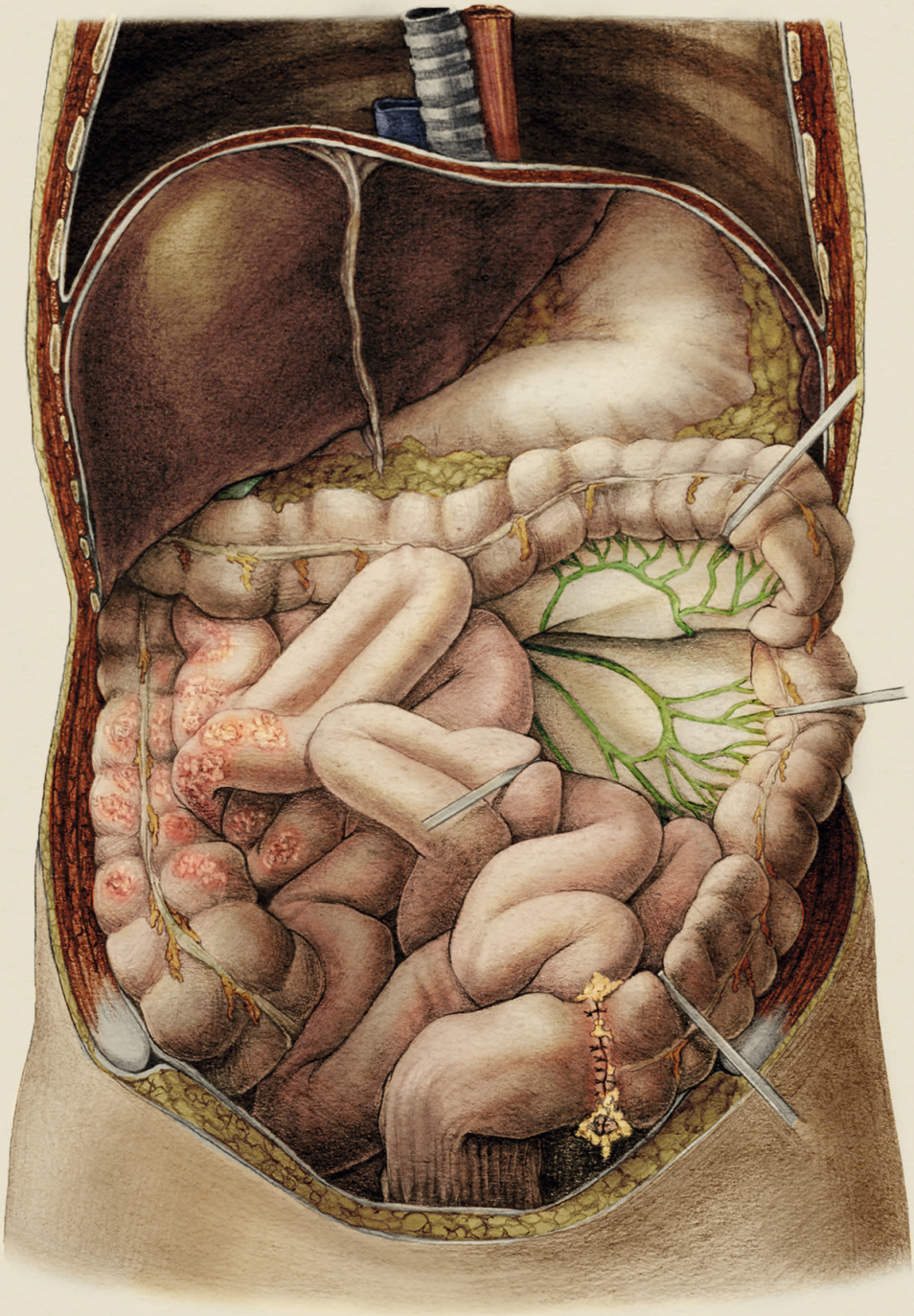
SUPPLEMENTARY

S1. Usability of PerfusiX-Imaging for intestinal perfusion assessment questionnaire

A questionnaire was designed to assess the usability of PerfusiX-Imaging. The questionnaire consisted of six items, each addressing a specific aspect of usability. The items were answered using the Likert scale from 1 to 5, with the 1 representing the least favorable response and 5 indicating the most favorable response. The questionnaire can be found underneath.

1. I was able to identify the intestinal watershed area using PerfusiX-Imaging
2. PerfusiX-Imaging was able to visualize tissue perfusion intraoperatively
3. Was there latency during the perfusion imaging?
4. How easy was it to setup PerfusiX-Imaging?
5. PerfusiX-Imaging was easy to use during surgery
6. How was the display quality of PerfusiX-Imaging on displaying the blood flow?
7. The PerfusiX-Imaging perfusion information reflected the expected pattern of blood flow



CHAPTER 8

REAL-TIME QUANTIFICATION OF LASER SPECKLE CONTRAST IMAGING DURING INTESTINAL LAPAROSCOPIC SURGERY: SUCCESSFUL DEMONSTRATION IN A PORCINE INTESTINAL ISCHEMIA MODEL

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ABSTRACT

Background. Anastomotic leakage (AL) is a dreaded complication following colorectal cancer surgery, impacting patient outcome, and leads to increasing healthcare consumption as well as economic burden. Bowel perfusion is a significant modifiable factor for anastomotic healing and thus crucial for reducing AL.

Aims. The study aimed to calculate a cut-off value for quantified laser speckle perfusion units (LSPUs) in order to differentiate between ischemic and well-perfused tissue and to assess inter-observer reliability.

Methods. LSCI was performed using a porcine ischemic small bowel loop model with the PerfusiX-Imaging® system. An ischemic area, well-perfused area, and two watershed areas were selected based on the LSCI colourmap. Subsequently, local capillary lactate (LCL) levels were measured. A logarithmic curve estimation tested the correlation between LSPU and LCL levels. A cut-off value for LSPU and lactate was calculated, based on anatomically ischemic and well-perfused tissue. Inter-observer variability analysis was performed with 10 observers.

Results. Directly after ligation of the mesenteric arteries, differences in LSPU values between ischemic and well perfused tissue were significant ($p<0.001$) and increased significantly throughout all following measurements. LCL levels were significantly different ($p<0.001$) at both 60 and 120 minutes. Logarithmic curve estimation showed an R^2-value of 0.56 between LSPU and LCL values. A LSPU cut-off value was determined at 69, with a sensitivity of 0.94 and specificity of 0.87. A LCL cut-off value of 3.8 mmol/L was found, with a sensitivity and specificity of 0.97 and 1.0 respectively. There was no difference in assessment between experienced and unexperienced observers. Cohen's Kappa values were moderate to good (0.52-0.66).

Conclusion. Real-time quantification of LSPUs may be a feasible intraoperative method to assess tissue perfusion and a cut-off value could be determined with high sensitivity and specificity. Inter-observer variability was moderate to good, irrespective of prior experience with the technique.

Keywords: Anastomotic leakage; image-guided surgery; laparoscopic surgery; laser speckle contrast imaging; perfusion assessment.

INTRODUCTION

Anastomotic leakage (AL) is one of the most feared complications following colorectal cancer surgery. It negatively impacts surgical outcome, functional results, and quality of life due to reoperation, permanent diversion, or delayed ostomy reversal [1-3]. Besides, AL increases the total clinical and economic burden [4]. Despite advances in pre-operative risk assessment, operative techniques, and postoperative care, the overall incidence of AL has not significantly decreased over the last decades, with an incidence of 1.5 to 23% and mortality rates as high as 29% [1-3, 5, 6].

Several pre-, intra- and postoperative risk factors for colorectal AL have been described [7]. The consensus is that sufficient perfusion of tissue is a prerequisite to ensure appropriate anastomotic healing [8-10]. An accurate indication of the borderline between the viable and non-viable tissue, i.e. the watershed area, could help surgeons to create optimal anastomosis and mitigate ischemia-related complications [11]. Currently, the majority surgeons determine the location of anastomosis based on vital signs of the bowel (e.g., mucosal colour, pulsation in the mesenteric bed, bleeding from resection lines), a subjective strategy that does not take micro perfusion and collateral circulation into account [11-13]. Therefore, bowel perfusion assessment is a strategy employed to minimize the risk of AL.

At present, most research focuses on bowel perfusion assessment with intraoperative near-infrared fluorescence imaging (NIRF) using indocyanine green (ICG). However, a more recently developed technique is laser speckle contrast imaging (LSCI). Compared to NIRF, LSCI is a dye-free, non-invasive technique which provides real-time blood flow information by detecting the dynamic interference pattern of laser light on moving red blood cells, known as a speckle pattern [12, 14, 15]. Previous studies demonstrated the feasibility of using laparoscopic LSCI to evaluate real-time intraoperative intestinal perfusion [12, 16-18]. However, optimization and fine-tuning of the technology, supported by additional pre-clinical experiments, are necessary to further validate the anticipated clinical usefulness. Although LSCI generates an objective colourmap based on quantitative data to visualize perfusion differences, interpretation of the colourmap remains subjective. The colour on the map does not indicate tissue viability, but flow. Hypothesizing that quantification of data can enhance objectivity and reproducibility, reduce reliance on individual operators, and potentially improve patient outcomes [19-21], the current study was conducted.

The objectives of this study were twofold: firstly, to establish a cutoff value for laser speckle perfusion units (LSPUs) indicative of optimal tissue perfusion and viability, aiming to furnish surgeons with quantitative data to enhance clinical decision-making; and secondly, to evaluate inter-observer reliability among both LSCI experts and inexperienced clinicians. Given lactate's well-established role as a marker for both systemic and local ischemia [22-24], capillary lactate levels were utilized as a reference point in this study.

MATERIALS AND METHODS

Animals and surgical procedure

This animal study was performed at the Central Animal Facilities of Maastricht University (Maastricht, The Netherlands). A total of four mature female Landrace pigs were used for this study, in compliance with the regulations of the Dutch legislation concerning animal research, ARRIVE guidelines and with approval from a local animal ethics committee (DEC-UM; Number: 2017-021-001).

All surgical interventions were conducted with the administration of general anaesthesia. An intravenous combination of medications, including 6mg/kg Zoletil, 0.01 mg/kg/h sufentanyl (Hameln Pharma GmbH, Hameln, Germany), 9 mg/kg/h propofol (B. Braun Melsungen AG, Melsungen, Germany), and 1 mg/kg/h midazolam (Aurobindo, Baarn, The Netherlands), was administered for anaesthesia induction. Throughout the procedure, all animals underwent mechanical ventilation to ensure adequate respiration. Ventilation settings were adjusted when necessary to maintain optimal oxygenation and ventilation. Continuous infusion of sufentanyl and propofol was used to sustain anaesthesia, with additional doses administered as required during the procedure. At the conclusion of the experiment, euthanasia was performed using a lethal dose of 200 mg/kg Pentobarbital (AST Farma, Oudewater, The Netherlands).

A midline laparotomy was performed by an experienced surgeon and small bowel loops of approximately 20 cm in length were randomly selected. Subsequently, a minimum of eight consecutive mesenterial arteries feeding the loop were identified and ligated using an energy device (Thunderbeat, Olympus, Hamburg, Germany) to induce ischemia.

Laser Speckle Contrast Imaging

A PerfusiX-Imaging® device (LIMIS Development BV, Leeuwarden, The Netherlands) was used to perform laparoscopic LSCI, as described by Heeman et al. [17]. This is a laparoscopic perfusion imager that functions as an add-on with a range of widely clinically available laparoscopic video equipment. In this study, an OTV-S200 laparoscopic video system (Olympus, Hamburg, Germany) and a 30-degree chip-on-the-tip laparoscope (EndoEye, Olympus, Hamburg, Germany) were used in combination with the investigational device. This setup is capable of instantaneously providing real-time perfusion maps of intestinal tissue using a red laser source. A proprietary mechanism in the device allows switching between the original white light source and the visible red laser light.

LSCI is based on changes in the speckle pattern that arise when illuminated tissue contains moving particles [25]. The level of moving particles (i.e. red blood cells) affects the changes in speckle contrast, allowing for calculation and visualization of perfusion levels through 2D-perfusion maps on the surgical monitor. The colourmap shows a gradient between blue (relatively low perfusion) and yellow (relatively good perfusion), based on LSPUs (arbitrary units, or AU). LSPU's were calculated by the ratio of the standard deviation (SD) divided by

the mean intensity of the pixels in a window of 7x7 pixels. During the procedure, the surgeon was able to view live LSPU-values, as well as a graph plotting LSPU-values over time. For standardization purposes, the laparoscope was placed in a 3D-printed mount, 14 cm above the specimen, with the camera sensor placed perpendicular to the tissue.

Data acquisition and statistical analysis

LSPUs and lactate levels

Four timepoints were selected to acquire data during the procedure. At T_{-1}, prior to any manipulation of vascularization occurred, an LSCI recording of the untouched intestinal loops was made. Each loop was placed outside of the abdominal cavity on a black drape at the time of imaging for standardization purposes. During recording, lights in the operating room (OR) were turned off. Immediately after ligation of the arteries, the LSCI visualization mode was turned on and shown real-time to the operating surgeon (T_0). Following a concise explanation on the system's visualization of perfusion using a colour map, the surgeon designated four regions of interest (ROIs) accordingly: an ischemic area, a well-perfused area and two watershed areas (transition zones between well- and poorly perfused tissue; Figure 1).These ROIs were marked with a surgical tissue marker pen for reference during the image analysis. LSCI recording was repeated 60 (T_{60}) and 120 (T_{120}) minutes after devascularization. In addition to the LSCI recording, systemic lactate levels were taken at T_0, T_{60} and T_{120} for every loop to estimate the ischemic state of the pig. Also, local capillary lactate (LCL) levels in the intestinal serosa were measured at the four ROIs. For practical reasons, this was done at T0 for the watershed and ischemic ROIs only in 3 loops, but in all loops for the well-perfused ROIs. At T_{60} and T_{120}, LCL levels were taken at all four ROIs in all loops. The LCL measurement was done using a 23 Gauge needle and an EDGE lactate analyzer (ApexBio, Taipei, Taiwan, People's Republic of China) which allowed for instant lactate measurements.

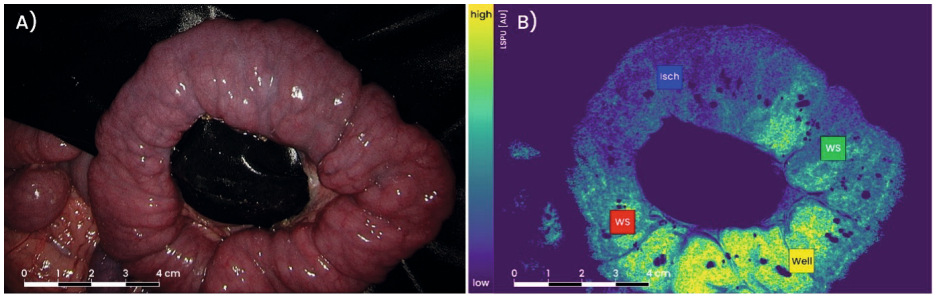

Figure 1. Small bowel tissue was placed in an extracorporeal loop on black drape. At T_0, arteries were ligated to induce ischemia. **A)** White light image as produced by a standard laparoscopic system. The mesenteric defect in the middle of the loop, is the result of cauterization of arterial vascularization**. B)** Visualization of perfusion levels in the same intestinal loop as seen by the surgeon during the procedure. Four regions of interest (ROIs) can be seen, representing the surgeon-selected perfusion areas: Well = well-perfused tissue (yellow); WS = watershed areas (red and green), Isch = ischemic tissue (blue). The scale bar on the left of the colourmap indicates the low flow (blue) to high flow (yellow) gradient.

Data quality assessment

Sequences were inspected for artefacts, such as erroneous ROI tracking or surgical instruments blocking a clear view on the intestinal tissue. Artefacts were excluded from further analysis. The middle 96 frames of each recording were used to equalize sequence length and prevent any selection bias. ROIs were placed, based on surgical pen markings, and measured 60x60 pixels.

Cut-off values

In addition to real-time quantitative perfusion values, a cut-off value for LSPUs was calculated, aiding in the identification of well-perfused and ischemic tissue. To estimate this cutoff value, the Youden index was used; a measure for evaluating the effectiveness of diagnostic tests based on sensitivity and specificity. The index ranges from 0 to 1, with a value of 1 indicating a perfect test with no false positives or false negatives, and a value of 0 indicating a test that performs no better than chance [26]. First, a cut-off value with the highest Youden Index for LSPU was calculated, based on anatomically ischemic and well-perfused tissue. Tissue with LSPU levels below the cut-off value were classified as ischemic. The same process was repeated to calculate the cut-off value for lactate levels, which was compared to existing evidence to validate the LSPU cut-off calculation.

Inter-observer reliability

In this study, the selection of ROIs was done subjectively by the surgeon based on real-time interpretation of the color map on the monitor. To assess the robustness and reliability of color map interpretation, an inter-observer variability analysis was performed after the experiment using the LSCI images taken during the surgery. Five LSCI experts and five physicians (surgical residents) with no experience in assessing LSCI images were asked to locate the watershed areas on the LSCI colourmaps, as well as the ischemic and well-perfused areas. The inexperienced physicians received an introductory training consisting of two slides on how LSCI works and how to interpret the colormaps, along with one training image and accompanying text on how to place ROIs. Inter-observer distance was registered in centimetres between all assessors, measuring over a midline on the small intestinal loop. A more detailed explanation can be found in supplemental 1. An expert was defined as someone having multiple years of experience in working with or developing LSCI systems.

Statistical analysis

Microsoft Excel (Microsoft Excel version 2312, Microsoft Corporation, Redmond, Washington, United States) and IBM SPSS statistics software package (IBM SPSS statistics version 27, IBM Corp, Armonk, New York, United States) were used to perform statistical analyses. A linear mixed effect model was built using a random intercept model with time and ROI location as fixed effects and an interaction term between time and ROI. A scaled identity covariance structure was used, and it was considered adequate to use Restricted Maximum Likelihood (REML) as estimation method, to prevent biasing by the used method. Logarithmic curve

estimation was performed to test the correlation between LSPUs and LCL-levels and plot a coefficient of determination, or R^2. Inter-rate reliability was analyzed using Cohen's Kappa [27]. Differences were considered significant when *P*<0.05. Figures were produced with PRISM (PRISM version 10.1.0 (316), GraphPad Software LCC, Boston, Massachusetts, United States). Mann-Whitney U tests were conducted for non-normally distributed variables, while T-tests were employed for normally distributed variables. Numerical variables are presented as mean ± SD or median [IQR 25%-75%] where appropriate.

RESULTS

The surgical procedures were performed without complications or adverse events. The average weight of the landrace pigs was 39 kilogram (range 36-42). A total of 18 intestinal loops were created, ranging between three and five loops per animal. The operating surgeon was able to interpret all LSCI derived perfusion colourmaps in real-time on the surgical monitor and to place the ROIs (Figure 2). Systemic lactate levels ranged from 12 to 30 mmol/L, indicating that none of the pigs experienced ischemia during the experiment.

LSPU values

At T_{-1}, there were no significant differences in mean LSPUs between the watershed, ischemic, and well-perfused areas as presented in Figure 3A. At T_0 (Figure 3B) LSPUs of the ROIs started to diverge. Mean ischemic LSPUs were not only significantly lower, compared to well-perfused areas (66.8 ± 19.4 versus 94.7 ± 18.7 AU, P≤.001), but also compared to the watershed areas (78.7 ± 18.3 AU, P=.038). This difference further increased at T_{60} and T_{120} in all measurements (P≤.004, Figure 3C-D).

Temporally, all ROIs showed a decrease in perfusion levels (Figure 3E). Mean LSPUs decreased significantly over time from respectively 96.9 ± 8.0 AU at T_{-1} to 45.8 ± 6.4 AU at T_{120} in ischemic areas (P≤.001). In watershed, a decrease from 88.9 ± 7.6 to 64.8 ± 14.8 AU was seen (P≤.001). However, there was no significant change in well-perfused areas over a two-hour period. In ischemic areas, there was a significant decrease of LSPUs in the first hour (66.8 ± 19.4 at T_0 versus 52.2 ± 12.6 at T_{60}, P=.014). Between T_{60} and T_{120}, the curve flattened out. Using a mixed model analysis, the interaction term between time and ROI was significant (P≤.001)

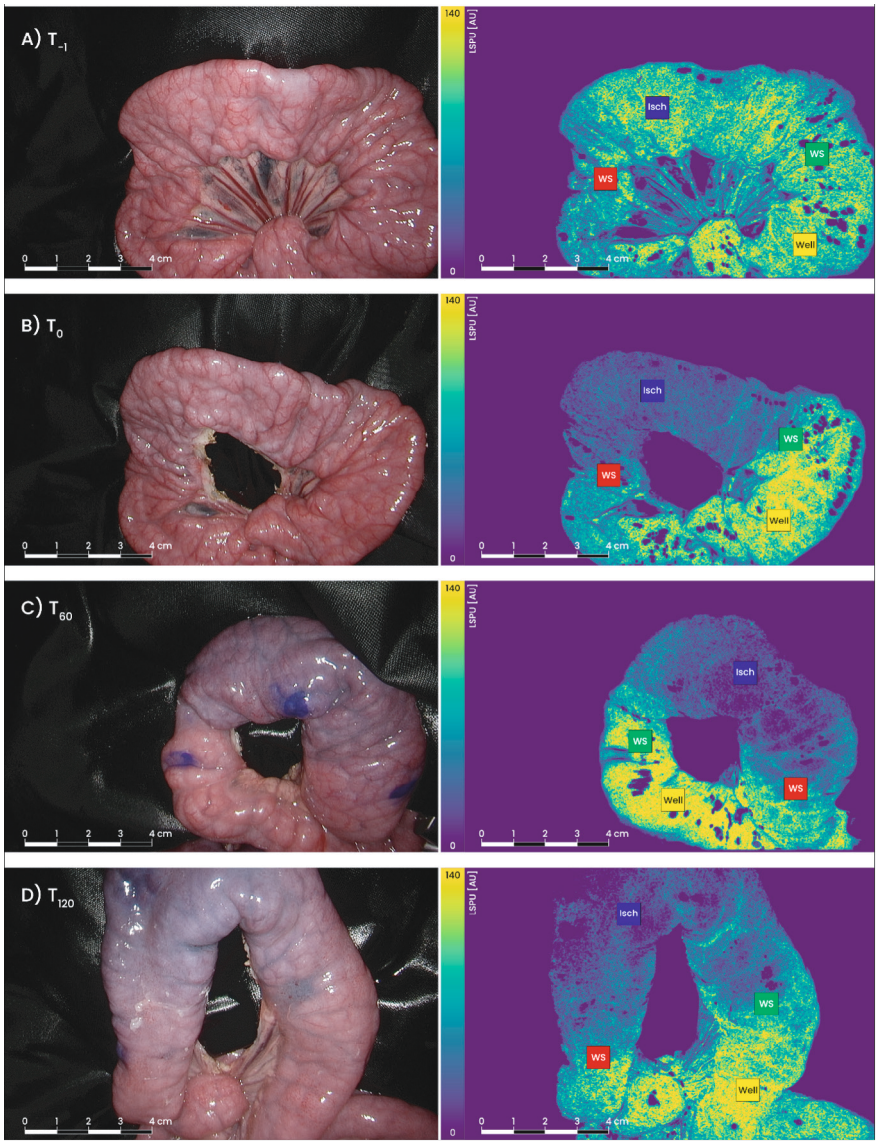

Figure 2. Progression of ischemia over time in both white light images (left), and Laser Speckle Contrast images (right). The Viridis colour scheme was used in the visualization of Laser Speckle Perfusion Units (LSPUs). The scale bar on the left of the colourmap indicates the low flow (blue) to high flow (yellow) gradient. **A)** T-1 images show the intestinal loop prior to any vascular manipulation (baseline). ROIs are placed, based on the selection location at the T0 measurement (image 2B), since no ischemic and watershed areas could yet be identified. **B)** Recording immediately after ligation (T0) and selection of Regions of Interest (ROI): Well = well-perfused tissue (yellow); WS = watershed areas (red and green), Isch = ischemic tissue (blue). Locations were marked with a surgical marker for reference. **C)** White light and LSCI recording 60 minutes after ischemic onset (T60). The blue dots in the white light image are the ROI markings from T0. **D)** 120 Minutes after ischemic onset (T120).

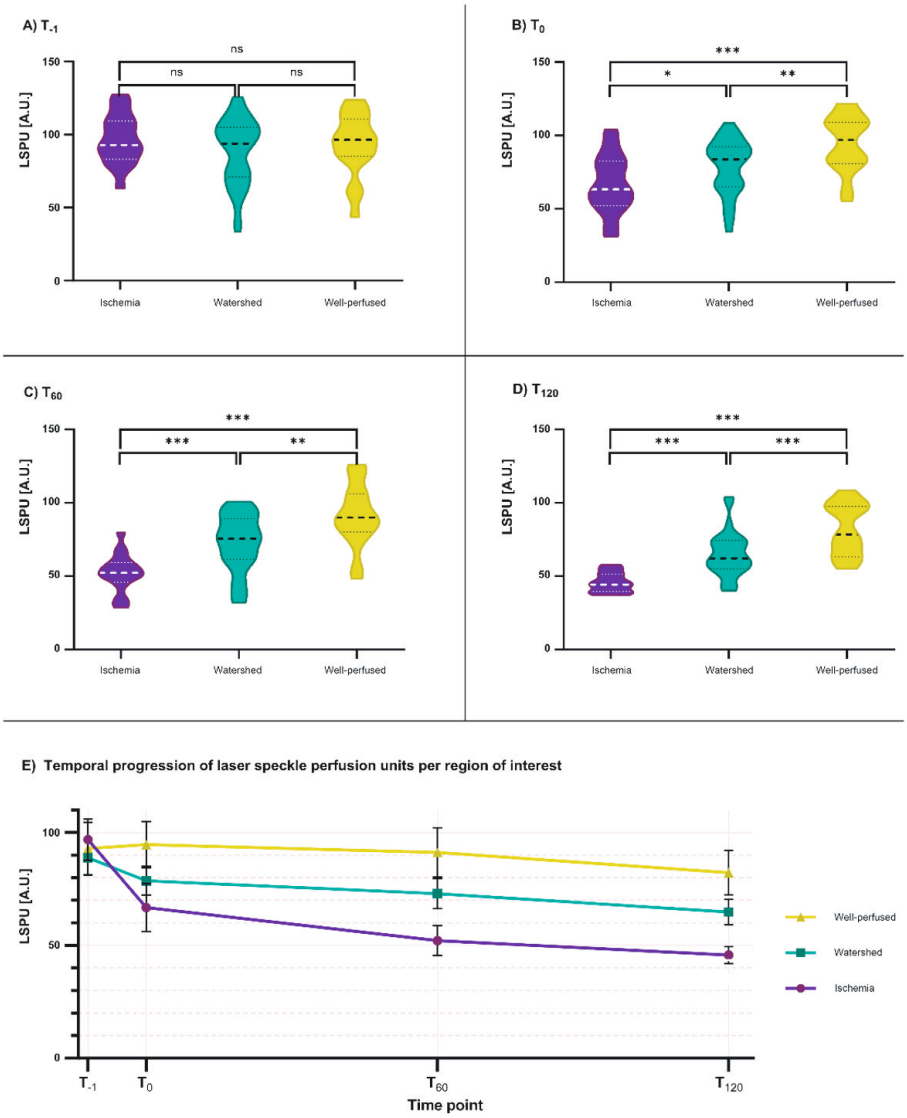

Figure 3. Overview of both spatial (A-D) and temporal (E) progression of average laser speckle perfusion units (LSPUs) per Region of Interest (ROI). **A)** LSPUs per ROI before devascularization (T-1). There was no significant difference between the ROIs. **B)** LSPUs measured at the ROIs directly after devascularization (T0). **C)** LSPU measurements after 60 minutes of ischemia (T60). Values further diverge and become more significant. **D)** At 120 minutes after inducing ischemia to the intestinal segment (T120), differences between all ROIs are highly significant. Dashed and dotted lines indicate mean values and quartiles respectively. **E)** Temporal development of LSPUs per ROI. A strong decline can be seen in ischemic tissue, shortly after inducing ischemia, further decreasing after this. As time progresses, the ischemic ROIs show increasingly low values compared to other ROIs. In addition, 95% CI bars narrow when ischemic time increases. Levels of significance in all images (P-values): *: ≤0.05; **: ≤0.01; ***: ≤0.001. An overview of all P-values can be found in Supplemental 2.

Lactate levels

Systemic lactate levels ranged from 12 to 30 mmol/L, confirming that none of the pigs experienced systemic ischemia during the experiment. Data collection of the LCL levels missed in one loop at T120, resulting in a total of 167 LCL values collected for analysis. At T_0, after ligation of mesenteric arteries, a significant difference in mean LCL levels between well-perfused and ischemic tissue (2.2 ± 0.6 mmol/L and 7.2 ± 1.9 mmol/L, P≤.001) was measured. This difference remained statistically significant over time, with P≤.001 at all timepoints, as can be seen in Figure 4A-D). Between T_{60} and T_{120}, a significant decrease in LCL levels was seen (10.3 ± 1.6 and 8.2 ± 2.8 mmol/L respectively, P=.015). Changes in mean well-perfused tissue lactate were not significant between T_{60} and T_{120} (2.0 ± 0.4 mmol/L versus 2.4 ± 0.9 mmol/L, P=.*195)*. Watershed area mean lactate levels showed an increase over time and were significantly higher than well-perfused LCL levels at all given timepoints. However, these values were significantly lower compared to those in ischemic areas at T_{60} and T_{120} (respectively 4.6 ± 2.8 versus 10.3 ± 1.6 and 5.0 ± 2.7 versus 8.2 ± 2.8mmol/L, both P≤.001).

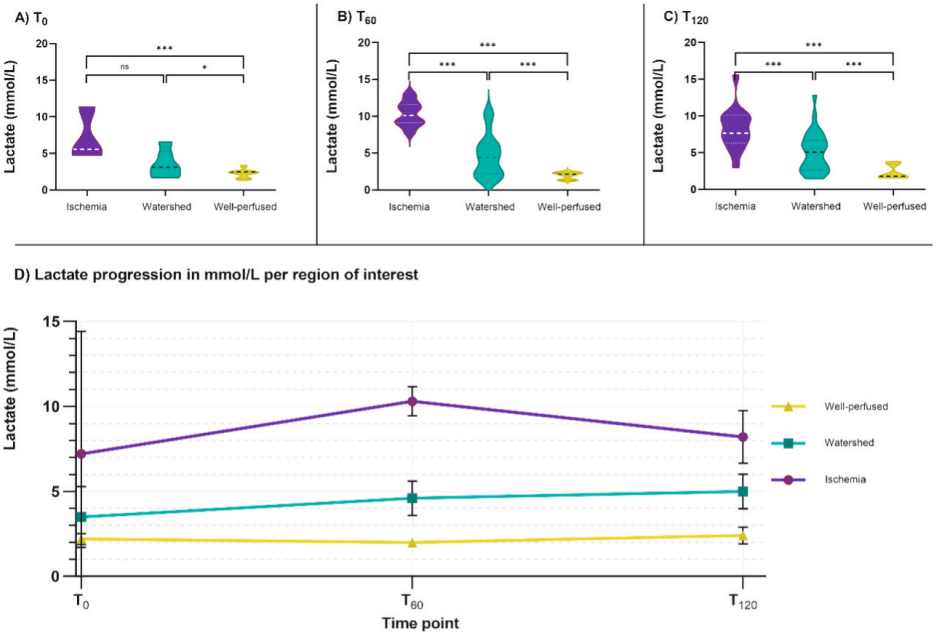

Figure 4. Overview of spatial and temporal differences in lactate (mmol/L) per region of interest (ROI). **A-C)** Violin plots representing lactate levels per Region of Interest (ROI) per timepoint. No measurements were taken at T-1 since ROIs were not yet identified at this moment. Dashed and dotted lines indicate mean values and quartiles, respectively. **D)** Temporal representation of mean lactate progression with standard deviation. In the first hour after inducing ischemia, the level of lactate rises significantly in the ischemic area. However, a decrease is seen in the second hour. Well-perfused lactate does not change significantly. Levels of significance in all images (P-values): *: ≤0.05; **: ≤0.01; ***: ≤0.001. An overview of all P-values can be found in Supplemental 2.

Cut-off values

Logarithmic curve estimation between LSPUs and LCL showed an R^2 of 0.56. A scatterplot of all ROIs from ischemic and well-perfused tissue with the coefficient can be found in Figure 5. The cut-off value for LSPUs was determined at 69 AU with a sensitivity of 0.94 and specificity of 0.87 (Youden index 0.81). Consecutively, a cut-off value of 3.8 mmol/L was calculated for lactate, with a sensitivity of 0.97 and specificity of 1.00 (Youden index 0.97).

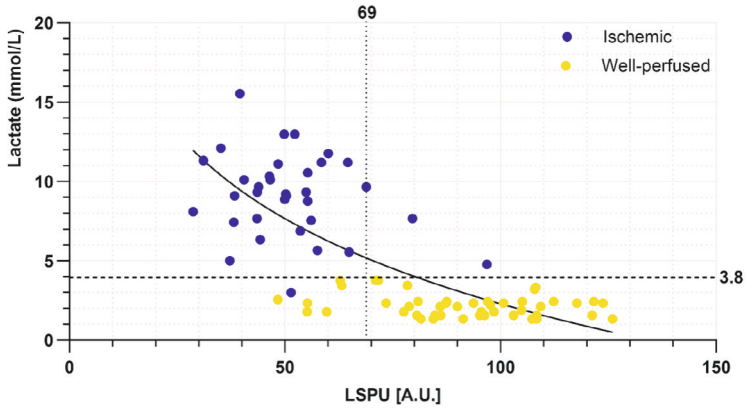

Figure 5. Scatterplot of Regions of Interest (ROIs) from ischemic (blue) and well-perfused (yellow) areas. Higher values indicate better perfusion. The horizontal black dashed line represents the cut-off value for lactate levels (3.8mmol/L), whereas the vertical dotted line represents the cut-off value for LSPUs (69 AU). In addition, logarithmic curve estimation was used to estimate an R2-value of 0.56 (solid black curve). A difference can be noticed between ischemic values and well-perfused values, as higher LSPUs are linked with lower lactate levels.

Inter-observer variability

All images were assessed by all experienced and non-experienced observers. No significant differences were observed between the groups regarding the time taken to point out a ROI. Experts failed to place one ROI in 17 cases (3.3%), and physicians in 12 instances (2.3%), citing indistinct transitions between well-perfused and ischemic tissue or broad gradients from high (yellow) to low (blue) values. Both experts and physicians showed similar decision making. A dot plot of all measurements is presented in Figure 6.

When tasked with identifying watershed areas, 66.3% of both experts and physicians placed the ROI more towards the ischemic side (average of 0.54 cm [IQR 0.26-1.41] and 0.66 cm [IQR 0.33-1.29] respectively), compared to the operating surgeon. A site towards better perfused tissue was chosen by 33.5% of experts and 33.7% of physicians (average of 0.44 cm [IQR 1.18-0.18] and 0.45 cm [IQR 1.11-0.18] respectively).

When comparing the placement of all ROIs to that of the operating surgeon, 72% of ROIs were located within one centimeter, whether proximal or distal. In observations within a two

centimeter range, this percentage increased to 89%. An inter-rater reliability analysis was conducted comparing the group of experts with the operating surgeon, yielding a substantial Kappa of 0.66 (95% CI 0.58-0.74). The comparison between physicians and the surgeon showed a moderate Kappa of 0.56 (95% CI 0.47-0.65). The comparison between the whole observer group and the surgeon showed a moderate Kappa of 0.52 (95% CI 0.44-0.61, P=.764).

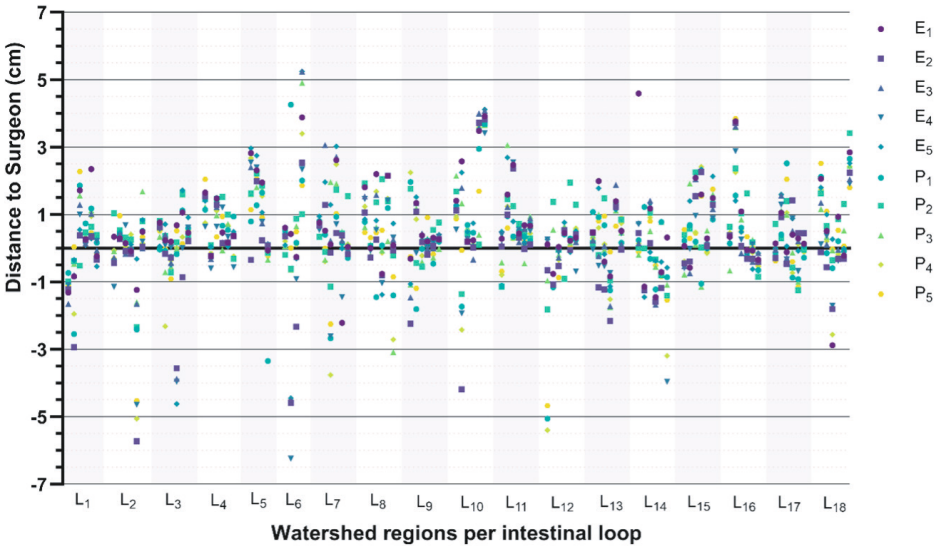

Figure 6. Intra Class Correlation (ICC) dot plot of all assessors per watershed region in all intestinal loops. Distances are measured from the Region of Interest (ROI) placed by the operating surgeon to the ROI placed by a different observer. Negative values are measured from the operating surgeon towards well-perfused tissue. Positive numbers are measured from the operating surgeon towards ischemic tissue. Within each loop, T0, T60 and T120 are shown from left to right, with two ROIs per timepoint (left and right watershed). A more detailed example of how ROI distances are calculated can be found in Supplemental 1. Ex = expert observer; Px = Physician, Lx = loop number.

DISCUSSION

To our knowledge, this is the first study to correlate quantitative LSPUs to lactate levels and providing a cut-off value for well-perfused tissue, thus adding to the increasing evidence that LSCI may serve as a suitable tool to guide the surgeon in the construction of an optimal anastomosis during laparoscopic surgery [13, 28-32]. Inter-observer agreement among physicians and experts was moderate to substantial, indicating that interpreting LSCI images is feasible even without extensive experience.

A distinct contrast in LSPUs between ROIs was seen at all timepoints following the creation of an ischemic segment. This underscores PerfusiX-Imaging®'s efficacy in visualizing perfusion

differences, consistent with previous studies [12, 16-18]. Although there was a clear distinction between areas, all LSPUs continued to decrease over time. In ischemic tissue, this decline is due to diminished flow post-ligation of feeding arteries, restricting blood inflow. Conversely, in well-perfused tissue, intestinal perfusion restriction may result from systemic illness response following prolonged ischemia. In contrast to the LSPUs in ischemic tissue, we surmised that lactate levels decreased after the first hour. This may be attributed to small overlapping vessels on the serosa, originating from a more adequately perfused intestinal segment, facilitating a modest collateral reperfusion effect [33]. This phenomenon may also explain a marginal surge in systemic lactate levels, as collateral vessels transport lactate into the systemic circulation. Additionally, oxygen deprivation from devascularization induces anaerobic fuel consumption, initiating fermentative glycolysis and an initial rise in lactate within ischemic tissue [34]. Compromised metabolic flux in ischemic tissue may deplete glucose supplies, reducing cellular energy consumption and halting lactate formation. Moreover, despite commonly perceived as a waste product, lactate can serve as an alternative fuel source within the tricarboxylic acid (TCA) cycle [35].

Based on our current findings, LSPUs above 69 AU indicate well-perfused tissue, with high sensitivity and specificity, highlighting the robustness of LSCI for perfusion visualization. Nevertheless, extensive additional research is required to extrapolate the use of a cut-off in LSCI in different tissues and humans. The cut-off value for lactate was primarily determined to validate the perfusion areas derived from the PerfusiX-Imaging® device. Values exceeding 3.8 mmol/L appeared indicative of ischemia. Although a well-defined cut-off value for Landrace pigs exists, our results aligned with existing literature, with systemic lactate levels typically below 2.0 mmol/L in a neutral state, while levels exceeding the determined cut-off value were observed in ischemic tissue [12, 33, 36-39].

All evaluators observed clear differentiation between adequately and inadequately perfused tissue. Agreement in identifying watershed regions ranged from moderate to substantial, with no significant difference between experts and unexperienced physicians. However, observers did encounter challenges in interpreting images lacking a clear-cut watershed line. This scenario occurred in case of a more gradual transition between well-perfused and poorly perfused tissue. Consequently, images displaying such transitions exhibited decreased inter-observer agreement, stipulating the importance of quantitative assessment methods to aid in the identification of viable and non-viable tissue.

In contrast to fluorescence angiography, LSCI does not require any pharmaceuticals or dyes, reducing risks of adverse reactions as seen with ICG [40]. Moreover, absence of these substances eliminates the need for timing and dosing calculations and facilitates repeated measurements without the interference of wash-out effects or residual signals [41, 42]. Quantifying ICG poses challenges, as it demands the standardization of numerous factors to obtain meaningful quantitative data [20]. While maximizing standardization in measurements is essential across

all modalities, LSCI could emerge as a more direct, real-time, and repeatable approach in providing quantitative information on tissue perfusion [28, 40, 42-45].

Strengths and limitations

This study maintained standardized conditions [20, 46]. The laparoscope was set at a perpendicular position at 14 cm to tissue to mitigate the effects of camera angulation and laser intensity [13]. PerfusiX-Imaging® utilized algorithms to compensate for motion-induced pixel contrast variations [47]. However, caution is advised in interpreting the findings, and further human investigation is required to assess reproducibility in clinical settings. The device is currently limited to research applications and the intraprocedural LSPU graph presentation is developed specifically for this study. While most of the elective abdominal clinical procedures involve laparoscopic approaches, the bowel loops were created during laparotomy and the laparoscopic system was used in an open setting to maximize standardization (and minimize the procedural time). Nonetheless, a prior technical demonstration has affirmed that the camera system was working appropriately in a total laparoscopic setting [18]. This study specifically addresses ischemia of the small intestine instead of colon surgery, which is more prevalent in daily clinical practice, acknowledging the impracticality of generating ischemic bowel loops in the porcine colon due to its spiral-like orientation [12]. Additionally, colorectal resection in a human population is often complicated by a higher presence of visceral fat compared to a porcine model. Nevertheless, prior studies have demonstrated favorable outcomes utilizing the same device for assessing colonic perfusion in human subjects [16].

Despite the modest sample size, we considered it adequate to address our hypothesis while adhering to the principles of the 3R framework (replace, reduce, refine) in animal research [48]. However, this precluded examination of inter-animal differences from the mixed model analysis. A larger-scale study could offer insights into variations in baseline perfusion and enhance understanding of cut-off values and LSCI quantification.

The next phase would be to further quantify measurements to precisely identify (non-) viable tissue and safe resection zones. Interpreting subtle perfusion differences is crucial for assessing tissue viability, particularly when ischemia is not evidently clear or when achieving this necessitates a profound comprehension of perfusion variations within tissues and across patients, emphasizing the importance of continued studies on the quantification of LSCI. Furthermore, future research should focus on evaluating LSCI in clinical trials to assess its impact on surgical outcomes, including AL rates, and compare its effectiveness with conventional white light imaging.

CONCLUSION

This study demonstrates the accuracy of Laser Speckle Contrast Imaging in visualizing and distinguishing between ischemic and well-perfused tissue. Changes in Laser Speckle Perfusion Units corresponded with alterations in lactate levels in both types of tissue. A cut-off value of 69 for LSPU showed high sensitivity and specificity. LSCI holds promise as an adequate and objective perfusion visualization tool, but further research on real-time quantification of LSPUs and clinical applicability is imperative.

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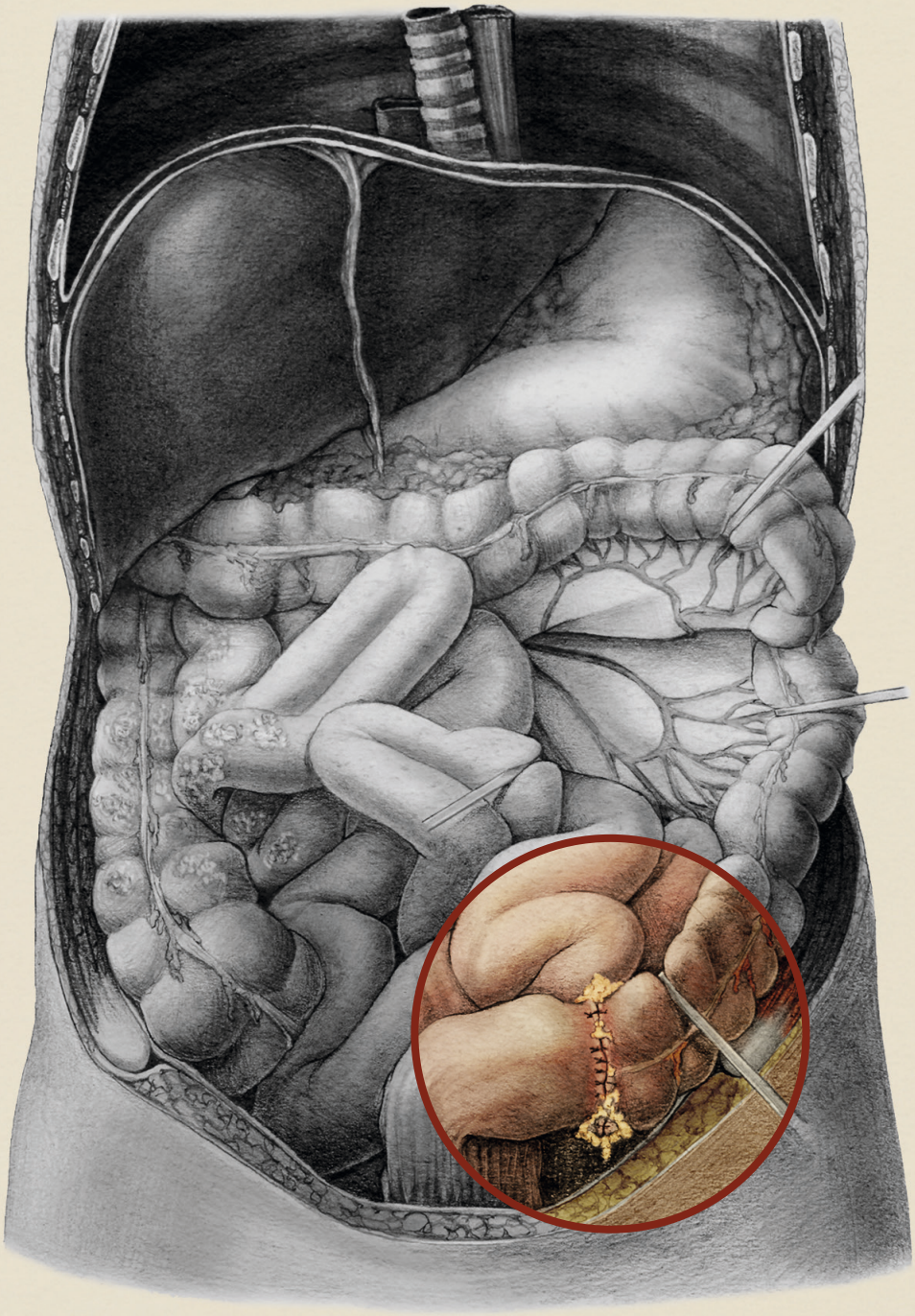
SUPPLEMENTARY

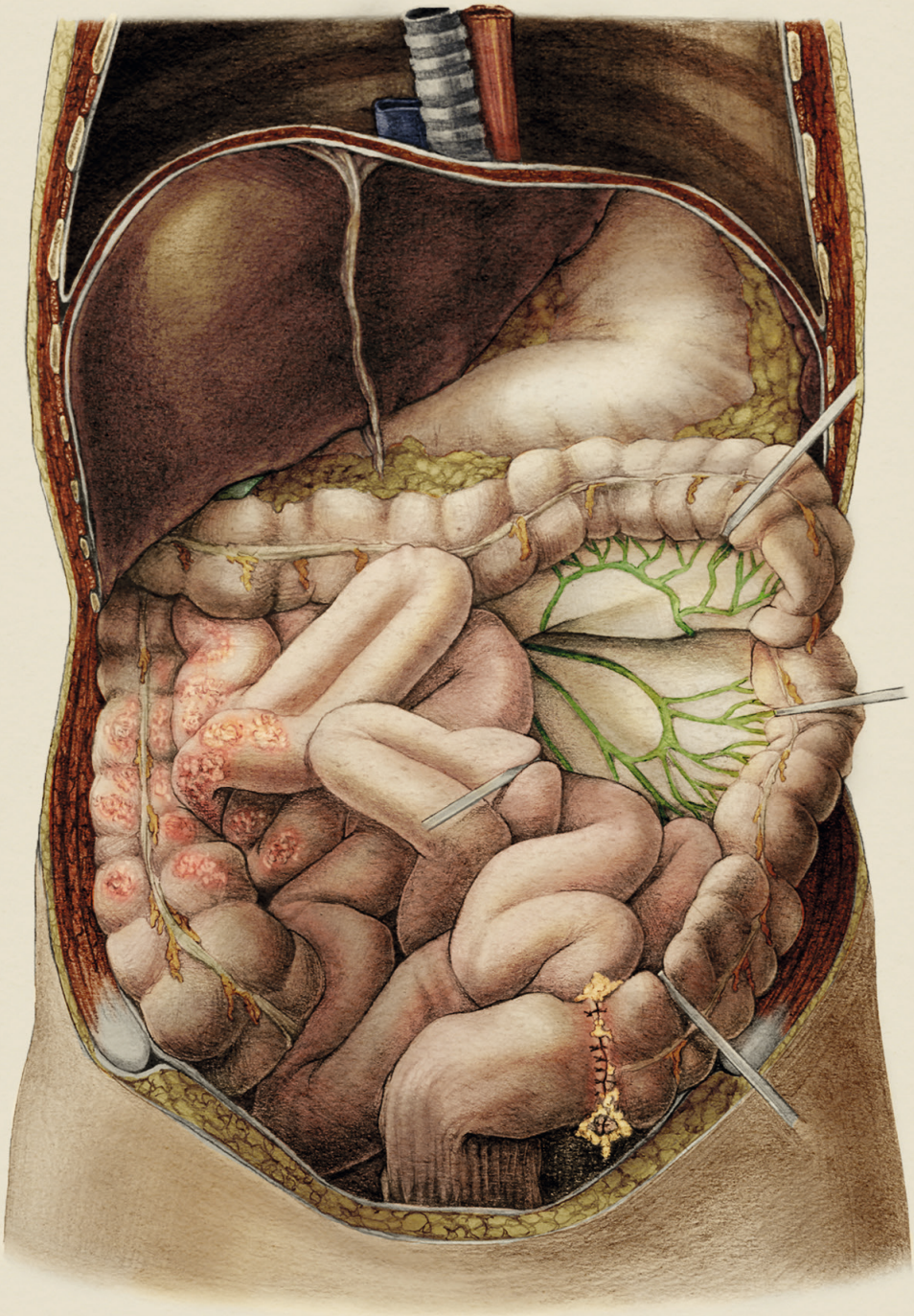
The following supplementary material can be downloaded from:

- *Figure S1-S4: Inter-observer measurements and calculations*
- *Table S1: Overview of P-values for LSPU and lactate levels*



PART III

PATIENTS' PERSPECTIVES ON COLORECTAL ANASTOMOTIC LEAKS



CHAPTER 9

IMPACT OF ANASTOMOTIC LEAKAGE AFTER COLORECTAL CANCER SURGERY ON QUALITY OF LIFE: A SYSTEMATIC REVIEW

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ABSTRACT

Background. Colorectal anastomotic leakage remains one of the most frequent and dreaded post-operative complications following colorectal resection. However, limited research has been conducted on the impact of this complication on quality of life of patients undergoing colorectal cancer surgery.

Objective. The aim of this systematic review was to identify, appraise and synthesize the available evidence regarding quality of life in patients with anastomotic leakage following oncological colorectal resections in order to inform clinical decision-making.

Data sources and study selection. PubMed, Embase and the Cochrane library were searched for studies reporting on quality of life using validated questionnaires in patients with anastomotic leakage after oncological colorectal resections. The literature search was performed systematically and according to PRISMA guidelines.

Outcomes. Outcomes of quality of life questionnaire scores of patients with and without anastomotic leakage were analyzed.

Results. Thirteen articles reporting on 4618 individual patients were included, among which 527 patients developed AL. Quality of life was evaluated utilizing ten distinct questionnaires administered at various postoperative time points, ranging from 1 month to 14 years. Quality of life outcomes differed across studies and timepoints, but overall scores were most negatively affected by AL up to twelve months postoperatively.

Limitations. There was a high heterogeneity between the included studies based on used questionnaires and time of assessment.

Conclusion. The published evidence suggests that anastomotic leakage following oncologic colorectal resection is associated with impaired quality of life, especially within the first postoperative year. The impact of anastomotic leakage on quality of life warrants further evaluation and discussion with patients.

Keywords: Colorectal cancer surgery, anastomotic leakage, Quality of Life (QoL).

INTRODUCTION

Oncological colorectal resection with or without primary anastomosis remains the cornerstone in the treatment of colorectal cancer (CRC). In patients undergoing restorative procedures, anastomotic leakage (AL) remains one of the most frequent and dreaded postoperative complications with reported incidence varying from 1.5-20% [1-4]. This wide ranging incidence in the literature may be due to differences in surgical risk among different study populations and variability in surgical techniques, but also reflects significant differences in reporting standards for AL. Albeit several classifications and definitions of AL have been described in the literature, there is no consensus on definitive diagnostic or clinical criteria for AL [5-8].

Several important risk factors for AL have been identified over the past decades, such as active smoking, malnutrition, male gender, obesity, emergency surgery, operative time, postoperative use of non-steroidal anti-inflammatory drugs and neoadjuvant chemotherapy [9-11]. Despite innovations in surgical techniques, preoperative optimization and intraoperative interventions to further minimize the risk of AL, rates of anastomotic complications have not decreased. AL range in clinical severity from minor, subclinical, and contained leaks to fulminant sepsis, organ failure with increased short-term mortality rates [12].

A standardized consensus framework for defining, reporting and, grading colorectal AL is currently being developed by the Consensus on Reporting and Defining Colorectal Anastomotic Leaks (CoReAL). This expert group noticed gaps in knowledge about the short and long-term impact AL on functional outcomes and overall quality of life (QoL). As patients should be fully informed not only regarding the immediate surgical risks, but also on the impact surgical complications may have on long-term function and QoL, this systematic review was undertaken to address this important question about short and long-term impact of AL in CRC patients. The aim of this systematic review was to identify, appraise and synthesize the available evidence regarding short- and long-term QoL in patients undergoing oncological colorectal resections complicated by AL.

METHODS

Study protocol and Registration

This systematic review was conducted according to the latest edition of the "Preferred Reporting Items for Systematic Reviews and Meta-Analyses" (PRISMA) guidelines [13]. The study protocol was developed a priori and registered at PROSPERO (ID 411065).

Outcomes and Definitions

The primary outcomes were QoL and Health-related Quality of Life (HRQoL). QoL was defined according to the World Health Organization (WHO) as "an individuals' perception of their

position in life in the context of the culture and value systems in which they live and in relation to their goals, expectations, standards and concerns" [14]. HRQoL in cancer is often used interchangeably with QoL, since there is no consensus on a standardized definition. We have applied the definition of Testa et al. on HRQoL as the "physical, psychological and social domains of health, seen as distinct areas that are influenced by a person's experiences, beliefs, expectations and perceptions" [15].

AL was defined as a combination of clinical signs and symptoms (e.g., abdominal pain or tenderness, peritonitis, fever, tachycardia, purulent or faecal discharge from an abdominal drain or vagina, purulent discharge per anus), biochemical elements (elevated white cell count and/or C-reactive protein (CRP)), and radiological confirmation of an interruption of the anastomosis and/or a peri-anastomotic collection on computed tomography (CT) scan [6, 16].

Search and information sources

The literature search was performed on the 13th of March 2023 and repeated on the 14th of August 2023. PubMed, Cochrane library and Embase were searched with the use of MeSH-, Emtree- and free terms including 'colorectal neoplasms', '(adeno)carcinoma', 'colorectal surgery', 'anastomotic leak', 'complications', Quality of life (QoL)' and 'Health-Related Quality of Life (HRQoL)' (Supplementary S1). Reference lists of relevant publications were cross-checked to identify additional studies. This hand-search method was continued until no further relevant studies were identified.

Selection process

Eligibility criteria and selection process

All English or Dutch articles published in peer reviewed index journals reporting on QoL in patients over the age of 18 with AL after oncological colorectal resections were considered eligible for inclusion. Analysis of QoL after AL had to be identified as a predetermined aim in the 'methods' section of the study in order to be eligible for inclusion. Trials were included irrespective of blinding. Systematic reviews and secondary sources such as letters to the editor, technical descriptions, conference proceedings or commentaries were excluded. Articles reporting on fewer than ten patients, or solely reporting on outcomes after colorectal resections for benign indications were excluded. Since the first systematic review on the definition of AL has been published in the year 2001, all articles published before the 1st of January 2000 were excluded [8]. Furthermore, articles were excluded when no validated (HR)QoL instrument had been applied. All search results were imported into an online tool designed for systematic reviews (Rayyan) [17]. After removal of duplicates, articles were screened for eligibility by two independent researchers (AG and DH) according to the predefined criteria. First, articles were screened based on title and abstracts. Definitive article inclusion followed if the eligibility criteria were met after full-text screening by both reviewers. Disagreements were resolved through discussion. All references were stored in the Endnote Reference Management Tool (version 20.4, Clarivate, Chandler, United States).

Data extraction and synthesis of results

Two independent researchers (AG and DH) performed a qualitative analysis and extracted data from the main text, tables and figures using a predefined and standardized data extraction table. Extracted data included first author, year of publication, country, study design, study period, inclusion and exclusion criteria, aim of the study, number of patients, general patient characteristics, indication for surgery, surgical procedures performed, the applied (validated) QoL questionnaires, time of assessment, and secondary outcomes. Furthermore, definitions, timeframe and criteria for diagnosis of AL were collected. Data acquired via the outlined search strategy were summarized in tables. Findings were described in a narrative approach, i.e., primarily words and text were used to summarize and explain the findings. Because of the heterogeneity among included studies in terms of definition of AL and questionnaires used to assess QoL, pooling in a meta-analysis was impossible.

Assessment of risk of bias in individual studies

To ascertain the validity of the included studies, the risk of bias of each study was assessed by two reviewers (AG and DH) with a revised ROBINS-I [18] tool to assess risk of bias in non-randomized studies. All types of bias were evaluated for every study and judged 'low risk', 'moderate risk' or 'high risk'. Possible confounding domains were a priori defined as active smoking, malnutrition, male gender, Body Mass Index (BMI), comorbidities or higher American Society of Anesthesiologists (ASA) classification, emergency surgery and longer operative time.

RESULTS

Study selection

The electronic literature search generated 1323 articles, and 980 unique articles after removing duplicates. Of these, 865 were excluded after title and abstract screening. Full-text screening of the resulting 115 articles was performed and another 104 were excluded. Cross-reference checking generated one additional article, and one additional publication was identified after repeating the search before submission. Ultimately, 13 articles were included in the analysis (Figure 1A).

Study and patient characteristics

All 13 included articles were cohort or case matched studies and comprised 4596 individual patients, with study sample size ranging from 32 to 1207 patients (Table 1). Four studies reported on colorectal resections [19-22], all other studies focused on rectal resections. All studies included patients diagnosed with CRC, with only two studies also including patients with benign indications for colorectal resections (e.g., diverticulitis or inflammatory bowel disease). As benign indications were presented separately, the outcome of these patients were excluded in this review [20, 21]. The final population consisted of 4618 patients, of which

2946 (64%) were male and 1672 (36%) female, with a mean age of 61.9 years. Among these patients, 527 (11%) developed AL and 4091 (89%) recovered without clinical, radiological or biochemical signs of AL (Table 2). The median time of follow-up was 4.3 years (4.8 months to 14.4 years). Additional study information on perioperative care of each study is provided in Supplementary S2.

Table 1. Characteristics of included studies

Reference	Year	Country	Study design	Study period	No. of patients	Inclusion criteria	Exclusion criteria	Aim of the study
Arron et al.	2023	The Netherlands	Observational cohort study	2010-2019	1197	Patients ≥ 18 years, diagnosed with stage I-III CRC	History of CRC, IBD, hereditary CRC syndromes, non-Dutch speaking or diagnosed with a mental condition limiting the ability to fill out questionnaires, non-elective surgery, resection without primary anastomosis or with end stoma reconstruction	To assess whether AL is associated with HRQoL at 6 months and 2 years post-diagnosis and whether AL is associated with a clinically relevant decrease in HRQoL at 6 months and 2 years post-diagnosis compared to the time of diagnosis.
Ashburn et al.	2013	United States of America	Retrospective cohort study	1980 - 2010	864	Restorative resection for rectal cancers for tumors less than 15 cm from the anal verge	Patients with IBD, familial adenomatous polyposis, hereditary and non-polyposis colon cancer; patients undergoing nonrestorative resections	To evaluate the impact of AL, when intestinal continuity can still be maintained, on bowel function and QoL in patients undergoing rectal cancer resection with low colorectal or colo-anal anastomoses.
di Cristofaro et al.	2014	Italy	Prospective cohort study	2020 – 2011	116	Patients admitted for elective CRC surgery	Emergency surgery, explorative laparotomy, inoperable CRC or recurrence at follow-up	To investigate how postoperative complications after surgery for CRC affect patients' QoL and satisfaction with care.
Hain et al.	2016	France	Case-matched study	2005 – 2014	135	All patients undergoing laparoscopic sphincter saving partial or TME	Patients with temporary or permanent stoma, no minimal follow-up of 1 year, no current chemotherapy, and patients who were included in a previous study from the group that evaluated a postoperative program	To assess long-term functional outcomes after laparoscopic, sphincter-preserving operative intervention for rectal cancer, according to the type of AL.

Table 1. Continued

Reference	Year	Country	Study design	Study period	No. of patients	Inclusion criteria	Exclusion criteria	Aim of the study
van Kooten et al.	2022	The Netherlands	Observational cohort study	1996 - 1999	1207	Clinically resectable adenocarcinoma with an inferior tumour margin below the level of S1/S2 and within 15cm from the anal verge without evidence of distant metastasis	Not having filled out the baseline HRQoL questionnaire, deceased within 30 days after surgery	To objectify the difference in short- and long-term HRQoL between uncomplicated and complicated postoperative recovery after TME for rectal cancer.
Lim et al	2006	United Kingdom	Observational cohort study	2000 - 2014	138	All patients undergoing surgical procedures with low rectal anastomosis (<10 cm from the anal verge).	NA	To evaluate the effect of (sub)clinical AL on QoL and the significance of features seen on water-soluble contrast enemas in prediction of subsequent anastomotic healing.
Marinatou et al.	2014	Greece	Retrospective case-matched study	2007 - 2012	75	Biopsy-proven CRC	Age < 18 or > 80, distant metastatic disease at presentation, initially managed on an emergency basis, history of IBD or hereditary cancer	To explore the effect of clinically evident AL on HRQoL.
McGiffin et al.	2022	Australia	Retrospective cross-sectional study	2004 - 2018	224	Patients undergoing minimally invasive proctectomy with a low extra-peritoneal anastomosis and without a temporary diverting ileostomy.	Age <18 years and the inability to give consent for survey participation, patients with temporary diverting ileostomy	To analyse the timing and management of AL and to evaluate the effect on long-term QoL and functional outcomes using validated instruments.

Table 1. Continued

Reference	Year	Country	Study design	Study period	No. of patients	Inclusion criteria	Exclusion criteria	Aim of the study
Miura et al.	2017	Japan	Retrospective cohort study	2000 - 2012	275	Low rectal adenocarcinoma	Diverting stoma	To evaluate permanent stoma formation and defecation in long-term follow up after surgery for low rectal cancer without a diverting stoma.
Mongin et al.	2014	France	Retrospective case-matched study	2004 - 2010	63	Laparoscopic TME for rectal cancer with a follow-up of ≥ 6 months and consent to fill in the questionnaires	Permanent or temporary stoma	To assess the influence of AL on long-term functional results and QoL after laparoscopic TME for cancer.
di Re et al.	2022	Australia	Retrospective case-matched study	2010 - 2020	122	- AL - Adult population - Benign or malignant colorectal disease	IBD, pouch formation and redo resections	To assess bowel function and QoL after AL from rectal resections.
Riss et al.	2011	Austria	Retrospective case-matched study	1995 - 2006	32	- TME for rectal cancer - Control group: uneventful postoperative course	Died during follow-up and no response to questionnaire	To investigate the impact of AL after rectal resection for malignancies on overall pelvic organ function and QoL.
Westerduin et al.	2021	The Netherlands, Belgium and France	Retrospective comparative cohort study	2007 - 2017	170	- TME for rectal cancer - All indications for redo anastomosis - Control group: successful primary anastomosis	Partial mesorectal excision, stoma at the time of questionnaire, unable to read or understand the questionnaires	To compare functional outcomes and the QoL between redo anastomosis and primary successful anastomosis following TME for rectal cancer.

**Post-hoc analysis of a prospective multicentre randomized controlled trial*
AL, anastomotic leakage; CRC, colorectal cancer; HRQoL, Health-related Quality of Life; IBD, Inflammatory Bowel Disease; QoL, Quality of Life; TME, Total Mesorectal Excision.

Table 2. Patient characteristics

Reference	Indication for surgery	Type of surgery	AL rate Yes : No	Sex (M : F)w		Age (years, mean ± SD or median (IQR))		Comorbidities/ASA			Clavien Dindo
				AL	No AL	AL	No AL	ASA	AL	No AL	
Arron et al.	Colon cancer (n=895) Rectal cancer (n=302)	Open and laparoscopic ileocecal resection, right and left hemi-colectomy, transverse resection, sigmoid resection, subtotal colectomy and LAR	63 : 1134 (5.26%)	45 : 18	706 : 428	64.8 (59.7-69.7)	66.6 (61.7-71.6)	I II III+ Unknown	20 32 10 1	343 635 122 35	NA
Ashburn et al.	Rectal cancer	Any open or laparoscopic restorative rectal resection	52 : 812 (6.02%)	43 : 9	519 : 293	56.9 ± NA	61.3 ± NA	I II III IV V	232 1910 26 1 0	8 395 369 18 0	NA
di Cristofaro et al.	Colon cancer (n=82) Rectal cancer (n=34)	Any open and laparoscopic colorectal resection	5 : 111 (4.31%)	71: 45		69.5 (61-75)		NA			Grade I: 3 Grade II: 17 Grade III: 9 Grade IV: 1
Hain et al.	Rectal cancer	Laparoscopic sphincter saving PME or TME according to the principles of extrafascial dissection	46 : 89 (34%)*	35 : 11	65 : 24	60.9 ± 8.3	63.5 ± 9.4	I II III	13 29 3	22 62 3	NA
van Kooten et al.	Rectal cancer	LAR, APR and Hartmann resection	79 : 1128 (6.55%)	767 : 440		A: 64 (23-88) B: 65 (41-86) C: 66 (26-92) D: 67 (43-88)[1]		NA			NA

Table 2. Continued

Reference	Indication for surgery	Type of surgery	AL rate Yes : No	Sex (M : F)w		Age (years, mean ± SD or median (IQR))		Comorbidities/ASA			Clavien Dindo
				AL	No AL	AL	No AL	ASA	AL	No AL	
Lim et al.	Rectal cancer (n=126), adenoma (n=4), endometriosis (n=2), M. Crohn (n=1), diverticulitis (n=3) or post-endomucosal resection (n=2)	TME with anastomotic distance of no more than 10 cm from the anal verge	23 : 115 (16.67%)	11 : 12	72 : 43	*Clinical* 66 (54-81)	*Subclinical* 62 (51-75)	II III IV	*Clinical* AL 6 5 2	*Subclinical* 4 5 1	NA
Marinatou et al.	Rectal cancer	TME for lower and mid rectum tumors, and PME with transection of the mesorectum at least 5 cm distal to the tumor for upper rectum tumors.	25 : 50 (33.33%)*	15 : 10	30: 20	62 ± 15.2	61 ± 16.3	I II III IV	1 9 13 2	5 20 23 2	NA
McGiffin et al.	Rectal cancer	Laparoscopic and robotic LAR with an extra-peritoneal anastomosis	24 : 200 (10.71%)	15 : 9	119 : 81	62 (52-69.8)	65 (56.3-73)	I II III-V	9 14 1	56 106 32	Used but not specified
Miura et al.	Rectal cancer	LAR (n=157) and ISR (n=118)	60 : 215 (21.81%)	199 : 76		64		ASA III-IV 30			Grade I/II: 62 Grade III: 66 Grade IV: 6 Grade V: 1
Mongin et al.	Rectal cancer	Laparoscopic sphincter-saving TME	21 : 42 (33.33%)*	4 : 17	11 : 31	61 ± 9	60 ± 11	NA			NA

Table 2. Continued

Reference	Indication for surgery	Type of surgery	AL rate Yes : No	Sex (M : F)w		Age (years, mean ± SD or median (IQR))		Comorbidities/ASA			Clavien Dindo
				AL	No AL	AL	No AL	ASA	AL	No AL	
di Re et al.	Colon cancer (n=16), rectal cancer (n=55), benign (n=28)[2]	Laparoscopically, open, robotically, or with conversion LAR (n=25), ultralow LAR (n=50) and high anterior resection (n=25)	61 : 61 (50%)*	43 : 18	41 : 20	62.4± 12.3	64.1± 8.6	Cardiovascular disease n=14 Diabetes mellitus n=24 Renal failure n=4 Lung disease n=10 Liver disease n=7			Grade I: 0 Grade II: 39 Grade IIIa: 49 Grade IIIb: 32 Grade IV: NA Grade V: 0
Riss et al.	Rectal cancer	ISR, LAR and APR	16 : 16 (50%)*	11 : 5	11 : 5	67 ± 11.2	70 ± 8.4	NA			NA
Westerduin et al.	Rectal cancer	Open and laparoscopic TME with anastomosis within 3cm from the dentate line	52 : 118 (30.59%)*	34 : 18	79 : 39	63 ± 8.9	68 ± 9.9	NA			NA

*1Group A: no complications, group B: surgical complications, group C: non-surgical complications, group D: surgical and non-surgical complications; ² Benign indications for resection were irresectable polyps or diverticulitis. **AL rates do not reflect incidence of AL due to the study design.*
AL, anastomotic leakage; APR, Abdominoperineal resection, CRC, colorectal cancer; ISR, Intersphincteric rectal resection; LAR, low anterior resection; NA, not applicable; TME, total mesorectal excision.

Risk of bias in studies

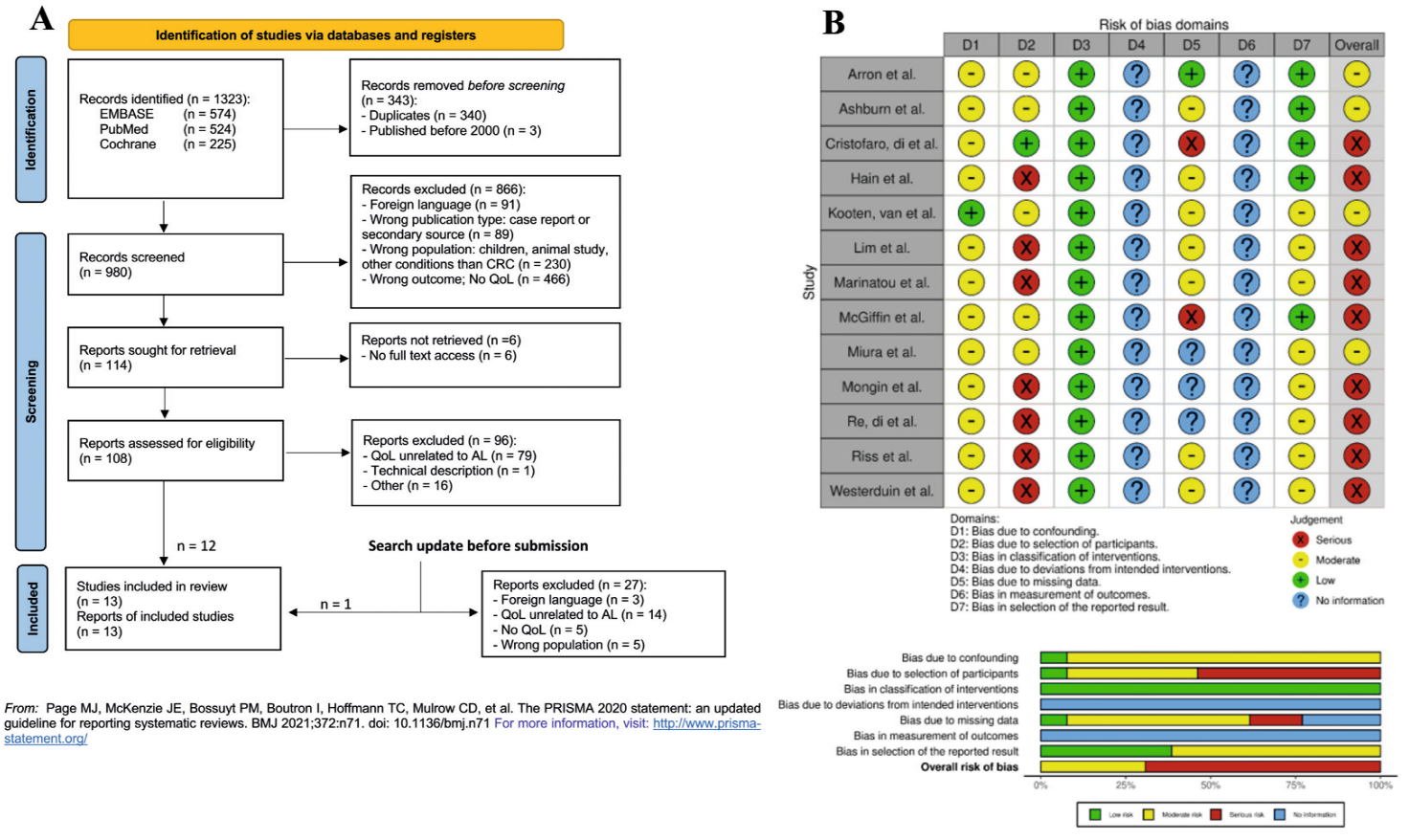
The relevant categories from the ROBINS-I tool were used to assess the risk of bias (Figure 1B). We reported a serious risk of bias in ten studies, primarily attributed to the non-randomized design of these studies [19-21, 23-28] and a moderate risk of bias in the other 4 studies [22, 29-31].

AL characteristics

All details on AL and specific characteristics reported by each study are summarized in Supplementary S3. The reported definitions and diagnostic modalities for AL varied widely among the studies reviewed. Four studies (33%) did not report any specific definition for AL [19, 26, 27, 32]. Furthermore, none of the studies applied the same definition for AL. The severity of AL was assessed using various classifications across the included studies. Four studies applied the International Study Group of Rectal Cancer (ISREC) classification [22, 25, 27, 31], while two studies utilized the Clavien-Dindo classification [19, 24]. Four studies divided AL cases into symptomatic and asymptomatic, or clinical and subclinical manifestation [20, 21, 23, 26]. The other studies did not provide a specific classification or grading of severity of AL. The timeframe in which AL was suspected or diagnosed was reported in four articles, with the latest reporting time being six months after surgery [20, 22, 23, 31]. One study reported biochemical characteristics that might indicate surgical complications [20]. Eight studies (67%) used CT-scan with or without contrast to confirm the diagnosis of AL [20, 21, 23, 24, 26, 29-31]. Four studies reported performing radiological assessment, and subsequent AL assessment, only when clinical symptoms occurred [22, 24, 29, 31]. Three other studies additionally performed routine scanning for AL before ileostomy closure (range 6 weeks – 3 months after surgery) [20, 23, 26]. The type of re-interventions was specified in ten studies [19-27, 31] and ranged from antibiotic treatment to reoperation (laparotomy) with takedown of anastomosis and end-colostomy construction.

Questionnaires

A total of ten validated QoL questionnaires were administered at different time points within the studies. Four validated instruments were administered across the majority of studies (Supplementary S4): The European Organization for Research and Treatment of Cancer Quality of Life Questionnaire (EORTC QLQ) -C30 (Core) and -CR29 (CRC specific), the Short-Form 36 questionnaire (SF-36) encompassing both a physical component (PCS) and a mental component score (MCS), and the Fecal Incontinence QoL (FIQL) questionnaire. Six additional questionnaires were used in only one study (Supplementary S5). These included the Cleveland Global QoL (CGQL), the EORTC IN-PATSAT32 questionnaire for assessing cancer care satisfaction, the Gastrointestinal Quality of Life Index (GIQLI) addressing digestive disorders with both physical and emotional components , the EuroQoL visual analogue scale (EQ-VAS) for patient self-rated health, the Short-Form-12 (SF-12) evaluating health impact on daily life, and the Rotterdam Symptoms Check List (RSCL) questionnaire, which generally evaluates HRQoL.



From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71 For more information, visit: http://www.prisma-statement.org/

Figure 1. (A) PRISMA flowchart outlining study selection strategy. **(B)** Adapted ROBINS-I tool Risk of Bias from included studies.

Quality of Life scores

QoL was evaluated at different time points. Almost all studies compared QoL scores at specific time points between AL and non-AL patients, but not always relative to baseline assessment (Figure 2, Table 3).

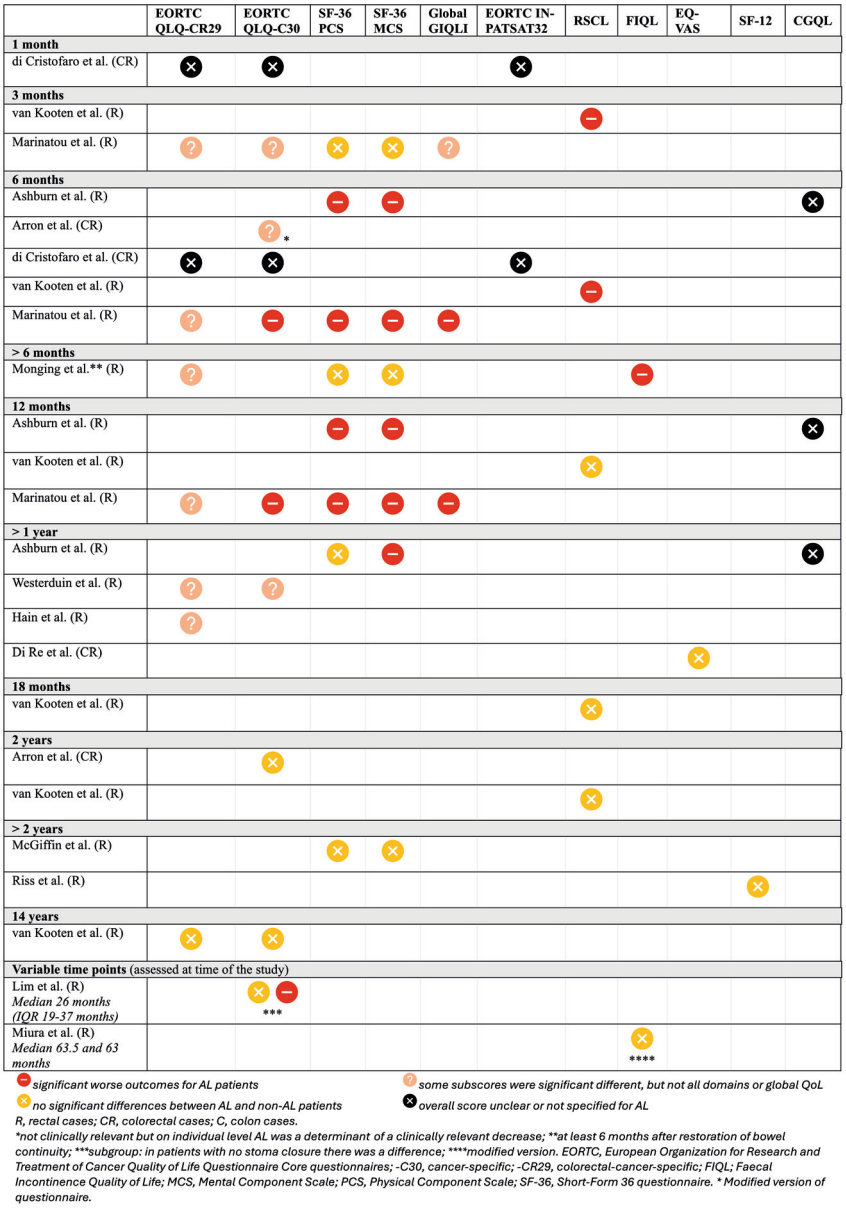


Figure 2. Schematic overview of results in Quality of Life Questionnaires based on different time points.

Table 3. Detailed overview of questionnaires and impaired domains if applicable

Reference	Time of assessment after surgery	Questionnaire	Significant impaired domains *(in favor of non-AL patient)*	P values	Groups compared
Arron et al.	6 months	EORTC QLQ-C30	Summary score*/** Role function Social function Global QoL* Physical function* Emotional function*	p = 0.00 p = 0.00 p = 0.00 p = 0.00 p = 0.01 p = 0.01	AL vs non-AL after colorectal surgery at time of assessment and compared to baseline
			Multivariable linear regression analysis	NS	
	2 years	EORTC QLQ-C30	No significant outcomes	/	
			Multivariable linear regression analysis	NS	
Ashburn et al.	6 months and 1 year	SF-36 PCS	NA	p = 0.01	AL vs non-AL after restorative proctectomy at time of assessment (not compared to baseline)
		SF-36 MCS	NA	p = 0.007	
		CGQL	Unclear	Unclear	
	> 1 year (range 1 – 6 years)	SF-36 PCS	NA	NS	
		SF-36 MCS	NA	p = 0.02	
		CGQL	Unclear	Unclear	
di Cristofaro et al.	1 month	EORTC QLQ-CR29 EORTC QLQ-C30 EORTC IN-PATSAT32	Outcomes of questionnaires not specified for AL patients		Patients without complications vs with complications at time of assessment (not compared to baseline)
			Multivariate analysis for AL: β = 0.42	p < 0.01	
	Six months	EORTC QLQ-CR29 EORTC QLQ-C30 EORTC IN-PATSAT32	Outcomes of questionnaires not specified for AL patients		
			Multivariate analysis for AL: β = 0.52	p = 0.004	

Table 3. Continued

Reference	Time of assessment after surgery	Questionnaire	Significant impaired domains *(in favor of non-AL patient)*	P values	Groups compared
Hain et al.	Variable, at time of the study (Ranging from 1 – 10 years; Mean 46 months ± 26 after restoration of bowel continuity)	EORTC QLQ-CR29	Blood and mucus in stool Frequent bowel movements per day Frequent urination per day	p = 0.045 p = 0.04 p = 0.03	Symptomatic AL patients vs. non-symptomatic and non-AL patients after rectal surgery at time of assessment (no baseline assessment performed)
van Kooten et al.	3, 6, 12, 18 and 24 months	RSCL	Global health Activity level	p < 0.01 p < 0.01	AL vs non-AL after rectal surgery compared to baseline
	14 years	EORTC QLQ-C30	NA	NS	
		EORTC QLQ-CR29	NA	NS	
Lim et al.	Variable, at time of the study (Median 26 months; IQR 19-37 months)	EORTC QLQ-C30	NA (only global score given)	p = 0.03	Clinical leaks without stoma closure compared to subclinical leaks, clinical leaks with stoma closure and non-AL patients after rectal surgery at time of assessment (no baseline assessment performed)

Table 3. Continued

Reference	Time of assessment after surgery	Questionnaire	Significant impaired domains *(in favor of non-AL patient)*	P values	Groups compared
Marinatou et al.	3 months	EORTC QLQ-C30	Physical function	p = 0.008	AL vs non-AL after rectal surgery at time of assessment (not compared to baseline)
		GIQLI	Physical function	p = 0.03	
		EORTC QLQ-CR29	Abdominal and pelvic pain Stoma- related problems Sore skin	p = 0.03 p = 0.03 p = 0.04	
		SF-36	NA	NS	
	6 months	EORTC QLQ-C30	Physical function Global health status/QoL	p = 0.03 p = 0.002	
		GIQLI	Emotional function Physical function Global score	p = 0.008 p = 0.004 p = 0.01	
		EORTC QLQ-CR29	Stoma- related problems Sore skin	p = 0.002 p = 0.03	
		SF-36	Role limitations due to physical health Role limitations due to emotional problems Social functioning General health	p = 0.01 p = 0.02 p = 0.008 p = 0.03	
	12 months	EORTC QLQ-C30	Global health status/QoL	p = 0.004	
		GIQLI	Emotional function Physical function Global score	p = 0.007 p = 0.03 p = 0.005	
		EORTC QLQ-CR29	Sore skin	p = 0.005	
		SF-36	Physical functioning Role limitations due to physical health Role limitations due to emotional problems Social functioning General health	p = 0.04 p = 0.001 p = 0.003 p = 0.009 p = 0.002	

Table 3. Continued

Reference	Time of assessment after surgery	Questionnaire	Significant impaired domains *(in favor of non-AL patient)*	P values	Groups compared
McGiffin et al.	> 2 years (Median 6.4 (IQR 3.1–8.6) and 4 years (IQR 2.7–8.5) for no AL and AL group respectively	SF-36 PCS	NA	NS	AL vs non-AL after rectal surgery at time of assessment (no baseline assessment)
		SF-36 MCS	NA	NS	
Miura et al.	Variable, at time of the study (Median 63.5 and 63 months for no AL and AL group respectively)	mFIQL	NA	NS	AL vs non-AL after rectal surgery at time of assessment (no baseline assessment)
Mongin et al.	> 6 months after restoration of bowel continuity (Median 33 months (IQR 6 – 75) and 30 months (IQR 6-70) for no AL and AL group respectively)	SF-36 PCS	NA	NS	AL vs non-AL after rectal surgery at time of assessment (no baseline assessment)
		SF-36 MCS	NA	NS	
di Re et al.	Variable, at time of the study (Range 1 to > 5 years)	EQ-VAS	NA	NS	AL vs non-AL after colorectal surgery at time of assessment (no baseline assessment)
Riss et al.	Variable, at time of the study (Median 106.8 months (range 32.4-170.4))	SF-12	NA	NS	AL vs non-AL after rectal surgery at time of assessment (no baseline assessment)

Table 3. Continued

Reference	Time of assessment after surgery	Questionnaire	Significant impaired domains *(in favor of non-AL patient)*	P values	Groups compared
Westerduin et al.	> 1 year (Median of 41 months (IQR 23–71) and 27 months (IQR 16–43) for no AL and AL group respectively)	EORTC QLQ-C30	role function	p = 0.049	AL vs non-AL after rectal surgery at time of assessment (no baseline assessment)
			social function	p = 0.006	
			overall global health	p = 0.002	
			fatigue	p = 0.04	
			pain	p = 0.002	
		EORTC QLQ-CR29	body image	p = 0.03	
			anxiety	p = 0.02	
			abdominal pain	p = 0.03	
			buttock pain	p = 0.005	
			flatulence	p = 0.008	
			fecal incontinence	p = 0.03	
			sore skin	p = 0.0007	

**not clinically relevant; [1]Multivariable linear and logistic regression; [2]Wilcoxon rank sum tests; [3]Mann–Whitney U-test; [4]Kruskal–Wallis ANOVA; [5]Multivariate analysis; [6]Univariate and multivariate logistic regression; [7]Wald's tests using linear mixed-effects model and univariable Poisson regression analysis; [7]Paired samples t-test; [8]Statistical analysis unclear. EORTC, European Organization for Research and Treatment of Cancer Quality of Life Questionnaire Core questionnaires; -C30, cancer-specific; -CR29, colorectal-cancer-specific; EQ-VAS, EuroQoL Visual Analogue Scale score; FISI, Fecal Incontinence Severity Index; FIQL, Faecal Incontinence QoL; Gastrointestinal QoL Index; GIQLI, The Gastrointestinal Quality of Life Index; -IN-PATSAT32, patient satisfaction; NA, not applicable; NS, not significant; SF-12, Short-Form-12 health survey; RSCL, Rotterdam Symptom Checklist; QoL, Quality of Life; SF-36, Short-Form 36 questionnaire*

Quality of Life up to six months after surgery

Based on EORTC QLQ-CR29 and -CR30 scores at one and 6 months postoperatively, di Cristofaro et al. identified AL was an independent predictor of lower QoL in multivariate analysis ($p < 0.001$ and $p = 0.004$ respectively) [19]. van Kooten et al. found that patients who developed AL reported a decrease in RSCL global health status and activity level within the first three months compared to preoperative scores, with some improvement at six months [30]. In contrast, Marinatou et al. did not document any improvement based on GIQLI and EORTC QLQ-C30 questionnaires administered at three and six months [24]. Instead, significant decline in physical functioning, global and overall QoL scores were documented among AL patients relative to non-AL patients at six months [24]. Additional results from EORTC QLQ-C29 demonstrated significantly worse scores with respect to pain, stoma and perianal skin-related complaints at three and six months in AL patients. Also, SF-36 scores demonstrated significantly worse function among AL vs non-AL patients at six months, especially along emotional and social domains, which was not seen at three months. Impairment in functional outcomes based on SF-36 scores were also reported by Ashburn et al. among AL patients compared to non-AL patients after proctectomy [29]. Arron et al. demonstrated that the decrease in EORTC QLQ-C30 scores observed among AL patients at 6 months relative to non-AL patients did not meet the threshold for clinical relevance, and AL status was not associated with the observed decrease. Among patients with clinically relevant decrease in their 6 months scores relative to baseline, AL was an independent predictor of this decrease based on multivariate regression analysis [22].

Quality of life at 12 months after surgery

Three studies reported QoL at one year following colorectal cancer resection [24, 30, 33]. van Kooten et al. demonstrated that HRQoL scores returned to baseline preoperative levels among rectal cancer patients with and without complications [30], while Marinatou et al. demonstrated persistently significant differences between AL and non-AL groups for perianal skin soreness and worse overall EORTC QLQ-C30, global GIQLI and SF-36 scores [24]. Ashburn et al. also documented significantly worse SF-36 scores along the PCS and MCS domains at one-year postoperatively in patients with AL compared to those without AL following restorative proctectomy [29].

Beyond one year after surgery

Monging et al., evaluated QoL in patients undergoing restoration of bowel continuity at least 6 months prior to the assessment [26]. Given that median time of QoL assessment was 33 vs 30 months in patients with vs without AL, results were interpreted as representing longer-term QoL. No difference is SF-36 scores were found between the two groups. However, 'blood and mucus in stool' scores of the EORTC QLQ-CR29 indicated significantly worse function in AL patients, as did depression/self-perception FIQL scores. Ashburn et al. noted that although the SF-36 PCS scores did not show significant differences beyond 12 months postoperatively (median 3.2 years), MCS scores were still significant worse in AL patients

after proctectomy [29]. Westerduin et al. identified five domains of the EORTC QLQ-30 as well as two functional and five emotional domains of the -CR29 which were significantly better beyond one year postoperatively in patient with AL compared to the patients without AL [28]. Hain et al. reported additional impaired -CR29 outcomes (more blood and mucus in stool, and frequent bowel movements and urination per day) in patients with symptomatic AL compared to the combined groups of patients with no or asymptomatic AL [23]. Di Re et al. also demonstrated lower mean EQ-VAS scores among patients with AL vs non-AL patients in a matched cohort, at one year after surgery (range up to 5 years), although the difference did not reach statistical significance [21].

At 18 and 24 months postoperatively, van Kooten et al. found no differences in RSCL scores between AL and non-AL patients [30]. Arron et al. found no difference in overall HRQoL scores between AL and non-AL patients at two years relative to baseline EORTC QLQ-C30 scores [22]. Similar results were described when SF-36 scores were compared more than two years after surgery between patients with AL (median of 4 years postoperatively) and without AL (median of 6.4 years postoperatively) [25]. Riss et al. described no significant difference in mental and physical QoL scores measured by the SF-12 questionnaire at a median follow-up time of 106.8 months after rectal surgery (range of 32.5–170.4 months) comparing AL to AL patients from a matched cohort [27].

Two additional studies evaluated longer-term impact of AL on QoL [20, 31]. Lim et al. assessed the EORTC QLQ-C30 in patients without AL, with subclinical leaks, and with clinical leaks with and without ileostomy closure (overall median follow-up time of 26 months; IQR 19-37 months) [20]. They found worse scores in patients with clinical leaks in whom ileostomy reversal was not possible. Miura et al. did not find significant differences in overall modified FIQL scores when comparing AL and non-AL patients at a median time of 63 months after low rectal cancer surgery [31].

Van Kooten et al. conducted a supplementary analysis on EORTC QLQ-C30 and -CR29 outcomes 14 years post-surgery, with no statistically significant differences between AL and non-AL patients [30].

Other outcomes related to QoL

Some additional outcomes that might influence QoL are summarized in Supplementary S6. Neo-adjuvant treatments were described by nine studies [20-24, 26-29], which showed to be significantly different between AL and non-AL patients in one study for chemoradiation therapy and in another study for radiotherapy only [24, 28]. Diverting stoma rates between AL and non-AL patients were compared in six studies [21, 22, 24, 27-29], of which two found significant differences (more diverting in AL group) [27, 29]. Stoma status during follow-up was clearly described by two studies [22, 24], which all showed significant differences between AL and non-AL patients within the first year after surgery. Two additional studies described permanent stoma rates related

to AL [20, 31]. Di Re et al. additionally analysed oncological outcomes as disease free survival at one, three and five years after surgery [21], which were not significantly different between AL and non-AL patients. Overall, there was a lack of comparing type if (re-)interventions.

DISCUSSION

This systematic review appraised and synthesized the evidence on the impact of AL on QoL following oncological colorectal resections. In total, the studies comprised 4596 individual patients, with an overall incidence of AL of 12.4% (N=572). QoL was assessed using ten validated questionnaires administered at postoperative time points ranging from one month to 14 years. Overall, AL was found to negatively impact QoL at 6 and even 12 months postoperatively, with variable degree of subsequent improvement.

The heterogeneity in questionnaires administered and variable times of assessment hindered our data analysis and may account for some of the conflicting results across studies. In a comprehensive systematic review of research studies on QOL and HRQOL, Haraldstad et al. concluded that the majority suffered from conceptual and methodological challenges with no clear consensus on how QoL should be measured. The use of various assessment tools and questionnaires in different studies hinders meaningful comparisons between similar study populations [34]. Adoption of standard set of outcomes for colorectal cancer proposed by the International Consortium for Health Outcomes Measurements (ICHOM) may avoid some of these issues [35]. In this consortium, it was recommended to use the EORTC QLQ-C30 tool to capture overall QoL and the -CR29 to capture colorectal cancer specific outcomes. The optimal time for QoL assessment was also addressed, with recommendations to administer questionnaires at baseline (prior to surgery), 6 months after surgery, and then annually up to 10 years. Our research team suggests following the ICHOM recommendations.

Other patient and treatment variables such as the American Society of Anesthesiology (ASA) score, body mass index, anastomotic height, adjuvant radiotherapy and others that may impact QoL after colorectal cancer resections [36-39]. Only two out of the 13 included studies performed multivariate logistic regression analyses to investigate whether differences in QoL scores observed between AL and non-AL groups were due to the leak, or driven by other factors like neo-adjuvant treatment, surgical procedure, or re-intervention [22, 23]. Ideally, all studies should have performed such an analysis verify if AL is an independent factor that influences QoL. Besides, not all studies comparing outcomes relative to baseline function, which weakens the interpretation of the functional scores at subsequent postoperative time points. As a result, it was difficult to draw valid conclusions comparing the included studies.

The observed decline in QoL scores reported among AL patients in the first six, and even 12 months, may be due to several reasons. AL delays recovery, result in additional postoperative

complications, higher rates of re-intervention, and increase mortality within the first 30 days after surgery [4, 40]. This often prolongs length of hospital stay and adversely impacts mobility and the ability for patients to care for themselves [41-43]. Furthermore, some patients require stoma construction which impairs role and social functioning scores [44]. In the current study, there was a lack of correlation of stoma status and QoL outcomes. One study excluded patients who had a stoma [26], while others did include them but did not draw strong conclusion on any association between stoma formation and QoL scores. AL has also been associated with higher rates of local recurrence and distant metastases in CRC patients [45, 46]. Although smaller cohort studies have not found the same association between AL and colon cancer outcomes, the fear of (local) recurrence as well as additional treatments required to mitigate higher risk of recurrence, may further negatively impact QoL [47, 48]. Moreover, AL has been shown to be an independent risk factor for worse defecatory function (LARS), sexual function after CRC resections [49-52]. Although these functional outcomes were not specifically assessed in the current study, it is crucial to consider their impact on overall QoL [53].

To our knowledge, this is the first systematic review on the effects of AL on QoL in patients undergoing oncological colon and rectal resections. This study has limitations. A high heterogeneity in AL reporting was found in the included articles. It was often unclear what type of intervention and re-operation was performed to manage leaks. Since these elements are important when comparing outcomes, standardizing the reporting and management of leaks would be helpful. Subsequently, some studies only included rectal cancer patients, while other included all types of colorectal surgeries. Secondly, a wide range of QoL questionnaires and timeframes for assessment was used across the different studies. Although only studies using validated instruments were included, the heterogeneity of questionnaires used creates challenges when comparing outcomes across studies and interpreting results. The use of patient-centred methods, like patient-reported outcome measures (PROMS) may be even more informative to gain more insight in overall changes [54]. Due to the heterogeneity of the included studies, comparisons across studies are limited and a meta-analysis was not possible to perform. Lastly, all included studies demonstrated a moderate to serious risk of bias, which results in a low level of evidence and caution is warranted by the presented findings.

CONCLUSIONS

This systematic review demonstrated that QoL of CRC patients may be compromised after AL up to one year, but assessment and reporting of QoL needs to be standardized to draw clear conclusions. In addition to exploring strategies for preventing and effectively managing AL, it is crucial to investigate long-term sequelae on patients' QoL in future research. We recommend incorporating a standardized QoL assessment for CRC patients who have experienced AL and integrating this outcome measure into a core outcome set for research focused on AL in the colorectal field. Continuous assessment and monitoring of QoL in patients undergoing CRC resection is essential to better support for patients throughout their recovery. We emphasize the relevance of uniform reporting of AL outcomes to facilitate comparisons of results in future research. To reach this goal, we advise to follow the proposed questionnaires and timepoint as described by the colorectal cancer ICHOM working group [35] and work on a standardized reporting framework for AL-related research within the CoReAL project.

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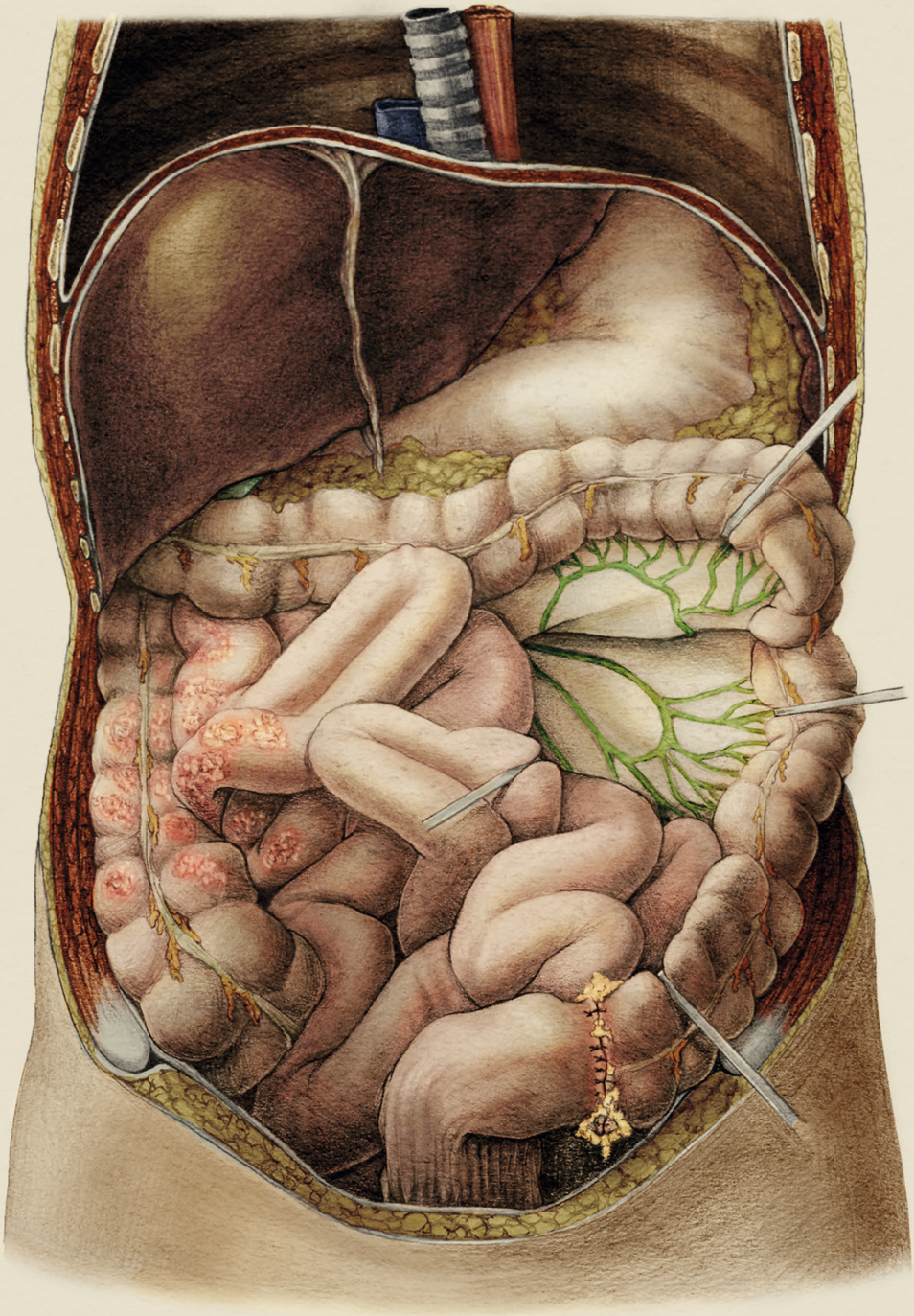
SUPPLEMENTARY

The following supplementary material can be downloaded from:

- *S1. Search Strategy*
- *S2. Additional study information on perioperative care*
- *S3. Characteristics of anastomotic leakage described by the included studies*
- *S4. Detailed information of questionnaires used in more than one study*
- *S5. Links to questionnaires used in only one study*
- *S6. Additional reported outcomes*



CHAPTER 10

THE PATIENT PERSPECTIVE ON COLORECTAL ANASTOMOTIC LEAKS: A QUALITATIVE STUDY

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ABSTRACT

Background. Anastomotic leakage is a well-known complication following colorectal surgery, however, there is limited knowledge about patients live their experiences with leaks across the care continuum, from initial diagnosis and treatment to long-term recovery.

Objective. To explore patients' experiences and perspectives related to an anastomotic leak after colorectal resection, to inform the 'Consensus on Reporting and defining colorectal Anastomotic Leaks (CoReAL)' project

Design. Qualitative descriptive study reported using the COnsolidated criteria for REporting Qualitative research (COREQ).

Settings. Online semi-structured interviews conducted with patients from five countries (Australia, Canada, Netherlands, United Kingdom, and United States).

Participants. Ten participants (six male, median age 53 [range 39 –65 years]) who experienced an anastomotic leak following a colorectal resection.

Main Outcome Measures. Themes emerging from thematic analysis until saturation was reached.

Results. Four main themes were identified: (1) physical impact, (2) emotional impact, (3) coping mechanisms, and (4) anastomotic leak care. Within these themes, participants detailed their lived experience during the different phases of the care continuum: diagnosis, treatment, early and late recovery. Additionally, participants indicated the elements of anastomotic leak care that they deemed most important, including preoperative education, communication, support from medical staff and peers, sharing of information, aftercare, shared decision making, and case management.

Conclusions. Patients having experienced a colorectal anastomotic leak reported a physical and emotional impact, applied different coping strategies, and emphasized the importance of clear communication, comprehensive care, and sustained attention beyond the early postoperative and treatment phase.

Keywords. Anastomotic leakage, qualitative study, participant reported outcomes, colorectal surgery, Burden of disease, semi-structured interviews.

INTRODUCTION

Anastomotic leakage (AL) is one of the most dreaded complications following colorectal resection. The severity of AL varies based on its management, ranging from small defects, which may be managed conservatively with antibiotics and drainage of potential abscesses, to major dehiscence leading to peritonitis and sepsis, requiring reoperation, intensive care unit (ICU) admission, prolonged hospitalization and even death [1, 2]. Moreover, AL often leads to delays in adjuvant therapy and stoma reversal, which negatively impacts oncological, functional and quality of life (QoL) outcomes [3-6].

Despite the extensive evidence on the prevalence, etiology, risk factors, treatment algorithms, and outcomes of colorectal AL, patients' experiences with this complication have not been fully explored in qualitative studies. This approach is essential for capturing the complexities of patients' perspectives, which quantitative methods cannot fully address [7], in the co-design of effective surgical care pathways [8]. Understanding patients' perspectives and experiences throughout this complication will provide insight into optimizing AL care. This includes managing expectations, communicating the diagnosis, deciding on a treatment plan, ensuring patients understand the anticipated outcomes, and providing multidisciplinary support for patients and their families throughout the entire care continuum [3, 9].

The aim of this qualitative study was to explore patients' experiences and perspectives with the diagnosis, treatment, and recovery from a colorectal AL. This preliminary study was conducted with patient partners from the 'Consensus on Reporting and defining colorectal Anastomotic Leaks (CoReAL)' project, an American Society of Colon and Rectal Surgeons (ASCRS) initiative to create a standardized framework for AL reporting after colorectal cancer surgery and served as an informative step for the framework.

METHODS

Study design

This was a qualitative study using semi-structured interviews conducted online from August-September 2023 [10]. This inductive methodology resulted in independence from pre-existing theoretical or philosophical commitments, aligning with our primary aim of describing patients' experiences [11, 12]. This study was reported according to the Consolidated Criteria for Reporting Qualitative Research (COREQ) checklist [13, 14].

Participants and recruitment

Members of the CoReAL collaborative identified eligible patient partners from surgical societies, individual institutions or patient organizations. Adult patients (≥ 18 years old) who had experienced an AL following a colorectal resection for benign or malignant indications,

were eligible to be a CoReAL patient partner. We used a maximum variation sampling strategy, ensuring diversity in age, gender, and the severity of AL based on the International Study Group of Rectal Cancer (ISREC) definition and classification [14]. Severity of AL ranged from small abscesses treated with antibiotics, to peritonitis requiring admission to the ICU. Non-Dutch or non-English speaking patients were excluded. Subsequently, the lead researcher (DH) shared the study details via email and obtained written informed consent. Purposive sampling continued until data saturation was reached, defined as the point at which no new information or themes emerged from the data [15].

Data collection

One-on-one semi-structured interviews were conducted online to ensure the capture of in-depth individual perspectives on specific topics, while also allowing participants the freedom to express any additional information they wished to share. The interview guide (Supplementary S1) was developed by two authors (DH and MK), with input from experts in colorectal surgery, qualitative research and patient-centered research. The guide included three core content areas: diagnosis, treatment, and impact. Participants' demographic characteristics (e.g., age, gender) and clinical data (e.g., type of surgery, AL treatments) were obtained during the interview. All interviews lasted approximately one hour and was conducted by the lead investigator (DH) on an institutional version of Zoom. DH is a female physician who was completing a PhD at the time of the interviews and had undergone formal training in qualitative interviewing with assistance from an experienced qualitative researcher (MK). She had no prior clinical relationship with any of the participants. Verbal informed consent was obtained before commencing each interview. While the interview guide was not pilot tested, it was iteratively refined after three interviews.

Data analysis

The interviews were audio recorded,transcribed verbatim, and deidentified before analysis. Two researchers (DH and MBos) extracted and analyzed the data manually using Braun & Clarke (2006)'s thematic analysis approach [17, 18]. Analysis was data-driven, with no pre-existing coding scheme or theoretical framework applied. The researchers read the first five transcripts and independently coded them by identifying and marking relevant sections within the text and subsequently allocating a code to each section using a qualitative analysis software (ATLAS.ti, Berlin, Germany) [19]. Emotions were described using an emotion/feeling wheel [20, 21]. All codes were compared, and a coding tree was created to inform the analysis of the remaining transcripts (Supplementary S2), which was discussed with the larger team. Any discrepancies in coding were resolved by consensus or consulting the senior researcher (MK). Throughout the analysis process, new codes were iteratively added to the coding tree as appropriate. All coded segments were analyzed, and similar concepts were grouped into themes. Saturation was assessed on an ongoing basis using a saturation grid and considered to have been reached when two consecutive interviews produced no new themes [22]. The

research team comprised members with diverse perspectives (Supplementary S3) to limit the role of preconceptions and pre-study beliefs [23].

RESULTS

Study participants

Ten participants who underwent colorectal resections for benign or malignant conditions were included in the study (Table 1). The participants were from the Netherlands (n=3), Canada (n=1), Australia (n=1), the United States (n=3), and the United Kingdom (n=2). Six were male, with a median age of 53 (range 39-65) years. The wife of one participant (P6) joined the interview. Analysis of the interviews showed that no new codes emerged by the ninth interview. However, one additional interview was analyzed to ensure thematic saturation. AL occurred from 3 days to one month after the index operation. Eight participants underwent percutaneous drainage and 7 underwent a reoperation, including six with a new ostomy. The median time since the AL diagnosis was 2 years (range 4-88 months). Interviews lasted between 30-45 minutes.

Table 1. Participant characteristics

Participant, gender, **age** (years)	**Surgical procedure**	**POD of AL diagnosis**	**Time since AL diagnosis** (months)	**Treatment of AL**	**Outcomes** (at the time of the interview)
P1, F, 45	Ileostomy revision after open procedure (unclear which one)	3 days	24	Reoperation with stoma creation and percutaneous drainage	Not yet medically fit for stoma reversal
P2, F, 64	Laparoscopic sigmoid resection without diverting stoma for metastatic colon cancer	9 days	40	Reoperation with stoma creation, and percutaneous drainage	Permanent stoma, wound complications, and parastomal hernia
P3, F, 39	Ileostomy revision after open subtotal colectomy with diverting ileostomy	± one week	88	IV antibiotics	Recto-vaginal fistula which required another surgery and new ileostomy
P4, M, 42	Colostomy revision after laparoscopic sigmoid resection	3 days	36	Reoperation (without stoma creation), and percutaneous drainage	NA
P5, M, 54	LAR with ileostomy	± 2 weeks	36	Percutaneous drainage	Stoma reversal done, LARS

Table 1. Continued

Participant, gender, age (years)	**Surgical procedure**	**POD of AL diagnosis**	**Time since AL diagnosis** (months)	**Treatment of AL**	**Outcomes** (at the time of the interview)
P6, M, 65	Robot LAR without diverting stoma	6 days	19	Reoperation with stoma creation, and percutaneous drainage	Stoma reversal done, incisional hernia at stoma closure site
P7, M, 63	Laparoscopic colon resection (no other details provided)*	7 days	6	Laparoscopic reoperation with stoma creation, and percutaneous drainage	Stoma reversal planned
P8, M, 51	Robot assisted LAR without diverting stoma	8 days	24	First antibiotics and VAC; after 5 weeks reoperation with ileostomy	Stoma reversal and incisional hernia repair done, colonic stricture
P9, M, 51	Sigmoid resection without diverting stoma*	8 days	4	Reoperation with stoma creation, and percutaneous drainage	Stoma reversal done
P10, F, 55	Ultra LAR without diverting stoma	One month	33	Percutaneous drainage	LARS

**Benign resection, no malignancy. F = female; M = male; LAR, Low Anterior Resection; LARS, low anterior resection syndrome; VAC, vacuum-assisted closure; POD, postoperative day.*

Themes and subthemes

Four main themes were identified in the interviews (Figure 1): (1) physical impact, (2) emotional impact, (3) coping mechanisms, and (4) important elements of AL care. These themes were relevant to participants throughout the AL care continuum (from the time of diagnosis to long-term recovery). Illustrative quotes within each theme are presented in Table 2.

Physical impact

Participants reported a wide range of physical symptoms at initial presentation. Some described feeling *'lethargic, really tired'* (P7) or *'weak'* (P1), others endorsed experiencing '*pelvic*' (P5) or *'stabbing abdominal pain'* (P2). Some participants had a 'fever' (P10), experienced *'loss of appetite'* (P6) or had a *'bloating feeling'* (P4), while others noticed their recovery was not evolving as planned based on a change in their bowel habits or decreased mobility due to pain.

During the treatment phase, percutaneous (transgluteal) drainage was reported as a painful experience, leading to limitations in mobility and daily activities. Additionally, participants faced challenges with narcotic use, both in achieving adequate pain relief and in managing

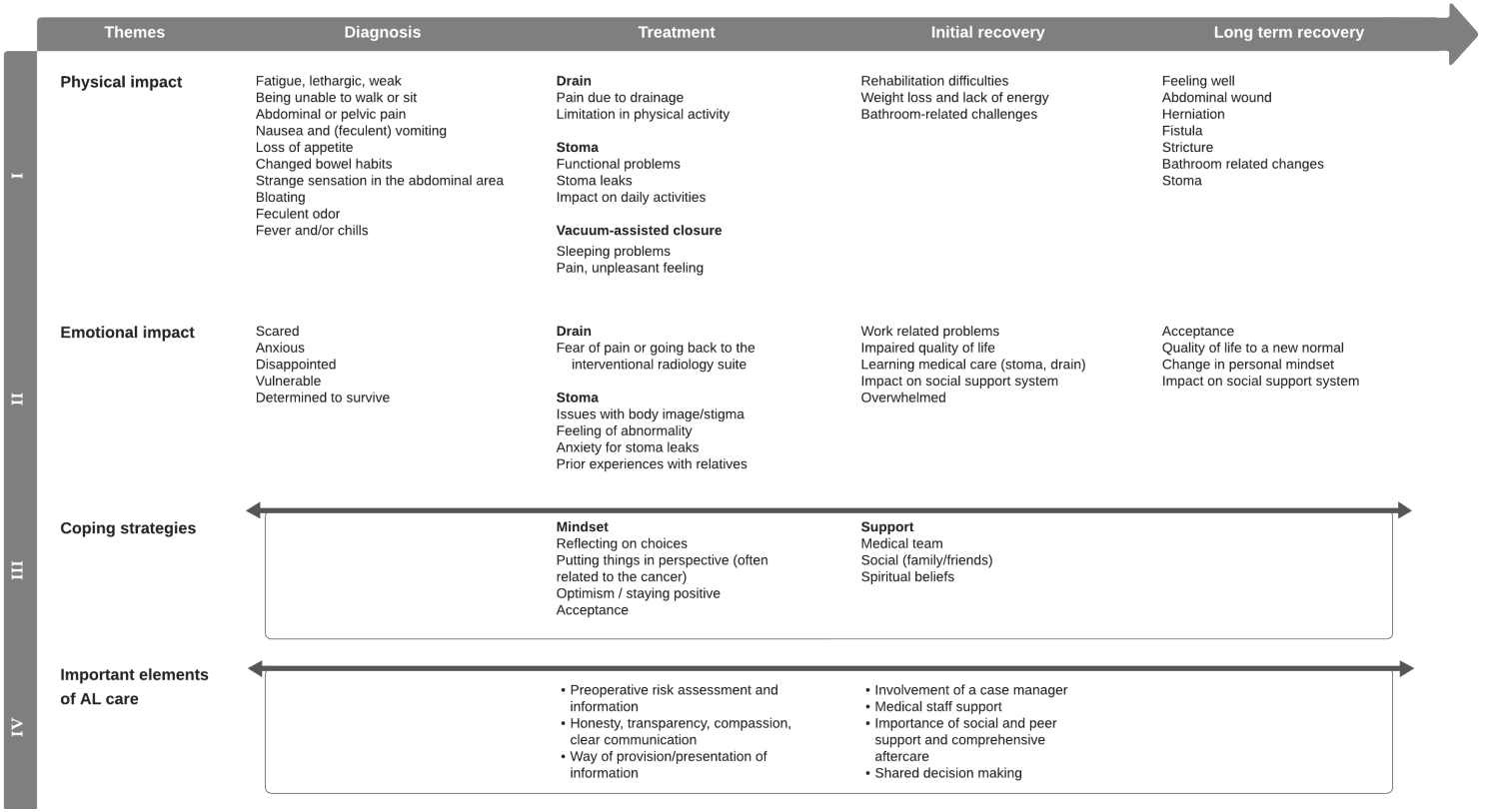

Figure 1. Schematic overview of themes, subthemes and timeline

tapering of medications afterwards. One participant (P8) reported a negative experience with transrectal vacuum-assisted drainage of their AL due to pain, which resulted in difficulty sleeping.

Physical rehabilitation proved challenging as participants struggled with weight loss and a lack of energy, finding it difficult to resume exercise post-illness. While some physical symptoms persisted beyond AL treatment, participants generally expressed having learned to live with and manage the physical symptoms. Ongoing and more long-lasting physical issues included *'abdominal wound problems'* (P2), *'parastomal or incisional herniation'* (P2/6), *'fistula'* (P3), and *'stricture'* (P8), requiring additional interventions. Participants who did not have a stoma created at their index surgery, and those who underwent stoma reversal described experiencing defecatory issues including incontinence, urgency, and incomplete bowel evacuation. These participants were all diagnosed with low anterior resection syndrome (LARS), for which some required educational, medical, or operative management.

Emotional impact

Participants felt fearful, anxious, scared, vulnerable, and disappointed when they were informed that they had developed an AL, with some expressing a sense of determination to overcome the complication. The voiced reasons for these emotions included concerns about having to return to the hospital shortly after being discharged home and experiencing pain. Other participants were concerned about the possibility of requiring a permanent stoma or another surgery, and some expressed concerns about the risk of dying.

Participants reported feeling anxious about the potential for escalating or new pain. Some reported fear associated with going to the bathroom due to diarrhea, obstipation, or pain during defecation. Several participants who underwent cancer resections expressed greater fear of cancer recurrence than of the consequences of an AL. Lastly, some participants expressed a renewed sense of determination to survive and overcome the AL.

Participants who required reoperation for stoma creation described feeling insecure about their body image. Many expressed concerns about their stoma bag leaking, especially when away from their homes. While some participants expressed minimal concern about having a temporary stoma to solve AL, most feared a permanent one. For some participants, this was informed by prior negative functional and emotional experiences reported by family members who had a stoma (P5). Despite having learned how to manage their stomas, many participants opted for stoma reversal, driven by a desire to regain a sense of normalcy. The fear of complications during reversal surgery was generally low, with participants expressing confidence in their surgical team, despite the previous AL complication.

Work-related issues due to the AL diagnosis were also described, primarily delayed return to work. Learning how to manage drains or a stoma added to the physical difficulties. It was

clear that AL initially impacted patients' QoL, but the longer-term impact was generally minor. One participant noted increased anxiety about returning to the hospital in general, which she attributed to her extensive AL treatment (P10). While participants experienced both emotional and physical limitations in the initial recovery period, most participants accepted the situation and adapted.

Coping strategies

Participants described how they navigated the physical and emotional challenges associated with the AL continuum. Many adopted an optimistic outlook, trying to stay positive throughout the diagnosis and treatment despite the pain (P8). Participants emphasized the importance of accepting their 'new normal', putting their disease into perspective, and moving on with their lives. While some found comfort in having undergone curative resection despite the AL, one participant with metastatic disease (P2) noted that fixing the leak did not aid recovery as the cancer persisted. Participants emphasized the crucial role of a support system including surgeons, nurses, stoma specialists, and other related health care professionals, and appreciated receiving information about their ongoing treatment. Support from family, friends, and patient support groups helped with coping. Some participants also found relief in their spiritual beliefs.

Important elements of AL care

Participants were asked to reflect on their experience to identify important elements of AL care of which seven were identified (Figure 1 and illustrative quotes in Table 3).

First, participants felt that sharing all-encompassing and detailed information about possible postoperative complications before surgery was essential. They recommended that healthcare professionals provide patients with more information regarding the symptoms that would raise suspicion for AL in the early postoperative period. This may help patients recognize the signs of AL earlier and seek medical attention sooner. Second, honest, transparent, compassionate, and clear communication during the whole journey was highlighted as a very important factor. Third, some participants felt that the amount of information received and the way it was presented at the time of AL diagnosis was overwhelming. One participant endorsed that the most difficult time for them was during the initial phase in hospital as they felt very ill and were not sleeping well due to the environment (i.e., multiple visits from medical team, equipment alarms). Fourth, the use of case managers throughout the recovery period to help patients navigate the care continuum was emphasized. Fifth, medical staff support was highlighted as very critical during this time. Sixth, participants stressed the importance of adequate nursing and social support. They recommended that patients receive postoperative information and resources for additional social and peer support. Also, the importance of timely referral to stoma and/or physical therapists for comprehensive aftercare was emphasized. Finaly, participants appreciated being involved in the decision-making process regarding management options (shared decision making).

Table 2. Illustrative quotes per theme and timeframe

Theme	Time	Quotes
Physical impact	*Diagnosis*	• *'I felt sort of bubbly, popping feeling, something I've never felt before, so I knew something was wrong.'* - P3 • *'You know, the incontinence and the diarrhea was just out of control and I just felt like I was getting sicker and sicker.'* - P10
	Treatment	• 'I had 5 drains at one point and that was constant going down to radiology and having them drained. Honestly, that was the most difficult part of everything. It wasn't supposed to be painful, but it was. (…) Like at times they'd say, well, we need to go back in, either we need to relocate it, or we need to drain it again, I was already completely panicking. So that was a very difficult part of the illness, very difficult. It was so painful.' - P7 • *'Oh, the drain, it was really painful. I could hardly walk with that. The pain was horrendous.'* – P1 • *'The drain, it was like a dog walking behind me, so annoying. (…) The impact of waking up with a stoma did not affect me that much, but the pain of the drainage did. They say it does not hurt. They said it's just a small puncture, but they should call it ram or crash it in.'* – P2 • *'I did find having a drain extreme. Just going to the toilet and anytime I had to like move (…) irritation on that bag area was very painful. It gave me anxiety. And I just kept wanting it to be gone. You know, it was so painful. It just seemed such a long time.'* – P10 • '*The internal VAC did so much pain. It's stitched to your rectum and the outside of your buttocks. The sound was annoying; the alarm went off all the time, so my wife put a thick blanket over it and multiple pillows, so it wouldn't wake us up. It was terrible.'* – P8
	Recovery phase	• *'I hated my colostomy, but got used to it very quickly in, in some respects, but it still disturbed my sleep. It leaked occasionally and that was always in the middle of the night or somewhere else. I just did not like having this. (…) I did not want to think about the fact it could happen.'* – P5
	Longer term	• *'I'd rather live my life with a permanent stoma, then the thought of having to go through the fistula again.'* – P3 • *'I end up having a hernia greater my right side was greater than 4 inches. My hernia stuck out over 4 inches, so it was a huge complication and it became more and more and impacted my mobility.'* - P8
Emotional impact	*Diagnosis*	• '*Having to go back into hospital when you've just come out is always negative, isn't it?'* – P3 • *'I just wanted the best chances of not having to have another surgery.'* – P5 • *'I was scared to death to get a stoma. I was like, anastomotic leaks who cares, it's just going to heal! That was my mindset. (…) But I was at some point I did become fearful of pain because it was so painful. (…) There was a time I was ready to, I was ready to go to heaven.'* – P8 • *'I was just thinking: okay I'm maybe going to wake up with a stoma, as long as I survive. I just want to survive.'* – P4

Table 2. Continued

Theme	Time	Quotes
	Treatment	• *'I want the rejoin of my stoma. I think it's just to get a bit of body security back, a bit of normality, so I don't have to worry about leaks. It's a bit scary, but I know I'm going to be alright.'* – P1 • *'I mean my mother had ovarian cancer in the 90s, and it metastasized into her bowel. And then she had a permanent colostomy and it really made her miserable. And I think that experience me seeing her go through, that affected me negatively in terms of definitely not wanting a permanent colostomy, so I did not want to have a permanent colostomy.'* – P5 • *'The stoma just has to be managed. I do not have problems. But still I want to feel normal again.'* - P7 • *'The stoma did not bother me in daily activities, it did not affect me that but. But still… It's just nice to not have it; if it can be removed then remove it.'* – P6 • *'I didn't really like the stoma, it was depressing. Uh, and it limited my ability to go outside, do things. At least psychologically. I didn't want to be walking around with a bulge in my T-shirt or whatever. It's just annoying: poop in a bag. There's some, you know, body illusions of a body autonomy that are involved there maybe in terms of what we can control and what we can't.'* - P9
	Recovery phase	• *'Yes it impacted my quality of life cause I couldn't work.'* – P1 • *'Learning all this medical care sucked. These were not really the things that I wanted to learn about or things that I wanted to do. Again, due to my wife's support we made it work. She was all over the Internet looking for information…'* – P9.
	Longer term	• *'I'm a very stoic person. The complication did give me compassion for people that are dealing with chronic diseases. It gave me a whole different viewpoint and I'm actually glad about that.'* – P8 • *'I don't know that it's from the leak itself, but I guess the experience contributed to my anxiety and my anti-anxiety medication now. My anxiety going to any doctor or specialist is really quite high because I'm not a very forceful person by nature, and so advocating for myself when I was so ill was very hard.'* – P10
Coping strategies		• *'It's just staying positive and figuring out what are the best treatment options to stop the leak and how to deal with it. You know, I mean, in hindsight.'* – P8. • *'I was so focused that the tumor was gone and that part of the operation was successful, that I kept on being so positive, maybe too much, despite the fact that I developed a leak'* – P6. • *'Obviously it can turn badly so there was a small part of me that perhaps was thinking that this could be pretty dangerous. But then once I was in the hospital, I felt reassured. I was in the right place with the right people around me. Honesty and transparency of the medical team are so important, and this made me feel safe.'* – P3 • *'I have always felt massively well looked after by my medical team and I was fully informed at all times about what they thought it could be. Therefore, I felt fine, this was my most positive experience.'* – P4 • *'Luckily, my wife was nearby and taking care of me. She helped out, thankfully. (…) I prayed and read my Bible and all that it was at that point of my thoughts'* – P9

Table 3. Illustrative quotes on important elements of AL care (theme 4)

Quotes
• *'I went into the surgery knowing what the outcomes were and so my risk assessment and the honesty of the team was correct. It's important to let participants know what possible side effects are there. I don't feel like I didn't know any of the possible side effects.'* – P5
• *'What's really important is the awareness of potential symptoms that can occur when developing a leak like preoperative, that is important somebody knows. (…) It's not necessarily the gravity of a leak they should talk about, but the symptoms involved with it so a participant knows where to look at'.* – P9
• *'I know that not everybody has the same sort of communication level and care that I've had. So that is an important thing for me; be honest, transparent and just tell and inform the participants.'* - P3
• *'Sometimes it's overwhelming. If someone doesn't have a relative or somebody that's advocating for them, that it could be very confusing. (…) The hottest spot is that when you're really sick, and they come in the morning, the surgical team starts talking to you and they start explaining things to you while you're too sick. That part of it is kind of tough to keep track of where you are day-to-day. I was lucky to have my wife came in every day for 30 days and I was lucky to have her to kind of get the right information and make the right decisions as we went along.'* – P7
• *'For participants it's way easier to understand and follow everything if there is the same person in front of them instead of completely different people saying other things, and to make sure to talk to family as well if possible.'* - P7
• *'I liked the fact that I was included in decision making, treatment options and available evidence.'* – P4

DISCUSSION

This qualitative study highlighted perspectives of patients who developed AL following a colorectal resection, providing an in-depth understanding of the experiences they lived, from the initial diagnosis to long-term recovery. Four main themes were identified: physical impact, emotional impact, coping mechanisms, and important elements of AL care. Participants' experiences with the development of AL varied based on the phase in the AL care continuum, with initial treatment and early recovery described as the most impactful.

In this study, the effect of AL on patients' physical status was evident at every stage of the journey. A prominent impact of AL that emerged was pain, whereby participants experienced significant procedure-related pain such as percutaneous drainage and reported difficulties managing their pain medication in the early phases. It has been described that transgluteal drainage can be particularly painful, necessitating appropriate analgesia to ensure patient tolerance [24, 25]. Although participants stated that the physical impact of AL was less prominent in the long-term phase, they reported chronic sequelae, including abdominal wound complications, stoma herniation, fistula, and stricture formation. Additionally, patients reported functional problems, such as incontinence and LARS. These issues, build on literature, affirm close follow-up, and extended care in participants with AL, well beyond the early postoperative phase [26]. The negative emotional impact of AL observed in

our study corroborate findings from a cohort study (1,197 patients), where patients with colorectal AL experienced a clinically significant reduction in quality of life (QoL) at 6 months relative to baseline, while long-term QoL scores were similar to patients without an AL [3]. This emphasizes the necessity for increased support for patients during the initial phase of AL and up to 6 months post-diagnosis. Additionally, our participants expressed concerns about adjusting to a stoma. Despite prior studies demonstrating good QoL scores in older patients living with a stoma [27], the stigma and disability of stomas are well documented[28]. The interviews highlighted the different ways participants coped with AL using their support network and developing resilience. Various studies indicated that interventions aimed at enhancing optimism, social support, and active coping strategies like acceptance and positive reappraisal, could foster positive changes in the aftermath and enhance QoL [29, 30]. The identified coping mechanisms may explain why participants experienced minimal disruption in their daily lives in the longer-term period. The key aspects of AL care identified by our participants emphasized the importance of social support, patient- and family-centered care, including communication strategies and shared decision-making, and approaches such as nurse-led case management. These elements have all been shown to improve patient outcomes in general, particularly psychological well-being[31-39]. We developed five clinically applicable recommendations, displayed in Table 4, to apply the findings of this study in improving perioperative care for colorectal patients at risk of an AL.

Table 4. Recommendations for perioperative care

1.	Complete a preoperative risk assessment and provide clear and realistic information regarding AL (including associated signs and symptoms);
2.	Apply a patient- and family-centered care approach (including clear communication, information provision, and shared-decision making);
3.	Make timely referrals to supportive care (e.g. referrals to a stoma therapists, physio therapists, wound care specialists, and social work);
4.	Emphasize the importance of streamlining information (not too many people involved in the treatment plan);
5.	Optimize pain management, particularly as it relates to the need for further interventions like percutaneous drain management.

The findings of this study were used to inform the ASCRS' CoReAL project, which aims to create an evidence-, patient- and expert-informed standardized framework for reporting colorectal AL [40]. The CoReAL framework includes the reporting of AL-related factors at different stages: preoperative, intraoperative, and postoperative short-term (<90 days) and long-term (> 90 days). Based on the current work, we integrated specific patient-identified reporting elements into the framework. In the preoperative phase, patients emphasized the importance of risk assessment and the need for clear information about the risk of AL. This is reflected in the CoReAL framework where all modifiable (obesity, smoking, albumin levels and alcohol consumption) and non-modifiable risk factors for AL (tumor characteristics and comorbidities) were compiled as preoperative reporting elements. We recommend assessing these factors preoperatively and providing a detailed individualized preoperative

risk assessment for AL, including a comprehensive discussion with each patient to ensure they are fully informed. Lack of preparation for a potential stoma was another issue identified in our study. As a result, two stoma-related reporting elements were included in the preoperative assessment: (1) whether potential need for a postoperative or permanent stoma was discussed, and (2) whether the patient received preoperative education about a possible stoma. To better capture the occurrence and impact of short- and long-term sequelae of AL, we included the reporting of all complications related to AL (e.g., hernia, stricture, fistula, stoma complications) in the early and late phases of the framework, respectively. Finally, to address the impact of AL on function and QoL, we integrated the LARS score [41], Wexner Fecal Incontinence Score [42], and QoL measures in the early and long-term phases of the framework. The framework includes follow-up on all emotional and physical outcomes for at least one year.

Strengths, limitations and future perspectives

We used a robust qualitative approach to gain insights into the physical and emotional burdens experienced by patients with an AL. While this study included a diverse group of participants, highlighting a range of perspectives, the number of participants included was small. However, unlike quantitative studies, qualitative studies do not aim for generalizability based on sample size but strive for plausibility based on thematic saturation. The researchers' comprehension and interpretation of the data could have influenced the results. To mitigate this, two trained researchers independently coded the data and themes were discussed with the larger research team. The possibility of socially desirable responses regarding treatment satisfaction or the omission of sensitive issues cannot be ruled out. Nevertheless, interviews were conducted without prior clinical associations with participants. Lastly, respondent validation (i.e., member checking) was not performed, but the rigorous application of thematic analysis ensured that interpretations remained closely aligned with participants' perspectives as expressed in the interviews.

This initial exploration could guide future research involving a larger, more diverse patient population worldwide. Within future research and the use of the CoReAL framework, it may also be beneficial to compare patient perceptions according to the grade of AL [43]. Yet, this study may serve as a foundation for creating a universally accepted questionnaire for patient-reported outcomes (PROs).

CONCLUSIONS

This study provides multidimensional and novel insights into the lived experiences of patients with anastomotic leakage. The findings highlight the physical and emotional impact of AL, as well as identified coping strategies, emphasizing clear communication, comprehensive care, and sustained attention beyond the early postoperative and treatment phase. These findings highlight the broad impact of AL and its treatment on patients' lives and the importance to raise more awareness among clinicians, urging them to consider patients' experiences and values when making AL treatment decisions. The findings could inform future larger research and the development of a patient-reported outcome measure to systematically assess AL-related outcomes in future research and clinical practice.

CONTRIBUTORS, ACKNOWLEDGEMENTS AND STATEMENTS

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SUPPLEMENTARY

S1. *Interview guide*

We've invited you for this interview because you have undergone a colorectal operation with an anastomosis, after which you developed an anastomotic leak. Therefore, we would like to ask you some questions around this event.

Introduction: general questions
- Would you please briefly introduce yourselves? When you were diagnosed with colorectal cancer and what kind of operation you got?
- Can you tell me something about your experience around your operation?
- Where you informed by your health care professional (specialist nurse or medical specialist) about the risk of any complications?

Diagnosis
- What complains or symptoms did you have after the operation/around the time of the leak, how did you feel?
- What did the medical team do to diagnose and confirm your AL? How did you experience this?
- What were your feeling or expectations after you heard you developed a leak?

Treatment
- Did you undergo a re-intervention for the leak? If yes, can you tell me how you've experienced this.
  - Did the treatment went well? Did you still had complains afterwards?
  - Did the treatment influence your daily life?
  - What do you feel was the most positive aspect of the treatment? Did you also have any negative experiences?
- If applicable:
  - How did you feel about having a stoma? / How do you feel about having a stoma?
  - How did you feel about having a drain? / How do you feel about having a drain?

Impact
- How are you doing now?
  - Do you still experience anything in relation to the anastomotic leakage or the treatment of it? Do you still have any complains? How do these complains influence your daily life? / Do you feel hindered in daily activities by these symptoms?
- Do you feel that your general quality of life has changed due to the anastomotic leak and the treatment this? If so, how do you notice?
- To what extent are you happy with how the postoperative phase after the anastomotic leakage went? What are you most disappointed about? And most satisfied?
- In the future we want to be able to evaluate the most important outcomes and aspects of anastomotic leakage to get a better understanding of the impact, your wellbeing and health status. Based on your experience what aspects and outcomes would be necessary to discuss with your doctor?
  - If a participant is going to the hospital after the development of an anastomotic leakage, what are for you the most important items to discuss with your doctor? What is the most important to you?

End
- Are there any issues that we haven't discussed that you would like to tell me about?
- Do you have any final questions?

S2. *Coding tree*

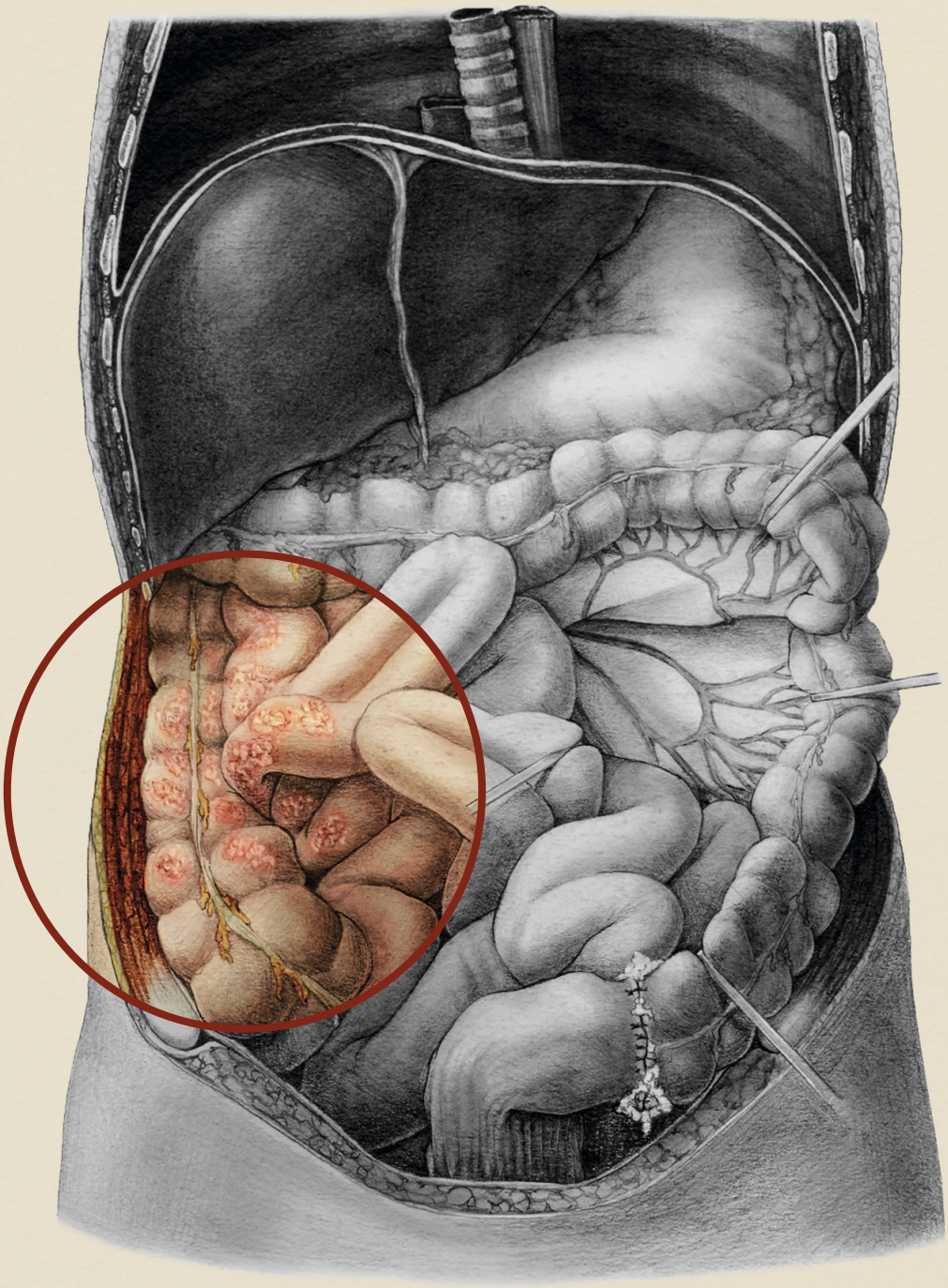
1. Experiences in regards to anastomotic leaks
    a. Participant experience
        i. Physical experience
            1. Symptoms due to AL
                a. Feeling weak, tired and lethargic
                b. Being unable to walk or sit down
                c. Loss of appetite
                d. Experiencing cold and shaking
                e. Diarrhoe / High output followed by no output i.c. stoma
                f. Bloating
                g. Nausea and (fecal) vomiting
                h. Abdominal pain
                i. Feeling of dying
                    i. Not afraid of dying anymore
            2. Complications *(i.e. physical consequences)*
                a. Fistula
                b. Persisting herniations
                c. Persisting incontinence, diarrhea, hardly sleeping
                d. Anal stricture
            3. No complaints anymore
        ii. Mental experiences
            1. Anxiety
                a. For pain
                b. For going back to the hospital / seeing a HCP
                c. For surgery resulting from leak
                    i. Confidence due to prior surgeries
                    ii. (Blinding) optimism
                c. For recurrence
                d. For death
            2. Acceptance *(after some time)*
                a. Disappointment not getting life back as it was
    b. Informal caregiver experience
        i. Negative emotional impact *(i.e. trauma)*

2. Experiences in regards to stoma
    a. Negative experiences
        i. Impact on daily life
            1. Frequent leakages
            2. Dietary limitations
        ii. Stigma with stoma
            1. Body security/normality
            2. Prior experiences with family members
        iii. Emotional impact
            1. (Bathroom) anxiety
            2. Gaining continence after rejoint
    b. Positive experiences
        i. Satisfied with stoma
            1. Being able to sport
            2. Being able to work
            3. Being able to travel
            4. Feeling relieved of getting another stoma *(knowing what to expect)*
    c. Deliberations on rejoints *(linked with negative experiences in regards to stoma)*
        i. Looking back: was it worth it?

3. Experiences in regards to drain
    a. Pain due to drainage
4. Experiences in regards to care
    a. Pre-surgery
        i. Risk deliberation
            1. Focus on removal of cancer and possible complications
    b. Post-surgery
        i. Aid in tapering pain medication
        ii. Rehabilitation
            1. Limitations in ADL
            2. Mobility
        iii. Work related problems
        iv. Peer contact
    c. Continuity in staff (*i.e. medical team consistency)*
        i. Availability of healthcare professionals
    d. Positive attributes of health care professionals
        i. Honesty and transparency
            1. Feeling acknowledged and listened to
        ii. Compassion and caring
        iii. Education on stoma and/or drain
            1. Support by visualisations
        iv. Importance of informal caregivers
        v. Using Shared decision making techniques
        vi. Affirmation of participants' positive attributes

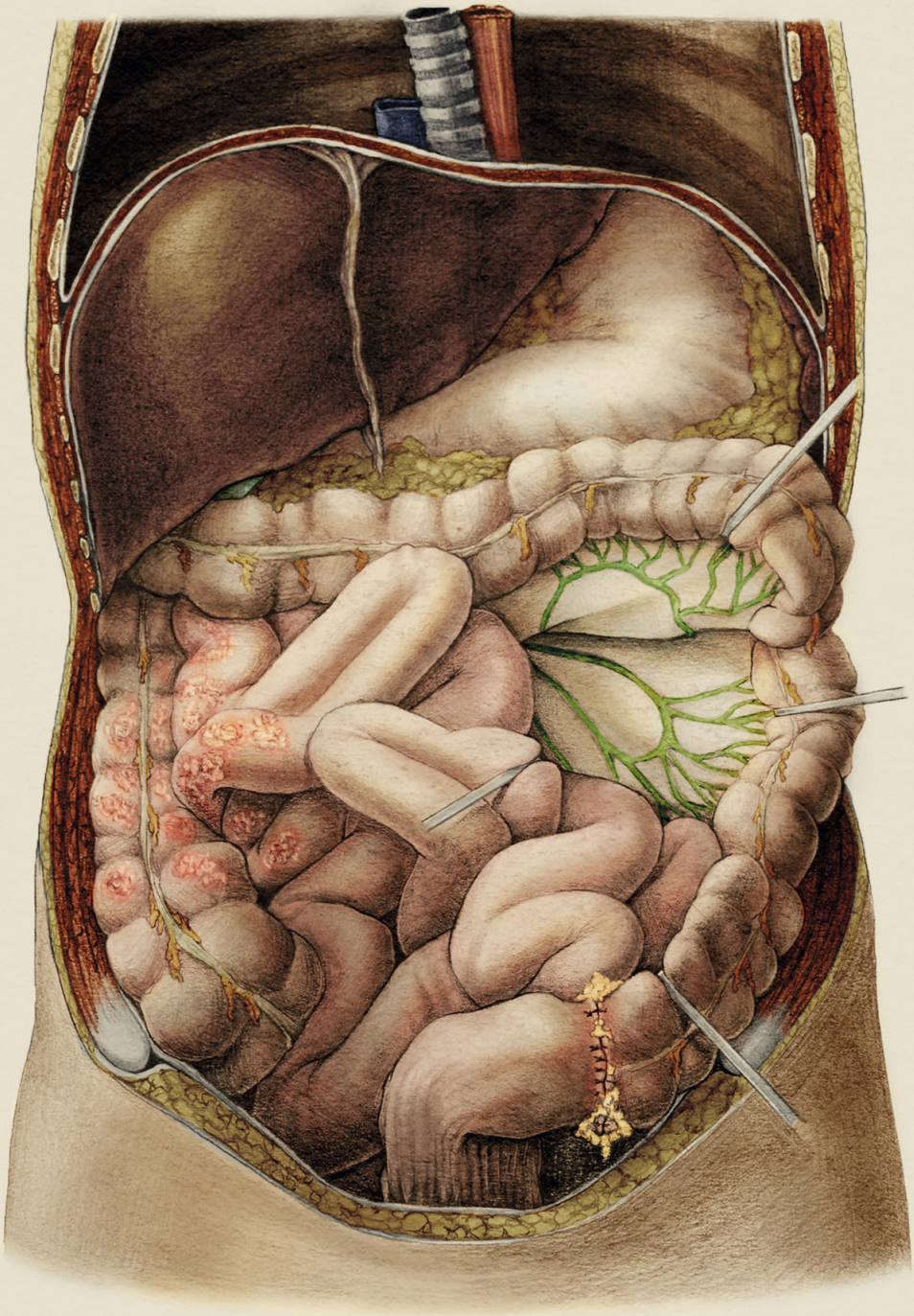
S3. *Reflexivity details*

DH and AG are female medical doctors and PhD students at the department of Surgery, focusing on improving outcomes after CRC surgery. MBos is a male medical doctor who has worked in an emergency department as general physician and holds a master's degree in healthcare policy, innovation, and management. He pursues a PhD focusing on active participant participation in education and self-management support. O.M. is a female medical doctor, working as a surgical resident, and completed a Master of Science in Epidemiology with experience in qualitative research. JF is male medical doctor and associate professor with postdoctoral experience in qualitative research. MK works as a senior researcher within the department of clinical epidemiology and medical technology assessment (KEMTA) after obtaining a master in Health Sciences (Health Policy, Economics and Management) and a PhD in Health Technology Assessment at Maastricht University. MBou is a colorectal surgeon and heads the JGH Colon and Rectal Surgery Research Program, and multiple projects focused on innovation and outcome research. NF is a professor of surgery and conduced an educational PhD in assessment of surgical skills. PS is professor of surgery and System Chief of the Division of Colon and Rectal Surgery at the Mount Sinai Health System in New York City. NB is a professor of innovative surgery and has conducted a PhD into metabolic and oncological consequences of laparoscopic surgery. MBou, NF, PS and NB are principal investigators of the Consensus on defining and Reporting colorectal Anastomotic Leaks (CoReAL) projects, of which this study is part. All members of the collaborative group are surgical experts or surgical researchers on AL.



PART IV

PREVENTION OF METACHRONOUS PERITONEAL METASTASES AFTER COLORECTAL CANCER SURGERY



CHAPTER 11

DNA AND RNA ALTERATIONS ASSOCIATED WITH COLORECTAL PERITONEAL METASTASES: A SYSTEMATIC REVIEW

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ABSTRACT

Background. As colorectal cancer (CRC) patients with peritoneal metastases (PM) have a poor prognosis, new treatment options are currently being investigated for CRC patients. Specific biomarkers in the primary tumor could serve as a prediction tool to estimate the risk of distant metastatic spread. This would help identify patients eligible for early treatment.

Aim. To give an overview of previously studied DNA and RNA alterations in the primary tumor correlated to colorectal PM and investigate which gene mutations should be further studied.

Methods. A systematic review of all published studies reporting genomic analyses on the primary tissue of CRC tumors in relation to PM was undertaken according to PRISMA guidelines.

Results. Overall, 32 studies with 18,906 patients were included. *BRAF* mutations were analyzed in 17 articles, of which 10 found a significant association with PM. For all other reported genes, no association with PM was found. Two analyses with broader cancer panels did not reveal any new biomarkers.

Conclusion. An association of specific biomarkers in the primary tumors of CRC patients with metastatic spread into peritoneum could not be proven. The role of *BRAF* mutations should be further investigated. In addition, studies searching for potential novel biomarkers are still required.

Keywords: Biomarkers; colorectal cancer; genetic mutations; peritoneal metastases; systematic review

INTRODUCTION

Colorectal cancer (CRC) is the third most prevalent type of cancer worldwide and a common cause of morbidity and mortality, which is generally attributable to metastatic disease [1, 2]. At initial diagnosis, almost one-fourth of patients with CRC present with synchronous metastases [2, 3]. Liver metastases (LM) occur most frequently, followed by peritoneal metastases (PM) [2, 4]. Colorectal PM are found in 5–15% of patients at primary diagnosis (synchronous PM) [2, 4-6]]. One can also develop PM after curative resection of the primary tumor (metachronous PM), usually within the first 3 years after the primary diagnosis [3]. Metachronous PM are reported in 4–12% of colon cancer patients and in 2–19% of rectal cancer patients [4, 6]. However, the true incidence of PM might be underestimated. The preoperative diagnosis is mostly made by CT scan, but this has limited diagnostic accuracy for the assessment of the extent of PM [2, 6, 7].

CRC patients with PM have a poor prognosis. Currently, the only potentially life-prolonging treatment option involves surgical debulking of all visible metastases (cytoreductive surgery; CRS) followed by Hyperthermic Intraperitoneal Chemotherapy (HIPEC). Only a highly selected group of patients are eligible for this intervention. Patients with a poor physical condition and/or a too extensive metastatic disease are generally excluded and will undergo palliative systemic treatment or best supportive care only [2, 8, 9]. Without any treatment, the average life expectancy is 6 to 12 months after diagnosis [5, 8, 10].

Recently, research has been ongoing to develop new treatment options for locally advanced CRC patients [11]. Since these new treatment techniques could be invasive to a certain degree and be expensive, it would not be desirable to implement these routinely for all patients. A diagnostic tool able to identify patients who are at high risk of developing metachronous PM would allow targeted treatment in a preventive and/or curative setting [2]. According to previous research, a molecular profile of the primary tumor might help identify patients who are at high risk. It is hypothesized that specific biomarkers identified in the primary tumor can be incorporated in a prediction tool to estimate the risk of distant metastatic spread [12, 13]. In patients with synchronous PM, genetic alterations could be interesting to determine prognosis or to predict response to therapy.

It is known that several pathogenic mutations occur during adenoma-to-carcinoma transformation in CRC. Important oncogenes are *adenomatous polyposis coli* (*APC*), *tumor suppressor gene TP53*, *Kirsten rat sarcoma virus* (*KRAS*), *transforming growth factor beta* (*TGF-β*), and *phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha* (*PIK3CA*) [14, 15]. Recent data suggest mutations may also affect the metastatic dissemination of tumors [16]. Different omics techniques, such as genomics (e.g., next-generation sequencing (NGS), polymerase chain reaction (PCR), pyrosequencing (PS), Sanger sequencing (SS)) and transcriptomics (e.g., NGS), could be used to elucidate DNA markers and RNA transcripts, respectively. Furthermore, individual omics techniques can be integrated into multi-omics

analyses, which capture the complexity of diseases on multiple levels. As sequencing technologies have become less expensive, tumor genotyping has become standard practice for metastatic CRC (mCRC) [14, 16]. As a result, clinicians now often have information on the mutational status of several oncogenes, and investigating molecular changes in primary tumors concerning metastatic potential is becoming more common [16, 17]. We hypothesize that specific biomarkers, based on DNA/RNA alterations identified in the primary tumor, might characterize colorectal PM patients. Once identified, these alterations can be incorporated into a prediction tool to estimate the risk of PM development, prognosis, and be helpful in choosing the appropriate treatment options [12, 13].

In this paper, the authors aim to systematically review the available literature to: (1) create an overview of previously investigated DNA and RNA alterations in the primary tumor correlated to colorectal PM and (2) investigate which gene mutations are of potential biomarker value and should be further studied. This study focuses solely on CRC (stages I–IV) and does not include other types of neoplasms.

METHODS

Study Protocol and Registration

This systematic review was conducted and reported according to the guidelines of the "Preferred Reporting Items for Systematic Reviews and Meta-Analyses" (PRISMA) [18]. The study protocol was registered at PROSPERO (registration number CRD42021297366).

Search and Information Sources

A literature search was performed on the 6 January 2022 and repeated before submission on the 3rd of November 2022. PubMed, Embase, the Cochrane Library, and CINAHL Database were searched with the use of MeSH-, Emtree-, and free terms including ''colorectal neoplasms'', ''peritoneal neoplasms'', ''mutations'', ''genetic testing'', ''genetic association studies'', ''gene expression profiling'' and ''biomarkers, tumor'' and additional search terms such as ''colorectal'', ''adenocarcinoma'', ''carcinomatosis'' and ''predictive biomarker''. The full search strategy is displayed in Appendix A. A professional clinical librarian was involved to ensure an appropriate search strategy. Reference lists of all relevant publications were hand-searched for additional studies. This method of cross-referencing was continued until no further relevant publications were identified.

Selection Process

Inclusion and Exclusion Criteria

Articles containing original data concerning genomic analyses on patients with CRC and PM were considered eligible. The primary outcome measure was specific mutations on the DNA or RNA level in the primary colorectal tumor that might be associated with PM. Studies were

excluded if the tumor samples were not from primary tumor tissue origin or if the researchers only investigated metastases other than peritoneal ones. The method of genomic analysis was not a criterion for exclusion. Secondary sources such as technical descriptions, letters to the editor, conference proceedings, and commentaries were not considered. Only articles in English, Dutch, French, Italian, or German were eligible.

Study Selection

All search results were imported into a free web tool designed for systematic reviewers (Rayyan) [19]. All duplicates were removed. The screening of studies for eligibility was performed by two reviewers (DH, JL) independently, using the predefined inclusion and exclusion criteria. First, articles were screened based on title and abstract. Disagreements between reviewers were resolved by initial discussion to create consensus. If the eligibility criteria were met after full-text screening by both reviewers, article inclusion followed. All references were stored in the Endnote Reference Management Tool.

Data Items and Collection Process

Two reviewers (DH, JL) independently extracted data from the text, tables, and figures in a standardized, predefined datasheet. Data extraction for each article included first author, year of publication, country, study design, study period, inclusion and exclusion criteria, aim of the study, number of patients and genes, general patient information, methods of genomic analysis, methods of tissue collection and sample information, and outcome of genetic analysis. Data acquired via the outlined search strategy are summarized in tables.

Study Risk of Bias Assessment

To assess the validity of the included studies, the bias risk was assessed independently by two reviewers (DH, JL). Since there is no standard bias assessment tool for the type of included studies, a suitable tool was designed based on the Risk of Bias using the Quality In Prognosis Studies (QUIPS) tool. All types of bias were evaluated and judged as low, moderate, or high risk.

RESULTS

Study Selection

The electronic search yielded 1751 articles after removing duplicates. After abstract reading, 64 potentially eligible articles remained, based on the predefined inclusion and exclusion criteria. Full-text assessment from ten articles was not possible (e.g., language restrictions, congress submissions), whereafter 54 articles remained eligible. Reference checking resulted in one additional study, attaining 55 articles for full-text assessment. As 23 articles did not fulfill inclusion criteria, 32 studies were included for final analysis. No additional publications were identified after repeating the search before submission. The study selection process is summarized in Figure 1.

Study Characteristics

All 32 studies are observational cohort or case control studies published between 2008 and 2021. The number of subjects per study ranged from 15 to 5967, with a total of 18,906 patients. The main characteristics of the included studies are summarized in Table 1. In 21 studies, the tissue samples were retrieved retrospectively [20-40]. Two studies collected tissue samples at time of surgical resection [41, 42], and in nine studies, there was no need for tissue collection because the mutation status of genes of interest had already been analyzed as part of diagnostic reasons [43-51]. Most tissue samples used in the studies (*n* = 24) were formalin-fixed paraffin-embedded (FFPE), while the remaining eight studies used fresh frozen tumor samples [22, 31, 32, 34, 38, 40-42]. All characteristics of patients and tissue samples are summarized in Table 2. Only two articles reported the time of PM occurrence, i.e., metachronous or synchronous metastases [23, 44]. All other studies did not specify the time of onset of PM or only included synchronous metastases. Because of the heterogeneity among the included studies in terms of the study population, genetic analyses methods, level of genetic testing, and (number of) genes, pooling in a meta-analysis was not possible.

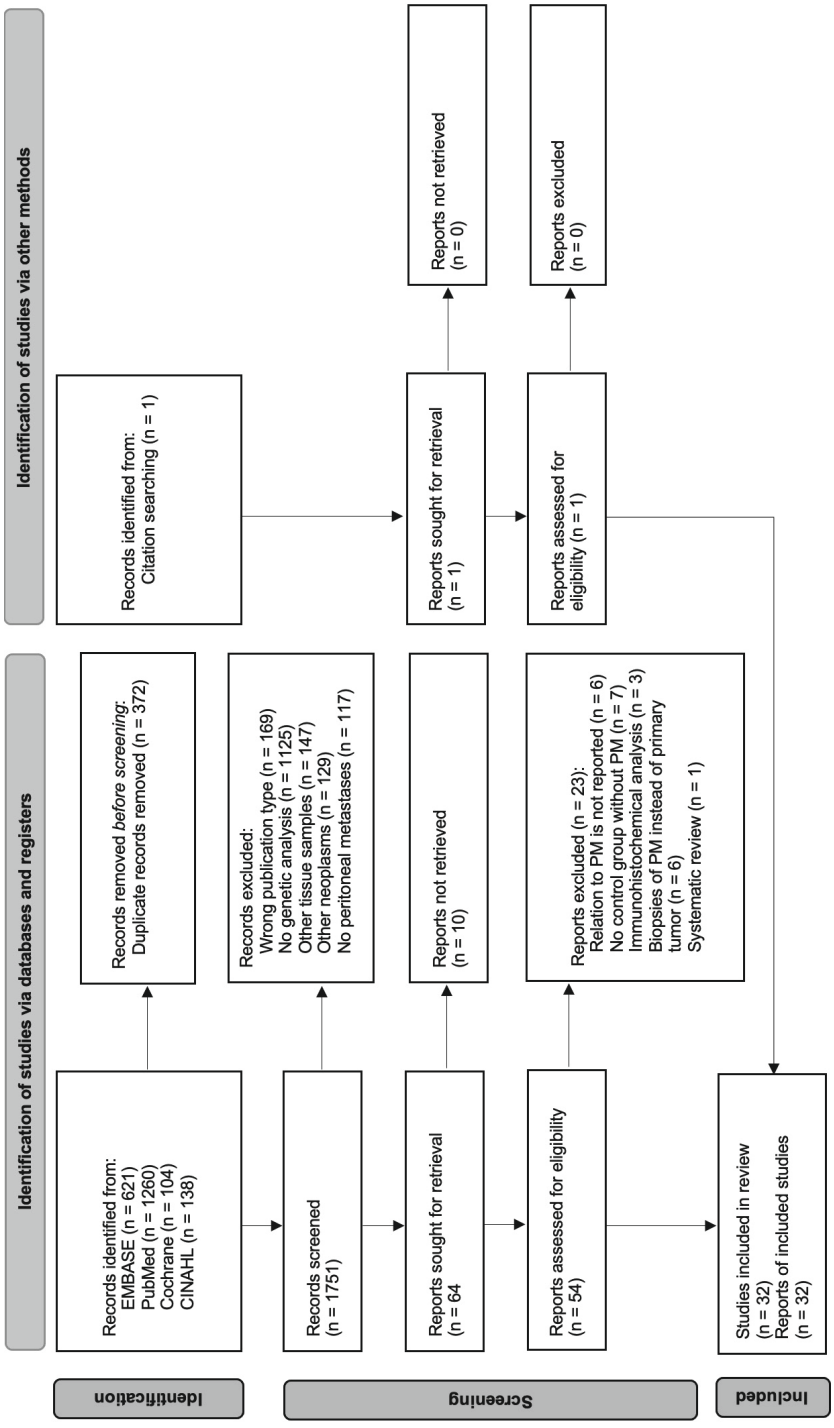


Figure 1. PRISMA flowchart outlining study selection strategy [18].

Table 1. Characteristics of included

Reference	Year	Country	Study Design	Patient Inclusion Period		Reasons for Patient Exclusion	No. of Subjects	No. of Genes Investigated	Aim of the Study
				Start	End				
Astrosini et al. [20]	2008	Germany	CH	-	-	-	63	1	To investigate if *REG1A* is upregulated in CRC patients with unfavorable clinical outcome.
Atreya et al. [43]	2016	USA	MCH	01/2013	09/2015	-	120	1	To investigate if *BRAF* V600E mutation is associated with sites and radiographic appearance of metastatic disease in patients matched by primary tumor location.
Bruzzi et al. [21]	2019	France	CH	12/2005	11/2009	-	1650	2	To assess recurrence patterns according to microsatellite instability, RAS and *BRAF*V600E status in stage III.
Cheng et al. [22]	2018	Taiwan	CH	2000	2013	History of other malignancies, inflammatory bowel disease or death within 30 days after surgery.	1969	1	To evaluate clinicopathological features, metastatic patterns, and prognostic value of CRC with the *BRAF*V600E mutation.
Christensen et al. [44]	2018	Denmark	CH	01/2005	08/2008	Presence of other active tumors and no tissue sample or medical charts available.	448	3	To investigate associations between mutations and pattern of metastases.
He et al. [23]	2020	China	CH	12/2015	02/2020	Non-metastatic synchronous CRC; neo-adjuvant therapy; location cecum, appendix or ileocecal junction; neuroendocrine components.	194	3	To investigate the connection between mutant *KRAS*, *NRAS*, and *BRAF* and clinicopathological characteristics in therapy-naïve synchronous mCRC in Chinese populations.

Table 1. Continued

Reference	Year	Country	Study Design	Patient Inclusion Period		Reasons for Patient Exclusion	No. of Subjects	No. of Genes Investigated	Aim of the Study
				Start	End				
Heublein et al. [24]	2018	Germany	CH	1988	2012	-	23	754 miRNAs	miRNA profiling of primary CRC tissue to identify miRNAs potentially associated with defining the site of metastatic spread in CRC.
Jacob et al. [25]	2021	Germany	CC	01/2005	12/2014	Lack of any of the baseline variables or specimens, HNPCC or FAP co-malignancies.	18	770	To identify genes associated with the metastatic route in CRC.
Jacob et al. [26]	2021	Germany	CH	-	-	Missing FFPE tissue of the primary tumor, co-malignancies, Lynch-syndrome or other hereditary diseases.	18	770	To elucidate the link between immunosurveillance and organotropism of metastases in CRC by evaluating different gene signatures and pathways.
Kawazoe et al. [27]	2015	Japan	CH	01/2013	06/2014	-	264	4	To evaluate mutations in Japanese mCRC patients and assessing their corresponding effects on the efficacy of anti-EGFR therapy.
Lan et al. [28]	2015	Taiwan	CH	03/2000	01/2010	No tissue sample available in the biobank.	1492	7	To analyze mutation spectra of the *PI3K* and *RAS* pathways in CRC and the associations with sites of metastases of recurrence.
Lan et al. [29]	2021	Taiwan	CH	-	-	Patients who had stage I–III CRC, received emergent surgery, or who did not have available tumor or preoperative serum samples in the biobank.	95	10	To evaluate the concordance of mutation patterns between tumor tissue DNA and circulating cell-free DNA in stage IV CRC patients and to analyze relationship between the mutational patterns and site of metastases.

Table 1. Continued

Reference	Year	Country	Study Design	Patient Inclusion Period		Reasons for Patient Exclusion	No. of Subjects	No. of Genes Investigated	Aim of the Study
				Start	End				
Lee et al. [30]	2019	Korea	CH	2004	2008	-	15	409	Analyzing genetic mutations which may be presage PM.
Nagahara et al. [31]	2011	Japan	CH	1993	2000	Chemotherapy or radiotherapy before surgery.	113	1	To investigate if *Kif18A* has a role in the progression of CRC.
Prasanna et al. [45]	2018	Australia	CH	01/2005	12/2015	-	5967	2	To explore the outcome of patients with mCRC based on their site of metastases at diagnosis and to explore the association between tumor characteristics and site of metastases.
Roberto et al. [46]	2020	Italy	CH	2008	2019	-	207	1	To evaluate the outcome of right CRC patients according to *BRAF* status and the treatment performed.
Sakuraba et al. [32]	2009	Japan	CH	-	-	-	38	1	To evaluate the correlation between *Tip60* expression and the clinicopathological findings.
Sasaki et al. [33]	2016	Japan	CH	02/2006	10/2011	Previous chemotherapy for advanced disease.	526	3	To compare the prognostic impact of modern chemotherapy or *anti-EGFR* monoclonal antibody between CRC patients with and without PM.
Sayagués et al. [34]	2018	Spain	CH	-	-	-	87	4	To investigate the frequency of mutations in primary sCRC tumors and their impact on patient progression-free survival and overall survival.

Table 1. Continued

Reference	Year	Country	Study Design	Patient Inclusion Period		Reasons for Patient Exclusion	No. of Subjects	No. of Genes Investigated	Aim of the Study
				Start	End				
Schirripa et al. [47]	2020	Italy	CH	01/2010	12/2018	-	499	1	Description of the clinicopathologic features and prognosis of *KRAS G12C*-mutated metastatic CRC.
Shelygin et al. [35]	2014	Russia	CH	11/2012	02/2014	-	58	7	To describe the epithelial–mesenchymal transition in terms of gene expression profile and somatic changes in CRC patients with or without PM.
Shirahata et al. [42]	2010	Japan	CH	-	-	-	52	1	To examine the expression of the *MACC1* gene in primary tumors and to evaluate the correlation between the *MACC1* expression and the clinicopathological findings.
Shirahata et al. [41]	2009	Japan	CH	-	-	-	48	1	To examine the methylation status of the Vimentin gene in CRC patients and to evaluate the correlation between this status and the clinicopathological findings.
Sjo et al. [36]	2011	Norway	CH	1987	2006	Stage I–III or stage unknown, distant metastases without PM, no surgery or diverting procedures.	57	1	To evaluate the incidence of PM in CRC patients and to compare clinicopathological characteristics, survival and *TP53* mutation status in primary tumors.

Table 1. Continued

Reference	Year	Country	Study Design	Patient Inclusion Period		Reasons for Patient Exclusion	No. of Subjects	No. of Genes Investigated	Aim of the Study
				Start	End				
Smith et al. [37]	2013	UK	CH	-	-	Patients with resectable disease.	2161	4	To study the somatic molecular profile of the epidermal growth factor receptor pathway in advanced CRC, its relationship to prognosis, the site of the primary and metastases, and response to cetuximab.
Takahashi et al. [38]	2013	Japan	CH	1992	2002	-	180	1	To investigate the clinical significance of *NEK2*/miR128 expression in CRC.
Taniguchi et al. [39]	2020	Japan	CH	08/2014	04/2016	RAS-mutant tumors or unknown RAS status.	331	2	To present institutional experience with patients with CRC who underwent clinical mutation profiling and to evaluate the differences in patient characteristics with *BRAF* mutations.
Tran et al. [48]	2011	Australia	CH	-	-	-	524	1	To investigate whether *BRAF* mutant CRC is further defined by metastatic spread and evaluation of the impact of this mutation on prognosis.
Yaeger et al. [49]	2014	USA	CC	2009	2012	-	515	1	To determine the clinicopathologic characteristics, *PIK3CA* mutation frequency, and outcomes after metastasectomy in patients with *BRAF*-mutant mCRC.

Table 1. Continued

Reference	Year	Country	Study Design	Patient Inclusion Period		Reasons for Patient Exclusion	No. of Subjects	No. of Genes Investigated	Aim of the Study
				Start	End				
Yang et al. [50]	2021	China	CH	01/2015	03/2020	Chemo- or radiotherapy before NGS and no follow-up information.	582	1	To evaluate the frequency and phenotypic characteristics of mCRC with somatic RET mutation.
Yokota et al. [40]	2011	Japan	CH	2002	2010	-	229	2	To investigate the clinicopathological features and prognostic impact of *KRAS*/*BRAF* mutation in advanced and recurrent CRC patients.
Zihui Yong et al. [51]	2018	Singapore	CH	01/2010	12/2014	Appendiceal tumors, other stages than stage 4 and patients without metastases.	363	1	To describe the metastatic pattern of advanced CRC by assessing the interaction between the *KRAS* mutational status and the location of primary tumors.

CC, case control study; CH, cohort study; CRC, colorectal cancer; CRS, cytoreductive surgery; FAP, familiarly adenomatous polyposis; HNPCC, hereditary non-polyposis colon cancer; HIPEC, hyperthermic intraperitoneal chemotherapy; MCH, matched cohort study; mCRC, metastatic colorectal cancer; PM, peritoneal metastases.

Table 2. Characteristics of patients and tissue samples in included studies.

Reference	Patients' Specification	No. of Patients for Analysis		Tissue Samples	Tissue Collection
		Without PM	With PM		
Astrosini et al. [20]	Nonpretreated CRC patients.	54	9	Primary tumor tissue	Retrospect, archives of pathology department
Atreya et al. [43]	Cohort of patients with *BRAF* mutant mCRC was matched 1:2 to patients with *BRAF* WT mCRC.	75	45	Not specified	Not performed, *BRAF* status was already known
Bruzzi et al. [21]	Patients with signed informed consent for biological sample collection from the PETACC-8 trial (stage III CRC patients) were included.	1446	38	FFPE tumor tissues	Retrospect, archives of pathology department
Cheng et al. [22]	Patients who underwent surgery for CRC. Stage IV patients are included for genetic analysis.	260	76	Frozen tumor tissue in liquid nitrogen	Retrospect, hospital biobank
Christensen et al. [44]	Patients with mCRC who had received multiple treatment line (irinotecaban and cetiuximab).	405	43	FFPE tumor tissue blocks from primary tumors	Not performed, *BRAF*, RAS and *PIK3CA* status was already known
He et al. [23]	Therapy-naïve synchronous mCRC patients at first diagnosis.	168	26	FFPE tumor tissue blocks	Retrospect, archives of pathology department
Heublein et al. [24]	Patients underwent surgical resection and were divided into three groups according to metastases location.	10	13	FFPE tissue from primary tumors	Retrospect, archives of pathology department
Jacob et al. [25]	Patients undergoing surgery. Four groups; patients without metastases, with LM, with PM and with LM and PM.	12	6	FFPE tissue from primary tumors	Retrospect, national database and biobank
Jacob et al. [26]	CRC patients surgically treated and divided in three groups: patients without metastases, with LM and with PM.	12	6	FFPE tissue from primary tumors	Retrospect, archives of pathology department
Kawazoe et al. [27]	CRC patients with histologically confirmed adenocarcinoma and presence of unresectable metastatic disease.	212	52	FFPE cancer specimens (239 primary tumors and 25 metastases)	Retrospect, archives of pathology department

Table 2. Continued

Reference	Patients' Specification	No. of Patients for Analysis		Tissue Samples	Tissue Collection
		Without PM	With PM		
Lan et al. [28]	Patients with stages I–IV CRC who underwent surgery.	1388	104	Surgery tissue samples	Retrospect, hospital biobank
Lan et al. [29]	Patients with stage IV CRC who underwent surgery.	63	32	Surgery tissue samples	Retrospect, hospital biobank
Lee et al. [30]	Small obstructive colorectal cancer group compared to large non-obstructing colorectal cancer group (contrast group).	5	10	FFPE surgical specimens	Retrospect, archives of pathology department
Nagahara et al. [31]	Patients identified as having primary CRC based on the clinicopathologic criteria described by the Japanese Society for Cancer of the Colon and Rectum.	107	6	Frozen tissue specimens in liquid nitrogen from colorectal tumor tissue and paired healthy tissue at least 10 cm distal from primary tumor	Retrospect, archives of pathology department
Prasanna et al. [45]	Patients with proven mCRC who were registered in the included databases.	4755	1212	Unknown	Not performed, *BRAF* and *KRAS*/RAS status was already known
Roberto et al. [46]	Patients with right mCRC with known *BRAF* mutation status.	154	53	Unknown	Not performed, *BRAF* status was already known
Sakuraba et al. [32]	Patients undergoing surgery for CRC.	33	5	Frozen tumor specimens and corresponding normal tissues	Retrospect, archives of pathology department
Sasaki et al. [33]	Patients with mCRC treated with systemic chemotherapy, combined with or without bevacizumab, cetuximab or panitumumab.	409	117	FFPE tumor samples	Retrospect, archives of pathology department
Sayagués et al. [34]	Caucasian patients diagnosed with CRC who underwent surgical resection of primary tumor tissues.	80	7	Freshly frozen primary tumor tissues	Retrospect, archives of pathology department

Table 2. Continued

Reference	Patients' Specification	No. of Patients for Analysis		Tissue Samples	Tissue Collection
		Without PM	With PM		
Schirripa et al. [47]	Patients with presence of a *KRAS* mutation with the focus of the specific variant.	391	108	FFPE tissue from primary tumors and/or paired metastases	Not performed, *KRAS* status was already known
Shelygin et al. [35]	Patients undergoing surgery for colorectal cancer.	38	20	Tissue samples from primary tumor, peritoneal metastases, and healthy tissue	Retrospect, archives of pathology department
Shirahata et al. [42]	CRC patients who underwent surgery.	47	5	Frozen colorectal cancer tissue and corresponding normal tissues	Were collected at surgical resection and stored at the pathology department
Shirahata et al. [41]	CRC patients who underwent surgery.	43	5	Frozen primary tumor specimens and corresponding normal tissue	Were collected at surgical resection and stored at the pathology department
Sjo et al. [36]	CRC patients with PM.	57	148 *	FFPE tumor samples	Retrospect, archives of pathology department
Smith et al. [37]	Patients with measurable metastatic of locally advanced colorectal adenocarcinoma and unresectable disease.	1667	283	FFPE tumor samples (adenocarcinomas)	Retrospect, archives of pathology department
Takahashi et al. [38]	Patients with CRC who underwent surgical treatment.	174	6	Frozen resected tumor samples in liquid nitrogen	Retrospect, archives of pathology department
Taniguchi et al. [39]	Patients with CRC who received any treatment at one of the 15 study hospitals that participated, and who had RAS WT tumors.	62	281	FFPE tumor samples	Retrospect, archives of pathology department
Tran et al. [48]	Patients with mCRC with known *BRAF* mutation status from two institutional databases.	385	139	Tumor tissue	Not performed, *BRAF* status was already known
Yaeger et al. [49]	Patients with mCRC with available tumor sequencing.	431	84	FFPE primary tumor samples or metastatic tissue	Not performed, *BRAF* status was already known

Table 2. Continued

Reference	Patients' Specification	No. of Patients for Analysis		Tissue Samples	Tissue Collection
		Without PM	With PM		
Yang et al. [50]	Cohort of patients with mCRC.	412	170	Unknown	Not performed, RET status was already known
Yokota et al. [40]	Cohort of patients with CRC.	175	54	Frozen or FFPE tissues	Retrospect, archives of pathology department
Zihui Yong et al. [51]	Stage 4 CRC patients with metastases to the liver, lung, and/or peritoneum.	266	89	FFPE surgical specimens	Not performed, *KRAS* status was already known

*CRC, colorectal cancer; FFPE, formalin-fixed paraffin embedded; LM, liver metastases; mCRC, metastatic colorectal cancer; PM, peritoneal metastases; * Use of a control group with non-PM from a previous published study.*

Risk of Bias in Studies

The relevant categories from the QUIPS tool were used to access the risk of bias; a score per domain per study is presented in Figure 2A. We reported a high risk of bias for five studies [20, 33, 35, 37, 45] and a moderate risk of bias for all other studies. The overall lowest risk of bias was found in the statistical and outcome measurement domains, while the highest was found in the confounding domain (Figure 2B).

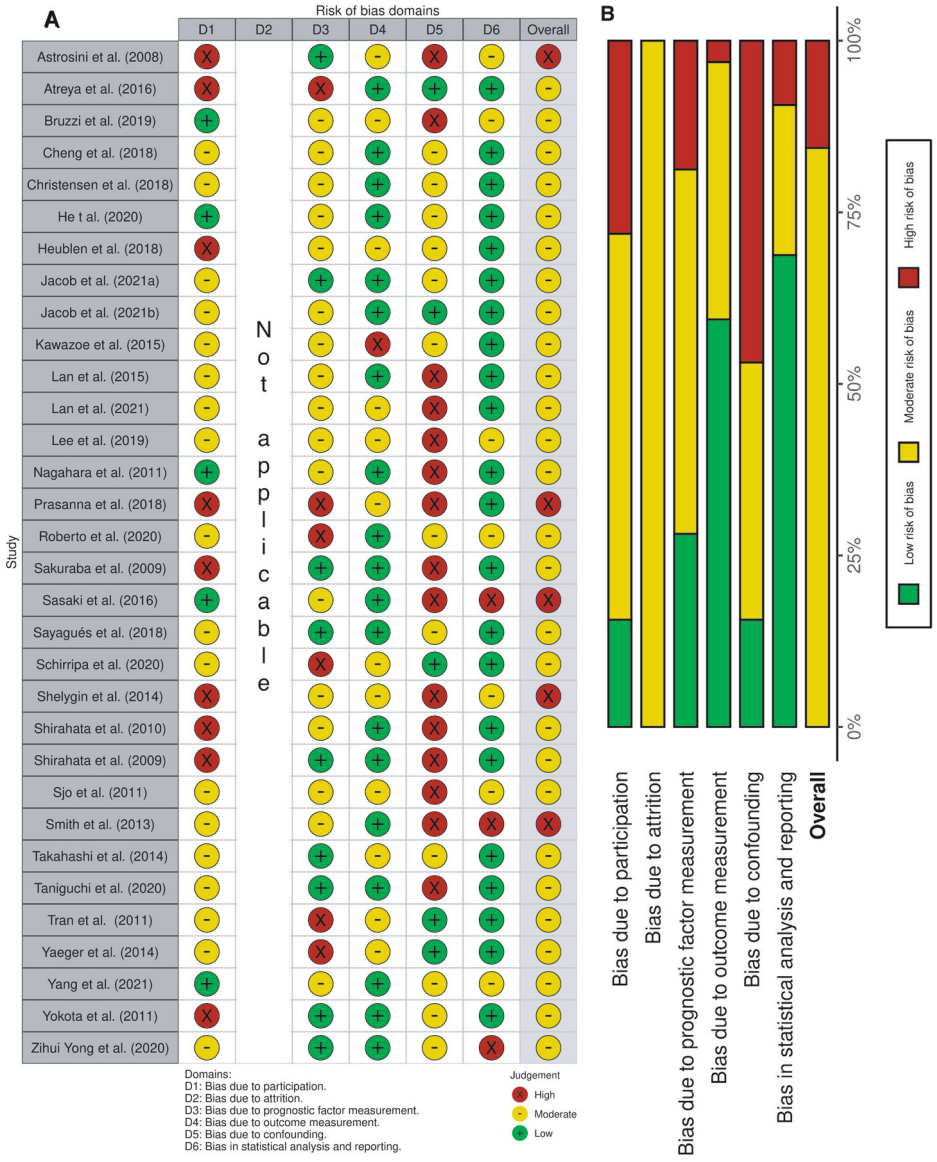


Figure 2. Risk of bias based on the QUIPS tool. (**A**) Summary of the domain–level judgements for each study. (**B**) Risk–of–bias judgements within each bias domain.

Reported Genes

Most studies focused on a selected predefined group of genes (Figure 3). Genes that were predominantly studied were *RAS* (*KRAS/NRAS*), *PIK3CA*, *TP53*, and *BRAF*. The remaining 13 genes (e.g., *androgen receptor* (*AR*), *ASXL Transcriptional Regulator 1* (*ASXL1*), *AT-Rich Interaction Domain 1A* (*ARID1A*), *NIMA Related Kinase 2* (*NEK2*), *MET Transcriptional Regulator MACC1* (*MACC1*), *Paired Box 5* (*PAX5*), *Ubiquitin Protein Ligase E3 Component N-Recognin 5* (*UBR5*), *Vimentin*, *Ret Proto-Oncogene (RET), Histone acetyltransferase* (*Tip60*), *PKHD1 Ciliary IPT Domain Containing Fibrocystin/Polyductin* (*PKHD1*), *Regenerating Family Member 1 Alpha* (*REG1A*), and *Kinesin Family Member 18A* (*Kif18A*)) were, except for *ARID1A*, all separately examined by individual studies (Figure 4). Three studies did a broader comprehensive genomic analysis on the tissue samples. Jacob et al. performed a PanCancer Progression Panel in 2 studies [25, 26] including 770 genes, and Lee et al. used a Comprehensive Cancer Panel covering 409 genes [30]. All details about the reported genes are displayed in Table 3.

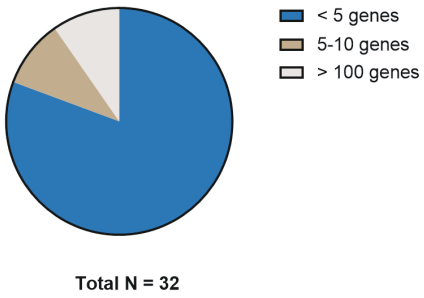


Figure 3. Distribution of number of genes investigated.

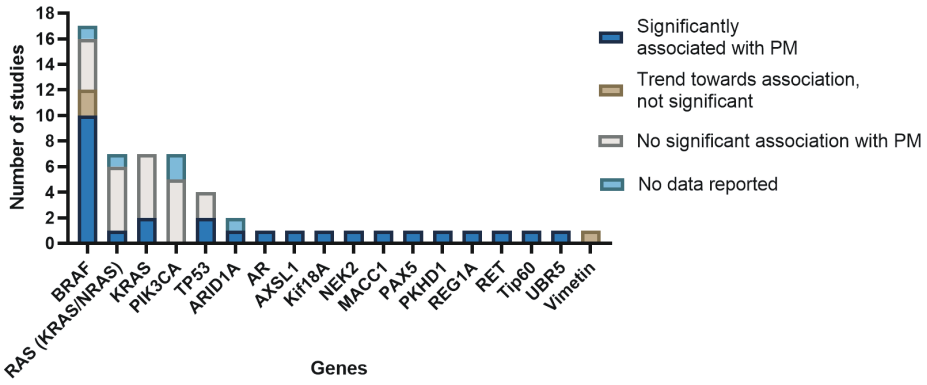


Figure 4. Number of studies investigating specific genes.

Table 3. Overview of genetic analysis and outcomes.

Reference	Level of Testing	Name Genes, Molecules or Panel Investigated	Type of Analysis Performed	Gene or Molecule Name and Mutation or Expression Status (n)	No. of Patients with PM (N) and Outcomes (n)	No. of Patients without PM (N) and Outcomes (n)	MMR Status (MSI/MSS)	Findings as Reported by Authors in Studies
Astrosini et al. [20]	RNA	*REG1A*	RT-PCR		*N* = 9	*N* = 54	N/A	*REG1A* expression levels highly correlated with formation of PM (median relative amount of 10.36 vs. 0.94, $p = 0.0039$ [a]).
				REG1A expression	-	-		
Atreya et al. [43]	DNA	*BRAF*	-		*N* = 45	*N* = 75	Total: 10/68 PM: 2/23	No significant differences in metastatic sites were observed, although PM were more common in *BRAF* mutant patients ($p = 0.045$ †[b]).
				BRAF mutant (40)	20	20		
				BRAF wild-type (80)	25	55		
Bruzzi et al. [21]	DNA	*BRAF*, *RAS* (*KRAS* and *NRAS*)	RT-PCR		*N* = 38	*N* = 1446	Only MSS included.	There is a trend for a higher rate of PM in *BRAF*V600E mutant compared to *RAS* mutant and wild-type patients (12.2% vs. 7.44% vs. 9.96% respectively, $p > 0.05$ [c,d]).
				BRAF V600E mutant (127)	15	112		
				RAS mutant (748)	56	692		
				Double wild-type (609)	61	548		
Cheng et al. [22]	DNA	*BRAF*	PCR or SNP genotyping assay		*N* = 76	*N* = 260	N/A	Stage IV CRC patients with a *BRAF*V600E mutation had a higher frequency of PM (41.7% vs. 21.2%, $p = 0.04$ [d]).
				BRAF V600E mutant (312)	66	246		
				BRAF wild-type (24)	10	14		

Table 3. Continued

Reference	Level of Testing	Name Genes, Molecules or Panel Investigated	Type of Analysis Performed	Gene or Molecule Name and Mutation or Expression Status (n)	No. of Patients with PM (N) and Outcomes (n)	No. of Patients without PM (N) and Outcomes (n)	MMR Status (MSI/MSS)	Findings as Reported by Authors in Studies
Heublein et al. [24]	miRNA	miRNAs	RT-PCR		*N* = 10	*N* = 13	N/A	A set of 31 miRNAs was significantly upregulated in the PM group, while 10 miRNAs were repressed as compared to LM. A set of 2 miRNAs was significantly upregulated in the PM group, while 25 were repressed as compared to M0. hsa-miR-31-5p was significantly overexpressed in PM patients ($p = 0.002$ [b,d]).
				hsa-miR-215-5p	Induced 17-fold	Compared to LM		
				hsa-miR-31-3p	Induced 8.9-fold	Compared to LM		
				hsa- miR-31-5p	Induced 5.4-fold	Compared to LM		
				hsa-miR-483-5p	Repressed 0.04-fold	Compared to LM		
				hsa-miR-1226-5p	Repressed 0.29-fold	Compared to LM		
				hsa-miR- 296-5p	Repressed 0.32-fold	Compared to LM		
				hsa-miR-215-5p	Induced 3.6-fold	Compared to M0		
				hsa-miR-148a-3p	Induced 2.8-fold	Compared to M0		
Jacob et al. [25]	mRNA	PanCancer Progression Panel	NanoString analysis		*N* = 6	*N* = 12	N/A	In PM patients, 18 genes demonstrated significant different expression rates ($p < 0.05$ [b,d]).
				Not specified	-	-		

Table 3. Continued

Reference	Level of Testing	Name Genes, Molecules or Panel Investigated	Type of Analysis Performed	Gene or Molecule Name and Mutation or Expression Status (n)	No. of Patients with PM (N) and Outcomes (n)	No. of Patients without PM (N) and Outcomes (n)	MMR Status (MSI/MSS)	Findings as Reported by Authors in Studies
Jacob et al. [26]	RNA	PanCancer Progression Panel	NanoString analysis		*N* = 6	*N* = 12	N/A	The analysis between patients with PM and M0 did not show a significant down- or upregulation of distinct gene sets.
				Not described	-	-		
Kawazoe et al. [27]	DNA	*KRAS*, *NRAS*, *BRAF* and *PIK3CA*	PCR		*N* = 52	*N* = 212	N/A	*BRAF* mutant tumors were more likely to have PM in comparison with *BRAF* wild-type tumors (50.0% vs. 18.0%, $p = 0.009$ [c]). No significant differences for PM according to *RAS* mutation ($p = 0.64$ [d]).
				BRAF mutant (14)	7	7		
				RAS pathway mutant (21)	17	4		
				KRAS exon 2 mutant (90)	75	15		
				PIK3CA	N/A	N/A		
Lan et al. [28]	DNA	RAS pathway (*KRAS*, *NRAS*, *HRAS*, *BRAF*) *PI3K* pathway	PCR		*N* = 104	*N* = 1388	Total: 154/1492	PM was significantly higher in RAS pathway mutated patients compared to wild-type tumors ($p = 0.009$ [d]). Tumors with *KRAS* mutation had a trend toward a higher proportion of PM ($p = 0.061$ [d]). There was no association between PM and the presence of a *PI3K* pathway mutation ($p = 0.408$ [d]).
				PI3K pathway mutant (213)	12	201	36/177	
				RAS pathway mutant (706)	62	644	91/615	
				BRAF mutant (70)	8	62	N/A	
				KRAS mutant (602)	51	551		
				NRAS mutant (49)	5	44		
				HRAS mutant (21)	4	17		

Table 3. Continued

Reference	Level of Testing	Name Genes, Molecules or Panel Investigated	Type of Analysis Performed	Gene or Molecule Name and Mutation or Expression Status (n)	No. of Patients with PM (N) and Outcomes (n)	No. of Patients without PM (N) and Outcomes (n)	MMR Status (MSI/MSS)	Findings as Reported by Authors in Studies
Lan et al. [29]	DNA	*TP53*, APC, *KRAS*, *FAT4*, *ARID1A*, *FBXW7*, *SMAD4*, *PIK3CA*, *NRAS* and *BRAF*	NGS		*N* = 32	*N* = 63		For patients with PM, the frequency of genetic mutations was the highest in *TP53*. The authors conducted analysis to compare left- and right-sided CRC with mutation status but not between the non-PM and PM group.
				TP53 mutant (59)	19	40		
				KRAS mutant (35)	7	28		
				APC mutant (45)	17	28		
Lee et al. [30]	DNA	Life Technologies Ion AmpliSeq Comprehensive Cancer Panel	NGS		*N* = 10	*N* = 5	N/A	*ARID1A*, *PKHD1*, *UBR5*, *PAX5*, *TP53*, *ASXL1* and *AR* were detected more frequently in the SOC group with PM (*p* values of 0.002, 0.019, 0.002, <0.001, 0.007, 0.047, 0.019 respectively [d]). *TNFRSF14, VHL, MTRR, MLLT10, BIRC2, EP400, IRS2, PER1, TCF3 and CYP2D6* were detected more frequently in the LNOC group without PM (*p* values of 0.019, 0.004, 0.047, 0.022, <0.001, 0.022, <0.001, 0.022, 0.026, 0.004 respectively [d]).
				ARID1A mutant	9	0		
				PKHD1 mutant	7	0		
				UBR5 mutant	9	0		
				PAX5 mutant	10	0		
				TP53 mutant	8	0		
				ASXL1 mutant	8	1		
				AR mutant	7	0		
				TNFRSF14 mutant	5	3		
				VHL mutant	4	0		
				MTRR mutant	4	2		
				MLLT10 mutant	3	0		
				BIRC2 mutant	5	0		
				EP400 mutant	3	0		
				IRS2 mutant	5	0		
				PER1 mutant	3	0		
				TCF3 mutant	5	3		
				CYP2D6 mutant	4	0		

Table 3. Continued

Reference	Level of Testing	Name Genes, Molecules or Panel Investigated	Type of Analysis Performed	Gene or Molecule Name and Mutation or Expression Status (n)	No. of Patients with PM (N) and Outcomes (n)	No. of Patients without PM (N) and Outcomes (n)	MMR Status (MSI/MSS)	Findings as Reported by Authors in Studies
Nagahara et al. [31]	RNA	*Kif18A*	RT-PCR		*N* = 6	*N* = 107	N/A	*Kif18A* overexpression in CRC significantly correlated with PM ($p = 0.02$ [d]).
				Kif18A low expression	0	38		
				Kif18A high expression	6	69		
Prasanna et al. [45]	-	*BRAF*, RAS	-		*N* = unknown	*N* = unknown	PM: 29/239 M0: 77/940	*BRAF*-mutated colorectal cancer showed higher incidence of PM with a relative risk of 1.8 ($p < 0.001$ [e]). *KRAS*-mutated patients showed no higher incidence of PM with a relative risk of 0.95 ($p = 0.63$ [e]).
				RAS mutant (965)	199	766		
				RAS wild-type (1271)	274	997		
				BRAF mutant (143)	51	92		
				BRAF wild-type (1058)	208	850		
Roberto et al. [46]	-	*BRAF*	-		*N* = 53	*N* = 154	Total: 19/66	*BRAF* mutant right colorectal cancer was significantly more likely to occur with peritoneal metastases (38.1% vs. 22.4%, $p = 0.003$ [d]).
				BRAF mutant (42)	16	26		
Sakuraba et al. [32]	RNA	*Tip60*	RT-PCR		*N* = 5	*N* = 33	N/A	The authors found that *Tip60* downregulation (compared to healthy tissue expression) showed significant correlation with PM ($p = 0.0053$ [d]).
				Downregulation of *Tip60* expression	3	2		

Table 3. Continued

Reference	Level of Testing	Name Genes, Molecules or Panel Investigated	Type of Analysis Performed	Gene or Molecule Name and Mutation or Expression Status (n)	No. of Patients with PM (N) and Outcomes (n)	No. of Patients without PM (N) and Outcomes (n)	MMR Status (MSI/MSS)	Findings as Reported by Authors in Studies
Sasaki et al. [33]	DNA	*BRAF*, *KRAS*, *PIK3CA*	PCR		*N* = 117	*N* = 409	N/A	The PM group had a significantly higher incidence of the *BRAF* V600E mutation than the non-PM group (27.7% vs. 7.3%, $p < 0.01$ [d]). In contrast, no differences were observed between the two groups in *KRAS* and *PIK3CA* mutations (p 0.42 [d] and 0.76,[d] respectively).
				KRAS wild-type	54	163		
				KRAS mutation	46	115		
				BRAF wild-type	34	115		
				BRAF mutation	13	9		
				PIK3CA wild-type	53	181		
				PIK3CA mutation	5	*20*		
Sayagués et al. [34]	DNA	*KRAS*/*NRAS*, *BRAF* and *TP53*	NGS		*N* = 7	*N* = 80	Total: 6/48	*BRAF*-mutated CRC tumors were significantly associated with PM ($p = 0.006$[d]).
				KRAS mutant (24)	1	23	0/16	
				NRAS mutant (1)	0	1	0/0	
				BRAF mutant (6)	3	3	3/2	
				TP53 mutant (29)	2	*27*	1/21	
Schirripa et al. [47]	-	*KRAS*	Sanger sequencing, Sequenom MassArray		*N* = 108	*N* = 391	N/A	Compared to other *KRAS*-mutated cases, *KRAS G12C* mutations had a lower frequency in PM patients (13.5% vs. 25%, $p = 0.008$ [d]).
				KRAS mutant (694)	90	276		
				KRAS G12C mutant (145)	18	115		

Table 3. Continued

Reference	Level of Testing	Name Genes, Molecules or Panel Investigated	Type of Analysis Performed	Gene or Molecule Name and Mutation or Expression Status (n)	No. of Patients with PM (N) and Outcomes (n)	No. of Patients without PM (N) and Outcomes (n)	MMR Status (MSI/MSS)	Findings as Reported by Authors in Studies
Shelygin et al. [35]	DNA and RNA	*KRAS*, *BRAF* and *EMT* *	RT-PCR		*N* = 20	*N* = 38	PM: 2/18 M0: 6/32	Mutations in *KRAS* and *BRAF* with PM was 70%, compared to 42.1% in M0 patients ($p = 0.04$ [d]). The frequency of wild types in both genes was 57.9% in CRC without PM compared to 30% with PM ($p = 0.04$ [d]). No differences were observed between the two groups in *KRAS* and *BRAF* mutations solely.
				KRAS mutant	11	15		
				BRAF V600E	3	1		
				KRAS/*BRAF* wild-type	6	22		
				KRAS/*BRAF* mutant	14	16		
				EMT + (13)	7	6		
				EMT – (45)	13	32		
Shirahata et al. [42]	RNA	*MACC1* expression	RT-PCR		*N* = 5	*N* = 47	N/A	*MACC1* expression showed significant correlation with PM compared to the group without PM (5.75 ±4.58 vs. 2.57 ±3.09, $p = 0.042$ [b]).
				MACC1 expression	-	-		
Shirahata et al. [41]	DNA	Vimentin methylation	qMSP		*N* = 5	*N* = 43	N/A	A trend was shown toward preferentially developing PM with Vimentin methylation ($p = 0.080$ [d]).
				Vimentin +	5	26		
				Vimentin -	0	17		

Table 3. Continued

Reference	Level of Testing	Name Genes, Molecules or Panel Investigated	Type of Analysis Performed	Gene or Molecule Name and Mutation or Expression Status (n)	No. of Patients with PM (N) and Outcomes (n)	No. of Patients without PM (N) and Outcomes (n)	MMR Status (MSI/MSS)	Findings as Reported by Authors in Studies
Sjo et al. [36]	DNA	*TP53*	PCR		*N* = 49	*N* = 148	N/A	Univariate analyses demonstrated that PM was significantly associated with mutations in the *TP53* gene ($p = 0.05$ [f]). Multivariate analyses confirmed the previous finding (OR = 2.4; 95%CI = 1.2-4.8, $p = 0.013$ [g]).
				TP53 mutant	-	-		
				TP53 wild-type	-	-		
Smith et al. [37]	DNA	*KRAS*, *NRAS*, *BRAF*, and *PIK3CA*	PS and Sequenom		*N* = 283	*N* = 1667	Co-occurred with *BRAF* wild-type tumors	*BRAF* mutations were more common in patients with PM-only compared to LM-only (22.2% vs. 6.7%, $p = 0.00092$ [d]), although this association with PM did not withstand correction for multiple testing. *BRAF* mutations were significantly associated with PM ($p = 0.018$), which did not remain significant after Bonferroni correction ($p = 0.36$). For *KRAS*, *NRAS*, and *PIK3CA*, there was no association found for PM.
				KRAS mutant	131 (/282)	693 (/1667)		
				BRAF mutant	36 (/283)	193 (/1663)		
				NRAS mutant	7 (/283)	62 (/1656)		
				PIK3CA mutant	40 (/280)	293 (/1627)		

Table 3. Continued

Reference	Level of Testing	Name Genes, Molecules or Panel Investigated	Type of Analysis Performed	Gene or Molecule Name and Mutation or Expression Status (n)	No. of Patients with PM (N) and Outcomes (n)	No. of Patients without PM (N) and Outcomes (n)	MMR Status (MSI/MSS)	Findings as Reported by Authors in Studies
Takahashi et al. [38]	RNA	*NEK2*	RT-PCR		*N* = 6	*N* = 174	N/A	The high *NEK2* expression group showed greater PM then the low *NEK2* mRNA expression group ($p = 0.004$ [b,d]).
				NEK2 low (90)	0	90		
				NEK2 high (90)	6	84		
Taniguchi et al. [39]	DNA	*RAS*, *NRAS*, *BRAF* and *PIK3CA*	PCR		*N* = 62	*N* = 281	N/A	The frequencies of RAS/*BRAF*V600E wild-type over either *BRAF* or *PIK3CA* mutations were higher for PM (35% vs. 15%, $p = 0.003$ [d]).
				RAS and *BRAF* wild-type (291)	44	247		
				RAS wild-type + *BRAF* or *PIK3CA* mutation (52)	18	34		
Tran et al. [48]	DNA	*BRAF*	RT-PCR		*N* = 139	*N* = 385	Total: 40/310	*BRAF* mutant tumors had significantly higher rates of PM (46% vs. 24%, $p = 0.001$[d]).
				BRAF mutant (57)	26	31	12/30	
Yaeger et al. [49]	DNA	*BRAF*	Sanger sequencing, Sequenom MassArray		*N* = 84	*N* = 431	N/A	PM was significantly more common at the time of diagnosis of metastatic disease in the *BRAF*-mutant cases (26% vs. 14%, $p < 0.01$[d]).
				BRAF mutant (92)	24	68		
Yang et al. [50]	DNA	*RET*	NGS		*N* = 170	*N* = 412	Total: 24/558	The presence of *RET* mutations was associated with PM compared to wild-type tumors (56.2% vs. 28.4%, $p = 0.024$[d]).
				RET mutant (16)	9	7	6/10	

Table 3. Continued

Reference	Level of Testing	Name Genes, Molecules or Panel Investigated	Type of Analysis Performed	Gene or Molecule Name and Mutation or Expression Status (n)	No. of Patients with PM (N) and Outcomes (n)	No. of Patients without PM (N) and Outcomes (n)	MMR Status (MSI/MSS)	Findings as Reported by Authors in Studies
Yokota et al. [40]	DNA	*KRAS* and *BRAF*	PCR		*N* = 54	*N* = 175	N/A	60.0% of CRCs with *BRAF* mutation develops PM compared with 15% of CRCs with other subtypes ($p = 0.0062$ [b,d]).
				KRAS/*BRAF* wild-type (135)	30	105		
				*KRAS*G12X mutant (53)	11	42		
				*KRAS*G13X mutant (26)	4	22		
				*BRAF*V600E mutant (15)	9	6		
Zihui Yong et al. [51]	DNA	*KRAS*	PCR		*N* = 89	*N* = 266	N/A	After stratification, PM was associated with mutant *KRAS* tumors (26.6% vs. 15.1%, $p = 0.02$ [d]).
				KRAS mutant (126)	37	89		

Table 3. Continued

Studies with synchronous and metachronous peritoneal metastases population								
Reference	**Level of Testing**	**Genes or Panel Investigated**	**Type of Analysis**	**Gene or Molecule Name and Mutation or Expression Status (n)**	**No. of Patients with PM (N) with Outcomes (n)**		**No. of Patients without PM (N) with Corresponding Outcomes (n)**	**MMR Status (MSI/MSS)**	**Findings as Reported by Authors in Studies**
					Synch.	**Metach.**			
Christensen et al. [44]	DNA	RAS (*KRAS* and *NRAS*), *BRAF* and *PIK3CA*	NGS or Mutation kit and PS		*N* = 43	*N* = 33	*N* = 372	N/A	*PIK3CA* mutations were significantly associated with absence of PM (OR = 0.10; 95%CI = 0.01–0.79, *p* = 0.028 [g]) and with a decreased hazard of developing PM (HR = 0.31; 95%CI = 0.11-0.86, *p* = 0.024 [g]). The hazard ratio of developing PM and having *BRAF* mutations were not associated with PM (OR = 2.07; 95%CI = 0.60-6.19, *p* = 0.192 [g] and (HR = 1.82; 95%CI = 0.81–4.08, *p* = 0.146 [g]).).
				RAS mutant (206)	21	16	169		
				BRAF V600E mutant (30)	7	3	20		
				PIK3CA mutant (61)	1	3	57		

Table 3. Continued

Reference	Level of Testing	Genes or Panel Investigated	Type of Analysis	Gene or Molecule Name and Mutation or Expression Status (n)	No. of Patients with PM (N) with Outcomes (n)		No. of Patients without PM (N) with Corresponding Outcomes (n)	MMR Status (MSI/MSS)	Findings as Reported by Authors in Studies
					Synch.	Metach.			
He et al. [23]	DNA	*KRAS*, *BRAF*, *NRAS*	NGS		*N* = 26	*N* = 0	*N* = 174	N/A	Mutant *KRAS* tumors had a significant relevance with PM ($p = 0.017$ [d]). *KRAS* codon 12 mutation was more likely to present with PM ($p = 0.014$ [d]). Patients with PM had the tendency to carry mutant *KRAS* G12D ($p = 0.052$ [d]). Tumors with mutated *BRAF* were more likely to develop PM ($p = 0.052$ [d]).
				Any mutation (108)	20	-	88		
				KRAS mutant (77)	15	-	62		
				NRAS mutant (8)	0	-	8		
				BRAF mutant (23)	5	-	18		
				All wild-type (86)	6	-	80		

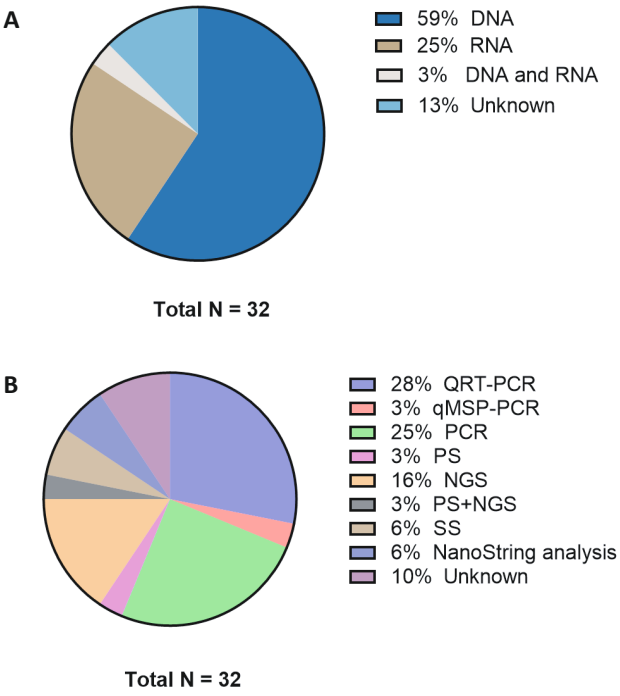
EMT, epithelial–mesenchymal transition; FFPE, formalin fixated paraffin embedded; LNOC, large non-obstructing colorectal cancer; LM, liver metastases; MS, microsatellite; MMR, mismatch repair; MSS, microsatellite stable; MSI, microsatellite instable; N/A, non-applicable; NGS, next-generation sequencing; PCR, polymerase chain reaction; PM, peritoneal metastases; PS, pyrosequencing; qMSP, quantitative methylation-specific polymerase chain reaction; RT, quantitative real time; SOC, small obstructing colorectal cancer; SNP, single nucleotide polymorphism.

** EMT, epithelial–mesenchymal transition (EMT) = ZEB 1, ZEB 2, SNAI1 and VIM overexpression and CDH1 downregulation.*

† No longer met the criteria for statistical significance. [a] Kruskal–Wallis test; [b] Student's t-test; [c] Mann–Whitney U test; [d] Chi-square test or Fisher's exact test; [e] Mantel–Haenszel chi-squared test; [f] Univariate analysis; [g] Multivariate analysis.

Genetic Analysis Methods

Primary tumor genetic analysis was performed on DNA level in 19 studies and on RNA level in seven studies (Figure 5). One study described the analysis on both levels [35]. Heublein et al. investigated MicroRNAs (miRNA) and the corresponding overexpression profiles [24]. Four articles did not specify if they performed testing on DNA or RNA level [43, 46, 47, 52]; three of these articles did not even specify which method they used for genetic testing [43, 46, 52]. Nine articles reported the use of real-time polymerase chain reaction (RT-PCR) [20, 21, 24, 31, 32, 35, 38, 42, 48]. One study specified the PCR tool as quantitative methylation-specific PCR (qMSP) [41]. PS and NGS were used in one [37] and five [23, 28, 30, 34, 50] studies, respectively. Christensen et al. reported the use of both methods [44]. Two studies analyzed the samples with SS [47, 49]. Jacob et al. described NanoString analysis in both their articles [25, 26]. All details about the genetic analyses are displayed in Table 3.



QRT, quantitative real time; PCR, polymerase chain reaction; qMSP-PC, quantitative methylation-specific; PS, pyrosequencing; NGS, next generation sequencing; SS, Sanger Sequencing

Figure 5. Distribution of (**A**) genetic analysis level and (**B**) different molecular techniques.

DNA/RNA Alterations Outcomes and Association with PM

All details about the reported alterations are displayed in Table 3.

Mitogen-Activated Protein Kinase (MAPK) Pathway Outcomes

BRAF and RAS are both involved in the *MAPK* pathway and were most commonly reported. *BRAF* mutations were analyzed in 17 articles [21-23, 27, 29, 33-35, 37, 39, 40, 43-46, 48, 49]. In ten studies, it was found on a statistically significant level that *BRAF* mutant tumors were more likely to develop PM and/or that patients with PM had more often *BRAF* mutated primary tumors compared to PM-free CRC patients [22, 27, 33, 34, 39, 40, 45, 46, 48, 49]. Most studies conducted the *BRAF* mutation analysis on codon 600, exon 15 (*n* = 12). Taniguchi et al. reported that the frequencies of *BRAF* mutations, in combination with *RAS* wild-type (WT) tumors, were significantly higher in CRC patients with PM [39]. Smith et al. showed a statistically significant association when *BRAF* status in unresectable CRC patients with PM was compared to other metastatic sites. This result, however, did not remain significant after a post hoc Bonferroni correction [37]. The authors also mention that *BRAF* mutations were significantly more common in patients with peritoneal-only metastases compared to patients with liver-only metastases. This, however, did not withstand a correction for multiple testing [37]. Atreya et al. and Bruzzi et al. reported no statistically significant difference in metastatic sites and *BRAF* mutation, although PM were more commonly observed in patients whose tumors harbored a *BRAF* mutation [21, 43]. He et al. investigated therapy-naïve synchronous mCRC patients and found no significant differences in mutation status [23]. Shelygin et al. found no association between PM and *BRAF* status when comparing patients, with and without PM, undergoing surgery for CRC [35]. Christensen et al. looked at the probability of developing PM while having a *BRAF* mutated tumor. The hazard ratio for developing PM and having a *BRAF*-mutated tumor was statistically not significant [44]. One article did not report any data about *BRAF* mutations and its relation to PM, although they intended to investigate this [28].

RAS pathway mutation analyses were reported in 14 studies. Seven studies focused on both *KRAS* and *NRAS* genes [21, 27-29, 34, 37, 44], and the other seven studies only described *KRAS* variants [23, 33, 35, 40, 45, 47, 51]. Lan et al. reported that the proportion of PM was significantly higher in stage I–IV CRC patients whose tumors carried a RAS pathway mutation, and *KRAS*-mutated tumors had a trend toward a higher proportion of PM, which was not significant [28]. Both Zihui Yong et al. and He et al. found a significant association between *KRAS* mutant tumors and PM [23, 51]. He et al. also stated that therapy-naïve synchronous PM patients tend to carry a mutant *KRAS* codon 12 [23]. One article did not report any outcomes, although they aimed to do so [28]. All other studies did not find a significant association or trend between *KRAS*/*NRAS* mutant tumors and the development of PM [21, 27, 33-35, 37, 40, 44, 45, 47].

To conclude, most articles (*n* = 10/17) state that *BRAF* mutant tumors are more likely to have PM and/or mutations in *BRAF* were more common in patients with PM compared to those

without. Almost all articles (*n* = 10/14) state that RAS pathway mutated tumors are not likely to have PM and were not more common in patients with PM compared to without PM.

PIK3CA Outcomes

The potential association of *PIK3CA* mutations with PM was analyzed in seven studies. In five studies, the *PIK3CA* mutations were not significantly associated with PM [28, 33, 35, 37, 44]. Christensen et al. even found that *PIK3CA* mutations were associated with the absence of PM and a decreased hazard of developing PM (HR = 0.31; 95%CI = 0.11–0.86, *p* = 0.024) in mCRC patients who had received chemo- or immunotherapy treatments [44]. Two studies did not report any outcomes, although *PIK3CA* mutations were investigated [29, 39].

TP53 Outcomes

TP53 mutations were analyzed in four studies. Two studies showed a significant association between PM and *TP53* mutations. Lee et al. detected more *TP53* mutations in patients with small obstructive CRC with PM compared to large non-obstructive tumors without PM [30]. Sjo et al. performed a multivariate analysis in stage IV CRC patients and showed that PM was significantly associated with *TP53* mutations [36]. Lan et al. stated that stage IV CRC patients with PM had a higher frequency of *TP53* mutations, although the authors did not perform statistical analysis on this association [29]. Sayagués et al. did not find a significant association between *TP53* mutational status and PM in Caucasian patients diagnosed with CRC [34].

Other DNA Outcomes

AR, *ASXL1*, *ARID1A*, *Kif18A*, *NEK2*, *MACC1*, *PAX5*, *PKHD1*, *REG1A*, *RET*, *Tip60*, and *UBR5* were mentioned as possible mutated genes associated with PM by several authors [20, 29-32, 38, 41, 50] but were, except for *ARID1A*, all investigated in only one study. NGS was performed by Yang et al. to detect RET mutations in mCRC without neoadjuvant treatment [50]. The presence of *RET* mutations was significantly associated with PM compared to WT tumors. *Tip60* regulation analysis was performed with RT-PCR in patients undergoing surgery for CRC by Sakuraba et al. [32]. The authors found that a downregulation of *Tip60* was significantly associated with PM. To conclude, all previous mentioned genes showed a significant association with PM, but all were studied by a single study only.

RNA Outcomes

Nagahara et al. report that *Kif18A* overexpression, measured by RT-PCR, in CRC patients without neoadjuvant treatment significantly correlates with PM [31]. The expression profile of *NEK2* was analyzed by Takahashi et al. in patients with CRC who underwent surgical treatment [38], demonstrating that the high *NEK2* expression group had significantly greater peritoneal dissemination compared to the low expression group. *MACC1* expression was found to be significantly associated with PM by Shirahata et al. [42]. The expression of *REG1A* was explored in non-pretreated CRC patients by Astrosini et al. and showed a positive e correlation with the formation of PM [20]. In addition, Heublein et al. analyzed MicroRNAs (miRNAs) expression

profiles and concluded that hsa-mri-31-5p seems to be overexpressed in patients with PM [24]. The authors reported a set of 31 miRNAs which were significantly upregulated in the PM group, while ten miRNAs were found to be repressed as compared to LM. Another set of two miRNAs was significantly upregulated in the PM group, while 25 were found to be repressed as compared to no metastases. Shirahata et al. discovered a trend toward preferentially developing PM in tumors with Vimentin methylation, although this was not significant [41].

Results of Broader Panel Analyses

Lee et al. performed a broader panel analysis of which the results (*ARID1A*, *PKHD1*, *UBR5*, *PAX5*, *TP53*, *ASXL1* and *AR*) are already described in Section 3.6.4 [30]. Jacob et al. explored gene expression profiles with a broad cancer "panel" comparing four groups (without metastases, with LM, with PM, and with both LM and PM) [25]. They report that "18 genes had significantly different expression rates", but they did not describe which genes. In another study, in which three groups were compared (without metastases, with LM, and with PM), the authors reported no significant down- or upregulation of distinct gene sets [26].

All details about the reported genes and corresponding conclusions are described in The Supplementary. A conclusive summary for all genes is displayed in Figure 6.

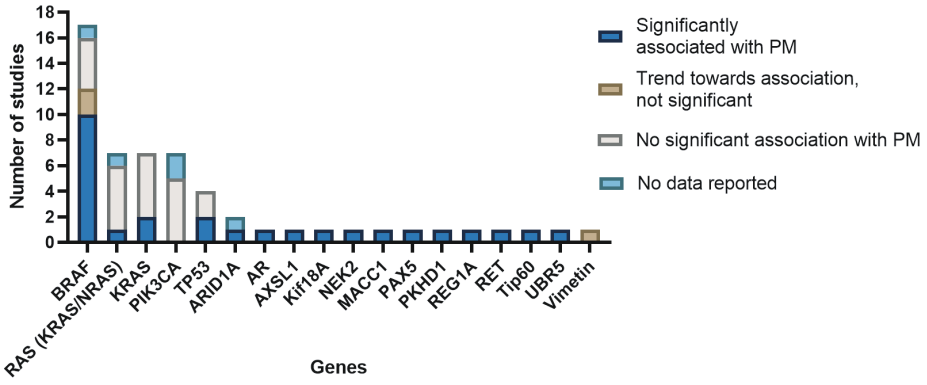


Figure 6. Overview of genes investigated with conclusions formulated by the authors of included studies.

MSI Status

In addition to DNA and RNA alterations, microsatellite instability (MSI) status was reported in ten articles [21, 28, 34, 35, 37, 43, 45, 46, 48, 50]. Tran et al. describe the impact of *BRAF* mutations in combination with MSI status on the pattern of metastatic spread and its prognosis [48]. The authors report that patients with MSI tumors show poorer survival in mCRC, and this is due to the association with *BRAF* mutations. Yang et al. state that MSI is associated with RET mutations [50].

DISCUSSION

This systematic review provides an overview of the results of studies which analyzed genomic DNA and RNA expression alterations correlated to PM with the goal of identifying alterations that could potentially serve as a predictive biomarker in patients with CRC. Of the 17 studies investigating *BRAF* mutations, ten studies reported a significant association with PM. Mutations in *ARID1A*, *ASXL1*, *Kif18A*, *NEK2*, *MACC1*, *PAX5*, *PKHD1*, *REG1A*, *RET*, *Tip60* and *UBR5* were also reported to be associated with PM [20, 29-32, 38, 41, 50], although these results were only described in maximum of one study. A recent analysis with a cancer panel of 770 genes from Jacob et al. did not show a significant down- or upregulation of distinct gene sets between CRC patients with PM and without distant metastases. Their sample size was, however, small (*n* = 18) [26].

BRAF Mutations

BRAF gene mutations occur in 5–15% of the mCRC cases; over 95% of these mutations consist of a substitution of valine to glutamic acid at codon 600 (V600E) [13, 16, 53]. *BRAF* is a serine/threonine protein kinase that plays an important role in the *MAPK* pathway. This pathway drives cell proliferation, differentiation, migration, survival, and angiogenesis, and therefore, changes in this pathway are associated with tumorigenesis [54]. *BRAF* mutations can be considered as an independent negative prognostic factor in early-stage microsatellite stable tumors and as a negative predictive factor for therapeutic approaches [54]. Due to its chemoresistance and resistance to *BRAF* inhibitor therapy, *BRAF*-mutated tumors are difficult to treat [54, 55]. Therefore, trials are currently going on with dual or triple drug therapy to enhance blockade of the *MAPK* pathway. Nowadays, CRC patients without metastases are not screened for *BRAF* mutations, and further molecular examination is only conducted in metastatic disease [56]. As only 55% of the studies reported a significant association between *BRAF* mutations and PM, we cannot conclude yet that *BRAF* mutations are specific enough to identify patients with colorectal PM.

Other Mutations

First, RAS pathway mutations are the most commonly investigated mutations in mCRC. Different codons of both *KRAS* and *NRAS* genes were included, thereby creating a broader overview of this pathway. *KRAS* is the most commonly activated oncogene in CRC, with mutations occurring in exon 2 codon 12 and 13, exon 3 codon 59 and 61, and exon 4 codon 117 and 146 [16, 57]. Approximately 30–50% of the CRC patients carry a somatic *KRAS* mutation [16]. *KRAS* mutations have been associated with lung metastases but not with PM [16]. *NRAS* is mutually exclusive with *BRAF* and *KRAS* and occurs in approximately 3% of CRC patients [16]. There has been no previously described association with PM, which is in line with the findings of this review. Second, *PIK3CA* (exon 9 and 20) gene mutations occur in 10–18% of CRC patients [53]. They commonly co-occur with *KRAS* or *BRAF* mutations. Approximately 70% of *PIK3CA* mutant patients have concurrent mutations [16, 58], although they have never been

described to be associated with PM. The results of our study demonstrate this as well. Third, *TP53* gene mutations are one of the most frequently described mutations as they occur in 35%–75% of the colorectal PM patients [13]. Previous research shows the contradictory result of *TP53* mutations and their prognostic value in CRC patients [53]. In this review, some authors showed a significant association, while others did not reach the significance.

MSI Status

Of the included studies, only 10 articles reported on MSI status, all without extensive analysis. This is unfortunate, as MSI status is the only prognostic molecular marker used in deciding adjuvant therapy options [56]. MSI originates from the inactivation of mismatch repair genes by either MLH1 hypermethylation or mutation. This results in the accumulation of somatic mutations and subsequent genomic instability, which is associated with nonhereditary CRC [53]. It is well reported that MSI is a good prognostic factor for some treatments in early-stage CRC [59]. We believe it is important to always report MSI status in biomarker research to incorporate all relevant characteristics.

Clinical Relevancy

Clinically, the known risk factors for metachronous colorectal PM are an advanced tumor stage, right-sided tumor, infiltrative or ulcero-infiltrative tumors, history of perforation, and obstruction [3, 8, 60]. A randomized trial (COLOPEC-1) investigating the therapeutic effectiveness of adjuvant HIPEC to prevent PM development in high-risk CRC patients showed that this treatment strategy did not improve PM-free survival [11]. In contrast, a Spanish study by Arjona-Sánchez et al. concluded that adjuvant HIPEC therapy might be useful in patients with T4 tumors [61]. Identifying genetic alterations in high-risk metachronous PM patients may have additional benefit on improving survival by additional targeted therapies such as adjuvant HIPEC. In synchronous PM patients, the alterations provide added value to determine prognosis or to predict response to therapy. For example, RAS pathway activating mutations are negative predictive markers for the efficacy of anti-epidermal growth factor receptor (EGFR) therapies [62], while MSI tumors with *BRAF* and *PIK3CA* mutations show survival benefit [39]. For CRS and HIPEC scheduled patients, a *BRAF* mutation is a marker for poor prognosis, whereas *KRAS* tumors do not influence the outcomes [63]. The choice of cytostatic in HIPEC can be based on mutation status, or specific therapy can be developed in the case of targetable mutations.

Unfortunately, most of the studies did not clearly specify whether the authors were using tumors from synchronous or metachronous PM patients. It was therefore hard to distinguish and separate these two scenarios in the results. Future studies should clearly specify the time of metastases onset, the aim of the genetic analysis, and clinical implications.

Techniques

In the studies evaluated in this review, several different genetic research techniques were applied. Since most studies used targeted PCR techniques to detect specific gene mutations, the number of studies that used comprehensive genetic analyses was scarce. The development and use of NGS technologies have revolutionized the speed and throughput of DNA and RNA sequencing [64, 65]. However, since the number of relevant cancer genes guiding targeted therapy in CRC is still limited and costs per sample are substantial, NGS sequencing is not yet commonly used in clinical decision making or limited to mutation hotspot target regions [66]. This has most likely influenced the research to unmap PM predictive biomarkers so far, and we believe that more comprehensive NGS analyses are needed for this purpose. When we critically look at the choice of techniques used in the included studies, we believe these were too restricted to identify DNA/RNA biomarkers in the primary tumor of CRC patients with synchronous or metachronous PM.

As mCRC is a highly complex genetic disease, an understanding of how all aspects interact is required to achieve the prediction and treatment of colorectal PM. Single target techniques, mostly used in the included articles in this paper, might be insufficient for this purpose. We believe that omics techniques (i.e., techniques that generate high-throughput data [67]) might be a promising method for new CRC biomarkers research instead of most of the methods used in this paper. The integration of multiple omics techniques, by combining genomic data with data from other modalities such as transcriptomics, epigenetics, and proteomics, to measure gene expression, gene activation, and protein levels, could be helpful to reveal this problem in further research. This integration might bring us much closer to the prediction, prevention and tailored treatment of PM in CRC [68].

Limitations

This is the first systematic literature review of DNA/RNA biomarkers in relation to colorectal PM to the author's knowledge. This study has also some limitations. First, almost all included studies were retrospective with a different number of patients and different patients' characteristics (T-stage, number of metastatic sites, treatments, etc.). Second, comparisons between the studies are limited due to heterogeneity, and a meta-analysis was therefore not possible to perform. The standardization of techniques and analysis and more insight in the individual analysis outcomes via FAIR data sharing would be helpful. Third, most studies focused on the most commonly analyzed CRC target genes, i.e., *KRAS*, *NRAS*, *BRAF*, *PIK3CA*, and *TP53* with simple sequencing methods and PCR technology. Only three studies performed a broader gene panel NGS analysis. Fourth, most of the included studies did not report if CRC patients received neoadjuvant systemic treatments and if they did, which type. Such treatments could namely affect the outcomes of the genetic analysis. Fifth, most of the studies lacked the MSI of the CRCs. Sixth, all studies showed a moderate to high risk of bias with a high risk for the confounding domain.

Future Perspectives

We believe the use of comprehensive genomic profiling with for example broader cancer gene panels is essential to identify new potential cancer genes for PM prediction. In addition to using an optimal technique, we recommend applying these in a homogenous patient population (e.g. strict synchronous or metachronous PM patients, tumor characteristics, etc.).

CONCLUSIONS

Increasing amount of data suggest that the presence of biomarkers in the primary tumor might have an impact on metastatic patterns. However, unfortunately, based on the given evidence, we cannot consider the genes (e.g., *BRAF*) possibly associated with PM as reliable enough to function as an individual biomarker in a clinical setting yet. Further investigation as well as more exploratory research questions leading to identify novel biomarkers, rather than performing analyses on panels consisting mostly of already established biomarkers, are still necessary. Techniques on DNA and RNA level are required to determine an association between genomic, epigenomic and transcriptomic changes and colorectal PM. Furthermore, future studies should include homogenous populations so that firm conclusions can be drawn. In that way, we might be able to identify biomarkers that can be incorporated in a prediction tool to estimate the risk of distant metastatic spread or to create targeted treatment options.

ACKNOWLEDGMENTS

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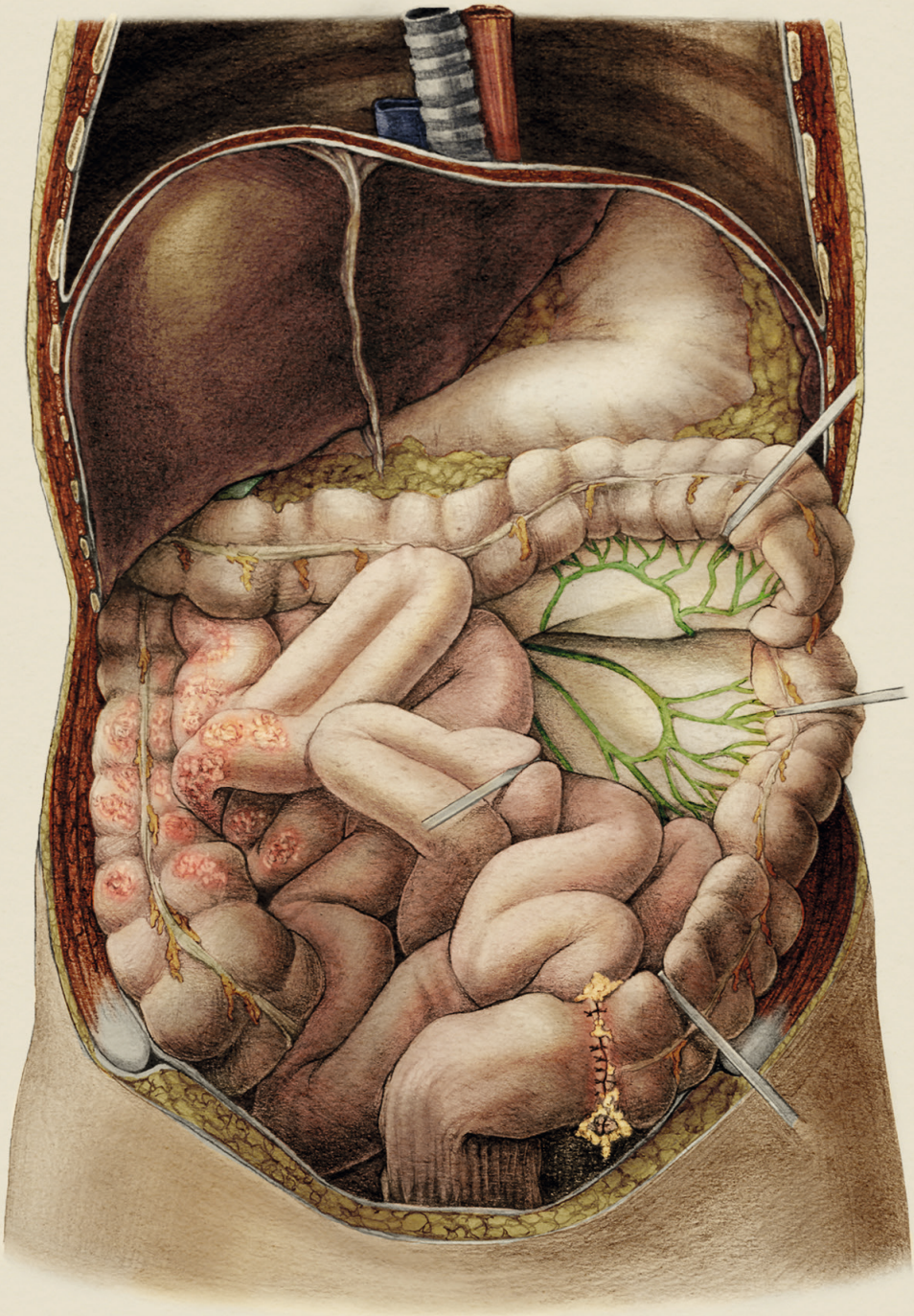
APPENDIX AND SUPPLEMENTARY

The following appendix and supplementary material can be downloaded from:

- *Appendix A: Search strategy*
- *Table S1. Overview investigated genes with conclusions*



CHAPTER 12

PREDICTIVE GENETIC BIOMARKERS FOR THE DEVELOPMENT OF PERITONEAL METASTASES IN COLORECTAL CANCER

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ABSTRACT

Background. Metastatic colorectal cancer (CRC) is a common cause of cancer-related mortality, of which peritoneal metastases (PMs) have the worse outcome. Metastasis-specific markers may help predict the spread of tumor cells and select patients for preventive strategies.

Aim. This exploratory pilot study aimed to gain more insight into genetic alterations in primary CRC tumors, which might be a predictive factor for the development of PM.

Methods. Forty patients with T3 stage CRC were retrospectively divided in three groups: without metachronous metastases during 5-year follow-up (M0, $n = 20$), with metachronous liver metastases (LM, $n = 10$) and with metachronous PM (PM, $n = 10$). Patients with synchronous metastases were excluded. Primary formalin-fixed paraffin-embedded tumor samples were analyzed via comprehensive genome sequencing (TSO500 analysis) to identify DNA alterations and RNA fusion transcripts in 523 genes and 55 genes, respectively.

Results. Thirty-eight samples were included for final analysis. Four M0 tumors and one PM tumor were microsatellite instable. *BRAF* mutations were uniquely identified in three microsatellite-stable (MSS) PM tumors (37.5%, $p = 0.010$). RNA analysis showed an additional *FAM198A-RAF1* fusion in one PM sample. *BRAF* p.V600E mutations were only present in PM patients with MSS tumors.

Conclusion. Greater attention should be paid to BRAF-mutated tumors in relation to the development of metachronous PM.

Keywords: Colorectal cancer; peritoneal metastases; biomarkers; genetic mutations; next generation sequencing

INTRODUCTION

Metastatic colorectal cancer (CRC) is a common cause of cancer-related mortality. At initial diagnoses, almost one-fourth of CRC patients present with metastases [1, 2]. Liver metastases (LMs) occur most frequently, followed by peritoneal metastases (PMs) [2, 3]. PMs are characterized by the development of solid tumor deposits on the peritoneal surface [4]. It is suggested that PMs develop through the shedding of tumor cells from the primary tumor, leading to intraperitoneal seeding [1]. Synchronous PMs are found in approximately 5–15% of patients with colorectal cancer at primary surgery [2-5], but PM may also develop metachronously after curative-intent treatment of the primary tumor. In clinical studies, these metachronous PMs are reported in 4–12% of patients following curative resection for colon cancer and in 2–19% of patients following curative resection for rectal cancer [5]. Routine imaging techniques frequently fail to detect PMs due to their small size along with the inherently low contrast resolution of the soft tissue in which they occur, resulting in an underestimation of their true incidence [2, 5, 6].

Since colorectal PMs occur less frequently than liver and lymph node metastases, they are considered less important from a prognostic perspective [7, 8]. Nonetheless, the consequences of PMs are significant. Without treatment, the average life expectancy is six to twelve months after diagnosis [4, 9, 10]. Currently, the only potential treatment to improve the survival of patients with colorectal PM is the surgical removal of all visible tumor deposits (cytoreductive surgery, CRS) followed by the application of heated chemotherapy, called hyperthermic intraperitoneal chemotherapy (HIPEC). Inquiries emerged concerning the requisite of adjuvant HIPEC subsequent to CRS, as CRS alone resulted in a survival advantage of over 40 months in the PRODIGE-7 trial [11].

Unfortunately, only a selection of physically fit patients with limited colorectal PM (peritoneal cancer index (PCI) below 20) are eligible for this therapy [2, 9, 10, 12]. With the changing perspective of this disease, many aspects of the biological and clinical understanding of this challenging disease process remain to be better understood [13].

In patients with synchronous PMs, genetic alterations are interesting as a biomarker to determine prognosis or to predict response to therapy [14-16]. In addition, genetic alterations in the primary tumor may also be useful for the prediction of PM occurrence. Several pathogenic mutations occur during adenoma-to-carcinoma transformation in CRC. Important oncogenes are adenomatous Polyposis Coli (*APC*), tumor suppressor gene *TP53*, *KRAS*, transforming growth factor beta (*TGF-β*), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), and loss of the chromosome arm 18q [17]. Additionally, some genetic alterations are described in relation to a specific metastatic site. For example, differences in *APC*, *BRAF*, *KRAS,* and *NRAS* are associated with the location of the primary tumor, whereby mutations in *KRAS* and *BRAF* seem to result in worse overall survival and the recurrence site in patients with PM [17, 18].

The aim of this study was to identify genomic changes in primary CRC that are associated with development of PMs, which would allow early detection and personal and early patient treatment. Such a study has not been reported yet [17, 19], despite the growing attention and possibilities for the genomic analysis of cancer using, for example, next-generation sequencing (NGS) techniques with broad gene panels investigating DNA and RNA alternations. In this explorative study, we identified specific DNA/RNA alterations (via TruSight Oncology (TSO) 500 analysis) in primary colorectal T3 tumors to predict metachronous PMs after curative resection.

METHODS

This study was conducted in a collaboration between the Maastricht University Medical Centre (MUMC+) and Catharina Hospital Eindhoven (CZE). The study was approved by the Institutional Medical Ethics Committee from MUMC+ (nr. 2021-2888) and CZE (nr. 2021-089) and conducted according to the Declaration of Helsinki.

Patients

The medical records of patients who underwent curative resection between 1 January 2012 and 31 December 2021 for colorectal adenocarcinoma were retrospectively reviewed. The research team deliberately chose to include a maximum of 40 patients in this pilot study, based on clinical prediction modeling which states at least 10 persons with the event (development of PM or LM) and 10 persons without the event (no metastases within 5 years) per included variable in the prediction model to obtain sufficient power [20]. Patients with T3 tumors were classified into three groups: those who had developed metachronous PMs (*n* = 10); those who had developed metachronous liver metastases (LM, *n* = 10); and those who never developed metastatic disease within 5 years after primary surgery (M0, *n* = 20). Patients with metachronous PMs were not allowed to be diagnosed with metachronous LMs and vice versa. As T4 tumors penetrate the surface of the visceral peritoneum and directly invade other organs or structures, the risk of spread into the peritoneal cavity is higher. Therefore, we only included T3 tumors and deliberately excluded T1 and T2 tumors to create a homogenous population. Patients with synchronous disease were excluded. Patients in the LM and PM group had no signs of metastases during resection of the primary tumor but were diagnosed with PM or LM during follow-up, at least 6 months after initial surgery. Patients in the M0 group did not develop any type of metastases during the follow-up period of at least 5 years. All in- and exclusion criteria are summarized in Table 1. Patient record files were screened, and the first 40 patients who met inclusion criteria were contacted. Informed consent was obtained from all participants. Demographics, pre-operative, operative, and follow-up data of all patients were retrospectively retrieved from medical records.

Table 1. In- and exclusion criteria for patient selection.

Inclusion Criteria	Exclusion Criteria
• Tumor histological type defined as an adenocarcinoma • Pathology report confirmed a radical resection with >15 lymph nodes • Pathological T3 classified according to the TNM classification • M0 group: Follow-up of 5 years without development of metastases	• Acute colorectal surgery with blow-out or proven perforation • Anastomotic leak after surgery • Patients with hereditary CRC • LM and PM group: Metachronous metastases > 6 months after primary surgery

M0: no metastases; LM, liver metastases; PM, peritoneal metastases.

Tumor Samples

Primary tumor FFPE tissue samples were obtained from MUMC+ and CZE. From each FFPE tissue specimen, 10 paraffin sections of 5 µm were cut. Hematoxylin and eosin (H&E) staining was performed. An experienced pathologist (J.B.) marked the tumor circumflex and estimated the tumor cell percentage under the microscope. Only samples with ≥10% tumor cell percentage were considered eligible for further analysis. Subsequently, microdissection with a pointed surgical blade was performed. DNA and RNA were extracted and isolated using a Maxwell RSC® System for Genomic DNA or RNA Extraction with a FFPE AS1450 kit and FFPE AS1440 kit, respectively (Promega, Madison, WI, USA). A blank control sample was analyzed in parallel to each set of samples. A minimum amount of 40 ng DNA or RNA was necessary for further analysis. DNA samples were stored at 4 °C and RNA samples at −80 °C. Fragment analysis of both DNA and RNA samples was performed as quality control. For DNA, a PCR was performed to visualize all DNA fragments. For RNA, the samples were assessed using a 4150 TapeStation system, which separates nucleic acids through electrophoresis. All fragments needed to be at least 200 bp in length.

TruSight Oncology 500 Analysis

TSO500 is an NGS assay that enables the comprehensive genomic profiling of tumor samples. The TSO500 panel (20028216; Illumina, Hayward, San Diego, CA, USA) was used to detect mutations and identify other relative pan-cancer genes in the tumor samples, as previously described by Verkouteren et al. [21]. The analysis includes 523 genes for mutations (all for single-nucleotide variants (SNVs)) and 59 for copy number variations (CNVs) (amplifications, insertions, and deletions). In addition, the assay allows for the identification of MSI and TMB. Besides DNA analysis, 55 genes are screened for fusion and splice variants on the RNA level. All genes included in the TSO 500 panel can be found in Supplementary Section S1, Figure S3. DNA and RNA processing and the generation of library preparations were performed according to the manufacturers' instructions. Data analysis was performed using the TSO500 Local App (Illumina, Hayward, San Diego, CA, USA). For DNA analysis, additional thresholds were maintained. First, for variant allele frequency, a percentage of ≥5% was maintained. Second, for classification as an amplification, a fold change of ≥3 was maintained. Third, the threshold for classification as MSI-high was ≥20% of microsatellite sites being unstable.

Fourth, a threshold of ≥15 mutations per megabase (mut/Mb) was used to define high TMB. Variants were classified subsequently using the inline Varsome application (access via https://varsome.com). Only pathogenic and likely pathogenic variants were included for further analysis; variants of uncertain significance (VUSs) were excluded.

Statistical Analysis

Gene mutation frequencies and associations between the found mutations and pathological patient characteristics were estimated. Analysis of both the total cohort (with MSI samples) as well as MSS samples only were performed. Numerical variables were presented as medians with interquartile range (IQR) as appropriate. For categorical variables, the number of patients and percentage were used. To evaluate the statistical significance of numerical variable differences observed between groups, non-parametric tests (Kruskal–Wallis and Mann–Whitney U-tests) were applied. Differences in categorical variables were tested using the Fisher–Freeman–Halton test and Fisher's exact test. Bonferroni correction for multiple comparison was applied to significant outcomes. All tests were two-sided, and differences were considered significant when the p value was <0.05. All the statistical analyses were performed with SPSS (IBM SPSS Statistics for Apple, Version 27, Armonk, NY, USA). In addition, an analysis with protein analysis through evolutionary relationships (PANTHER) was performed [22]. The latter was performed for Gene Ontology molecular functions and biological processes [23, 24], for PANTHER pathways, and for Reactome pathways [25]. For each sample, the significantly enriched terms were extracted for subsequent analyses using R (R core team, version 4.2.0., Vienna, Austria). Analysis and visualization of the genetic outcomes were carried out with Python (Matplotlib v3.7.0, Salt Lake, UT, USA) and GraphPad Prism (GraphPad software for Apple, version 8.0.0, San Diego, CA, USA).

RESULTS

Study Cohort

Initially, 40 cases were selected according to predefined in- and exclusion criteria. After revision of the CT, one of the patients was diagnosed with a synchronous metastatic lesion in the lung and excluded from further analysis. All patient characteristics and clinicopathological variables are summarized in Table 2. Most patients were males (64%), with a median age of 69 years (61.00–74.00) at the time of diagnosis of the primary colorectal tumor. There was an overall significant difference for differentiation grade (p value < 0.001) and neoadjuvant treatment (p value = 0.039). After pairwise comparison, a significant difference was found in the differentiation grade when primary tumors of patients with metachronous PM were compared to patients without metachronous metastases (M0) and with metachronous liver metastases (LM) (p value < 0.001 and 0.015, respectively). Patients in the LM group were more often treated with neoadjuvant therapy compared to the M0 group (p value = 0.030), which did not remain significant after Bonferroni correction.

Table 2. Comparison of patient characteristics and clinicopathological variables in the relation to the development of metastases.

Variable	M0 (N = 20)	LM (N = 10)	PM (N = 9)	*p* Value
Age at time of diagnosis (years) —*median (Q1–Q3)*	69.00 (62.00–74.90)	69.00 (63.75–74.25)	68.00 (58.00–74.00)	0.801 [a]
Gender–*n (%)*				
Male	12 (60)	8 (80)	5 (55.6)	0.514 [b]
Female	8 (40)	2 (20)	4 (44.4)	
Primary tumor location [†]—*n (%)*				
Right colon	10 (50)	2 (20)	2 (22.2)	0.433 [b]
Left colon	7 (35)	5 (50)	4 (44.4)	
Rectum	3 (15)	3 (30)	3 (33.3)	
Tumor size (cm) –*median (Q1–Q3)*	4.10 (3.28–5.38)	2.25 (1.80–5.43)	3.00 (2.40–3.50)	0.061 [a]
Differentiation grade—*n (%)* *				**<0.001 [b]**
Poor	4 (20)	0 (0)	0 (0)	
Poor/moderate	2 (10)	2 (20)	8 (88.9)	
Moderate	14 (70)	6 (60)	1 (11.1)	
Moderate/well	0 (0)	1 (10)	0 (0)	
Type of surgery—*n (%)*				
Open	10 (50)	2 (20)	5 (55.6)	0.153 [b]
Laparoscopic	10 (50)	6 (60)	4 (44.4)	
Robot assisted	0 (0)	2 (20)	0 (0)	
Positive lymph nodes—*n (%)*				0.389 [b]
No	11 (55)	8 (80)	5 (55.6)	
Yes	9 (45)	2 (20)	4 (44.4)	
Neoadjuvant treatment—*n (%)*				**0.039 [b]**
No	17 (85)	4 (40)	7 (77.8)	
Yes	3 (15)	6 (60)	2 (22.2)	
Adjuvant treatment—*n (%)* *				0.247 [b]
No	9 (45)	7 (70)	4 (44.4)	
Yes	11 (55)	2 (20)	5 (55.6)	
Oncological history—*n (%)*				0.882 [b]
No	18 (90)	8 (80)	8 (88.9)	
Yes	2 (10)	2 (20)	1 (11.1)	
Oncological family history—*n (%)* *				1.000 [b]
No	6 (30)	3 (30)	0 (0)	
Yes	12 (60)	5 (50)	1 (11.1)	
Time between surgery and metastases (months)—*median (Q1–Q3)*	N/A	18.09 (7.77–28.95)	16.42 (9.71–25.05)	0.744 [c]
PCI score—*median (Q1–Q3)*	N/A	N/A	3.50 (3.00–4.00)	N/A

*[a] Kruskal–Wallis Test; [b] Fisher–Freeman–Halton Exact Test; [c] Mann–Whitney test. [†] Right-sided = from caecum to transverse colon; left-sided = from the splenic flexure to sigmoid. * Missing data in differentiation grade (LM = 1), adjuvant treatment (LM = 1), and oncological family history (M0 = 2, LM = 2 and PM = 8). M0, no metastases; LM, liver metastases; PM, peritoneal metastases; Q1–Q3, quartile 1–quartile 3; N/A, not applicable; PCI, peritoneal cancer index.*

DNA Sequencing

In one LM sample, no (likely) pathogenic mutations were found, most probably due to the low residual tumor area after neo-adjuvant treatment. This outcome was considered unreliable, and the sample was excluded from further DNA analysis. The final study cohort thus consisted of 38 patients (Supplementary Section S1, Figure S1 and Table S1).

Microsatellite instability (MSI) analysis showed that a total of 5/38 samples (four M0 [20%] and one PM [11%]) were MSI with a median of 53.91% unstable MSI sites (Q1 32.55–68.11; Supplementary Section S1, Figure S2). These samples also showed a significant TMB with a median of 64.3 mut/Mb (Q1 49.45–Q3 180.60). All significant MSI and TMB patients had a right-sided primary tumor with poor or poor/moderate differentiation grade. The occurrence of MSI and TMB was not significantly different between the three groups. One of the MSI samples harbored a nonsense mutation in *MSH6* (i.e., c.3772C>T p.(Q1258*)), a DNA mismatch repair protein, which could explain the instability of the sample. All other four samples showed *MLH1* promotor hypermethylation.

Mutational signatures from each sample were individually analyzed. Base substitution of C>T and T>C were the most common ones in all samples. No specific profile was identified when comparing the three subgroups. A general overview of all variant type frequencies and amplifications is displayed in Supplementary Section S1, Table S2 and of all tumor mutations and amplifications in Table S3. Analysis of the total cohort did not identify significant gene mutations in the PM group nor other subgroups. As MSI samples showed a lot of passenger genes that were influencing analysis outcomes, all MSI samples were excluded for a separate analysis with only microsatellite stable (MSS) tumors. The analysis of the total cohort (MSI + MSS samples, *n* = 38) can be found in Supplementary Section S2 (Figure S4-5, Table S5).

MSS Samples Analysis

All MSI tumors were excluded for a separate analysis. This resulted in a study population of 33 patients with MSS tumors (M0 N = 16, LM N = 9, and PM N = 8). A total of 164 (likely) pathogenic genetic alterations were detected in 78 genes (Figure 1). Missense, frameshift, and nonsense mutations were most commonly detected. When comparing the occurrence of all variant types, no significant differences were found. The distribution among cancer genes related to CRC was investigated (Figure 2). APC mutations occurred most frequently; in 4/8 (50%) of the PM cases and 8/9 (89, 89%) LM and 14/16 (87, 50%) M0 patients (not significant). *BRAF* (c.1799T > A p.(V600E) exon 15) mutations were only present in PM patients in this cohort (3/8 = 37.5%, *p* value = 0.010). None of the M0 samples were carrying *PIK3CA* mutations after MSI exclusion, and none of the PM samples were carrying *NRAS* mutations, although these findings were not significantly different. A detailed overview of all MSS subgroup comparisons with statistical *p* values can be found in the Supplementary Section S1, Table S4.

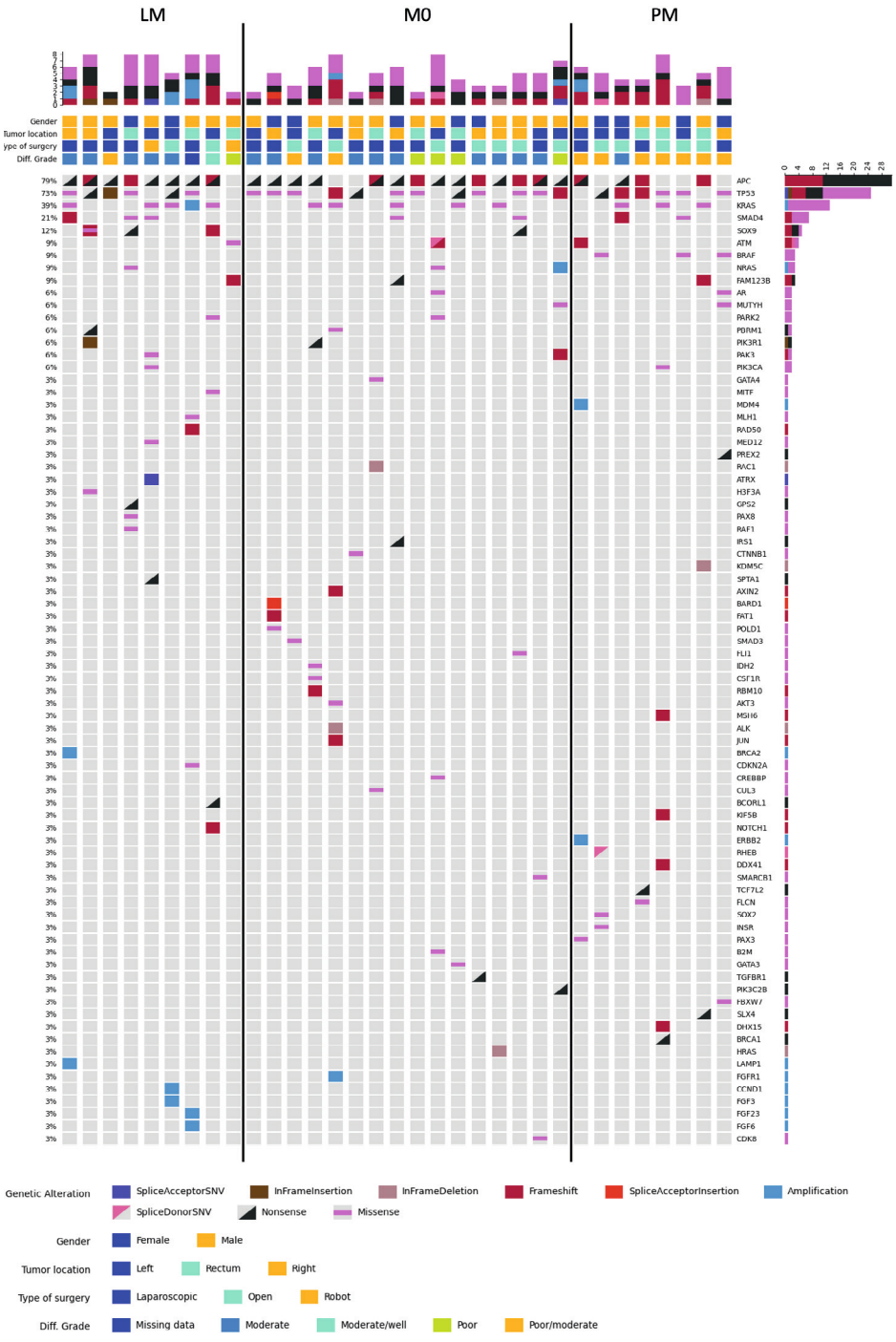

Figure 1. Oncoplot of variants across MSS samples. Genes on *y*-axis; samples on *x*-axis.

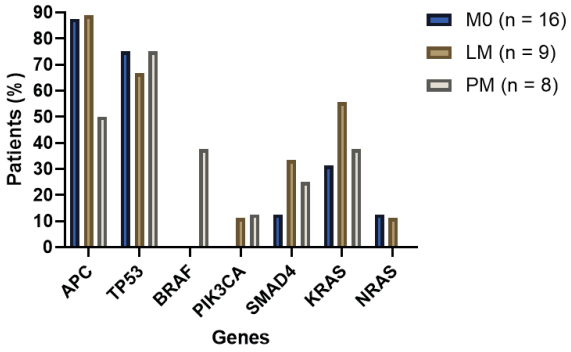

Figure 2. Distribution of well-known cancer genes related to MSS CRC.

Additional Analyses

Pathways, molecular functions, and biological processes were not significantly different between the three CRC subgroups. Also, after the additional inclusion of all identified variants of uncertain significance (VUSs), no significant differences were found between the subgroups. A detailed overview of all additional data analyses can be requested via the corresponding author.

RNA Sequencing

RNA sequencing was performed on 28 samples, divided as follows: M0 (*n* = 10), LM (*n* = 9), and PM (*n* = 9). Data analysis revealed no splice variants for the genes in the panel, whereas three samples (one M0 and two PM samples) showed gene fusion transcripts, which are summarized in Table 3. Interestingly, two gene fusions were identified which can be considered driving mutations, i.e., *FAM198A-RAF1* and *TARSL2-NTRK3*. The *NTRK3* fusion was confirmed via fluorescence in situ hybridization (FISH), using an *NTRK3* break-apart probe (Figure 3).

Table 3. Detailed output of RNA analysis.

M Group	Gene Pair	Breakpoint 1	Breakpoint 2	Fusion Supporting Reads
M0	*TARSL2-NTRK3*	Exon 18 chr15:102197123	Exon 14 chr15:88576274	19
PM	*FAM198A-RAF1*	Exon not found chr3:43101459	Exon 3 chr3:12653448	85
PM	*RPS6KB1-HSF5*	Exon 1 chr17:57970685	Exon 3 chr17:56544340	21

M0, no metastases; PM, peritoneal metastases.

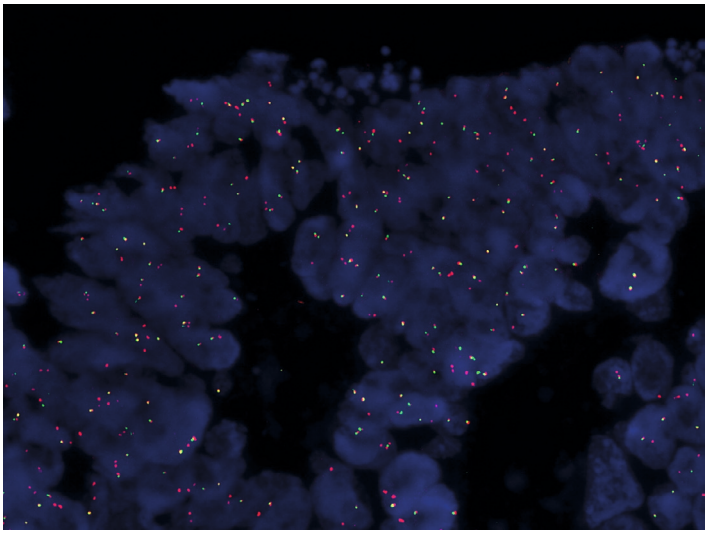

Figure 3. FISH analysis of the M0 sample harboring the *TARSL2—NTRK3* fusion, showing isolated green and red signals confirming an *NTRK3* gene rearrangement.

DISCUSSION

In this study, we performed an integrated pan-cancer oncology enrichment next-generation sequencing assay (TSO500 analysis) to assess DNA and RNA alterations in 523 and 55 genes, respectively, in primary colorectal adenocarcinomas with or without metachronous PM or LM. Our cohort showed a significant difference in differentiation grade when PM samples were compared to LM and M0 samples, and in the LM group for neoadjuvant treatment. Genetic analysis of all MSS tumors revealed that pathogenic *BRAF* exon 15 p.(V600E) mutations were exclusively identified in three *RAS* wildtype tumors with metachronous PM (37.5%, *p* value = 0.010). RNA sequencing identified a *FAM198A-RAF1* fusion in an additional tumor with PM, as well as a *TARSL2-NTRK3* fusion in a M0 sample.

Patient Characteristics and Clinicopathological Variables

We identified two clinicopathological characteristics that were significantly different between the three tumor groups. First, the PM group contained more poor/moderately differentiated tumors, while M0 and LM tumors were more often moderately differentiated. The latter was also shown in an extensive analysis of the association between metachronous PM and clinicopathological characteristics by Zhang et al. [26]. Tumor location is not mentioned in this analysis, although another study reports that right-sided primary colorectal tumors are associated with PM [2]. Only 22% of the PM tumors in our cohort were right sided. Second, the lowest tumor cell percentages were observed in the LM group, which may be explained by the fact that in this group, more patients received neoadjuvant treatment via chemoradiation

because of a low rectal primary origin. Other previously described clinicopathological risk factors for the development of metachronous PMs are advanced tumor stage, infiltrative or ulcero-infiltrative tumors, a history of perforation, and obstruction [1, 4, 27]. A clinical trial investigating the potential of adjuvant HIPEC in high-risk PM patients, based on these clinicopathological risk factors, showed that adjuvant HIPEC did not improve survival as compared to patients receiving systemic adjuvant chemotherapy [28]. In contrast, Arjona-Sánchez et al. concluded that adjuvant HIPEC therapy might be useful in patients with T4 tumors [29]. These outcomes suggest that specific biomarkers identified in the primary tumor might be helpful to further estimate the risk of metastatic spread and the need for preventive adjuvant treatments. As our study population has a semi-advanced tumor stage (T3) without (ulcero-)infiltrative or obstructing tumors, we exclude any influence of these possible clinical–pathological risk factors in our current study.

DNA and RNA Sequencing

The most frequently mutated cancer genes found in our study include *APC*, *TP53*, *KRAS, SMAD, NRAS, BRAF, PIK3CA*, and *SOX9*. These genes are well known to be involved in the tumorigenesis of CRC [17]. Prevalence data in the literature on these well-known oncogenes are in line with our findings [15, 30-51]. In addition, 12.5% of our tumors contained MSI, in four cases associated with MLH1 promoter hypermethylation, and in one case with an inactivating MSH6 mutation. This finding is in accordance with the literature, as was the finding that these tumors are often right-sided [52,53, 54]. MSI results from the inactivation of the mismatch repair genes (MMR), which leads to the accumulation of somatic mutations, genomic instability, and cancer-associated alterations [37]. TMB represents the total number of mutations per Mb found in the DNA of tumor cells and is therefore often significantly higher in MSI tumors. In this study, the five tumors with MSI all had a high TMB (IQR 49.45–Q3 180.60).

It has been suggested that MSI status may be useful as a predictor of the risk of developing metachronous CRC, because it can cause a further increase in metastatic potential [30, 52]. However, we did not observe a higher incidence of MSI tumors in our CRC cohort that developed metastases. Interestingly, *BRAF* p.V600E mutations were found to be exclusively present in PM patients with *RAS* wildtype MSS tumors (37.5%, *p* value = 0.010). Approximately 10–14% of all CRC cases have *BRAF*-activating mutations [30, 37, 55]. *BRAF* encodes a serine/threonine protein kinase, which plays an important role in the mitogen-activated protein kinase (MAPK) pathway. This pathway drives cell proliferation, differentiation, migration, survival, and angiogenesis, and therefore, changes in this pathway are associated with tumorigenesis [55]. The *BRAF* p.V600 mutation, caused by a transversion in exon 15 resulting in a valine amino acid substitution [56], accounts for more than 90–95% of *BRAF* mutations [37, 55] and is associated with poor overall survival [30]. In addition, we identified a *FAM198A-RAF1* fusion in one PM sample. Both RAF1 and BRAF belong to the *RAF* family of protein kinases playing a role in MAPK signaling. Previous studies suggested that *BRAF* p.V600 mutant tumors are more likely to develop PM [15, 38, 41-43, 57]. Therefore, we and some authors recommend analyzing *BRAF* mutation for its prognostic value in primary T3 CRC [37, 40].

The clinical significance of *NTRK3* fusion identified in our study, in the setting of CRC, as well as the possibility for targeted treatments should be explored in the future.

Prior to our explorative study, we performed a systematic review to summarize the current knowledge on genetics and genomics in CRC-PM [19]. An NGS analysis with 409 cancer genes showed several additional genetic mutations, i.e., *ARID1A*, *PKHD1*, *UBR5*, *PAX5*, *TP53*, *ASXL1*, and *AR*, presumably associated with PM [58]. In our TSO500 NGS panel, *ARID1A*, *PAX5*, *TP53*, *ASXL1*, and *AR* were included as well. *AR* and *ARID1A* mutations occurred in one PM (11%) and one M0 sample (5%). Only one *PAX5* mutation was found in one M0 patient, and *ASXL1* mutations were not detected. Thereby, the suggested genes related to PM by Lee et al. are not confirmed in our paper. The latter may be explained by the difference in study population; Lee et al. included patients with small obstructing adenocarcinomas (≤3 cm) with synchronous or metachronous PM and compared them with large non-obstructing tumors without PM. Another explanation could be our small sample size. Other authors describe *NEK2*, *MACC1*, *REG1A*, *KIF18A*, *RET*, and *TIP60* as possible PM-related cancer genes [59-64]. In our TSO500 panel, only *RET* was investigated. In contrast to the suggestion of Yang et al. concerning the association of *RET* mutations and PM, we did not identify any mutation in this gene in our cohort [63].

Another factor that can contribute to the difficulty of finding biomarkers is the genetic differences between the primary tumor and metastatic lesions. Studies investigating the differences between peritoneal lesions and their primary tumors reported some small unique differences [65], whilst other studies report high concordance [66-68]. A very recent study by Lenos et al. showed that peritoneal lesions seemed to have much more similarity to their primary tumor compared to other metastases, and these lesions seemed to retain both clonal heterogeneity and transcriptional profile [67].

A new way to look at CRC tumors is through dividing them into subtypes, for example, the previously described four consensus molecular subtypes (CMS 1-4). These subtypes aid in prognostication as well as in determining treatment strategies for individual patients based on the mutations, activated pathways, and phenotypic characteristics and responses to treatment of other tumors with similar signatures [69]. The majority of PMs in their study are of the CMS4 subtype, known as the mesenchymal subgroup [70, 71]. CMS4 is presented in 23% of CRC cases, which are most often distal tumors with poor relapse-free and overall survival and harbor prominent transforming growth factor β activation, stromal infiltration, and angiogenesis [72, 73]. CMS4 tumors have extremely low levels of hypermutation, MSS, and very high somatic copy number alteration counts [69]. The latter was also seen in our cohort. Unfortunately, we were not able to examine all of these characteristics in our study due to the limited content of our RNA NGS gene panel. Therefore, the translation to CMS subgroups was not possible in our study.

Treatment Options and Future Perspectives

BRAF mutations can be considered as an independent negative prognostic factor in early stage MSS tumors and as a negative predictive factor for therapeutic approaches [55]. The therapeutic approach to treat *BRAF*-mutated tumors is not straightforward due to its resistance to standard therapies [55]. Research into anti-epidermal growth factor receptor (EGFR) and anti-vascular endothelial growth factor (VEGF) antibodies has not shown statistical benefits in *BRAF*-mutated patients [55, 74]. *BRAF* inhibitors (i*BRAF*) have revolutionized the treatment of *BRAF* V600E metastatic melanoma, but so far, results in CRC patients are disappointing due to resistance [40, 55, 75]. Studies are currently ongoing with dual or triple drug therapy to blockade the *MAPK* pathway [55, 75]. Until now, partial activity of different combinations has been shown, but this is far from the promising results in melanoma patients. Ongoing research will hopefully demonstrate that combination strategies with *iBRAF* and other drugs can overcome the lack of efficacy [55]. As survival is about half as long as that of *BRAF* wildtype patients [74], there is an urgency to unravel new treatments that improve *BRAF*-mutant CRC patients' outcomes.

In current clinical practice, the classification of the MSI status is the only genetic test that is routinely performed in CRC patients to decide adjuvant therapy decisions [76]. Other genetic tests, such as *BRAF* mutation status, are only evaluated in metastatic tumors. Based on the results of this paper, we believe greater attention should be paid to *BRAF*-mutated tumors in relation to the development of metachronous PM in CRC patients without metastases. Standard clinical screening for *BRAF* mutations might feel too early as it does not offer any new treatment options, but a stricter follow-up in this population may be clinically beneficial. Based on new international guidelines, the first follow-up CT scan is not performed until 12 months after primary surgery. However, in a *BRAF*-mutated population, earlier follow-up imaging and more clinical monitoring for PM development may be warranted. Of course, future prospective research (e.g., with liquid biopsies) into the validation of *BRAF* mutations in relation to the development of metachronous PM is needed to substantiate this proposition.

Strengths and Limitations

A very homogenous group of tumors was selected for genetic analysis. To our knowledge, this is the first study investigating T3 tumors in relation to metachronous CRC metastases. Previous studies focused on T4 tumors with mostly synchronous PMs and had no other metastases group (LM) as a comparator. While PMs may develop from different cancer types, we specifically examined the colorectal origin and excluded appendiceal origin as it is known that gene expression from appendiceal tumors is distinct from CRC [65]. Due to refinements in DNA and RNA extraction techniques from formalin-fixed paraffin-embedded (FFPE) tissue material, the sensitivity of DNA and RNA testing has been increased. Our targeted TSO500 NGS technique accurately measures TMB, microsatellite instability, single-nucleotide variants, indels, copy-number/structural variation, and gene fusions in a single assay using relatively

small amounts of DNA and RNA as input. Combining DNA and RNA hybrid-capture with sophisticated informatics reduces errors and yields high-quality data, even from FFPE samples.

We did not perform an extensive sample size calculation due to the predictive and explorative character of this study. Despite the efforts made to create as much homogeneity between the three groups as possible, the number of patients in our cohort is small. A larger-scale study should be conducted to confirm the mutation differences in relation to PMs. Thereby, being a retrospective study, there is a likelihood of selection bias and information bias. Additionally, we performed a very broad cancer gene analysis with our TSO500 panel, although the method does not cover all genes. Through performing whole exome or genome sequencing (WES or WGS), potential candidate genes that can act as a predictive PM biomarker that are not included in the TSO500 panel may be identified. Unfortunately, WES and/or WGS are more expensive and have additional logistic limitations.

CONCLUSION

Over the last decade, the genetic analysis of CRC has evolved enormously, resulting in better tumor classifications, improved treatment decisions, and finally enabling personalized treatment options. Specific genetic changes and mutations that could predict PM remain largely unknown. In our cohort, we identified genes that have not been described in relation to metachronous PMs, or metastases in general, before. The clinical significance of this finding remains unknown due to the small sample size. BRAF V600E mutations were only present in PM patients with MSS tumors. We believe greater attention should be paid to BRAF-mutated tumors in relation to the development of metachronous PMs. Future prospective research into and validation of the molecular players identified here, specifically within non-synchronous tumors, might influence the efficacy of existing and future diagnostic (biomarker identification), prognostic (patient grouping and recurrence), and therapeutic (molecular) actions.

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The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Institutional Medical Ethics Committee from MUMC+ (nr. 2021-2888) and CZE (nr. 2021-089).

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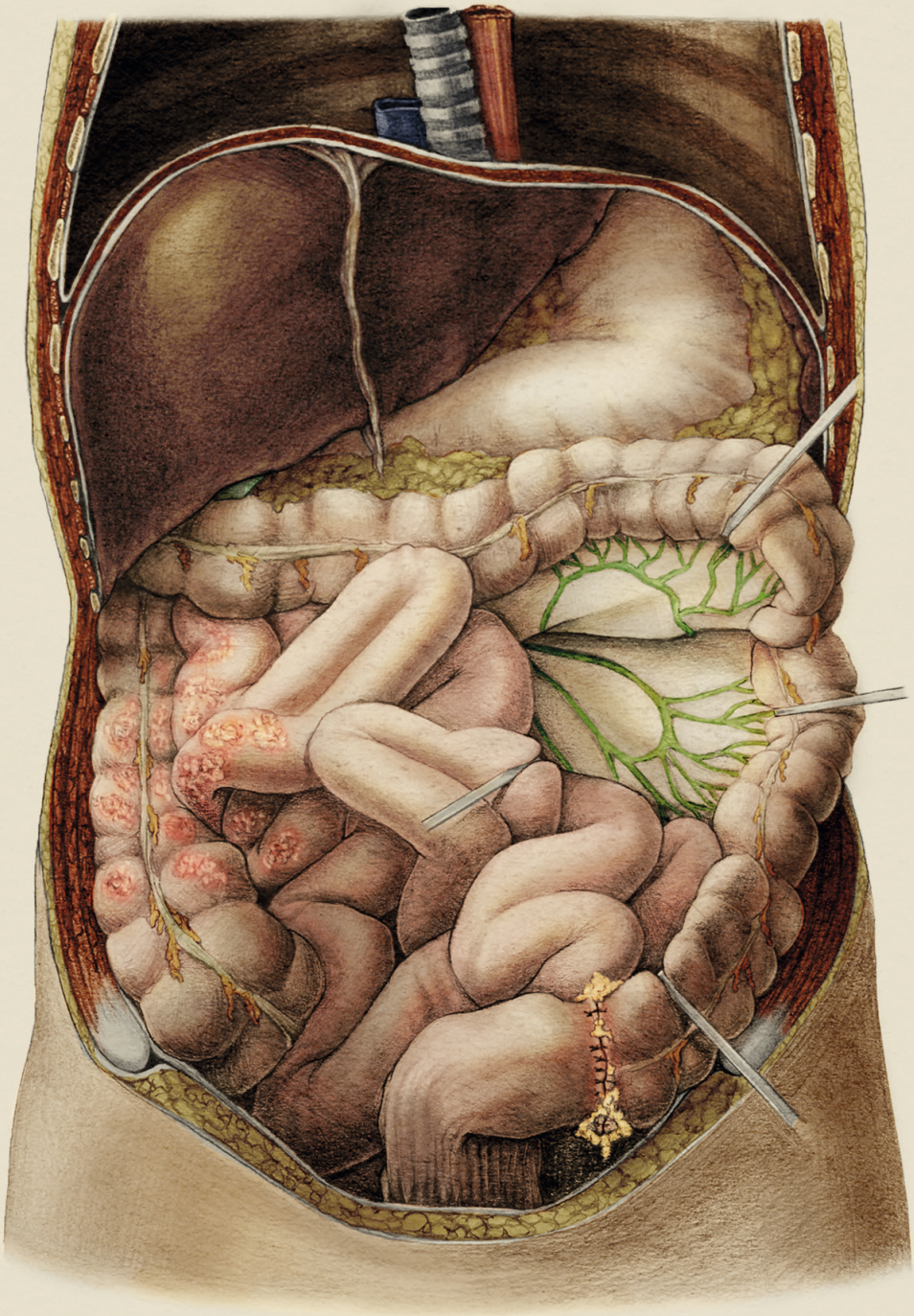
SUPPLEMENTARY

The following supplementary material can be downloaded from:

- *Figure S1. Details patient selection and data analysis*
- *Figure S2. Boxplots of median unstable MSI sites (%) and median TMB*
- *Figure S3. Overview of genes included in the TruSight Oncology 500 panel. [Illumina, Inc. (n.d.)]*
- *Table S1. Overview of tumor sample characteristics*
- *Table S2. (1) Overview of DNA sequencing (likely) pathogenic variant types and amplifications of total cohort (2) Overview of DNA sequencing (likely) pathogenic variant types and amplifications after MSI samples exclusion*
- *Table S3. Detailed overview of (likely) pathogenic DNA mutations and amplifications*

Supplementary Section S2: Analysis on total cohort (MSI + MSS samples)

- *Table S4. Detailed overview percentage of mutated/amplified gene in each subgroup after MSI sample deletion, with statistical analysis*
- *Figure S4. Oncoplot of variants across samples. Genes on y-axis, samples on x-axis*
- *Figure S5. (A) Distribution of well-known oncogenes related to CRC between subgroups. (B) Genes mutated or amplified* in PM group only. (C) Genes mutated or amplified* in LM group only. (D) Genes mutated in LM and PM group (not in M0)*
- *Table S5. Detailed overview percentage of mutated/amplified gene in each subgroup with statistical analysis*



CHAPTER 13

EVALUATION OF THE EFFECT OF AN INTRAPERITONEAL CYTOSTATIC-LOADED SUPRAMOLECULAR HYDROGEL ON INTESTINAL ANASTOMOTIC HEALING IN AN ANIMAL MODEL

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ABSTRACT

Background. The prognosis of colorectal cancer patients with peritoneal metastases is very poor. Intraperitoneal drug delivery systems, like supramolecular hydrogels, are being developed to improve local delivery and intraperitoneal residence time of a cytostatic such as mitomycin C (MMC).

Aim. To evaluate the effect of intraperitoneal hydrogel administration on anastomotic healing.

Methods. Forty-two healthy Wistar rats received a colonic end-to-end anastomosis, after which 6 animals received an intraperitoneal injection with saline, 18 with unloaded hydrogel and 18 with MMC-loaded hydrogel. After 7 days, animals were euthanized, and the anastomotic adhesion and leakage score were measured as primary outcome. Secondary outcomes were bursting pressure, histological anastomosis evaluation and body weight changes.

Results. Twenty-two rats completed the follow-up period (saline: $n = 6$, unloaded hydrogel: $n = 10$, MMC-loaded hydrogel: $n = 6$) and were included in the analysis. A trend towards significance was found for anastomotic leakage score between the rats receiving saline and unloaded hydrogel after multiple-comparison correction ($p = 0.020$, $\alpha = 0.0167$). No significant differences were found for all other outcomes. The main reason for drop-out in this study was intestinal blood loss.

Conclusion. Although the preliminary results suggest that MMC-loaded or unloaded hydrogel does not influence anastomotic healing, the intestinal blood loss observed in a considerable number of animals receiving unloaded and MMC-loaded hydrogel implies that the injection of the hydrogel under the studied conditions is not safe in the current rodent model and warrants further optimalisation of the hydrogel.

Keywords: peritoneal metastases; colorectal cancer; intraperitoneal delivery; injectable supramolecular hydrogel; mitomycin

INTRODUCTION

Colorectal cancer (CRC) is the third most prevalent cancer type worldwide and a common cause of morbidity and mortality generally attributable to metastatic disease [1, 2]. The prognosis of CRC patients with peritoneal metastases (PM) is very poor. For a selective group of patients, there are life-prolonging treatment options available. A common strategy for physically fit patients with limited disease burden is cytoreductive surgery (CRS) with or without adjuvant hyperthermic intraperitoneal chemotherapy (HIPEC) [1, 3]. Patients who are not considered eligible may undergo a new palliative treatment option that is currently being investigated called pressurized intraperitoneal aerosol chemotherapy (PIPAC) [4-8].

Despite the introduction of HIPEC and PIPAC, treatment failure is still a major issue in CRC patients with PM. As intraperitoneal delivery of cytostatic drugs is the preferred route for PM treatment, intraperitoneal drug delivery systems are being investigated [9]. One such system is a supramolecular hydrogel, which has shown potential in the field of PM therapy. The development of targeted therapies using drug-loaded hydrogels can help deliver drugs directly to the affected area, improving therapeutic outcomes [10, 11]. In recent years, our research team has conducted several experiments investigating the feasibility, safety, tissue compatibility and therapeutic efficacy of a supramolecular hydrogel loaded with mitomycin C (MMC) [12, 13]. The main function of this injectable hydrogel is to form an intraperitoneal depot of slow-releasing MMC, aiming to establish prolonged exposure of the PM to the cytostatic agent. The therapeutic efficacy in a PM rat model was investigated before and demonstrated that there is a clinically relevant survival benefit for MMC-loaded hydrogel compared to injection of free MMC [13].

Intraperitoneal chemotherapy is usually preceded by cytoreductive surgery, which frequently includes a colon resection to remove the primary tumor, often requiring a colonic or colorectal anastomosis. Anastomotic leakage (AL) is considered one of the most important complications after such a colon resection. It occurs in 1 to 19% of the cases and has a negative impact on survival [14, 15]. Chemotherapeutics, including MMC, that are administered intraperitoneally are suspected to have an effect on anastomotic healing after surgery [16, 17]. As the therapeutic benefit of MMC-loaded hydrogel in PM has been demonstrated by previous work, it is crucial to investigate its influence on anastomotic healing before considering this treatment option for in combination with CRS for PM of colorectal origin in humans.

The aim of this study was to investigate whether intraperitoneal administration of hydrogel (both unloaded and MMC-loaded) affects colonic anastomotic healing; specifically, whether it results in a higher incidence of AL in a rodent animal model. For this purpose, the previously investigated supramolecular hydrogel was intraperitoneally applied in healthy rats after creating a sufficient end-to-end colon anastomosis.

METHODS

Ethics and Safety Protocol

This animal study was performed at the animal center of Maastricht University (Maastricht, The Netherlands). The experimental protocol followed the Dutch Animal Experimental Act and was approved by the Animal Experimental Committee of Maastricht University Medical Center (project license AVD1070020198765). The ARRIVE guidelines [18] for reporting animal research were followed and additional information can be found in the Supplementary Materials. During the experiment, we maintained a local cytostatic protocol developed by the animal center of Maastricht University to ensure appropriate safety measures while working with chemotherapy.

Animals and Housing

A total of 42 healthy adult Wistar rats (21 males/21 females) aged 10–12 weeks with a body weight of 400 g–500 g (males) and 230 g–330 g (females) were used (all characteristics can be found in Supplementary Table S1). All rats were bred by Charles River Laboratories (Sulzfeld, Germany). An acclimatization period of at least one week was maintained. All animals were socially housed in individually ventilated cages in a temperature- and humidity-controlled room with 12 h light/dark cycles. All animals had ad libitum access to food (10 mm Sniff rat/mouse sterilized food compressed into pallets) and acidified drinking water. Postoperatively, animals were weighed daily, and welfare was scored systematically based on predefined standardized welfare scoring sheets (Supplementary Materials). Human endpoints (HEPs) were defined prior to the experiment.

Study Design, Randomization and Blinding

The aim of this study was to investigate if intraperitoneal administration of MMC-loaded and unloaded hydrogel affected the anastomotic healing compared to animals receiving a peritoneal injection with saline. To study the effect on anastomotic healing, all animals received a sufficient colon–colon anastomosis. Subsequently, the animals were randomly assigned to one of the three following intervention groups receiving a single injection with either saline (n = 6), unloaded hydrogel (n = 18) or MMC-loaded hydrogel (n = 18). The random allocation of the animals was performed by a computer-based random order generator. After a follow-up period of seven days (most ALs show up within the first week after surgery), the anastomotic healing was assessed. During the allocation, the conduct of the experiment and the outcome assessment, the research team, the veterinarian and the people working in the animal facility were blinded for the group allocation.

Supramolecular Hydrogel

For this experiment, we used a supramolecular hydrogel based on polyethylene glycol (PEG) chains end-modified with fourfold hydrogen bonding the ureido-pyrimidinone (UPy) units (UPy–PEG Hydrogel), previously described by Wintjens et al. [12, 13, 19, 20]. Identical to the previous

studies described by Wintjens et al., the rats received 20 mL/kg of hydrogel corresponding to a single intraperitoneal injection of 5 mL for female rats (±250 g) or 8 mL for male rats (±400 g).

Anesthesia, Surgical Procedure and Analgesia

A subcutaneous injection of 0.05 mg/kg buprenorphine (Richter Pharma AG, Wels, Austria) was given one hour prior to surgery as an analgesic. The surgical procedure was performed by experts (A.J. and N.B.) certified for performing anastomotic models in laboratory animals. All animals underwent general anesthesia using 4–5 vol.% isoflurane supplied with air (IsoFlo, Zoeties B.V., Rotterdam, The Netherlands) for induction which was maintained with 2–3vol.%. The body temperature was maintained by placing the animals on a heated plate with a temperature of ca. 36 °C. A 5 cm craniocaudal midline incision of the skin and abdominal musculature was performed with a scalpel, after removing the abdominal fur with electric clippers and local injection of bupivacaine (Aurobindo Pharma BV, Baarn, The Netherlands). The cecum and additional intestines were taken outside the abdomen onto sterile gauzes hydrated with sterile saline solution to prevent dehydration. The site for colon–colon anastomosis was identified at ca. 4 cm *ab ani*, whereafter the colon was fully transected with scissors. An end-to-end anastomosis was created using at least 9 interrupted polypropylene sutures (Prolene 6-0, Ethicon, Johnson & Johnson; Supplementary Figure S1). After the creation of a sufficient anastomosis, it was tested for leakage of water by injection of NaCl via the rectum. In case of water leaking through the anastomosis, additional sutures were placed until the anastomosis remained dry. Thereafter, the intestines were repositioned in the abdomen, and the abdomen was closed with a running suture for the muscle layer (Prolene 4-0, Ethicon, Inc., Johnson & Johnson) and interrupted sutures for the skin (Monocryl 4-0, Ethicon, Inc., Johnson & Johnson). Subsequently, the animals received a single intraperitoneal injection with 5 or 8 mL (F/M) saline, unloaded hydrogel or MMC-loaded hydrogel, corresponding to a volume-to-weight ratio of 20 mL/kg.

Postoperatively, a saline + 3% glucose solution (3–5 mL) was administered subcutaneously to prevent dehydration. General anesthesia using isoflurane was maintained for at least 20 min after intraperitoneal administration of either one of the three interventions, conforming previous experiments with a comparable hydrogel formulation [12]. Subcutaneous injections of 0.03 mg/kg buprenorphine were continued every six hours for 48 h for all animals, as most post-operative discomfort was expected in the first 48 h. In addition, 200 mg/kg paracetamol (Dafalgan, UPSA, France) was given in a separate drinking bottle during the entire experiment. If animals showed signs of discomfort based on the welfare scoring sheets, additional pain medication by subcutaneous injections of buprenorphine was administered and/or saline + 3% glucose solution in case of dehydration signs. After seven days, all animals were euthanatized via CO_2 asphyxiation. Afterwards, the intraabdominal cavity was inspected via laparotomy. If needed, blood samples were taken from the vena cava.

Study Outcomes

The primary study outcome was macroscopic scores including anastomotic adhesion and leakage scores. Secondary outcomes were bursting pressure, histological evaluation of the anastomosis (inflammation, fibroblast activity, neoangiogenesis and oedema) and changes in body weight.

Macroscopic Evaluation

After euthanasia of the animal, adhesions to the anastomotic site were assessed according to the method described by van der Ham et al. [21], in which the following classification was used: (0) no adhesions; (1) minimal adhesions, mainly between the anastomosis and omentum; (2) moderate adhesions, i.e., between omentum and the anastomotic site and between the anastomosis and a loop of the small bowel; and (3) severe and extensive adhesions, including abscess formation. The adhesion score was calculated per group by summing the scores per animal. Subsequently, AL was scored using a four-score system [22]. The latter is categorized as (1) no AL, (2) small abscess < 1 cm^3 at the anastomotic site, (3) large abscess of >1 cm^3 at the anastomotic site and (4) complete dehiscence with (fecal) peritonitis.

Bursting Pressure

Anastomotic strength was assessed by measuring the bursting pressure (Supplementary Figure S2), based on previously described methods [22, 23]. In short, a 4 cm segment of the colon including the anastomosis was resected *en bloc*, without removal of adherend adhesions to prevent iatrogenic damage. A plastic tube was inserted in the proximal end and ligated with polypropylene 6-0 suture (Prolene 6-0, Ethicon, Inc., Johnson & Johnson). The part distal of the anastomosis was clamped. The resected colon segment was immersed in water, while air was infused using a balloon connected to a manometer (Digitron, part of Rototherm Group). The pressure (mBar) was manually increased by pumping up the balloon and inflating the colon. Bursting pressure was defined as the intraluminal pressure at which air leakage was initially observed from the anastomosis.

Tissue Preparation and Histological Evaluation

After measuring the bursting pressure, the colon tissue including the anastomosis was placed in formalin. All samples were paraffinized within one week. From each formalin-fixed, paraffin-embedded (FFPE) tissue specimen, a 5 µm section was cut and stained with standard hematoxylin-eosin (H&E). Infiltration of inflammatory cells, fibroblast activity, oedema and neoangiogenesis at the anastomotic site were assessed by an experienced animal pathologist (MG). Inflammatory parameters were scored based on the modified 0-to-4 Ehrlich and Hunt numerical scale: 0 = no evidence, 1 = occasional evidence, 2 = light scattering, 3 = abundant evidence and 4 = confluent cells or fibers [24, 25]. All other characteristics were score on a 0-to-3 scale, meaning 0 = no evidence, 0.5 = minimal, 1 = mild, 2 = moderate and 3 = severe. Additionally, all other abdominal organs were collected as well and placed in formalin in case of need for further histological examination.

Statistical Analysis

General characteristics of the animals can be found in Supplementary Table S1. Statistical analysis was performed using SPSS (IBM SPSS Statistics for Apple, Version 27, Armonk, New York, NY, USA) and GraphPad Prism (GraphPad software for Apple, version 8.0.0, San Diego, CA, USA). Numerical variables were presented as median with interquartile range (IQR, Q1–Q3). To evaluate the statistical significance of numerical variables differences observed between groups, non-parametric tests (overall Kruskal–Wallis and post hoc Mann–Whitey U-tests for pairwise comparison) were applied. In case of significant overall tests ($\alpha = 0.05$), a Bonferroni correction was used for the pairwise comparisons ($\alpha = 0.0167$). The percentage of body weight change was calculated by subtracting the daily measured weight from the baseline weight of each animal. Group comparison of mean body weight was performed with mixed-effect models.

RESULTS

A total of 42 healthy rats underwent the surgical procedure (saline $n = 6$, unloaded hydrogel $n = 18$ and MMC-loaded hydrogel $n = 18$) of which 22 completed the follow-up period of 7 days (Supplementary Figure S3, saline = 6, unloaded hydrogel = 10, MMC-loaded hydrogel = 6) and were included in the final analysis.

Anastomotic Adhesion and Leakage Scores

The macroscopic anastomotic adhesion and leakage scores are displayed per intervention group in Figure 1A,B. Representative images of the anastomoses with corresponding scores are shown in Figure 1C–E. The median (IQR) adhesion scores were 1.5 (1–2), 1.5 (1–2) and 1 (1–2) for saline, unloaded hydrogel and MMC-loaded hydrogel groups, respectively. There were no significant differences between the groups (Table 1). Severe and extensive adhesions were only present in one animal that had unloaded hydrogel administered. The median (IQR) AL scores were 1 (1–1), 2 (1–2) and 1 (1–1.25) for saline, unloaded hydrogel and MMC-loaded hydrogel groups, respectively. A difference was observed for the AL score ($p = 0.034$), for which pairwise comparison showed a difference for AL score comparing the saline and unloaded hydrogel subgroup ($p = 0.020$, Figure 1B). This difference was not significant after Bonferroni correction ($\alpha = 0.0167$).

Table 1. Macroscopic adhesion and AL scores.

	Saline (*n* = 6)	**Unloaded Hydrogel (n = 10)**	**MMC-Loaded Hydrogel (*n* = 6)**	***p* Value**
Adhesion score – *median (Q1–Q3)*	1.5 (1–2)	1.5 (1–2)	1 (1–2)	0.741 [a]
AL score – *median (Q1–Q3)*	1 (1–1)	2 (1–2)	1 (1–1.25)	**0.034** [a,*]

*[a] Kruskal–Wallis test; * p = 0.020 after pairwise comparison of saline and unloaded hydrogel group with Mann–Whitney U test, which was not significant after Bonferroni correction.*

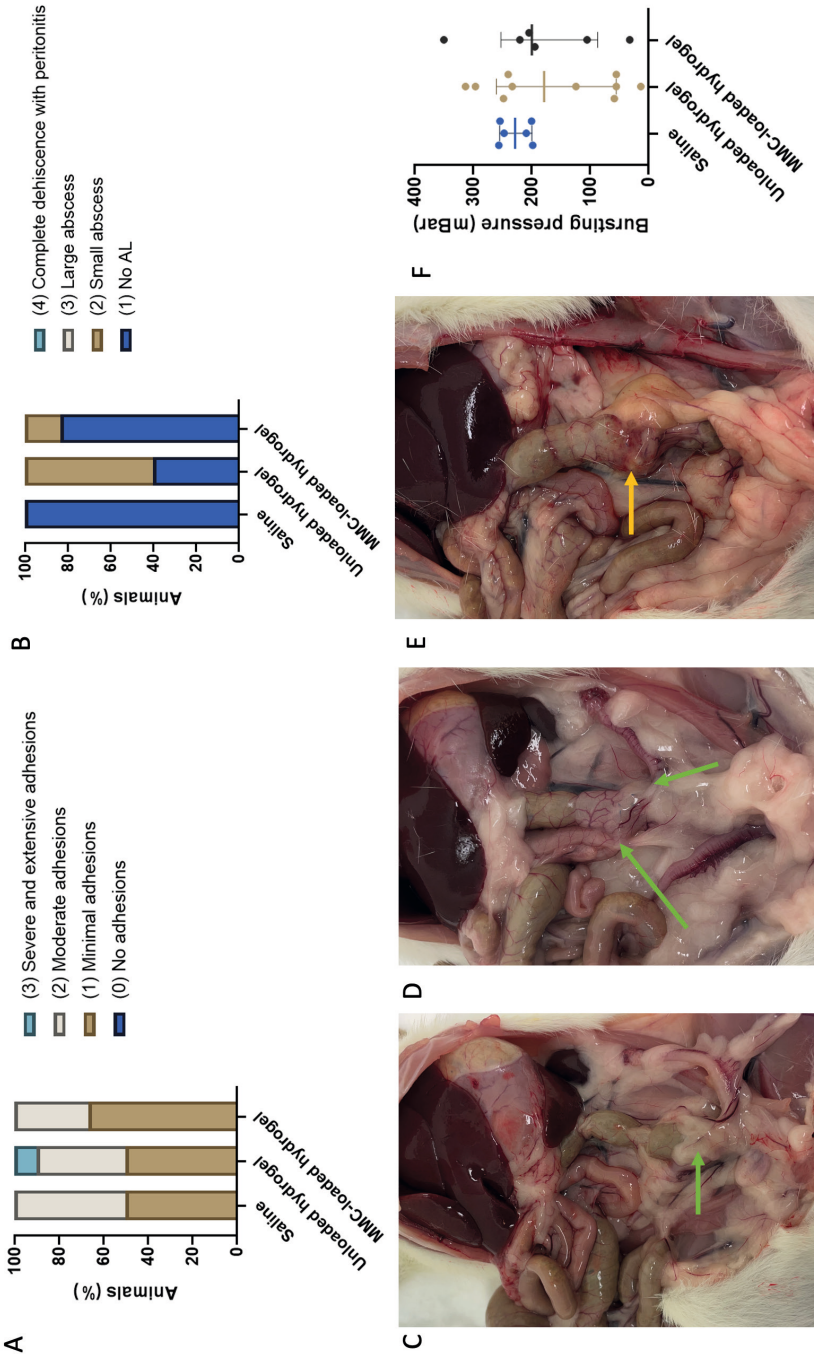

Figure 1. (**A**) Macroscopic anastomotic adhesion score. (**B**) Mascroscopic AL scores per group. (**C**) Minimal adhesions (green arrow) in a female rat treated with MMC-loaded hydrogel. (**D**) Moderate adhesions (green arrows) in a female saline-treated rat. (**E**) Small abscess (orange arrow) in a male rat treated with unloaded hydrogel. (**F**) Bursting pressures per group. Medians are indicated and whiskers show the Q1–Q3.

Bursting Pressure

The median bursting pressure was 228 (200–255) mBar, 179 (55–260) mBar and 200 (86–253) mBar for saline, unloaded hydrogel and MMC-loaded hydrogel group, respectively. This was not significant different between the groups (Figure 1F).

Microscopic Evaluation

The anastomotic site of the rats which completed the experiment was microscopically scored by an experienced animal pathologist. No significant differences were found for fibroblast activity, inflammation and neoangiogenesis scores at the anastomotic site (Figure 2A). In all animals of the experimental groups (treated with MMC-loaded and unloaded hydrogel), we observed lymphangiectasia, oedema in the muscularis propria and vacuolated macrophages around the anastomotic site and in the surrounding peritoneal fat that contained foreign material (Figure 2C,D showing representative images). These histological observations were not seen in the control animals receiving normal saline (Figure 2B).

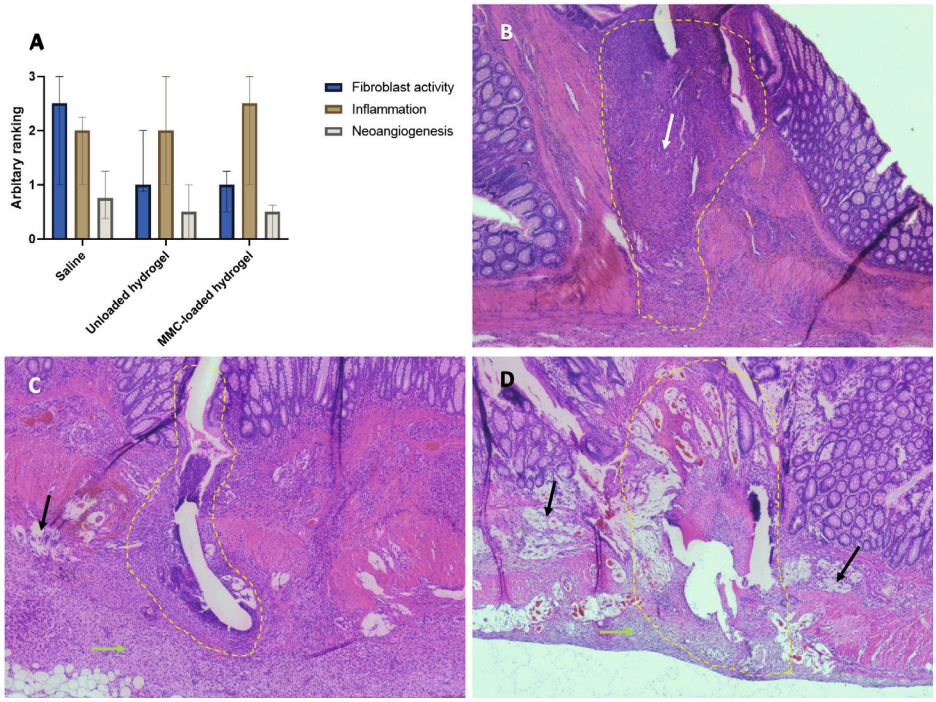

Figure 2. (**A**) Histological analysis of animals at postoperative day 7: scores of fibroblast activity, inflammation and neoangiogenesis. Medians are indicated and whiskers show the Q1–Q3. (**B**) Hematoxylin & Eosin (H&E) staining of anastomotic site (10 × 5) of a saline-treated animal. (**C**) H&E staining of anastomotic site (10 × 5) of an animal that received unloaded hydrogel. (**D**) H&E staining of anastomotic site (10 × 5) of animal that received MMC-loaded hydrogel. Yellow dashed line indicates site of anastomosis, white arrow highlights fibroblast activity, black arrows show the lymphangiectasia and green arrows point the area with vacuolated macrophages.

Drop-Out

A considerable number of rats, 20 out of 42, were prematurely excluded from the experiment. Twelve rats were prematurely sacrificed due to visible anal blood loss (n = 5 unloaded hydrogel, n = 7 MMC-loaded hydrogel). Five additional rats (n = 1 unloaded hydrogel, and n = 4 MMC-loaded hydrogel) were taken out because of a too high welfare score and reaching HEP before the end of the experiment. One female rat treated with unloaded hydrogel was excluded for further analysis due to technical error during the operation (too much blood loss during the creation of the anastomosis). Another female unloaded hydrogel animal was taken out of the experiment after one day because the animal had opened its fascia and peritoneum, and another one treated with MMC-loaded hydrogel because she had an incarceration of omentum in an abdominal hernia.

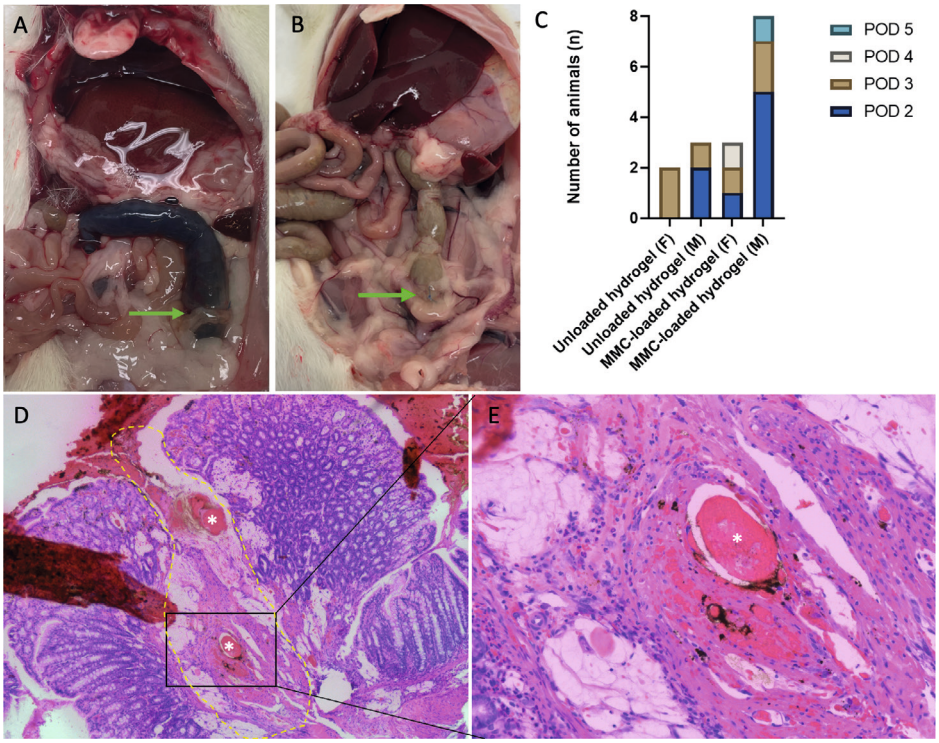

Figure 3. Anastomotic site (green arrows) in (**A**) an MMC-loaded hydrogel-treated rat with blood loss on POD 3 and (**B**) an MMC-loaded hydrogel-treated rat without blood loss on POD 7. Both show fat adhesions. (**C**) Occurrence of blood loss in relation to PODs. (**D**) H&E staining of anastomotic site (10 × 5) of an MMC-loaded hydrogel treated animal with intraluminal blood loss. A yellow dashed line indicates the site of anastomosis. Thrombi are annotated by asterisks, with surrounding signs of bleeding. (**E**) Enlargement (20 × 5) of the region of interest shown in (D), with hemorrhagic spots. M = male, F = female.

After obduction of the prematurely sacrificed rats, 16/20 rats (*n* = 5 unloaded hydrogel, *n* = 11 MMC-loaded hydrogel; 69% male) had signs of intraluminal blood loss at the anastomotic site (Figure 3A). It seemed like the blood clots accumulated at the site of the anastomosis, which was not seen in animals without blood loss (Figure 3B), and sometimes blood clots in the colon or small intestine proximal of the anastomosis were observed. We did not observe hemoperitoneum in any of the rats, nor did we identify any intraluminal blood loss in the animals surviving the whole experiment. In 50% (*n* = 8) of the animals that had intraluminal blood loss, the blood loss was present on postoperative day (POD) 2, and in 33% on POD 3; two other rats were diagnosed with blood loss on POD4 and 5 (Figure 3C). In 50% (*n* = 8), microscopic signs of blood loss around the anastomotic site were seen, e.g., necrotic blood vessels (Figure 3D,E), hemorrhagic spots in different layers (serosa, muscularis mucosa and the mucosa) or congestion in some villi. The small intestine and stomach did not show any signs to which the blood loss could be related.

Blood samples were taken from animals with (8 rats treated with MMC-loaded hydrogel) and without (8 rats treated with unloaded hydrogel and 3 with MMC-loaded hydrogel) intestinal blood loss (Supplementary S2, Figure S4 and Table S2). Thrombocyte numbers were not different between animals with and without blood loss. In addition, coagulation factors (prothrombin time, international normalized ratio, and activated partial thromboplastin time) were estimated in 5 of the previous animals (all MMC-loaded hydrogel) of which 4 were presenting with blood loss and one did not. No abnormalities related to coagulation outcomes were found.

Weight Loss and Welfare Scores

The results of the daily body weight monitoring in all animals who successfully underwent the operation are shown in Supplementary Figure S5. All animals had an initial weight gain on day 1 related to hydrogel and saline administration, followed by weight loss. From day 3, recovery to mean baseline weight was observed in saline treated animals, while animals who had hydrogel administered (unloaded hydrogel or MMC-loaded hydrogel) kept on losing weight. In both hydrogel groups, the course of the body weight was comparable, although we observed a little higher weight loss in the MMC-loaded hydrogel treated animals. A mixed-effects model showed a significant difference for both female and male rats ($p < 0.0001$ for both sexes) in favor of the saline-treated group.

Animals in both the unloaded hydrogel and MMC-loaded hydrogel had higher post-operative welfare scores, implicating more discomfort compared to animals treated with saline. Seven rats opened their laparotomy wound (*n* = 5 unloaded hydrogel, *n* = 1 MMC-loaded hydrogel, *n* = 1 saline).

DISCUSSION

In this experiment, 42 healthy rats underwent a laparotomy to create a sufficient colonic end-to-end anastomosis to investigate whether a single intraperitoneal administration of a (drug-loaded) hydrogel affects anastomotic healing compared to saline administration. Twenty-two animals who completed the follow-up period of the experiment were included in the analyses, investigating macroscopic and microscopic anastomotic healing. Adhesion scores were not significantly different between groups. A higher AL score was found in the animals treated with unloaded hydrogel as compared to saline-treated animals, which did not remain significant after correction for multiple testing. A wider range of bursting pressure values was found in the hydrogel-treated groups compared to the saline-treated group, but the differences were not significant. In addition, fibroblast activity, inflammation and neoangiogenesis scores were not different between groups. Unexpectedly, intraperitoneal administration of unloaded and MMC-loaded hydrogel after anastomotic surgery did not prove safe due to intestinal blood loss in nearly half of the hydrogel-treated animals under the current study conditions.

Animals that received unloaded hydrogel administered had a higher, but not significant, median AL score compared to animals treated with saline. The observed difference was attributed to the occurrence of small abscesses in several of the unloaded hydrogel-treated animals. However, no large abscesses or complete dehiscence with peritonitis were found in these animals. The hydrogel-treated animals demonstrated a wide range of bursting pressure values compared to the saline-treated animals. Previous studies reported wide ranges of bursting pressure values on different PODs [26, 27]. Bosmans et al. published a mean bursting pressure of 104.1 ± 40.8 mBar on POD 7 in their control group. In contrast, Kosmidis et al. reported a higher mean bursting pressure of 198.38 ± 12.80 mBar and de Castro Durães et al. even of 267.07 mBar in control animals on POD 7 [27, 28]. In our cohort, 18/22, 16/22 and 3/22 animals had a bursting pressure above 104, 198 and 267 mBar, respectively. Although our measured bursting pressures seem to be in line with previously reported absolute values, it is noteworthy that the range in both hydrogel-treated groups is wider compared to the saline-treated group. In a few animals, we measured rather low bursting pressures, which may indicate disturbed anastomotic healing. Still, no large abscesses or complete dehiscence with fecal peritonitis were found. Although using the bursting pressure is the most reliable method of mechanical power assessment of the anastomosis [29], the wide range of values in the literature and our study may suggest this method is not optimal for AL assessment.

Importantly, as this study involves an anastomotic safety model (in which normal healing is expected), we did not have to sacrifice an animal before the end of the experiment due to defective anastomotic healing or AL, nor did we identify animals with large abscesses or peritonitis. Already back in 1991, Fumagilli et al. investigated the effects of intraperitoneal chemotherapy on jejunal anastomotic healing in rats [16]. Although different types of rats,

location of the anastomosis and dose of MMC, histological examination of the anastomoses in rats given an intraperitoneal bolus of 2 mg/kg MMC showed significantly slower anastomotic healing, with an incidence of AL of 52.8% after 7 days. An investigated explanation for this impaired anastomotic healing is the affected collagen synthesis, as this is an essential feature of anastomotic healing in the intestine [16]. The strength of anastomosis is influenced by the interplay between newly-synthesized and deposited collagen, as well as the degradation of preformed collagen [17]. In the initial post-operative phase (3–5 days after surgery), there is a notable decrease of up to 40% in collagen concentration near the anastomosis site, primarily attributed to increased collagenase activity at the anastomosis site [16, 17]. However, starting from day 5 onwards, there is a gradual rise in collagen synthesis, leading to a progressive increase in the strength of the anastomosis. By the 7th day after surgery, the anastomosis achieves approximately 50% of its measured strength [16, 30]. MMC halts the proliferation of fibroblasts, which play vital roles in several crucial aspects of the previous wound-healing process [17]. Previous experiments showed that intraperitoneal MMC administered on or after the 5th day after anastomosis creation had no significant effect on the anastomotic healing anymore [17]. As the injectable hydrogel used in our study forms an intraperitoneal depot of slow-releasing MMC, we hypothesized less impaired anastomotic healing due to the slow-releasing characteristics. This was confirmed by the finding that we did not observe any rats suffering from AL as reported in other studies [16, 17]. No significant differences could be observed between the two experimental groups (unloaded hydrogel vs. MMC-loaded hydrogel) suggesting that slow-releasing but prolonged exposure of the chemotherapeutic does not impair wound healing, nor does the hydrogel. However, our results do show, although not significant, reduced fibroblast activity in unloaded hydrogel and MMC-loaded hydrogel treated animals. Despite the reduction in AL incidence in our study, we did not gain a clinical improvement due to the drop-out of almost half of the animals.

Active hemorrhage was only seen intraluminal at the site of the anastomosis and not in the abdominal cavity. Our previous study, in which this hydrogel was applied in a rat PM model, did not demonstrate intraluminal, extensive blood loss. The main reasons for intraluminal blood loss in animal experiments are (1) a *Clostridium piliforme* or *Clostridium perfrigens entertoxin* infection, (2) intestinal ulcer formation and (3) a systemic coagulation problem. In rats suffering from intestinal hemorrhage in the current experiment, these potential causes were all excluded by follow-up analysis of feces, tissue and blood samples. After ruling out several probable causes of blood loss in animal experiments, we propose an explanation based on microscopic findings. We observed lymphangiectasia, edema in the muscularis propria and vacuolated macrophages around the anastomotic site and in the surrounding peritoneal fat that contained foreign material, also reported by Wintjens et al. [12]. We hypothesize that the hydrogel is partly absorbed by the intestinal lymph system and macrophages, causing local congestion, which causes blood vessel damage around the anastomosis. The degree of damage ranged from larger necrotic blood vessels to hemorrhagic spots in different layers (serosa, muscularis mucosa and the mucosa). Although we did not

see these microscopic signs of damage in all sections of animals with intestinal blood loss, we did see large lymph congestion in all hydrogel-treated animals. Additionally, the presence of a considerable volume of hydrogel in the peritoneal space may lead to a high intraabdominal pressure which contributes to this local thrust in the first hours after injection. Based on this hypothesis, we can assume that of the animals that were taken out of the experiment due to the blood loss, the anastomotic healing was influenced due to the impaired blood supply as they were not included in the data analysis.

An important observation was the discomfort of the animals after the surgery. Our research team and the animal facility have ample experience with rat anastomosis research [22, 31] and with the intraperitoneal administration of the used hydrogel formulation [12, 13]. During this experiment, five rats treated with unloaded hydrogel, one treated with MMC-loaded hydrogel and one saline-treated animal managed to open their laparotomy wound resulting all in re-interventions and the sacrifice of one of the animals, suggesting abdominal discomfort. Therefore, in all subsequently operated animals, the skin was closed with metal clips in addition to the sutures. In addition to this discomfort, control animals stabilized their weight from 3 days postoperatively, while hydrogel-treated animals kept on losing weight and almost all reached a HEP on day 7 based on the weight loss. The drop-out, general discomfort and decreased body weight were more prominent in hydrogel-treated animals, and more specific in the male animals compared to female animals, which was also reported in previous experiments [12].

This is the first experiment to investigate the anastomotic safety of intraperitoneal administration of unloaded and MMC-loaded hydrogel (UPy–PEG). Outcomes of interest were compared with control animals receiving saline and undergoing identical study procedures and follow-up time. This study has some limitations. Due to the high number of dropouts, the sample size of animals that completed the experiment is small and lower than predefined in the power calculation. Given the small sample size, the study results should be interpreted with caution. Paradoxical to the observation that the MMC-loaded and unloaded hydrogel is not significantly causing more AL, we report unexpected signs of extensive intestinal blood loss in almost half of these animals in this model. As the cause of the intestinal blood loss at the anastomotic site after hydrogel injection is still hypothetical, further research to reveal this observation provides insight in anastomotic healing.

CONCLUSIONS

In this rodent model, we demonstrated the influence of an intraperitoneal injectable cytostatic (MMC) loaded hydrogel (UPy–PEG) on colon anastomoses. Although our preliminary results suggest that intraperitoneal administration of the hydrogel with or without MMC does not affect anastomotic healing based on the anastomotic adhesion and leakage score, bursting pressure and microscopic evaluation, we must conclude that injection of both unloaded and MMC-loaded hydrogel under the studied conditions is not safe in the current rodent model for colorectal anastomotic surgery because of the high number of rats which were prematurely sacrificed due to intestinal blood loss. This warrants future experiments to optimize the hydrogel for use in combination with colorectal surgery.

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This animal study was performed at the animal center of Maastricht University (Maastricht, The Netherlands). The experimental protocol followed the Dutch Animal Experimental Act and was approved by the Animal Experimental Committee of Maastricht University Medical Center (project license AVD1070020198765).

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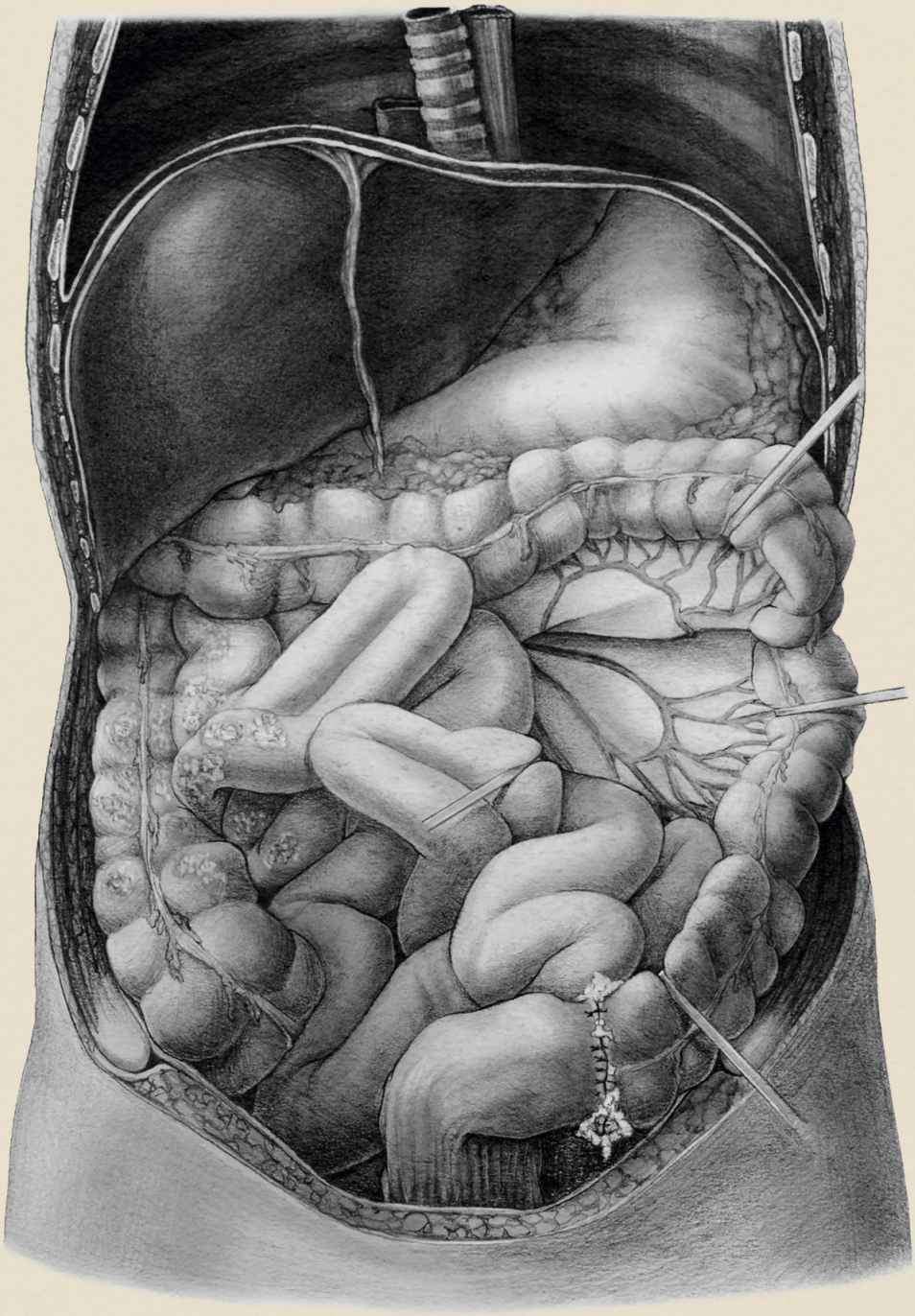
SUPPLEMENTARY

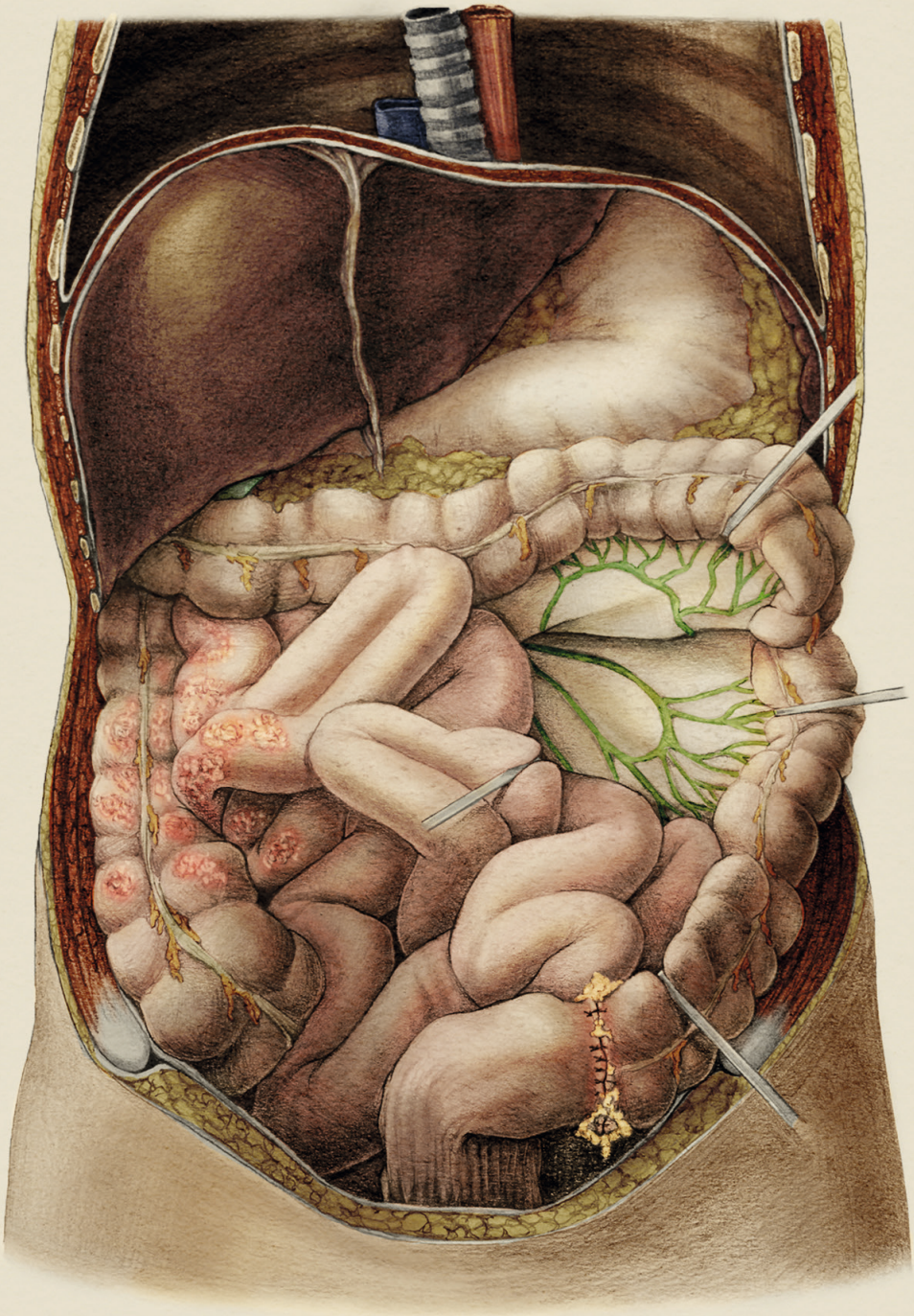
The following supplementary material can be downloaded from:

- *S1: Additional supplementary information according to ARRIVE guidelines*
- *S2: Supporting materials (results)*
  - *Table S1. Rat characteristics and outcomes*
  - *Table S2. Individual results of additional coagulation tests in four rats with (+) and one without (–) intestinal blood loss*
  - *Figure S1. Surgical procedure: creation of the anastomosis + closure of the fascia*
  - *Figure S2. Bursting pressure measurement*
  - *Figure S3. Survival proportions of the whole cohort*
  - *Figure S4. Thrombocyte values of animals with (+) and without (–) blood loss*
  - *Figure S5. Median body weight changes in percentages compared to mean baseline weight in the 7 days of the experiment*



PART V

SUMMARY, DISCUSSION,
FUTURE PERSPECTIVES,
AND IMPACT



CHAPTER 14

SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES

This thesis aimed to improve post-operative outcomes for colorectal cancer (CRC) patients by focusing on two key areas: increasing awareness on reporting and reducing anastomotic leakage (AL) and preventing the occurrence of metachronous peritoneal metastases (PM). More specifically the aims were to increase (I) insight in how AL is reported in high level evidence literature, and subsequently create an evidence-based reporting framework and radiological scoring system that can be used to standardize complication reporting of AL in the future, (II) investigate the feasibility and quantification of intestinal perfusion with innovative image-guided surgery techniques (both indocyanine green (ICG) and methylene blue (MB) and with Laser Speckle Contrast Imaging (LSCI), and (III) create an overview of the current knowledge of the impact on the Quality of Life (QoL) of patients after colorectal AL and obtain a more in-depth understanding of their experiences. Since there is increasing evidence suggesting that AL is also influencing oncological outcomes, this thesis additionally aimed to identify predictive biomarkers in primary colorectal tumors for metachronous PM and evaluate the safety of an intraperitoneal cytostatic-loaded supramolecular hydrogel as potential preventive strategy (part IV). The introduction (**Chapter 1**) provided an overview of the available evidence and current status with regard to the aims of thesis.

PART I: INCREASING INTERNATIONAL CONSENSUS ON CURRENT EVIDENCE AND REPORTING OF ANASTOMOTIC LEAKS AFTER COLORECTAL CANCER SURGERY

Reporting of AL has been challenging as the precise definition of what constitutes a leakage subsequent to colorectal anastomotic surgery has remained a subject of ongoing debate [1]. Yet, reporting complications is essential for promoting transparency, accountability, and continuous improvement in healthcare delivery, ultimately enhancing patients' outcomes and quality of care [2]. In the first section of the thesis, our focus was to enhance understanding of the diverse ways of reporting colorectal AL and subsequently develop a system to improve its reporting.

Chapter 2 comprised an overview in the form of a systematic review on how AL is currently reported in high-level evidence literature (randomized controlled trials (RCTs), systematic reviews, and meta-analyses). Among the 471 articles addressing AL as either a primary or secondary outcome, only 95 studies (comprising 45 randomized controlled trials, 13 systematic reviews, and 37 meta-analyses) provided a clear definition. This collective involved a total of 346,140 patients. Within these 95 articles, 68% provided a description of the clinical signs and symptoms of AL, 26% utilized biochemical criteria, 63% relied on radiological modalities, 62% considered radiological findings, and 13% described the findings during re-intervention. Notably, only 45% (n = 43) of the studies included in the analysis, reported the grading of AL severity or leak classification, and 41% (n = 39) specified a timeframe for AL diagnosis. A high degree of heterogeneity among the included studies was highlighted.

The outcomes underscored the pervasive issue of incomplete and inconsistent reporting of AL within the published CRC literature. To facilitate clearer communication regarding leaks, enable data comparison, and enhance clinical outcomes, there is an urgent need to develop and implement a consensus framework for defining, grading, and reporting AL.

Chapter 3 represented the collaborative consensus project "Consensus on Reporting colorectal Anastomotic Leaks (CoReAL)", which provided an evidence overview and reporting framework for AL after oncological colorectal surgery. The project consisted of a distinguished group of expert surgeons who were all members of international surgical societies. Firstly, the group analyzed all the available literature on AL. Based on an analysis of 477 high-level evidence (systematic reviews with or without meta-analysis, and RCTs) papers, a total of 33 evidence-based statements regarding AL after CRC surgery were formulated. In summary, we have identified pre-operative modifiable and non-modifiable risk factors associated with AL and acknowledged that preoperative oral antibiotics may reduce AL rates. Intraoperatively, we stated that factors such as the level of mesenteric artery ligation, conversion status, number of stapler firings, use of fluorescence angiography, anastomotic integrity tests, and prophylactic fecal diversion also impact AL occurrence. In the postoperative diagnostic phase, we found that serial C-reactive protein (CRP), CT-scan, or endoscopic examination are useful, and both minimally invasive and open re-interventions are feasible with proper patient selection to realize both earlier detection of AL and subsequent reduced morbidity. The evidence highlighted the long-term consequences of AL as increased mortality rates, overall complications, risk for permanent stoma, decreased overall survival and disease-free survival, higher local recurrence rates, and increased healthcare costs. All other formulated statements were on factors that did not influence AL rates based on the analyzed evidence. Secondly, the statements aimed to support the reporting framework presented in this paper, together with the input from an international group of experts as well as patients' perspectives. The final core reporting elements represent pre-operative (risk factors, antibiotics, mechanical bowel preparation, and potential need of a stoma), intra-operative (stoma creation, intraoperative difficulty, integrity testing, stapler loads, conversion, pitfalls, splenic flexure mobilization, inferior mesenteric artery ligation, and perfusion assessment), and postoperative short-term and long-term factors (re-interventions, stoma creation, diagnostic modalities, CRP measurement, readmission, length of hospital stay, ICU admission, anastomotic complications, oncological outcomes, functional outcomes, QoL outcomes and mortality) that should be documented and reported once a patient develops AL. For the postoperative course, these reporting elements were subdivided as reporting elements that should be reported during index admission, within 30 days up to 90 days and after 90 days during the follow-up period.

Increasing demand for healthcare escalating costs, resource constraints, and evidence of disparities in clinical practice have prompted a keen interest in assessing and enhancing the quality of healthcare delivery [2]. To conduct a meaningful quality assessment, it is imperative

to collect pertinent outcome data in a standardized and reproducible manner, enabling both comparisons among various centers, strategies/therapies, and within a center over time. The latter served as the background for the creation of the Clavien-Dindo classification in 2004 [2], which serves as a general way to report surgical complications and was also used to report AL in chapter 2. Later on, the International Study Group of Rectal Cancer (ISCREC) made an effort to define and grade AL in a more specific way [3]. This expert group stated that AL should be defined as an intestinal wall defect at the anastomotic site, encompassing suture and staple lines of neorectal reservoirs, resulting in communication between the intra- and extraluminal compartments. Additionally, they stratified severity based on its impact on clinical management, with grade A indicating no change, grade B requiring active therapeutic intervention but manageable without re-laparotomy, and grade C necessitating re-laparotomy. In our systematic review it became clear that worldwide adoption of this definition and reporting of this grading system has not been accomplished yet. Besides, this classification only focused on anterior resections for cancer and did not take all colorectal resections into account. Despite of debating on how we all should call a leak; we think it is more important to increase awareness and consensus on reporting of leaks in general. So, since the Clavien-Dindo classification is not specifically designed to document AL but rather complications in general, and there is no global standardization for reporting based on the ISREC definition and grading, we need to change this in order to enhance outcomes. Moreover, as numerous factors influence the occurrence of leaks and their subsequent reporting, reporting of leaks requires a broader framework, as outlined in Chapter 3. We believe this is necessary for several reasons. Standardizing reporting leaks across different institutions and countries will enhance the reliability of research findings and facilitating accurate comparisons between studies and improve care for colorectal surgical patients [4, 5]. We noticed in both chapter 2 and 3 that it was very difficult to compare outcomes due to heterogeneity in the way leaks were reported. Therefore, standardized reporting is also essential for conducting high-quality research in the future and generating reliable evidence to guide clinical practice and to assess the effectiveness of different interventions and identify factors associated with leaks [5]. Additionally, a standardized reporting protocol will help healthcare providers to benchmark their performance against international norms, identify areas for improvement, and implement targeted quality improvement initiatives to reduce the incidence of leaks and improve patient outcomes. We hope that in the end consistency will provide clinicians with more valuable information for optimal risk assessment, guiding clinical decision-making, enabling them to identify leaks early, initiate appropriate interventions promptly, and optimize patient management strategies. The evidence- and expert-based reporting framework as presented in Chapter 3 may also help promote transparency and enhances patient safety by providing patients with the information they need to make informed decisions about their treatment options and participate actively in their care, which will be also highlighted later in Chapter 10. By involving surgeons from the most important colorectal surgical societies throughout the world and aiming to publish this in their journals we expect that the impact of our framework will be more significant. The consensus group

is confident in the practicality of effectively incorporating the evidence-based reporting elements into local practices and anticipates their acceptance by stakeholders in the end.

During the CoReAL project, despite thoroughly reviewing all available literature on leaks, we encountered limitations in formulating evidence-based recommendations for all pertinent topics. Some of the evidence was too low to draw clear conclusions related to AL. For preoperative measures this included the comparison of preoperative selective decontamination to broad-spectrum antibiotics, the role of anemia correction and effect of oral nutritional supplements. Additionally, intraoperative evidence was scarce on potential human factors that influenced leak rates, the exact effect of anastomotic configurations and the role of intraoperative risk scoring systems. Lastly, the evidence on postoperative scoring systems, peritoneal biomarkers, postoperative laxatives or low fiber diet, incidence of chronic sequelae, financial consequences, and impact on Quality of Life (QoL) were too scarce to draw strong statements. We aimed to solve the knowledge gap of this last topic (QoL) in Chapter 9, but all other topics still need to be further addressed into systematic reviews with meta-analyses if possible, or large trials to be able to create additional recommendations.

While we acknowledge that accurate reporting of leaks using our proposed framework can potentially lead to prevention (through preoperative risk assessment and intraoperative measures), as well as earlier diagnosis (detailed reporting during the index admission), the utilization of algorithms for predicting AL is another intriguing aspect that may enhance patient outcomes. We are cognizant of the REVEAL study, a prospective observational investigation aimed at developing algorithms for assessing the risk of developing AL [6]. The two main goals of this study are to develop and validate an algorithm for predicting the pre-operative risk of AL by incorporating various risk factors along with inflammatory, immune-related, and genetic parameters, and to develop an algorithm for the post-operative diagnosis of AL at an earlier stage. If these algorithms work well, it would be from great value to additionally include them within our reporting framework. Final outcomes from the REVEAL study are expected in the short term and can hopefully help to predict AL and enhance early recognition and fast diagnosis. Also, research has shown that machine learning techniques have high predictive value for forecasting postoperative complications following CRC surgery in general [7-9]. As summarized in chapter 3, the risk of AL is influenced by modifiable and non-modifiable risk factors. It is therefore important to include both while developing predictive systems, and to gain insight into potential interactions. Additionally, the gut microbiome has recently emerged as playing a significant role in the pathophysiology of AL [10]. Certain pathogens, like *Enterococcus faecalis (E. faecalis)* and *Pseudomonas aeruginosa*, have been implicated in causing AL, but the mechanisms behind their proliferation remain yet unclear [11]. It is hypothesized that pathogens like *E. faecalis* can contribute to the development of AL due to their elevated collagenase activity and the activation of matrix metalloproteinase 9 (MMP-9), which are crucial factors in tissue degradation and intestinal inflammation [12]. It is also conceivable that a decrease in microbial diversity could prompt a shift towards a pathogenic

state among the remaining microbiota, potentially leading to anastomotic breakdown [10, 11]. Selective antibiotics, also known as selective decontamination, may effectively suppress mucosal-associated flora in the gastro-intestinal tract and, thus, prevent the contamination of the anastomosis [13]. Factors such as diet, antibiotic usage, surgical stress, and opioid consumption significantly influence the gut microbiome and may be adjustable at different stages of surgery [11]. Despite extensive research, much remains unknown about the normal composition and behavior of the gut microbiome compared to altered states. Therefore, targeting the gut microbiome as a modifiable factor in anastomotic healing could present a new approach for preventing AL.

Fast recognition and subsequent treatment of AL is necessary to minimize clinical consequences and chronic sequelae for these patients [14]. Current diagnostic methods often lack the ability to detect AL early enough to facilitate prompt intervention and mitigate severe morbidity and mortality [15]. Computerized tomography (CT) scanning and water-soluble contrast studies are currently the preferred techniques to diagnose AL [16]. We acknowledge CT scanning exhibit variable sensitivity and specificity, and we believe it is therefore pivotal to appropriately report radiological findings. The lack of reporting radiological features was highlighted in our systematic review in chapter 2, but also in previous research [1, 5]. Earlier, a panel of eight surgeons attempted to reach consensus not only on the definition of AL, but also on radiological criteria [17]. Consensus could only be achieved when a leak was radiologically defined as extravasation of contrast outside the intestinal lumen near the anastomosis on postoperative day (POD) 12 following laparoscopic sigmoidectomy; on POD 35 following low anterior resection (LAR); or when air bubbles around the anastomosis were seen on POD 35 following laparoscopic LAR. Consensus was not achieved for signs on earlier days, or when a leak was defined as radiological collections treated with antibiotics or those requiring percutaneous drainage (i.e., ISREC grade B leaks). Currently no consensus is published on how to report and manage leaks based on radiological examination [18]. Hence, the CoReAL group, comprising both surgeons and radiologists, acknowledged the necessity of establishing a method for radiological assessment and reporting of leaks. However, rather than attempting to reach a consensus on a specific radiological definition, the expert team proposed a scoring system for future reporting of AL. This concept was previously introduced by radiological colleagues as Reporting and Data Systems (RADS) of which the first published system was the breast RADS (BI-RADS) to assess breast cancer on mammography, magnetic resonance imaging (MRI), and ultrasound (US) [19]. It enables radiologists to communicate results to the referring breast surgeon clearly and consistently, with a final assessment and specific management recommendations. Many additional malignancy RADS were developed afterwards like C-RADS (colon cancer; CT colonography), LI-RADS (liver cancer; MRI, CT, US, and contrast-enhanced US), Lung-RADS (lung cancer; low-dose CT), NI-RADS (head and neck cancers; PET, CT, and MRI), O-RADS (adnexal masses; US), PI-RADS (prostate cancer; MRI), and TI-RADS (thyroid cancer; US) [19]. Additionally, systems to assess certain diseases instead of the likelihood of malignancy were developed as well like CAD-RADS (CT angiography) for coronary artery

disease and CO-RADS (chest CT) to assess coronavirus disease [19, 20]. Yet, this has not been developed to assess specific complications such as AL. However, it has not been designed to evaluate particular complications like AL. Our team has taken the initiative to develop such a scoring system to enhance diagnostic communication in cases where AL is suspected, aiming to facilitate clear communication, earlier recognition, and appropriate management.

Chapter 4 provides the protocol of the pre-clinical validation of a standardized score to assess potential leaks after colorectal surgery on CT scanning. The research team proposed a standardized CT assessment scheme for AL based on findings from 30 patients who developed an AL after colorectal surgery. Building on the standardization efforts seen in previous systems, the research team opted for the term Colorectal Anastomotic Leakage Reporting and Data System (CAL-RADS). The proposed score ranges from 0 to 5 and addresses the likelihood of AL. The aim of this study is to optimize the CAL-RADS classification, calculate interobserver variability, investigate the feasibility and clinical translation, and correlate its diagnostic value. A total of 150 CT scans of patients who had undergone colorectal surgery were recently scored with the proposed CAL-RADS score by 6 radiologists. Preliminary results suggest that the score is easy and feasible to assess the likelihood AL. Inter-observer variability is currently analyzed. Additional correlation analysis between the given scores and final interventions will be performed soon. Also, the influence of rectal or oral contrast in certain cases will be addressed.

PART II: IMPROVING BOWEL PERFUSION ASSESSMENT TO REDUCE THE RISK OF ANASTOMOTIC LEAKS

Typically, AL is attributed to factors such as technical errors in suturing by the surgeon or increased tension on the anastomosis. Nevertheless, it is increasingly evident that AL may occur regardless of surgical technique [21], as also demonstrated in the evidence summarized in the CoReAL project (Chapter 3). Insufficient blood supply to the transected intestinal edges has always been a known risk factor for the development of a leak, which can be assessed using near-infrared fluorescence (NIRF) imaging [22]. NIRF stands out as one of the most notable technical advancements in surgery over the past decade to improve patients' outcomes. Consequently, its clinical applications have proliferated significantly, encompassing various procedures such as fluorescence cholangiography, lymph node identification, ureteral delineation, and assessment of bowel anastomotic perfusion [23]. Improving imaging systems to optimize bowel perfusion assessment and simultaneous visualization of other structures is therefore essential to reduce the occurrence of AL and other possible complications.

Chapter 5 investigated the feasibility of simultaneous imaging of intestinal perfusion and the ureter using a commercially available near-infrared fluorescence (NIRF) imaging system. Six Landrace pigs underwent laparotomy under general anesthesia. Bowel perfusion was

assessed with an intravenous dose (IV) of 0.2 mg/kg indocyanine green (ICG). Ureteral visualization was investigated in two pairs, each receiving an IV injection of methylene blue (MB) at doses of 0.75, 0.50, or 0.25 mg/kg. NIRF imaging was conducted using the Quest Spectrum Fluorescence Camera (Quest Medical Imaging, Middenmeer, The Netherlands). The NIRF imaging successfully visualized ureters and bowel perfusion in all animals. Ureters became visible within five to ten minutes and remained clear throughout each experiment (120 - 420 min). A mixed model analysis did not reveal significant differences between the three doses groups or over time. Notably, bowel perfusion could be visualized using MB as well, and no interference was observed between ICG and MB. Additionally, MB exhibited an earlier washout time, which may be clinically advantageous in situations where repeated dye injections are necessary during a surgical procedure.

In **chapter 6**, a quantitative analysis of bowel perfusion assessment for both ICG and MB was performed in another animal model. Four mature female Landrace pigs underwent laparotomy under general anesthesia. An ischemic bowel loop with five regions of interest (ROIs) exhibiting varying perfusion levels was created in each animal. After 10 minutes, an intravenous injection of 0.25 mg/kg - 0.50 mg/kg MB was administered, followed by NIRF imaging in MB mode and measurement of local lactate levels in all corresponding ROIs. This procedure was repeated in ICG mode (IV dose of 0.2 mg/kg) after 60 minutes, utilizing the Quest Spectrum Fluorescence Camera (Quest Medical Imaging, Middenmeer, The Netherlands) for NIRF imaging of both MB and ICG. Intraoperative NIRF imaging of bowel perfusion assessment with MB and ICG proved successful in all studied animals. Ingress (i/s) levels were calculated and correlated with local lactate levels. Both MB and ICG ingress values exhibited a significant negative correlation ($r = -0.7709$; $p < 0.001$; $r = -0.5367$, $p = 0.015$, respectively) with local lactate levels. Notably, the correlation was stronger for MB compared to ICG, even though ICG analysis showed higher absolute ingress values. Therefore, this fluorescence quantification analysis validated the potential use of MB for bowel perfusion assessment alongside the well-established and widely used ICG.

The imaging technology discussed in chapters 5 and 6 provides encouraging results with NIRF during colorectal surgery, notably not just with ICG but also with MB. This study revealed a new potential application of MB in assessing bowel perfusion, a use previously unexplored. A thorough review prior to our study outlined existing applications of MB, covering its use in visualizing ureters, identifying parathyroid glands, imaging pancreatic tumors, detecting margins of breast cancer tumors, and facilitating breast cancer sentinel node biopsies [24]. A recent study also revealed its potential in identification of small intestinal neuroendocrine tumors and PM [25]. Overall, MB finds application in numerous clinical procedures with a relatively low risk for patients [24, 25]. Based on our analyses, it is evident that MB, when employed within a specialized imaging system, provides a variety of simultaneous and versatile functionalities including bowel perfusion assessment. However, the investigation of its fluorescent properties is still in its nascent stage, necessitating further pre-clinical

and clinical research to comprehensively elucidate its features in the field of colorectal surgery. In contrast, ICG has been studied extensively in the colorectal surgery setting. A recent consensus paper highlighted its potentials including visualization and identification of extra-hepatic biliary structures, lymphatic mapping, identifying liver tumors and metastases, sentinel node/lymph node procedures, and bowel perfusion assessment [23]. Both MB and ICG carry potential risks, including allergic reactions and organ toxicity. However, some experts express their worries to MB as they believe more adverse reactions to MB class products have been reported. It is important to notice that MB can only be used in patients with adequate renal function, and should be avoided in patients using serotonergic drugs like selective serotonin reuptake inhibitor (SSRIs) and serotonin-norepinephrine reuptake inhibitor (SNRIs) to avoid a serotonin syndrome [24, 26]. Mild adverse effects following MB administration include hypertension, dyspnea, hemolysis, methemoglobinemia, nausea, vomiting, and chest pain at doses ranging from 2 to 7 mg/kg. Refractory hypotension and skin discoloration may occur at doses between 20 and 80 mg/kg [27]. However, these doses far exceed those required for ureter delineation and bowel perfusion assessment. Therefore, such adverse events are not anticipated for the indications described in this thesis. MB is safely utilized for visualizing thyroid and parathyroid glands, pancreatic neuroendocrine tumors, and breast cancer tumors and sentinel nodes within therapeutic doses of less than 2 mg/kg [24], and we do not expect problems for bowel perfusion assessment as only low doses of MB are required for this purpose. Of course, careful consideration of patient factors and monitoring for adverse reactions are essential when using either contrast agent for fluorescence imaging.

The optimalisation of NIRF systems to reduce AL rates served as the general purpose of the work presented in chapter 5 and 6. Therefore, clinical trials in which direct patient benefit is explored are necessary to further implement NIRF in colorectal surgery and improve patients' outcomes. Pooled analysis of cohort studies has indicated that ICG fluorescence angiography decreases AL rates after colorectal resections, but comprehensive high-quality evidence has been insufficient [28]. Early studies focusing on colonic anastomoses are infrequent and have not demonstrated a notable decrease in AL rates with the use of ICG fluorescence angiography [28, 29]. Results on rectal resection are better described with more promising outcomes [30, 31]. Due to conflicting results in the past on AL outcomes, researchers stated that more multi-center RCTs with large sample size were required to further verify the value of NIRF [32]. A RCT involving 240 patients undergoing left-sided colon or rectal resection, revealed no significant difference in the AL rate between the ICG fluorescence angiography group and the control group [33]. The FLAG trial included 377 patients undergoing sigmoid or rectal resection and noted a lower AL rate in the ICG group [34]. Yet, this difference only occurred in grade A leaks, which include no effect on patient management. A third RCT (PILLAR III trial) including 347 patients did not report a significant reduction in AL in the ICG fluorescence angiography group compared to the control group either [35]. One of the main reasons to explain previous differences and negative results is the low incidence of AL and subsequent underpowered trials [28]. Besides, there is significant variability in clinical use and

technical details of use, such as dose, concentration, distance of target organ, and timing of dye administration [23]. The absence of standardization of these modifiable factors results in compromised outcomes and render the data incomparable. Therefore, it is important to standardize imaging protocols and validation and include more patients in future NIRF trials to compare AL outcomes.

Despite both conflicting results and necessary improvement in standardization and quantification, there is growing believe that assessment of colorectal anastomoses with NIRF is likely to be associated with lower risk of AL compared to traditional white light assessment [36]. The EssentiAL trail, which included 850 rectal cancer patients randomized to receive either ICG or standard care, showed that the incidence of AL was significant lower [37]. Overall, ICG fluorescence imaging significantly reduced the AL rate by 4.2%. Another phase III randomized controlled trial (AVOID trial) using ICG for the prevention of AL but in all colorectal resections, has recently reached its sample size of 978 CRC patients [38]. Preliminary results seem to be promising in reducing AL rates in case of left-sided resections when using ICG. Final outcomes from this trial are expected in the short term and could conclusively demonstrate the efficacy of NIRF in reducing AL rates. Subsequent, further investigation of the role of MB may be explored for simultaneous imaging during colorectal procedures and additionally reduce other complications like ureteral damage.

For both MB and ICG, we demonstrated feasibility of performing quantified fluorescence imaging. A specific limitation chapter 6 is the lack of a golden standard for quantification. Although we followed a standardized protocol within our study, this protocol is not world widely adopted. As previously mentioned, not only consensus on quantification of NIRF is essential to draw evidence-based conclusions, but also procedural standardization [39]. Several cohort studies have described quantified bowel perfusion using various methods, and most of these studies did not use a standardized imaging protocol (i.e., inconsistency in camera-to-target distance, angle of camera on target tissue, type of imaging system and its settings, etc.) [40-44]. A recent Dutch prospective cohort study has demonstrated a quantification approach to distinguish between various perfusion patterns (well perfused, transition zone, and poorly perfused) using a standardized imaging protocol [45]. By employing such a standardized imaging protocol, we expect to improve the reproducibility of quantification methods as suggested in this paper, which can also help to develop perfusion patterns for MB. Subsequent studies should further investigate the clinical significance of these perfusion patterns by correlating each pattern with the incidence of AL. Ideally, larger datasets can be used to develop prediction models providing risk rated of AL per tissue location in real-time.

In addition to fluorescence angiography, innovative imaging approaches that do not rely on dyes are currently another pertinent focus of research. Laser Speckle Contrast Imaging (LSCI) emerges as a promising technique in this field. As described in the introduction, LSCI offers real-time blood flow data through the detection of the dynamic interference pattern created

by laser light interacting with moving red blood cells, referred to as a speckle pattern [46]. Using a camera during surgery, fluctuations in the speckle contrast pattern can be interpreted as alterations in perfusion [47]. Although NIRF has been extensively studied in colorectal surgery setting as presented in the previous chapters, LSCI remains relatively untested in clinical environments. Nevertheless, LSCI offers inherent benefits such as ongoing assessment of tissue perfusion and independence from contrast agents and was therefore investigated in chapter 7 and 8.

Chapter 7 investigated the potential of LSCI to offer insights into tissue perfusion states and the real-time assessment of intestinal anastomotic perfusion. The study focused on a so-called red flag technique to guide surgeons in the creation an anastomosis using optimally perfused tissue. Utilizing a Landrace pig as the experimental subject, three small bowel loops with gradually varying perfusion levels were generated and shown to surgeons. The findings showcased a high level of accuracy in identifying compromised perfusion and discerning perfusion variations among the loops using LSCI feedback. Furthermore, the study assessed the influence of LSCI on the decision-making process related to anastomosis creation. The visual feedback provided by LSCI led all surgeons to advise against creating an anastomosis, underscoring its potential to steer surgeons away from inadequately perfused tissue segments. A survey on usability highlighted the satisfaction of senior surgeons with LSCI as a perfusion imager.

Chapter 8 aimed to establish a preliminary threshold for laser speckle perfusion units (LSPUs) to gauge tissue perfusion and viability, aiming to equip surgeons with quantitative data for clinical decision-making. Four mature female Landrace pigs were employed in this study. The surgeon identified ischemic and well-perfused areas, along with watershed regions, based on the color map provided by LSCI. Local capillary and systemic lactate levels were also measured alongside LSCI recordings. Mean LSPUs significantly decreased over time in ischemic areas ($P \leq .001$) and watershed areas ($P \leq .001$), while no significant change was noted in well-perfused areas over a two-hour period. Changes in LSPUs correlated with alterations in lactate levels in both ischemic and well-perfused tissues. Logarithmic curve estimation revealed an R^2 value of 0.56 for the correlation between LSPUs and local capillary lactate levels. The cut-off value for LSPUs was determined to be 69 AU with a sensitivity of 0.94 and specificity of 0.87 (Youden index 0.81), indicating well perfused tissue. Subsequently, a cut-off value of 3.8 mmol/L for lactate effectively indicated well perfused tissue, with a sensitivity of 0.97 and specificity of 1.00 (Youden index 0.97). A post-hoc inter-rater reliability analysis comparing a group of LSCI experts with the operating surgeon yielded a substantial Kappa of 0.66, and comparison between physicians and the surgeon resulted in a moderate Kappa of 0.56. The comparison between the whole observer group and the surgeon showed a moderate Kappa of 0.52 (95% 0.44-0.61). Overall, this study suggested that LSCI holds promise as a contender for current perfusion visualization techniques, but further research on real-time quantification of LSPUs and clinical applicability is imperative.

Parallel to and during the experiments in Chapter 7 and 8, we participated in the steps that were taken to optimize the LSCI device for intraoperative use, standardize LSCI protocols in research, and provide training for surgeons and healthcare professionals on interpreting LSCI data and integrating it into surgical workflows. In both our studies, we noticed that both observers with limited LSCI training and with more expertise can reliably select well-perfused and less perfused bowel tissue and the system seemed easy to use during surgery. This strengthened the believe that LSCI is a promising non-invasive technique for assessing tissue perfusion. It became clear that LSCI can influence decision making in an experimental setting; based on the LSCI images, surgeons were able to decide whether to create an anastomosis or not. Although we simulated an ischemic bowel model, the limitation of chapter 7 is the experimental setting, without performing a real surgical procedure. A recent prospective study imaged colonic perfusion using the same LSCI technique in 64 human participants [48]. Post-operatively, surgeons were questioned if the additional visual feedback would have led to a change in clinical decision-making. Overall, 17% of operating surgeons reported that they would shift the location of anastomosis based on LSCI feedback. While LSCI produces an objective color map derived from quantitative data to depict perfusion variances, the interpretation of this color map remains subjective. Ideally, surgeons can make their anastomosis decision based in a more objective way, thereby enhancing informed clinical decision-making. As LSCI feedback represent flow rather than tissue viability, it is important to gain insight into correlation of these two parameters. A similar issue was recently addressed in the field of burn wound care for measuring wound healing potential [49]. The research team created a LSCI color code in adult clinical burn patients and confirmed a good performance of the LSCI for prediction of wound healing potential. Its development was based on standard Laser Doppler Imaging (LDI) as a standard. Ideally, a similar model can be developed to estimate the potential of intestinal healing. As Chapter 8 described an initial threshold for LSPU to confirm tissue perfusion (with local lactate as a reference instead of LDI in the burn wound field), this information can be used to further develop such a quantitative real-time assessment model to discriminate between high and low AL risk regions.

While LSCI shows promising results as a non-invasive technique to assess tissue perfusion as demonstrated in our porcine studies, its clinical utility in correlation with patient outcomes remains largely unexplored. For now, given the experimental and observational nature of current available evidence on LSCI, we can only yield speculative conclusions regarding the impact on AL. Stronger evidence derived from larger human cohorts is required to substantiate the effectiveness of LSCI in improving patients' outcomes. By further improving real-time quantification of LSCI, concerns on the subjective aspect of LSCI will disappear and support its value compared to subjective white light assessment. Additionally, research that focus on the comparison with NIRF imaging might help to improve further clinical adoption of LSCI.

Chapter 5 to 8 focused on intraoperative bowel perfusion assessment to reduce the risk of leaks. Another interesting topic, although not covered in this thesis, is the preoperative identification of poor vascularization. Atherosclerotic calcification is one of the main causes of inadequate perfusion [50, 51], which can be assessed on CT scanning. Several studies have shown that the presence of atherosclerotic calcification in the aorta-iliac tract on preoperative CT scans is linked to a heightened risk of AL [52, 53]. While calcification in major arteries may reflect anastomotic perfusion, it is essential to note that the primary blood supply to the colorectal region comes from the inferior mesenteric artery (IMA). A retrospective study showed that preoperative mesenteric occlusive disease (70-100% stenosis) of the IMA is associated with a risk of AL in patients undergoing left-sided or rectal cancer surgery [54]. Therefore, it might be insightful to further investigate the role of preventive identification of mesenteric occlusive disease in larger trials. If this evidence strength the reduction of leakage rates, a combination of pre-operative and intraoperative perfusion assessment might become clinical practice.

PART III: PATIENTS' PERSPECTIVES ON COLORECTAL ANASTOMOTIC LEAKS

While 2-12% of AL patients may die within 90 days after the initial surgery, the majority of these patients survive [55]. AL significantly affects postoperative recovery, leading to severe morbidity and often necessitating reoperation. Therefore, it is crucial to understand the impact of this complication as much as possible to improve outcomes. Primarily, comprehending patients' experiences, preferences, and priorities facilitates the delivery of patient-centered care, wherein treatment strategies are customized to accommodate individual needs and preferences. Such patient-centered, has been demonstrated to enhance treatment adherence, satisfaction, and overall QoL, thereby playing an important role in improving medical outcomes [56, 57]. Up to now, there is conflicting evidence available regarding the impact on QoL after colorectal AL, and there is a lack of a clear overview to draw clear conclusions. As patients should be fully informed not only regarding the immediate surgical risks, but also on the impact surgical complications may have on long-term function and QoL, it is crucial to understand this impact. Additionally, to improve QoL outcomes, medical care providers should be aware of the factors that do influence and impact this QoL according to patients.

Chapter 9 synthesized available evidence concerning long-term QoL in patients experiencing AL after colorectal resections for oncological reasons. Studies that reported on QoL using validated questionnaires in patients with AL following oncological colorectal resections were included. Thirteen articles, encompassing 4596 individual patients, were summarized in this review, of which 566 patients experienced AL. Significant variability existed among the studies in terms of the questionnaires utilized and the timing of assessments. A total of ten validated QoL questionnaires were used, of which four were used in more than one study; The

European Organization for Research and Treatment of Cancer Quality of Life Questionnaire (EORTC QLQ) -C30 (Core) and -CR29 (CRC specific), the Short-Form 36 questionnaire (SF-36) encompassing both a physical component (PCS) and a mental component score (MCS), and the Fecal Incontinence QoL (FIQL) questionnaire. QoL was evaluated at different timepoints ranging from one month to 14 years post-surgery, and outcomes were not always compared to baseline assessments. In summary, synthesized evidence of the current work indicated that AL following oncologic colorectal resection is linked to diminished QoL, particularly during the initial six months and even one year following surgery, with variable degree of subsequent improvement. It emphasizes the need for additional scrutiny and dialogue with patients regarding the impact of AL and important outcomes.

Subsequently, **chapter 10** provided insights into the most important experiences, outcomes, and QoL features experienced by patients who developed an AL after colorectal surgery. Patients who encountered AL following colorectal surgery underwent interviews using a semi structured interview guide. All interviews were digitally recorded, transcribed verbatim, and coded. Data analysis employed a thematic approach to identify key themes that held significance for the interviewed patients. Ten patients (60% male, median age of 53 [39 – 65]) from three different continents participated in the interviews. Four main themes were identified in the interviews: (1) physical impact, (2) emotional impact, (3) coping mechanisms, and (4) important elements of anastomotic leak care. We noticed that pain due to treatments was an important factor that influenced patients' experiences, but also rehabilitation difficulties. Emotional topics included fear of treatments due to pain, but also mentally struggling with having a stoma. Many participants adopted an optimistic outlook as a coping strategy, forcing themselves to stay positive throughout the diagnosis and treatment process, and highlighted the importance of a supportive social system. Additionally, significant aspects according to the patients were underscored and recommendations for clinical care were formulated on preoperative information, communication, medical staff and peer support, presentation of information, aftercare, shared decision making and a potential case manager. The outcomes of this study were incorporated in the CoReAL framework, as described in Chapter 3.

As presented in this part, not only QoL assessment (chapter 9) but also qualitative research (chapter 10) is necessary to understand patients' experiences and perceptions, as they differ significantly in their approaches, goals, and methodologies. The overall synthesis of QoL evidence points towards a consistent finding in Chapter 9, namely that AL following oncological colorectal resections does impact patients' QoL, particularly in the initial year post-surgery. The observed decline in QoL scores reported among AL patients in the first six, and even 12 months, may be due to several reasons like additional postoperative complications, higher rates of re-interventions, prolonged length of hospital stay, etc. [58-62]. Yet, it is meaningful to ask the patients in a qualitative way what they thought was difficult during that period and what factors and outcomes contributed to the impaired QoL. By

the information gathered in chapter 10, it became clear that both emotional and physical factors influenced participants' well-being and many coping strategies were present during the AL experience. The reported elements of the patients were integrated in the CoReAL framework, described in Chapter 3. Combining our insights on QoL impact and the patient reported factors could be used for the development of a comprehensive patient-reported outcome (PRO) set.

PROs are measurements/insights provided by patients about any aspect of their own health status, QoL, or functional status in relation to the healthcare or treatment they have undergone [63, 64]. Integrating PROs into clinical practice enables healthcare providers to more accurately evaluate treatment efficacy and customize interventions to meet patients' individual needs and concerns, thereby enhancing medical outcomes [63]. Patient-reported outcome measures (PROMs) serve as instruments or tools utilized to capture these PROs [64]. Besides PROMs, patient-reported experiences measures (PREMs) do also exist. Through the use of PREMs, such as satisfaction scales, patients report their experiences, offering valuable insight into their care or the healthcare service they received [64]. Both measures can be used in healthcare for different specific purposes, but essentially, they are used to improve the quality of healthcare services, be it at the national level, the institutional level or the individual patient level. The growing international focus on PROMS and PREMS underscores their significance as indicators of patient care quality and safety. Currently, there is no PRO(M) for AL described. Adoption of standard set of (patient reported) outcomes for AL does not exist, but it does for CRC in general. The latter was proposed by the International Consortium for Health Outcomes Measurements (ICHOM) and does also includes QoL assessment [65]. According to this consortium, it is recommended to use the EORTC QLQ-C30 tool to capture overall QoL and the -CR29 to capture CRC specific outcomes, with recommendations to administer questionnaires at baseline (prior to surgery), 6 months after surgery, and then annually up to 10 years. Given the considerable variability observed in the reporting and assessment of QoL following AL, it is imperative to adhere more rigorously to this recommendation in order to gain a comprehensive understanding of its impact on AL patients. In the CoReAL reporting framework outlined in chapter 3, we integrated QoL assessment as a component of long-term reporting. We are convinced that adopting a standardized approach to assessing and reporting QoL after AL will enhance our comprehension of the ramifications of this complication and mitigate the uncertainties and points of contention raised in existing evidence (chapter 9). A standardized set of other patient-centered outcomes and measures to inform value-based health care during the experience of an AL can be additionally developed considering the gained knowledge in chapter 10.

After conducting the patient interviews, as described in chapter 10, it became clear that a lot of patients who experience AL emphasized the importance of family support. They also highlighted some important elements that are covered within this so-called patient-and-family centered care (PFCC) [66]. In recent years, there has been a heightened focus on

providing PFFC in hospitals in general. Family-centered care extends beyond simply having family members present during hospitalization; it encompasses active involvement and participation of families in all aspects of care delivery [67]. In the surgical realm, the active participation of family caregivers in essential care tasks holds promise for enhancing health-related outcomes, such as QoL and discomfort levels [68]. Based on the interpretations of part III of this thesis, we believe this care approach can positively influence patients' experiences during and after the development of a colorectal leak for several reasons [69]. By involving patients and their families in care decisions, empowerment and engagement are fostered, enabling active participation in treatment processes [70]. This engagement also promotes clear and transparent communication between healthcare providers, patients, and their family, ensuring that treatment decisions are aligned with patient preferences and values. The latter was mentioned as very important during the patient interviews in chapter 10. Moreover, a holistic approach to care is emphasized, addressing not only the physical health needs but also the psychosocial aspects of patient well-being [66]. These needs became clear while conducting the patient interviews. Additionally, PFCC supports patients in self-managing their care and recovery, facilitating better adherence to postoperative instructions and treatment plans, which were reported to be difficult sometimes by the patients. By gathering continuous feedback from patients and their family, healthcare providers can identify areas for improvement and implement quality enhancement initiatives, ultimately leading to better patient outcomes and experiences throughout the diagnostic, treatment and follow-up journey [66, 68]. This approach is not only necessary during the development of an AL, but during their whole oncological treatment journey.

As presented in the introduction of this thesis, current evidence regarding long-term oncological outcomes after AL is conflicting [71-79]. Whether it does or does not influence the risk of distant metastases, it is always important to minimize the risk of metachronous development of tumor deposits after primary surgery to improve patients' outcomes.

PART IV: PREVENTION OF METACHRONOUS PERITONEAL METASTASES AFTER COLORECTAL CANCER SURGERY

In part IV of this thesis highlighted the importance of discovering specific biomarkers for metachronous PM in primary colorectal tumors for deploying efficient preventive measures in patients. PM, which involve the dissemination of CRC cells to the peritoneal cavity, pose a substantial hurdle in cancer care owing to their aggressiveness and scant treatment choices. Nevertheless, early identification of particular biomarkers linked to PM might empower clinicians to investigate preventive strategies, thereby enhancing their efficacy in addressing high-risk patients.

The systematic review described in **chapter 11** aimed to evaluate (the quality of) sequencing studies reporting on specific biomarkers in primary colorectal tumors that could serve as a prediction tool to estimate the risk of peritoneal spread. The review identified 17 retrospective cohort studies that reported on potential biomarkers. The DNA analyses showed that most included articles (n = 10) described *BRAF* mutant tumors to be more likely to have PM and/or mutations in *BRAF* were more common in patients with PM compared to those without. Some additional genes were mentioned as possible mutated genes associated with PM by several authors but were, except for one, all investigated in only one study. Broader panel analysis did not show additional discoveries, and RNA outcomes were not consistent either. As almost all included studies were retrospective with a different number of patients, different patients' characteristics and different used sequencing methods, comparisons between the studies were limited due to this heterogeneity. Unfortunately, most of the studies did not clearly specify whether the authors were using tumors from synchronous or metachronous PM patients. It was therefore hard to distinguish and separate these two scenarios in the results. Based on the given evidence, we concluded that the summarized genes that were possibly associated with PM (especially *BRAF*), were not reliable enough to function as an individual biomarker in a clinical setting and future biomarkers research in a homogenous population was necessary. Subsequently, our research team performed such an analysis in the next chapter.

Subsequently, **chapter 12** explored and compared genetic alterations in primary colorectal tumours of patients without metastases, with metachronous PM and with metachronous liver metastases (LM) to potentially discover biomarkers for metachronous PM. This retrospective analysis involved forty patients with T3 stage CRC, categorized into the three metastatic groups based on a 5-year follow-up (M0 = 20, PM = 10; LM = 10). To ensure a homogenous population, patients with any synchronous metastases were excluded. A comprehensive genome sequencing (Trusight Oncology (TSO) 500 analysis) was conducted on primary formalin-fixed paraffin-embedded tumor samples, targeting DNA alterations in 523 genes and RNA fusion transcripts in 55 genes. Two patients were excluded (LM = 1 and PM = 1) from the final analysis, resulting in a final sample size of 38. Microsatellite instability (MSI) was identified in four M0 tumors and one PM tumor. There were new genes identified that had not been described in relation to metachronous PMs, or metastases in general, although the clinical significance remained unknown due to the small sample size. Notably, *BRAF* p.V600E mutations were exclusively present in PM patients with microsatellite stable (MSS) tumors (37.5%, $p = 0.010$).

The outcome of chapter 12 strengthened the suggestion made in chapter 11 and underscored significance of closely monitoring *BRAF* p.V600E mutated MSS tumors concerning the emergence of metachronous PM. In current clinical practice, the classification of the MSI status is the only genetic test that is routinely performed in CRC patients to decide adjuvant therapy decisions [80]. Other genetic tests, such as *BRAF* mutation status, are only

evaluated in metastatic tumors. Most research on *BRAF* mutation has been performed in case of metastatic CRC, and its therapeutic approach is complex due to their resistance to conventional therapies [81]. The treatment landscape for *BRAF* metastatic CRC has rapidly evolved in recent years, with efforts focused on combining therapies to improve outcomes [82]. Despite initial hopes based on outcomes in *BRAF*-mutant melanoma, monotherapy with BRAF inhibitors (*iBRAF*) has shown lower efficacy in metastatic CRC [81-84]. While the addition of anti-vascular endothelial growth factor (anti-VEGF) therapy to standard chemotherapy has shown limited benefit, combining *BRAF* inhibitors with anti-epidermal growth factor receptor (anti-EGFR) monoclonal antibodies has emerged as a promising approach [81, 85]. Ongoing research must still explore the role of triplet combinations with *MEK* or *PIK3CA* inhibitor, but also the wide variance in tumor response rates [82, 86, 87]. Transcriptomic signatures suggest potential responsiveness to immune checkpoint inhibitors, leading to ongoing investigations into combination therapies [82, 88]. Acquired resistance mechanisms pose challenges, with liquid biopsies offering a noninvasive means to identify and address resistance [82]. Ongoing studies are exploring novel combinations with *BRAF* inhibitors to overcome acquired resistance, underscoring the importance of continued research into *BRAF*-V600E-mutant biology to enhance patient care [81, 82]. With survival rates about half as long as those of *BRAF* wild-type patients [85], urgent exploration of new treatments is necessary to improve outcomes for *BRAF*-mutant CRC patients. Based on the results of Chapter 11 and 12, our research teams suggests that greater attention should be given to *BRAF*-mutated tumors regarding the development of metachronous PM in CRC patients without metastases. Therefore, an alternative approach could be to not only focus on *BRAF* status as a treatment option when metastases occur, but to use the *BRAF* status as a part of a prediction model to identify the patients at risk for metachronous PM and invent preventive strategies for these patients.

Alternatively, exploring biomarkers beyond the primary tumor focus may reveal some promising avenues. An interesting topic in this field is circulating cell-free DNA (cfDNA). Circulating tumor DNA (ctDNA) is a component of cfDNA that is shed by malignant tumors into the bloodstream and other bodily fluids, like the peritoneal cavity, and may be used as a marker for residual or recurrent disease [89]. Research has indicated the feasibility of detecting peritoneal cfDNA in ascites and peritoneal lavage fluid and conclude that peritoneal cfDNA's may be promising as a biomarker for postoperative monitoring and as an adjunctive tool for diagnostic laparoscopy in detecting peritoneal spread in high-risk CRC cases [90, 91]. It is therefore interesting to further investigate cfDNA and its potential in guiding high-risk PM patient selection for targeted therapies, as biomarkers in the primary tumor may be not sensitive enough.

Ideally, a minimally invasive preventive treatment option for patients at high risk to develop PM should exist. In recent years, considerable research has been dedicated to develop drug delivery systems that aim to prolong the intraperitoneal retention time of cytostatic agents without inducing systemic toxicity, and to enhance minimal invasive approaches [92]. Previous

work revealed that intraperitoneal administration of a supramolecular hydrogel loaded with mitomycin (MMC) resulted in extended peritoneal exposure and led to a clinically significant improvement in survival compared to treatment with just free MMC in rats with colorectal PM [93]. Therefore, our research team believed it was interesting to see if such a minimal invasive treatment is safe to use during colorectal surgery after primary resection of (e.g. *BRAF*-mutated) tumors as a preventive intervention.

Chapter 13 assessed the impact of intraperitoneal hydrogel administration in a colorectal surgery setting, especially on anastomotic healing. Forty-two healthy Wistar rats underwent a colonic end-to-end anastomosis, with subsequent intraperitoneal injections administered to 6 animals with saline, 18 with unloaded hydrogel, and 18 with mitomycin (MMC)-loaded hydrogel. After a 7-day period, the animals were euthanized, and primary outcomes, including anastomotic adhesion and leakage scores, were measured. Secondary outcomes encompassed bursting pressure, histological anastomosis evaluation, and changes in body weight. Twenty-two rats completed the follow-up (saline: n = 6, unloaded hydrogel: n = 10, MMC-loaded hydrogel: n = 6) and were included in the analysis. After multiple-comparison correction, a trend toward significance was observed for the AL score between rats receiving saline and those receiving unloaded hydrogel (p = 0.020, α = 0.0167). However, no significant differences were noted for all other outcomes. The primary reason for drop-out in this study was intestinal blood loss (n = 16), which only occurred in intervention animals. While the preliminary results suggest that MMC-loaded or unloaded hydrogel may not affect anastomotic healing, the observed intestinal blood loss in a substantial number of animals receiving both types of hydrogel indicates that the injection of the hydrogel under the studied conditions is not safe in the current rodent model. This underscores the need for further optimization of the hydrogel before it can be considered as a preventive strategy.

The promising results of applying a cytostatic loaded supramolecular hydrogel as a minimal invasive preventive treatment, turned out to be disappointing in chapter 13. Besides, as the strength of a good clinical biomarker lies in its ability to accurately and reliably predict a particular biological or clinical outcome, which is metachronous peritoneal spread in this case, the evidence found in chapter 11 and 12 is not strong enough to guide personalized therapeutic interventions that are more invasive. As we cannot offer the patients any treatment options, standard clinical screening for *BRAF* mutations in all primary colorectal tumors might feel too early. On the other hand, a stricter follow-up in this population may be clinically beneficial. While current international guidelines recommend the first follow-up CT scan 12 months after primary surgery, a *BRAF*-mutated population may warrant earlier imaging and increased clinical monitoring for PM development. Prospective research, including validation of *BRAF* mutations in relation to metachronous PM development, particularly through methods like liquid biopsies, is essential to support this proposal. Additionally, future research should focus on minimal invasive preventive strategies, whether it is optimizing the drug delivery system in our study or new innovations.

WRAP-UP AND FUTURE PERSPECTIVES

Numerous generations of clinical and experimental researchers have dedicated their efforts to address the challenge of improving patients' outcomes after colorectal surgery. Despite significant insights gained over the decades, AL continues to pose a substantial postoperative concern for a considerable number of patients. Furthermore, identifying patients at risk of metachronous PM and subsequently tailoring personalized treatment has posed challenges. While this thesis may not offer a conclusive solution to eliminate AL or metachronous PM, it signifies progress in confronting these issues.

We believe that enhanced reporting of AL will also reduce its incidence and impact. The proposed CoReAL framework for standardizing reporting of leaks after colorectal surgery is crucial to improve patients' outcomes. Not only will this framework help to enhance the reliability of research findings, but it will also promote early recognition and guide clinical decision making. Therefore, future steps will be taken to achieve worldwide adoption, including submitting our consensus statements and the reporting framework to surgical societies for evaluation of agreement and potential adoption into clinical practice, followed by surveys among physicians to monitor adherence. Additionally, factors contributing to potential lack of agreement will be systematically explored and discussed, with the expectation that evidence-based reporting elements will be effectively integrated into local practices and accepted by stakeholders. With the help of artificial intelligence to support clinicians, the reporting system will probably be self-generated in the future. The development and additional validation of our proposed CAL-RADS score will extra support the standardization of assessing and reporting leaks in the diagnostic phase.

The current thesis highlighted the use of NIRF or LSCI to assess bowel perfusion. To our expectation, bowel perfusion assessment will increase over the next decade, and eventually it will become standard of care in colorectal surgery under a few conditions. In the widespread search for reliable quantification of perfusion with both NIRF imaging and LSCI, standardization of quantification methods and surgical procedures and data acquisition is essential. Subsequent, clinical trials in which direct patient benefit is explored are necessary to improve worldwide adoption. Stronger evidence derived from larger human cohorts (ideally RCTs) is required to substantiate the effectiveness in reducing AL rates and improving patients' outcomes. Also, this will enhance comparison of both imaging techniques. Additionally, the role of MB in the landscape of NIRF should be investigated parallel to this. Larger datasets both for NIRF and LSCI should be used to develop prediction models providing risk rated of AL per tissue location in real time to alter surgical strategies. MB can probably also play an important role to reduce the number of iatrogenic ureter damage. Camera systems should therefore be adapted to visualize more wave lengths.

As QoL of AL patients showed an important decrease at six months post-diagnoses and even up to one year, we encourage that QoL should be assessed and reported when talking about leaks. We therefore advise to follow the ICHOM recommendations [65], and use the EORTC QLQ-C30 tool to capture overall QoL and the -CR29 to capture CRC specific outcomes at baseline (prior to surgery), 6 months after surgery, and then annually during follow-up. Ideally, a standardized set of patient-centered outcome measures to inform value-based health care in CRC patients with AL can be developed, in which both the physical and emotional impact mentioned by our participants can be considered.

Finally, this thesis also emphasized the importance of preventive strategies for PM after initial colorectal surgery. We revealed greater attention should be given to *BRAF*-mutated tumors regarding the development of metachronous PM after curative surgery. Future research should focus on validation of this mutation, alternative biomarker research, and minimal invasive preventive strategies, whether it is optimizing drug delivery systems or new innovative approaches.

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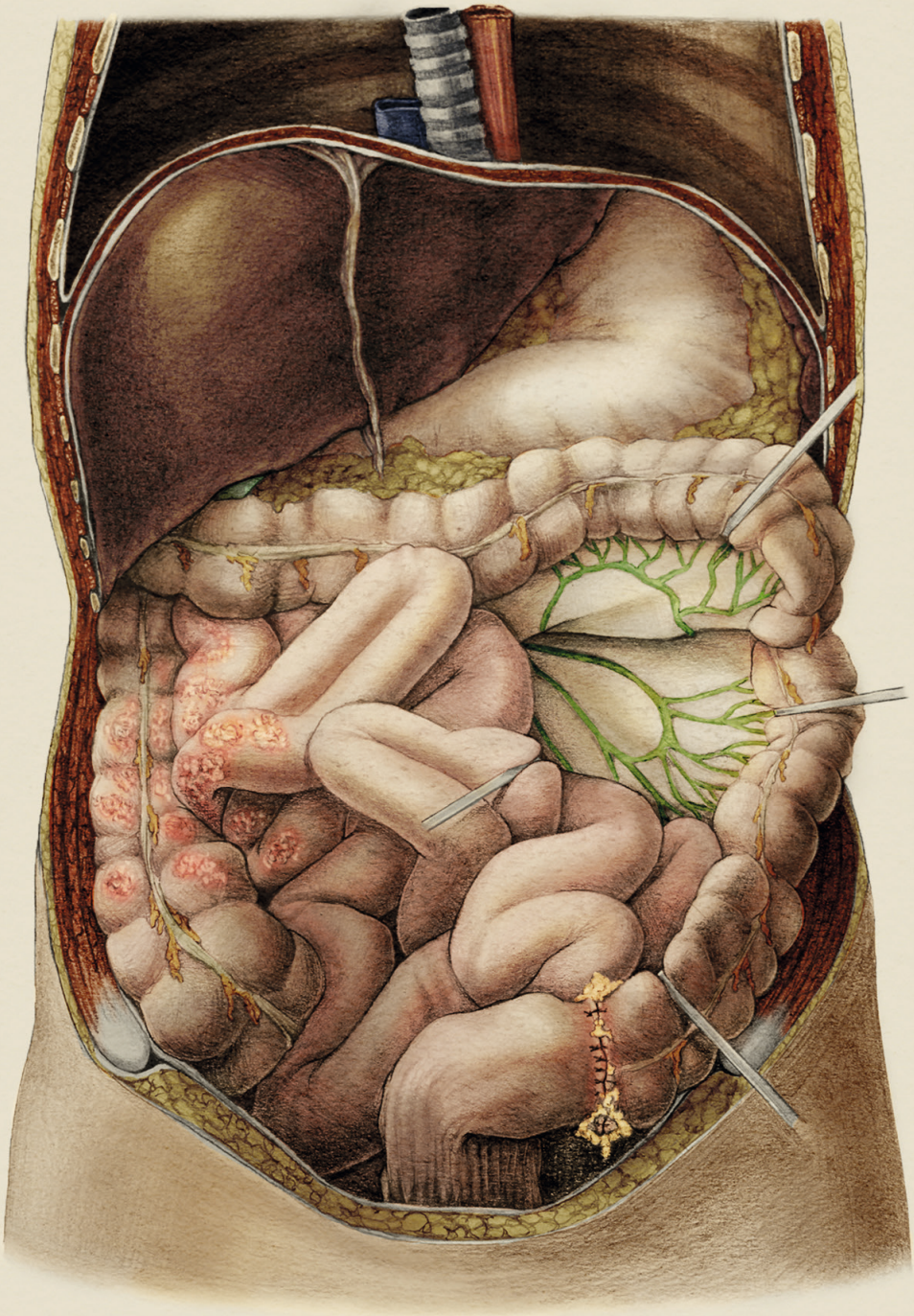

CHAPTER 15

IMPACT

Over the recent decades, the incidence of colorectal cancer (CRC) has exhibited dynamic trends [1]. This phenomenon has initiated research into potential etiological factors and environmental exposures. The escalating burden of CRC poses significant challenges to global healthcare systems. Consequently, research efforts on CRC are dedicated to investigating this burden and optimizing the allocation of medical resources and outcomes in a more equitable manner [2]. In essence, this thesis centered on enhancing patient outcomes following colorectal surgery, with the overarching goal of directly influencing patient well-being. This chapter delves into the societal and economic implications of the knowledge gathered from the various projects outlined in this thesis, while also analyzing their impact on the prevailing scientific priorities within the anastomotic leakage (AL) and peritoneal metastases (PM) field after CRC surgery.

SOCIETAL RELEVANCE

Firstly, reflecting on the work related to AL in this thesis, we believe better reporting of colorectal AL using the proposed reporting framework in this thesis (**part I**) can lead to a reduction for societal impacts in several ways. A worldwide issue of colorectal AL is that the lack of reporting them results in delayed recognition and treatment. Better reporting will ensure that leaks are identified promptly, allowing for early intervention and treatment. This can prevent acute complications such as sepsis and abscess formation, but also chronic sequelae like sinuses and fistulae, reducing the overall need for more extensive surgical procedures and hospitalizations over the long term [3]. Also, by accurately documenting the occurrence and severity of leaks, healthcare facilities can allocate resources more efficiently. Detailed reporting of leaks additionally provides valuable feedback to surgeons and healthcare teams, facilitating continuous improvement in perioperative care protocols, including adequate pre-operative assessment, intraoperative measurements, and postoperative diagnostic tools. This can ultimately reduce the incidence of leaks and improve patient outcomes over time, but also enhance more efficient care when a leak occurs. Furthermore, it is anticipated that the reporting framework will encourage closer collaboration between healthcare professionals and software developers in the coming years. This could involve facilitating electronic data collection as suggested by our reporting framework, including input from patients as presented in our qualitative study. These initiatives align with the current priorities of e-health outlined by the Dutch Ministry of Health, Welfare, and Sport [4]. By leveraging information technology, perioperative care stands to undergo further transformation. Not only by improving reporting of leaks, but also by optimizing bowel perfusion assessment (**part II**), we hope to reduce the risk of developing AL. Additionally, this thesis sheds light on the challenges faced by patients who developed AL post-surgery (**part III**), highlighting the importance of dialogue and additional support for patients dealing with AL. Also, it emphasizes the importance of psychological support and coping mechanisms for patients facing challenges such as pain, rehabilitation difficulties, and emotional distress.

Secondly, reflecting on the work related to PM in this thesis (**part IV**), we underscored the importance of accurate prediction tools for estimating the risk of peritoneal spread in CRC patients using genetic alterations. As PM is associated with a dismal prognosis and research into the prevention of PM, it is therefore crucial to improve outcomes. Our study investigating the safety of a preventive strategy in the form of an injectable hydrogel needs to be addressed here. Although the impact on patients may be primarily indirect, as it involves preclinical research conducted on rats rather than direct human subjects, the findings have important societal implications. As previous studies showed enhanced survival among animals subjected to this treatment, our study team strongly believed this hydrogel could influence patients' outcomes positively. Yet, the observed safety concerns, particularly the significant intestinal blood loss observed in animals receiving the hydrogel, highlight the importance of rigorous preclinical testing to ensure the safety and efficacy of new interventions before their translation into clinical practice. By identifying potential risks associated with the use of hydrogel, this study underscores the need for further optimization and refinement of the intervention to ensure patient safety in future clinical trials.

ECONOMIC RELEVANCE

Complications following colon or rectum surgery have a significant economic impact. For patients without complications, the average cost is €9,226 in the Netherlands (2015). However, for those with minor complications, the cost increases by €2,403, and for those with severe complications, it rises to €17,906 [5]. The occurrence of AL leads to a significant rise in healthcare resource utilization, primarily driven by longer hospital stays [6, 7]. The more complex the AL, the greater the associated treatment costs [6]. Subsequently, preventing or minimizing the occurrence of colorectal AL by enhancing uniform reporting of leaks (**part I**) can lead to significant cost savings within the healthcare system. Fewer complications mean shorter hospital stays, fewer readmissions, and reduced reliance on costly interventions such as reoperations, prolonged antibiotic therapy, and admission in the intensive care unit. Timely identification and management of leaks may help mitigate long-term complications, improving patients' quality of life (QoL) and reducing the burden on healthcare resources associated with ongoing care needs.

It is also important to address the potential benefits against the cost when using bowel perfusion assessment (**part II**) as a potential preventive strategy for AL. A recent article investigated the cost-effectiveness of utilizing indocyanine green fluorescence angiography (ICG-FA) as a preventive measure against AL in colorectal surgery [8]. It assessed the potential economic benefits and analyzed various factors, including the cost of ICG-FA equipment, procedure costs, and potential savings associated with reduced postoperative complications. According to this cost analysis based on recent studies on leak rates and the expenses of colorectal resections, incorporating fluorescence imaging for perfusion assessment as a

routine practice is economically advantageous from the hospital payer's standpoint, leading to cost savings. If future research provides strong evidence on the reduction of leaks by using fluorescence imaging, which has been widely discussed in Chapter 14 (discussion) of this thesis, we believe this immediately results in improved economic status. Future research is necessary to justify the use of laser speckle contrast imaging, but we expect similar results as for ICG-FA.

Additionally, **part III** underscores the potential economic burden associated with diminished QoL, particularly in the initial six months to one year following surgery, which may necessitate additional healthcare resources and support services. These findings highlight the importance of comprehensive preoperative information, effective communication, and peer support in optimizing patient outcomes, potentially reducing healthcare costs associated with postoperative complications and improving overall patient satisfaction and well-being. Addressing specific discussion points and exploring future perspectives, as outlined in Chapter 14, will facilitate the clinical implementation and translation of our research findings.

By enabling more accurate risk stratification and development personalized interventions, the research in **part IV** also intended to lead to cost-effective approaches for managing CRC patients at risk of PM. Investigating the safety and efficacy of an intraperitoneal hydrogel administration on anastomotic healing in a preclinical model, informs potential surgical practice and contributes to the ongoing efforts to develop treatment strategies to improve patient outcomes and minimize postoperative complications. The findings of this animal study have implications for healthcare costs by assessing the potential benefits and risks associated with intraperitoneal hydrogel use in colorectal surgery. Addressing the observed safety issues early in the development process help minimize potential healthcare expenses associated with adverse events and complications.

WORLDWIDE COLLABORATION AND SCIENTIFIC IMPACT

Over the past two years, our research group has fostered fruitful collaborations with various research teams both in the Netherlands and internationally. Notably, we have actively participated in the CoReAL project, a consensus expert group facilitating the exchange of ideas among clinicians and researchers to gain worldwide evidence and consensus on AL. This international collaboration has enabled the initiation of a standardized reporting framework for colorectal AL. Our systematic review (Chapter 2), consensus paper (Chapter 3), protocol for radiological reporting (Chapter 4) and systematic review on QoL (Chapter 9) and qualitative interview study (Chapter 10) have already demonstrated the power of these combined international efforts. The consensus paper in chapter 3, in which we used a modified Delphi analysis worldwide, engaged numerous esteemed researchers and experts to give their opinions and fostered collaborative discussions to reach consensus on this crucial

topic. Currently, we are in the final stages of achieving a universally accepted reporting framework for colorectal AL by gaining more feedback from members from different surgical societies all over the world. In parallel, we are working on a radiological reporting framework too. We are confident that the forthcoming work, resulting from this collective effort, will be embraced by researchers and clinicians worldwide. Ideally, better reporting practices contribute to the generation of robust data on colorectal surgical outcomes, supporting high-level evidence research and health initiatives aimed at reducing the overall burden of colorectal disease and improving population health. Additionally, the work presented in part III highlighted the importance of incorporating patients' perspectives after leak development into future research.

By synthesizing existing evidence on biomarkers associated with PM and analyze our own tumors, part IV contributes to the ongoing dialogue among healthcare professionals and researchers regarding personalized treatment approaches and patient care strategies. The observed safety concerns of a potential preventive treatment in the form of a hydrogel additionally underscore the importance of further optimization and research.

SUSTAINABILITY

Human health and the health systems we rely on face growing threats from the environmental crise, including climate change [9]. Ironically, the healthcare sector ranks among the one of the largest service industries with a significant carbon footprint, in which the operating room is particularly a resource-intensive component of the system [9, 10]. Surgical care demands substantial resources within healthcare, including costly and resource-intensive equipment, water and energy for sterilization procedures, advanced technology, and necessary life support systems. These processes consume significant amounts of energy and materials, while also generating substantial waste [10]. By trying to reduce the occurrence and impact of AL and PM, fewer resources such as hospital stays, medications, and surgeries are needed, leading to decreased energy consumption and waste production associated with healthcare facilities. Less surgical interventions and medical treatments mean reduced environmental impact associated with healthcare services, including transportation, equipment manufacturing, and hospital operations. Additionally, it leads to better patient health and reduced need for ongoing medical care, resulting in lower overall healthcare-related environmental impact over time. Lastly, improving the landscape of AL and PM frees up healthcare resources for other patients and medical needs, promoting efficient resource allocation and reducing strain on the healthcare system, which can indirectly contribute to environmental sustainability. Overall, preventing complications after colorectal surgery not only benefits patient health but also contributes to reducing the environmental footprint of healthcare systems, thereby improving environmental sustainability.

TARGET POPULATION

The data presented in this thesis are relevant for both doctors, researchers, healthcare insurance companies and patients to provide additional data on preventing leaks and current research into the prevention of metachronous PM after colorectal surgery. It also gives insights to society on what can nowadays be achieved with improving AL reporting, bowel perfusion assessment, incorporating patients' perspectives and biomarker research. Most importantly, it should motivate clinicians to adequately report AL, assess bowel perfusion with innovative techniques, take patients' experiences into account while diagnosis and treating AL, and acknowledge the ongoing debate of biomarkers research and the development of minimal-invasive preventive treatment options.

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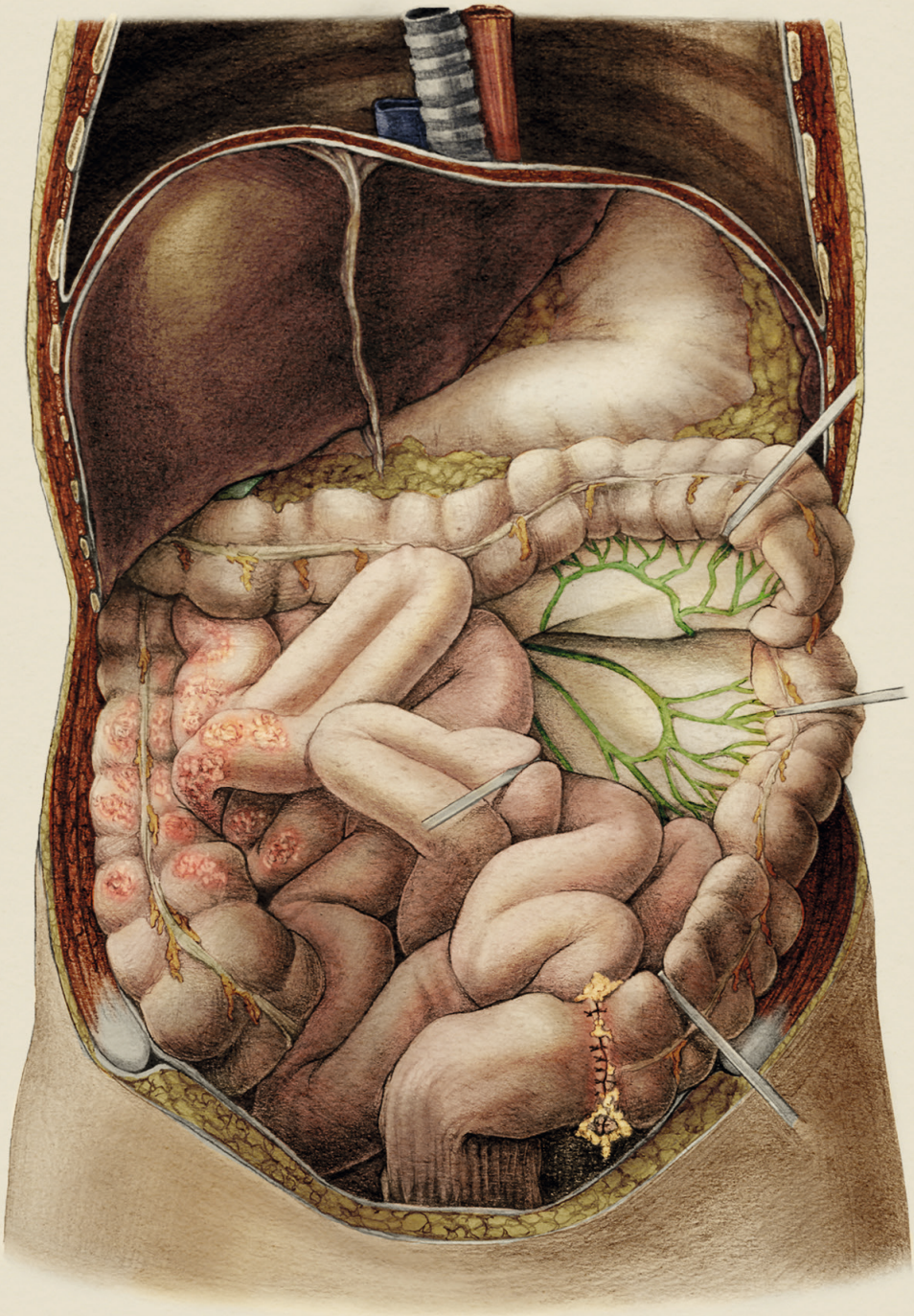


CHAPTER 16

DUTCH SUMMARY
NEDERLANDSE SAMENVATTING

ACHTERGROND

Darmkanker is wereldwijd de op twee na meest voorkomende vorm van kanker en goed voor ongeveer 10% van alle kankergevallen. Bovendien is het de op een na belangrijkste oorzaak van kanker gerelateerde sterfgevallen wereldwijd. De primaire curatieve behandeling van darmkanker omvat de chirurgische verwijdering van de tumor en aangrenzende lymfeklieren. De keuze voor de beste chirurgische procedure hangt af van de locatie van de tumor en de conditie van de patiënt waarbij meestal de tumor wordt verwijderd gevold door het aanleggen van een anastomose. Om de impact van colorectale chirurgie voor de patiënt te verminderen is het cruciaal om strategieën te implementeren die complicaties kunnen voorkomen en verminderen. De meest gevreesde complicatie na colorectale chirurgie met het aanleggen van een darmnaad is naadlekkage. Het is bekend dat patiënten met een naadlekkage een slechtere algehele overleving en oncologische prognose hebben, vooral na rectale chirurgie. Daarnaast is het meest levensbedreigende kenmerk van darmkanker het vermogen om metachroon uit te zaaien. Metachrone peritoneale metastasen (PM) hebben de slechte uitkomsten omdat de behandelingsmogelijkheden beperkt zijn. Het minimaliseren van het risico op zowel naadlekkage als metachrone PM na colorectale chirurgie is essentieel, aangezien hun ontwikkeling kan leiden tot o.a. een verminderde kwaliteit van leven en een slechtere prognose. Dit proefschrift heeft het doel de postoperatieve uitkomsten voor patiënten met darmkanker te verbeteren door zich te richten op deze twee belangrijke onderwerpen.

In **deel I** van dit proefschrift werd belicht hoe naadlekkages worden gerapporteerd in de literatuur en werd er vervolgens een rapportagesysteem en radiologisch scoresysteem ontwikkeld dat in de toekomst kan worden gebruikt om de rapportage van naadlekkage te standaardiseren. In **deel II** werd onderzocht of het beoordelen van de darmdoorbloeding geoptimaliseerd kan worden met innovatieve beeldvorming om het risico op naadlekkage te verminderen. Dit werd getest met zowel de contrastmiddelen Indocyanine Groen (ICG) en methyleenblauw (MB) met nabij infrarood licht, als met een nieuwere techniek genaamd Laser Speckle Contrast Imaging (LSCI). In **deel III** werd een overzicht gecreëerd van de huidige kennis over de impact op de kwaliteit van leven van patiënten na colorectale naadlekkages, en werd door middel van interviews met patiënten meer inzicht verkregen over patiëntperspectieven. Aangezien er steeds meer bewijs is dat naadlekkages ook de oncologische uitkomsten beïnvloeden, had dit proefschrift bovendien tot doel voorspellende biomarkers in darmtumoren voor metachrone PM te identificeren en de veiligheid van een injecteerbare chemogel als potentiële preventieve strategie te evalueren (**deel IV**).

INTERNATIONALE CONSENSUS OVER DE HUIDIGE WETENSCHAPPELIJK KENNIS EN DE RAPPORTAGE VAN NAADLEKKAGES NA DARMKANKER CHIRURGIE

Het correct rapporteren van naadlekkages blijft lastig omdat er geen consensus is over wat de exacte definitie van een naadlekkage is. Desondanks is het rapporteren van complicaties essentieel voor het bevorderen van de uitkomsten en de kwaliteit van zorg voor patiënten, als mede het interpreteren van wetenschappelijk onderzoek. In het eerste deel van dit proefschrift richtten we ons op de bewustwording van de heterogeniteit hoe naadlekkages worden gerapporteerd en het ontwikkelen van systemen om deze rapportage te verbeteren.

In **hoofdstuk 2** onderzochten we op een systematische manier hoe naadlekkages na colorectale oncologische chirurgie momenteel worden gerapporteerd in de literatuur. Van de 471 artikelen die een naadlekkage als primaire of secundaire uitkomst beschrijven, gaven slechts 95 studies (45 gerandomiseerde gecontroleerde onderzoeken, 13 systematische reviews en 37 meta-analyses) een duidelijke definitie van een naadlekkage. Verder keken we ook naar andere elementen die werden gerapporteerd. Van deze artikelen gaf 68% een beschrijving van de klinische tekenen en symptomen waarbij gedacht moet worden aan een naadlekkage en 26% rapporteerde welke aanvullende laboratorium onderzoeken werden gebruikt. Slechts 63% beschreef welke radiologische onderzoeken aanvullend werden gebuikt, en 62% de bevindingen op deze beeldvorming. Tot slot beschreef slechts 13% de bevindingen die ze vonden tijdens een verrichte interventie, zoals een nieuwe operatie. Opvallend is ook dat slechts 45% van de studies ernst van een naadlekkage classificeren en maar 41% gaf aan binnen welke tijd na de operatie ze de diagnose van naadlekkage gaven. Deze bevindingen onderstreepten het probleem van onvolledige en inconsistente rapportage van naadlekkages binnen de gepubliceerde literatuur. Om duidelijkere communicatie over naadlekkages te verkrijgen, uitkomsten van onderzoeken goed te kunnen vergelijken en uiteindelijke klinische uitkomsten te verbeteren, is er een dringende behoefte aan de ontwikkeling en implementatie van een consensus voor de definitie, gradatie en hoe we naadlekkages het beste kunnen rapporteren.

In **hoofdstuk 3** creëerden we het consensus project “Consensus on Reporting colorectal Anastomotic Leaks (CoReAL)”. Het project bestond uit een internationale groep chirurgen die allemaal lid waren van internationale chirurgische verenigingen. Allereerst analyseerde de groep alle beschikbare literatuur over naadlekkages. Op basis van deze analyses werden er in totaal 33 stellingen geformuleerd met betrekking tot naadlekkages na colorectale oncologische chirurgie. Deze stellingen werden verdeeld in de vier fases van de behandeling van de patiënt: factoren die belangrijk zijn voor de operatie (pre-operatief), tijdens de operatie (intra-operatief), kort na de operatie en tijdens de langere follow-up periode. Vervolgens werden de stellingen gebruikt om een rapportagesysteem te ontwikkelen, samen met input van de experts en van patiënten (hoofdstuk 10). In dit rapportagesysteem zitten elementen

die we verwachten dat gerapporteerd worden in de pre-operatieve, intra-operatieve en postoperatieve korte en lange termijn. Nieuw onderzoek werkt nu aan de implementatie van dit rapportagesysteem.

Om ook de communicatie tussen de radiologen en de chirurgen te bevorderen en duidelijkheid te scheppen over de bevindingen op een CT-scan wanneer er een verdenking is op een naadlekkage, ontwikkelde we in **hoofdstuk 4** een protocol om een eenduidige radiologische score te ontwikkelen. Dit protocol beschrijft de validatie van een gestandaardiseerde score om potentiële naadlekkages na colorectale chirurgie op CT-scans te beoordelen. Het onderzoeksteam stelde een gestandaardiseerd CT-beoordelingsschema voor naadlekkages voor, gebaseerd op bevindingen van 30 patiënten die een naadlekkage ontwikkelden. Gebaseerd op de standaardisatie-systemen van andere vakgebieden, kozen we om een Colorectal Anastomotic Leakage Reporting and Data System (CAL-RADS) te ontwikkelen. De voorgestelde score varieert van 0 tot 5 en geeft de waarschijnlijkheid van naadlekkage aan. Het doel van de studie is om de CAL-RADS-classificatie te optimaliseren, de variatie tussen de verschillende observatoren te berekenen, de haalbaarheid en klinische vertaling te onderzoeken en deze score te correleren aan de verrichte interventie. In totaal werden 150 CT-scans van patiënten die een colorectale operatie hadden ondergaan, recentelijk al gescoord met de voorgestelde CAL-RADS-score door 6 radiologen. Voorlopige resultaten suggereren dat de score eenvoudig en haalbaar is om de waarschijnlijkheid van AL te beoordelen. De inter-observer variatie wordt momenteel geanalyseerd. Aanvullende correlatieanalyses tussen de gegeven scores en de uiteindelijke interventies zullen binnenkort worden uitgevoerd. Ook zal de invloed van rectaal of oraal contrast in bepaalde gevallen worden onderzocht.

VERBETERING VAN DE BEOORDELING VAN DARMPERFUSIE OM HET RISICO OP NAADLEKKAGES TE VERMINDEREN

Naadlekkages worden vaak toegeschreven aan technische factoren zoals het foutief hechten van de darmnaad door de chirurg of een te hoge spanning hierop. Toch blijkt steeds vaker dat een naadlekkage ook kan optreden ongeacht de chirurgische techniek, zoals ook aangetoond werd in het CoReAL project in hoofdstuk 3. Onvoldoende doorbloeding van de doorgesneden darmranden is een bekend risico voor het ontstaan van naadlekkage. De mate van bloedvoorziening kan worden beoordeeld met behulp van nabij-infraroodfluorescentie (NIRF)-beeldvorming. De klinische toepassingen van NIRF zijn aanzienlijk toegenomen de afgelopen jaren, zoals voor visualisatie van lymfeklieren, de galwegen, de ureter en het beoordelen van de doorbloeding van een anastomose. Het verbeteren van beeldvormingssystemen om de beoordeling van darmperfusie te optimaliseren en gelijktijdig andere structuren te visualiseren, is belangrijk om het optreden van naadlekkages en andere mogelijke complicaties te verminderen.

In **hoofdstuk 5** onderzochten we of het mogelijk was om gelijktijdig de darmperfusie met ICG en de lokalisatie van de ureter met MB in beeld te brengen, met een nieuw camerasysteem. Zes varkens ondergingen een laparotomie onder algehele anesthesie. Darmperfusie werd beoordeeld met een intraveneuze (IV) dosis van 0,2 mg/kg ICG en uretervisualisatie met een IV-injectie van 0.75, 0.50 of 0.25 mg/kg MB. Tijdens de studie bleek het inderdaad mogelijk om met dit camerasysteem de ureters en darmperfusie bij alle varkens in kaart te brengen. De ureter was zichtbaar binnen vijf tot tien minuten en bleef gemakkelijk te identificeren gedurende elk experiment (120 - 420 min). Een aanvullende analyse toonde geen significante verschillen tussen de drie dosisgroepen MB. Opvallend was dat we de darmperfusie niet alleen met ICG konden beoordelen, maar ook met MB. Er werd tijdens de opnames geen interferentie waargenomen tussen ICG en MB. Bovendien zagen we dat het effect van MB eerder verdween, wat klinisch voordelig kan zijn in situaties waarin herhaalde perfusiebeoordelingen tijdens een chirurgische ingreep nodig kan zijn.

In **hoofdstuk 6** werd een aanvullende kwantitatieve analyse uitgevoerd op de darmperfusie beoordeling met zowel ICG als MB, gebruik makende van hetzelfde camerasysteem als in hoofdstuk 5. Vier varkens ondergingen opnieuw een laparotomie onder algehele anesthesie. In elk varken namen we de bloedvoorziening van een stukje dunne darm door, waardoor een deel van de darm ischemisch werd. Dit zogenoemde ischemische darmloop segment werd vervolgens gemarkeerd met vijf interessegebieden (ROIs) met verschillende perfusieniveaus (slecht doorbloed, 2 stukken matig doorbloed en een nog goed doorbloed gedeelte). Na 10 minuten werd een intraveneuze injectie van 0.25 mg/kg – 0.50 mg/kg MB toegediend, gevolgd door NIRF-beeldvorming. Ook werden er van elke ROI lokaal lactaat bepaald om de mate van ischemie in te schatten. Deze procedure werd herhaald in ICG-modus (IV-dosis van 0.2 mg/kg) na 60 minuten. De NIRF-beeldvorming van darmperfusie met MB en ICG was succesvol bij alle bestudeerde dieren. We correleerden de fluorescentie 'ingress (i/s)' levels met de lokale lactaatniveaus. Zowel de ingress levels van MB als ICG vertoonden een significante correlatie met lokale lactaatniveaus. Opvallend was dat de correlatie sterker was voor MB vergeleken met ICG, hoewel ICG-analyse hogere absolute ingress levels liet zien. Daarom concludeerden we met deze fluorescentie kwantificatieanalyse dat het potentiële gebruik van MB voor de beoordeling van darmperfusie voordelen kan hebben boven het veelgebruikte ICG.

In **hoofdstuk 7** onderzochten we de mogelijkheid van LSCI om de doorbloeding van de darm en anastomose tijdens de operatie te beoordelen. De studie richtte zich op een zogenaamde 'red flag'-techniek om chirurgen te begeleiden bij het creëren van een anastomose met optimaal doorbloed weefsel. Met behulp van een varken als proefdier werden drie dunne darm-lissen met geleidelijk variërende perfusieniveaus gegenereerd en aan chirurgen getoond. We onderzochten of de chirurgen vervolgens in staat waren de verschillende mate van perfusie met LSCI te benoemen. De bevindingen toonden dat dit mogelijk was met behulp van LSCI-feedback. Bovendien keken we in de studie naar de invloed van LSCI op het

besluitvormingsproces met betrekking tot het maken van een anastomose. Op basis van de LSCI-beelden, besloten alle chirurgen geen anastomose te creëren. Een aanvullende enquête benadrukte de tevredenheid van de chirurgen met LSCI als een perfusiebeeldvormer.

Vervolgens, had **hoofdstuk 8** tot doel LSCI beter te kwantificeren in relatie tot darmperfusie beoordeling. Om dit te realiseren, onderzochten we of we een afkapwaarde voor laser speckle perfusion units (LSPU's) konden berekenen die een goed geperfundeerde darm weerspiegelde. Dit met als doel de chirurgen te voorzien van kwantitatieve gegevens voor klinische besluitvorming. Vier varkens werden in deze studie gebruikt. Een chirurg met LSCI ervaring, identificeerde ischemische en goed geperfundeerde gebieden, evenals overgangsgebieden, op basis van de kleurenkaart die door LSCI werd verstrekt. Als gouden standaard werden lokale capillaire en systemische lactaatniveaus gemeten om deze aan de LSPU's te correleren. We zagen dat de gemiddelde LSPU's significant afnamen in ischemische gebieden en overgangsgebieden, terwijl er geen significante verandering werd waargenomen in goed geperfundeerde gebieden gedurende een periode van twee uur. Veranderingen in LSPU's correleerden met veranderingen in lactaatniveaus in zowel ischemisch als goed geperfundeerd weefsel. We vonden een logaritmische curve correlatie R^2-waarde van 0.56 voor de correlatie tussen LSPU's en lokale capillaire lactaatniveaus. De afkapwaarde voor LSPU's werd bepaald op 69 AU met een sensitiviteit van 0.94 en specificiteit van 0.87 (Youden index 0.81), wat goed geperfundeerd weefsel aangeeft. Vervolgens werd een afkapwaarde van 3.8 mmol/L voor lactaat effectief aangegeven voor goed geperfundeerd weefsel, met een sensitiviteit van 0,97 en specificiteit van 1.00 (Youden index 0.97). Een post-hoc analyse naar de mate van overeenstemming tussen verschillende beoordelaars werd uitgevoerd om de opererende chirurg te vergelijken met een groep zonder en met LSCI ervaring. Een Kappa van 0.66 weerspiegelde de vergelijking tussen experts en de opererende chirurg, en een Kappa van 0.56 die tussen artsen zonder ervaring met LSCI en de chirurg. De vergelijking tussen de gehele observatorgroep en de chirurg toonde een Kappa van 0.52 (95% 0.44-0.61). Over het algemeen suggereerde deze studie dat LSCI veelbelovend is als een concurrent voor de huidige perfusie-visualisatietechnieken, maar verder onderzoek naar de realtime kwantificering van LSPU's en klinische toepasbaarheid is noodzakelijk.

NAADLEKKAGES VANUIT HET PATIËNTPERSPECTIEF

Hoewel 2-12% van de patiënten met een naadlekkage binnen 90 dagen na de initiële operatie kan overlijden, overleeft de meerderheid van de patiënten deze complicatie. Naadlekkage heeft een aanzienlijke invloed op het postoperatieve herstel, wat leidt tot ernstige morbiditeit en nieuwe interventies noodzakelijk maken. Daarom achtten we het belangrijk om de impact van deze complicatie zo goed mogelijk te begrijpen om de uitkomsten te verbeteren. Tot op heden was er tegenstrijdig bewijs beschikbaar over de impact op kwaliteit van leven na colorectale naadlekkage. Aangezien patiënten volledig geïnformeerd moeten zijn, niet alleen over de

directe chirurgische risico's, maar ook over de impact die chirurgische complicaties kunnen hebben op langere termijn, is het cruciaal om deze impact beter in kaart te brengen. Daarnaast moeten medische zorgverleners, om kwaliteit van leven en uitkomsten van hun patiënten te verbeteren, zich bewust zijn van de factoren die volgens patiënten deze kwaliteit beïnvloeden.

Hoofdstuk 9 creëerde op een systematische manier een overzicht van alle eerdere studies die de impact op kwaliteit van leven na een colorectale naadlekkage onderzochten. Dertien artikelen, met in totaal 4596 patiënten, werden samengevat in dit review, waarvan 566 patiënten een naadlekkage hadden. Er was een opvallende variabiliteit tussen de studies in termen van de gebruikte vragenlijsten en het tijdstip van afname. In totaal werden tien gevalideerde vragenlijsten gebruikt, waarvan er vier in meer dan één studie werden gebruikt; de European Organization for Research and Treatment of Cancer Quality of Life Questionnaire (EORTC QLQ) -C30 (Core) en -CR29 (kanker specifiek), de Short-Form 36 vragenlijst (SF-36) met zowel een fysieke component als een mentale component score, en de Fecal Incontinence QoL (FIQL) vragenlijst. Kwaliteit van leven werd geëvalueerd op verschillende tijdstippen, variërend van één maand tot 14 jaar na de operatie, waarbij de uitkomsten niet altijd werden vergeleken met een afname voor de operatie (baseline). Samenvattend, concludeerden we dat een naadlekkage na een oncologische colorectale resectie gepaard gaat met verminderde kwaliteit van leven, vooral tijdens de eerste zes maanden en zelfs tot één jaar na de operatie, met een variabele mate van daaropvolgende verbetering. De heterogeniteit in de geïncludeerde studies, benadrukt de noodzaak van verdere aandacht en dialoog met zowel patiënten als onderzoekers over de impact van naadlekkages en de belangrijke uitkomsten.

Vervolgens werden er in **hoofdstuk 10** patiënten geïnterviewd die een naadlekkage hebben meegemaakt om meer inzicht te krijgen in hun ervaringen, uitkomsten, kwaliteit van leven maar ook verbeterpunten voor de zorg. Patiënten die een naadlekkage ontwikkelden na colorectale chirurgie werden geïnterviewd met behulp van een semigestructureerde interviewgids. Alle interviews werden digitaal opgenomen, letterlijk getranscribeerd en gecodeerd. Vervolgens werd een thematische benadering gebruikt om belangrijke thema's te identificeren die van betekenis waren voor de geïnterviewde patiënten. Tien patiënten (60% mannelijk, mediane leeftijd van 53 [39 – 65]) uit drie verschillende continenten namen deel aan de interviews. Vier hoofdthema's kwamen uit de analyse van de interviews: (1) fysieke impact, (2) emotionele impact, (3) coping strategieën en (4) aandachtspunten tijdens de zorg. We merkten dat pijn door behandelingen een belangrijke factor was die de ervaringen van patiënten beïnvloedden, evenals revalidatieproblemen. Emotionele onderwerpen omvatten angst voor behandelingen door pijn, maar ook mentaal worstelen met het hebben van een stoma. Veel deelnemers namen een optimistische houding aan als coping strategie, dwongen zichzelf positief te blijven gedurende het diagnose- en behandelingsproces, en benadrukten het belang van een ondersteunend sociaal systeem. De uitkomsten van deze studie werden meegenomen en geïmplementeerd in het CoReAL project, zoals beschreven in hoofdstuk 3.

PREVENTIE VAN METACHRONE PERITONEALE METASTASEN NA COLORECTALE CHIRURGIE

In het laatste deel van dit proefschrift werd gezocht naar voorspellende biomarkers voor metachrone PM in colorectale tumoren. Omdat uitzaaiingen naar het buikvlies een grote uitdaging vormen vanwege hun agressiviteit en beperkte behandelingsopties, is het belangrijk om patiënten met een hoog risico vroegtijdig te identificeren. Specifieke biomarkers voor PM zouden clinici in staat kunnen stellen preventieve strategieën te onderzoeken, waardoor de behandeling van bepaalde risicogroepen kan worden verbeterd.

Het systematische review in **hoofdstuk 11** onderzocht eerdere verrichte genetische analyses naar biomarkers die peritoneale verspreiding voorspellen. Zeventien retrospectieve cohortstudies die potentiële biomarkers beschreven werden geanalyseerd. De DNA-analyses uit enkele studies (n = 10) toonden aan dat er potentieel een relatie was tussen *BRAF*-mutaties en PM. Hierbij werd vaak geen onderscheid gemaakt tussen synchrone (aanwezig tijdens het diagnosticeren van de darmkanker) en metachrone metastasen. Andere genen werden ook genoemd, maar deze waren meestal slechts in een enkele studie onderzocht. Uitgebreidere analyses leverde geen aanvullende ontdekkingen op, als ook niet voor RNA-uitkomsten. Er werd daarom geconcludeerd dat de onderzochte genen en de associatie met PM (vooral *BRAF* mutaties), niet betrouwbaar genoeg zijn als individuele biomarker voor klinische toepassing. We adviseerde daarom dat er toekomstig onderzoek noodzakelijk was in een homogene populatie, met gedetailleerde analyses.

Hoofdstuk 12 voerden wij zelf dergelijke analyse uit. We vergeleken genetische veranderingen in primaire colorectale tumoren van 10 patiënten zonder metastasen, met 20 patiënten met metachrone PM en 10 patiënten met metachrone levermetastasen (LM) om potentiële biomarkers voor metachrone PM te vinden. Deze retrospectieve analyse betrof 40 patiënten met T3 stadium darmkanker. Om een homogene populatie te waarborgen, werden patiënten met synchrone metastasen niet meegenomen in deze studie. Een uitgebreide genoomsequentie (Trusight Oncology (TSO) 500-analyse) werd uitgevoerd op de primaire formaline-gefixeerde paraffine-ingebedde tumormonsters, gericht op DNA-veranderingen in 523 genen en RNA-veranderingen in 55 genen. Twee patiënten werden uiteindelijk niet meegenomen in de analyse (LM = 1 en PM = 1), resulterend in een definitieve populatie van 38 patiënten. Tijdens de analyse bleek dat er sprake was van microsatellietinstabiliteit (MSI) in vier M0-tumoren en één PM-tumor. In de volledige onderzochte groep werden nieuwe genen geïdentificeerd die niet eerder waren beschreven in verband met metachrone PM's of metastasen in het algemeen, hoewel de klinische relevantie onbekend bleef vanwege de kleine steekproefgrootte. Opmerkelijk was wel dat *BRAF* p.V600E-mutaties uitsluitend aanwezig waren bij PM-patiënten met microsatellietstabiele (MSS) tumoren (37,5%, p = 0.010). Daarom raadde wij aan om deze genmutatie in de toekomst verder te onderzoeken in relatie tot metachrone PM.

Om te achterhalen of patiënten met een hoger risico op PM, al dan niet door middel van dergelijke *BRAF*-mutatie, baat zouden hebben bij een nieuwe chemo-geladen intraperitoneale hydrogel, werd in **hoofdstuk 13** zo'n gel onderzocht. Specifiek, werd de impact van deze hydrogeltoediening in een darm anastomose model onderzocht. Tweeënveertig gezonde ratten kregen een colorectale anastomose, met daaropvolgende intraperitoneale injecties. Zes dieren kregen middels deze injectie een zoutoplossing (placebo), 18 dieren kregen een niet-geladen hydrogel (lege gel), en 18 dieren kregen we met mitomycine (MMC)-geladen hydrogel. De dieren weren 7 dagen lang geobserveerd, waarna ze werden opgeofferd voor verdere analyse. Primaire uitkomsten die bij obductie werden bekeken waren adhesie- en naadlekkagescores. Verder werd er gekeken naar hoe sterk de colorectale naad was door deze op te pompen onder druk, en werd de naad histologisch bekeken. Tweeëntwintig ratten voltooiden het experiment (zoutoplossing: n = 6, niet-geladen hydrogel: n = 10, MMC-geladen hydrogel: n = 6) en werden meegenomen in de analyse. Er werd slechts een trend richting significantie waargenomen voor de naadlekkage score tussen ratten die zoutoplossing kregen en degenen die niet-geladen hydrogel kregen. Er werden geen significante verschillen gevonden voor alle andere uitkomsten. De belangrijkste reden dat 16 dieren het einde van het experiment niet haalden, was het waarnemen van intestinaal bloedverlies. Dit trad alleen op bij de dieren die een lege of geladen hydrogel kregen. Dus, hoewel de voorlopige resultaten suggereerden dat de MMC-geladen of niet-geleden hydrogel mogelijk geen grote invloed heeft op de naadgenezing, wijst het waargenomen intestinale bloedverlies bij een aanzienlijk aantal dieren toch op een interactie. Daarom bleek in het huidig onderzochte diermodel dat het toedienen van de gel niet veilig is. Concluderend lijkt het noodzakelijk om de hydrogel verder te optimaliseren en onderzoeken, voordat deze als potentiële preventieve gel kan worden overwogen.

CONCLUSIE EN TOEKOMSTPERSPECTIEVEN

Al vele generaties van klinische en experimentele onderzoekers hebben zich ingespannen om de resultaten voor patiënten na colorectale chirurgie te verbeteren. Ondanks de aanzienlijke inzichten die in de afgelopen decennia zijn verkregen, blijven naadlekkages een substantiële postoperatieve zorg voor een aanzienlijk aantal patiënten. Daarnaast brengt het identificeren van patiënten met risico op metachrone PM en het daaropvolgend afstemmen van gepersonaliseerde behandelingen ook veel uitdagingen met zich mee. Hoewel dit proefschrift misschien geen definitieve oplossing biedt om naadlekkages of metachrone PM volledig te elimineren, biedt dit proefschrift wel potentiele vooruitgang in de behandeling van deze patienten.

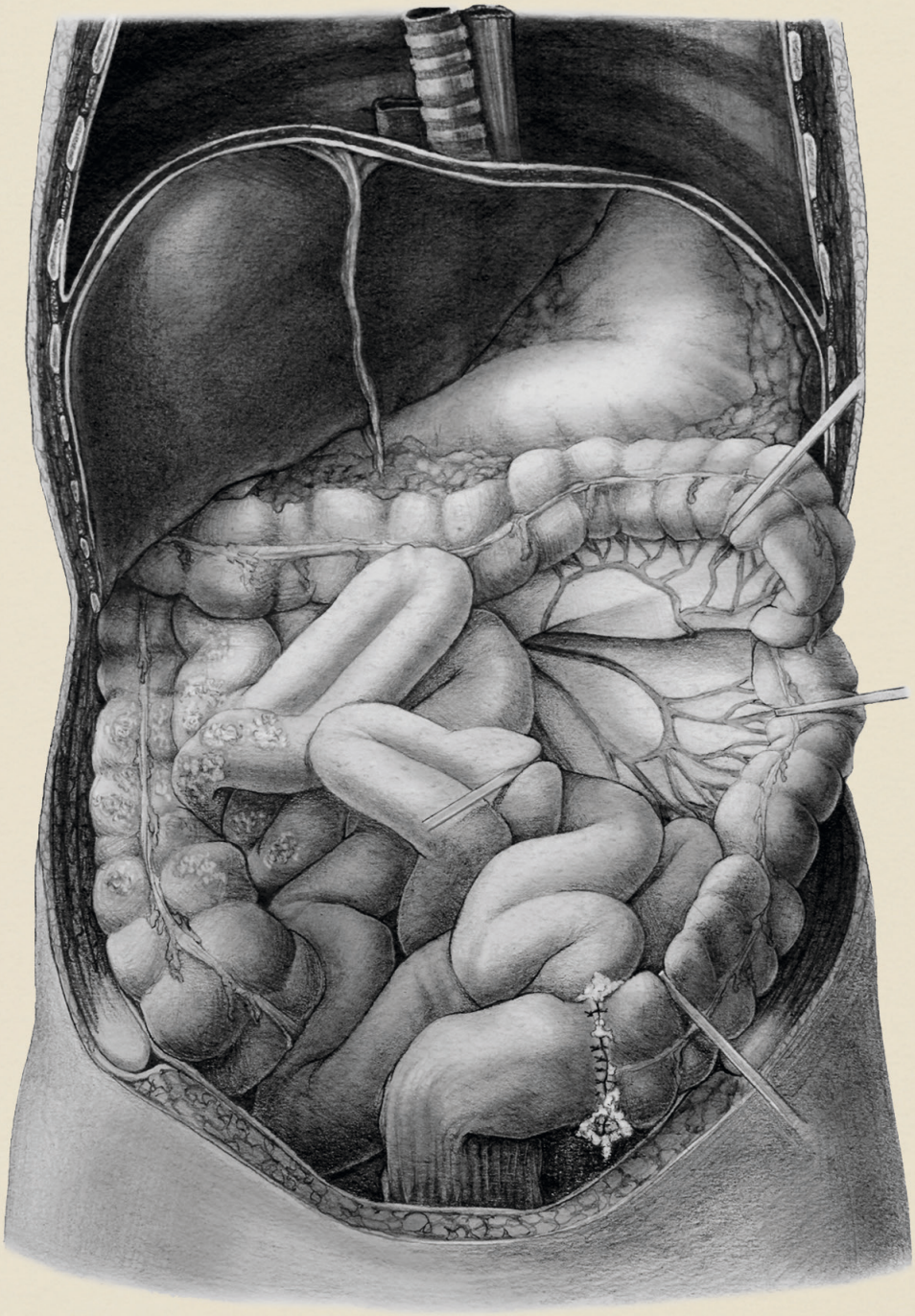
Na het uitvoeren van de onderzoeken in dit proefschrift, geloven wij dat verbeterde rapportage van naadlekkages ook de incidentie en impact ervan zal verminderen. Het voorgestelde CoReAL rapportagesysteem voor het standaardiseren van de rapportage

van lekkages na colorectale chirurgie is cruciaal om de uitkomsten voor patiënten te verbeteren. Dit rapportagesysteem zal niet alleen helpen om de betrouwbaarheid van onderzoeksbevindingen te vergroten, maar het zal ook vroege herkenning bevorderen en klinische besluitvorming begeleiden. Daarom zullen toekomstige stappen worden genomen om de wereldwijde implementatie van dit systeem te realiseren. De ontwikkeling en aanvullende validatie van onze voorgestelde CAL-RADS-score zullen de standaardisering van het beoordelen en rapporteren van lekkages in de diagnostische fase extra ondersteunen.

Dit proefschrift belicht het gebruik van NIRF of LSCI om darmperfusie te beoordelen. Naar onze verwachting zal de beoordeling van darmperfusie in het komende decennium toenemen en uiteindelijk standaardzorg worden bij de colorectale chirurgie. Grotere datasets voor zowel NIRF als LSCI moeten in de toekomst worden gebruikt om voorspelende modellen te ontwikkelen die in real-time het risico op bijvoorbeeld een naadlekkage per weefsellocatie kunnen weerspiegelen. Daarnaast moet de rol van MB hierbij ook verder worden onderzocht. Tevens kan MB ook een belangrijke rol spelen bij het verminderen van het aantal iatrogene ureterbeschadigingen. Camerasystemen zullen daarom moeten worden aangepast om meer verschillende golflengtes te visualiseren.

Aangezien de kwaliteit van leven van patiënten na een naadlekkage lager leek te zijn tijdens de eerste zes maanden na deze complicatie en deze impact zelfs tot een jaar merkbaar kon zijn, adviseren wij de kwaliteit van leven te beoordelen en rapporteren in het kader van naadlekkages. Hierbij lijkt het verstandig om de ICHOM-aanbevelingen te volgen en de EORTC QLQ-C30-tool te gebruiken om de algemene kwaliteit van leven vast te leggen en de -CR29 om darmkanker specifieke uitkomsten vast te leggen, voor de operatie, 6 maanden na de operatie en vervolgens jaarlijks tijdens de follow-up. Idealiter kan een gestandaardiseerde set patiëntgerichte uitkomstmaten worden ontwikkeld, waarbij zowel de fysieke als emotionele impact wordt meegenomen.

Ten slotte benadrukt dit proefschrift ook het belang van preventieve strategieën voor PM na colorectale resecties. We toonden aan dat meer aandacht moet worden besteed aan BRAF-gemuteerde tumoren met betrekking tot de ontwikkeling van metachrone PM na curatieve chirurgie. Toekomstig onderzoek moet zich richten op de impact van mutaties, onderzoek naar alternatieve biomarkers en minimaal invasieve preventieve strategieën, of het nu gaat om het optimaliseren van geneesmiddeltoedieningssystemen zoals de onderzochte hydrogel, of andere innovatieve behandelingen.



PART VI

APPENDICES

ACKNOWLEDGMENTS | DANKWOORD

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ABOUT THE AUTHOR

Curriculum Vitae

Danique Heuvelings was born on November 13th in Roosendaal, The Netherlands. After completing grammar school at the Sint-Jozef Instituut in Essen, Belgium, in 2014, she pursued a degree in Biomedical Sciences at the University of Antwerp. While enjoying her time in Antwerp, she successfully applied to medical school at Maastricht University and began her medical studies at the Faculty of Health, Medicine, and Life Sciences (FHML) in 2016.

Throughout her studies, Danique developed a strong interest in surgical specialties. During her third year of medical school, she joined the local board of the Dutch Surgical Society for Medical Students and continued her involvement on the national board during her Master's. She also worked as a research assistant at the Department of Surgery at Maastricht University Medical Centre (MUMC+) under the supervision of Prof. Dr. Nicole Bouvy. In her final year, she focused on surgery, completing an elective clinical rotation at the Surgery Department at Zuyderland Medical Center and a combined clinical and research rotation at MUMC+, again under Prof. Dr. Bouvy's supervision. After graduating from medical school in the summer of 2022, Danique continued her surgical research as a PhD candidate, which culminated in this thesis. Alongside her PhD work, Danique contributed to the organization of the annual Pelerin Arts-Assistenten Symposium and the first International Fluorescence Guided Surgery Congress. Currently, Danique works as a resident not in training at the department of surgery of MUMC+.