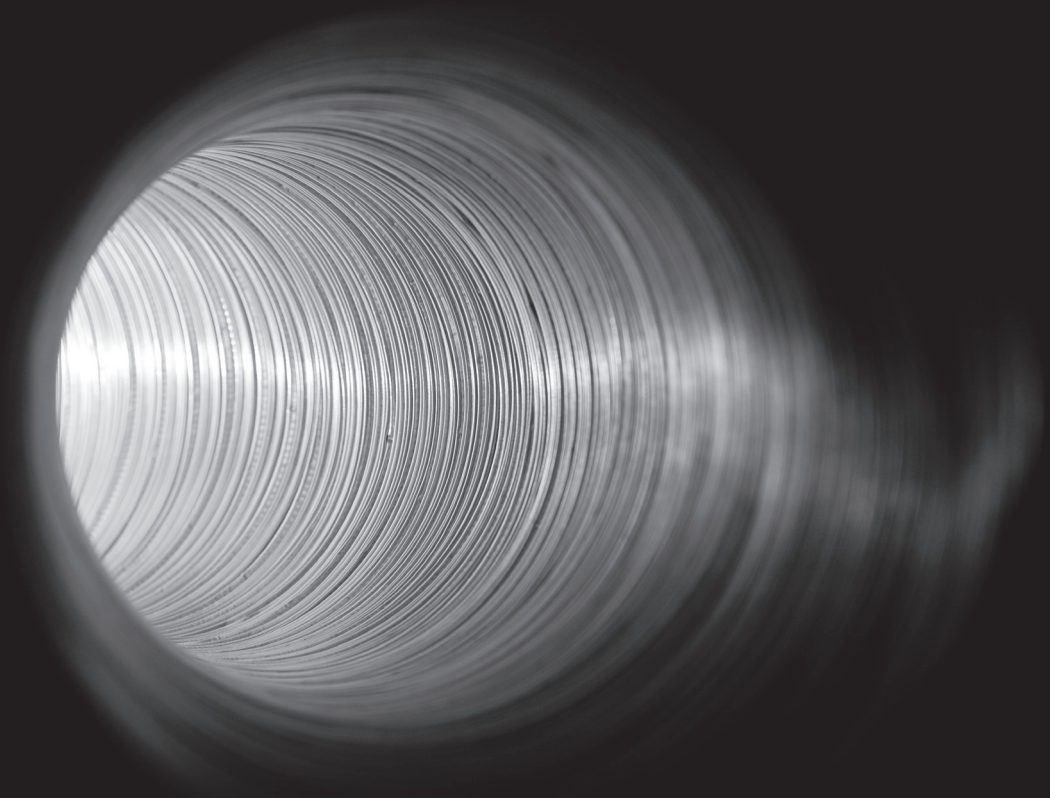
Diclofenac-related Leakage of Experimental Anastomoses



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1.
INTRODUCTION

Burden of anastomotic leakage

Worldwide, intestinal resection and anastomosis is required for a few million patients annually. The greatest number of patients undergoing this procedure have colorectal cancer, followed by those suffering from morbid obesity, drug-resistant inflammatory bowel diseases (e.g. Crohn's disease and ulcerative colitis) and diverticular disease [1–3]. Intestinal resections and anastomosis are also made in cases of ischaemia, perforation and obstruction of the gastro-intestinal tract. Intestinal anastomoses are also constructed during urological procedures, in particular bladder reconstruction surgery [4].

Anastomotic leakage (AL) is the main cause of major morbidity and mortality after intestinal surgery [5]. Leakage of intestinal content through a defect in the anastomosis and into the abdominal cavity can cause abscess, peritonitis, sepsis and death. It therefore requires rapid and appropriate intervention, such as percutaneous abscess drainage, reoperation, breakdown of the anastomosis, stoma construction and intensive care unit (ICU) admission [6–9]. A stoma constructed after leakage is often permanent, and if it is reversed, the operation to do so is associated with a risk of complications and functional decline [10, 11]. More readmissions, longer hospital and ICU stays, and additional interventions double the healthcare costs for patients with AL [12, 13]. In spite of rigorous efforts to manage leakage in the past decade, mortality rates remain high, varying from 2% to 37% [7, 8, 13]. Quality of life following AL is significantly reduced and remains lower even after 10 years compared to patients without a leak (Daams et al., in press). Finally, leakage has been associated with an increase in local and distant cancer recurrence and lower survival, possibly as a result of a higher proportion of cancelled or delayed administration of adjuvant chemotherapy [14–17].

Despite advances in surgical techniques and improvements in perioperative care, the AL rate has not declined over the past decades, and leakage still occurs in 3–14% of cases [5, 13, 18, 19]. The incidence of leakage is relatively low after small-bowel anastomoses (0–6%), but high after both left and right hemicolectomies (6–11%), and highest for low rectal anastomoses (8–14%) [8, 20–22]. Incidences may even be higher since substantial underreporting has been demonstrated, causing an underestimation of the true effects of leakage on patient outcomes [23]. The protective loop ileostomy or transversostomy that is routinely constructed during low rectal surgery reduces the morbidity of leakage and possibly its incidence, but it is in turn associated with stoma-related morbidity [24–26]. There is little evidence that surgical technique (i.e. single- or double-layer suturing; stapled or handsewn) significantly affects leak rates, though stapling may be beneficial in ileocolic anastomoses [27–29].

Combined with the devastating clinical consequences, prolonged hospital stay and doubled healthcare costs, the high incidence of AL imposes a huge burden upon the individual patient and society [13, 30].

Risk factors for anastomotic leakage

Multiple risk factors have been identified from a large number of studies (see Table 1) [31–34]. This knowledge, however, has not led to a satisfying reduction in leak rates, for several reasons. First, evidence about risk factors is often ambiguous or only of moderate strength, as data are primarily derived from retrospective analyses and experimental studies of questionable translational value [13, 35]. Second, leakage also occurs in the absence of known risk factors [5]. This indicates a gap in our knowledge of risk factors. The altered gut microbiome before and after surgery, for example, seems a factor which potentially constitutes a large risk but has seldom been investigated [36, 37]. Third, many risk factors, such as male sex and low tumour site, cannot be altered prior to surgery [38, 39]. An important factor is the lack of comprehensive knowledge of underlying pathophysiologic mechanisms, such as detailed knowledge of normal and disturbed anastomotic healing pathways. This hampers the translation from the phase of risk factor identification to the phase of appropriate (preventive) treatment recommendations. A good example of such a risk factor is the perioperative use of nonsteroidal anti-inflammatory drugs (NSAIDs).

Table 1 Potential risk factors for anastomotic leakage.

Patient	Perioperative care	Surgery	Other
Factors not or difficult to modulate			
Male sex	Blood transfusion	Emergency surgery	Low tumor site
(High comorbidity)	(Vasopressor use)	(Intraoperative events)	(Large tumor size)
(Diabetes)		(Long operation time)	
(Age – young and old)		(High blood loss)	
(IBD)			
(Previous surgery)			
(Ongoing anticoagulants)			
Factors possible to modulate			
Corticosteroid use	NSAID use	Multiple staple firings	Radiotherapy
Nutritional status	(Excessive fluid therapy)	(Open surgery)	(Chemotherapy)
(Alcohol)	(Low body temperature)	(Surgeon experience)	(Hospital beds)
(Smoking)	(Low blood pressure)		
(Obesity)	(Hyperglycemia)		

Text in parentheses indicate that evidence is equivocal or of low quality. IBD, inflammatory bowel disease; NSAID, non-steroidal anti-inflammatory drugs.

Non-steroidal anti-inflammatory drugs and anastomotic leakage

NSAIDs are frequently used as perioperative analgesics and are recommended in guidelines on perioperative care and 'fast-track' surgery [40]. In the past decade, several experimental studies have assessed their safety in visceral surgery (see Table 2) [41–44]. In 2006, de Hingh

et al. demonstrated that celecoxib (a cyclooxygenase-2 [COX-2] inhibitor) causes leakage of ileal anastomoses in rats [43]. In subsequent rat studies, both carprofen and diclofenac, and to a lesser extent naproxen, also caused leakage [45–47]. The use of NSAIDs has been associated as a risk factor for AL in several clinical studies (see Table 2). Rosenberg et al. found an alarming increase in leak rates from 3.9% to 20.5% following introduction of diclofenac in protocols on perioperative care [48]. Similar increases were seen after the introduction of celecoxib (15.1% leakage compared with 1.5–3.3% in non-celecoxib cohorts) [49]. In 2012, two clinical series related diclofenac and ibuprofen use to increased leak rates [20, 50]. Reviews of clinical and experimental studies similarly revealed higher leak rates in NSAID groups, although not all were statistically significant [51, 52]. One study reported no evident risk of leakage after ketorolac use, but another found a higher risk of leakage specifically in the ketorolac group [53, 54].

Altogether, evidence regarding perioperative NSAID use is worrisome and continues to increase [55]. Nevertheless, further research is needed in this field because most evidence has been derived from experimental and retrospective clinical studies, and a few studies reported no adverse effects [56–58]. In these studies selection bias may have affected outcomes; for example, those suffering from leakage are more likely to use analgesics. Furthermore, the relevance of NSAID subtype, dose, time of administration and the location of anastomosis at risk is still unclear. It is also not known to what extent NSAIDs increase the risk of leakage and whether this outweighs the clinical benefits of NSAIDs, such as reduced opioid use [59]. Most fundamentally, it is not known through which mechanisms NSAIDs cause leakage. An understanding of the underlying pathophysiology is needed to support and specify clinical treatment recommendations.

Table 2 Studies on the influence of NSAIDs on anastomotic healing.

Author, year	Study type (number of animals/patients)	NSAID type	Summary of results
		Experimental	
Mastboom, 1991 (60)	Rat ileum/colon model (n=100)	celecoxib, piroxicam, aspirine, indomethacin	Higher peritonitis + mortality rates in NSAID groups but no evident increase in AL.
De Sousa, 1991 (44)	Rabbit ileum model (n=48)	diclofenac	AL ileum: 21% in diclofenac group vs 0% in controls, p<0.05
Cahill, 2004 (61)	Rat colon model (n=40)	rofecoxib	AL: 5/20 in rofecoxib group vs 0/20 in controls, p=0.048.
De Hingh, 2006 (43)	Rat ileum/colon model (n=95)	celecoxib	AL ileum: 6/12, 5/12, 7/12b in celecoxib groups; 0/12 in controls (p<0.05). No AL in distal colon.
Inan, 2006 (62)	Rat colon model (n=36)	diclofenac	No AL. Bursting pressure lower after diclofenac.
Esen, 2008 (63)	Rat colon model (n=48)	diclofenac, metamizol, tenoxicam	No AL. Bursting pressure lower after diclofenac at day 7.

Neuss, 2009 (64)	Rabbit colon model (n=80)	resveratrol, metamizol, valdecoxib	AL: valdecoxib 0/20, metamizol 3/20, resveratrol 1/20, Control 0/20 (p>0.05)
Benjamin, 2010 (65)	Rat colon model (n=48)	etodolac (with propanolol)	No leak occurred
Klein, 2010 (66)	Rat colon model (n=32)	diclofenac	No AL data. No difference in breaking strength.
Klein, 2011 (67)	Rat colon model (n=60)	diclofenac	No AL data. Lower collagen content after diclofenac
Van der Vijver, 2012 (45)	Rat ileum/colon model	carprofen	AL ileum: 6/10, 8/10 and 12/15 in carprofena groups vs 0 in control groups (p<0.001). No AL in distal colon.
Van der Vijver, 2013 (46)	Rat ileum/colon model	diclofenac, naproxen	AL ileum: Ctrl 0/28; diclofenaca 19/28 (p<0.001); Naproxen 4/28. No AL in distal colon.
Reisinger, 2017 (68)	Mouse colon model	diclofenac	AL: Ctrl 25%, diclofenac 100% p<0.001 COX2 knockout 92%
		Clinical	
Rosenberg, 2007 (48)	Retrospective cohort, before/after design (n=230)	diclofenac	AL: 20.5% in diclofenac group, 3.9% before diclofenac cohort, p<0.001.
Holte, 2009 (49)	Retrospective cohort (n=502)	celecoxib	AL: 15.1% in period of celecoxib use vs 3.3% before and 1.5% after this period (p<0.001)
Klein, 2009	Retrospective cohort (n=75)	diclofenac	AL: 7/33 in diclofenac group, 1/42 in non-diclofenac (p=0.018)
Klein, 2012 (50)	Retrospective cohort (n=2,756)	diclofenac, ibuprofen. At least 2 days within 7 days postopera-tively	AL: diclofenac 12.8% vs Ibuprofen 8.2% vs controls 5.1% (p<0.001). OR after diclofenac 7.2(3.8-13.4; p<0.001).
Gorissen, 2012 (20)	Retrospective cohort (n=795)	non-selective (diclofenac and others) vs selective (meloxicam, celecoxib), any use within 5 days postoperatively	AL: any NSAIDs 13.2% vs no NSAIDs 7.6%, OR 1.86 (1.16-2.96) (p=0.010). Strongest effect in non-selective NSAID group. Longer duration of NSAID use associated with higher leak rate.
Burton, 2013 (51)	Review of RCT's with AL as 2nd outcome (n=562)	celecoxib, diclofenac, flurbiprofen ketorolac, valdecoxib	AL: any NSAID 5.2% vs no NSAID 2.4%, OR 2.16 (0.85-5.53; p=0.11)
Bhangu, 2014 (52)	Review of human (n=4464) + 12 animal studies	any	OR for AL 2.14 (p<0.001) after any NSAID; 2.37 (p<0.001) after non-selective, and 2.32 (p=0.170) after selective NSAID
STARSurg coll, 2014	Multicenter snapshot study (n=1503)	ibuprofen (>80%), diclofenac and other	AL (2nd outcome): NSAID 6.3%, no NSAID 4.9% (p=0.384)

Saleh, 2014 (53)	Restrospective cohort (n=731)	ketorolac	AL: ketorolac 3.4%, no NSAID 3.2%, OR 1.06 (0.43-2.62; p=0.886)
Subendran, 2014 (54)	Restrospective cohort (n=262)	ketorolac (>90%), ibuprofen, celecoxib, ketoprofen.	OR for AL: any NSAID OR 1.81 (0.98–3.37; p=0.06. ketorolac OR 2.09 (1.12-3.89; p=0.021)
Hakkarainen, 2015 (55)	Restrospective cohort (n=9,864)	ibuprofen, naproxen, ketorolac, caldolor, celecoxib, diclofenac	Adjusted OR 1.24 (1.01-1.56; P = .04). In non-elective colorectal surgery, AL: 12.3%in NSAID group vs 8.3%in non-NSAID group (OR 1.70 (1.11-2.68; P = .01).
Bakker, 2016 (69)	Restrospective cohort (n=856)	diclofenac, namebutone, ibuprofen	AL: NSAID 9.2 vs no NSAID 5.3%, p = 0.038. Diclofenac, for colonic resections, 11.8 vs. 6.0%, p = 0.016; for rectal resections, 13.1 vs. 0%, p = 0.017.
STARSurg coll, 2017 (70)	Multicenter snapshot study (n=1951)	mainly ibuprofen	AL (2nd outcome): NSAID 3.7%, no NSAID 3.8% (p=0.877)
Kverneng, 2017 (71)	Retrospective database (n=1495)	ibuprofen (70%), namebutone, diclofenac	AL NSAID 11%, no NSAID 14%. COX2-selective 17% (NS)
Haddad, 2017 (72)	Posthoc analysis of prospective cohort (n=533)		AL NSAID 14%, no NSAID 11% (p=0.26). NSAIDs in colonic anastomosis 30%. (p=0.03)
AL – anastomotic leakage, NS – non-significant, NSAID – nonsteroidal anti-inflammatory drug, COX2 – cyclooxygenase-2.			
Colors represent the overall risk of anastomotic complications in NSAID groups:		green – no increased risk	
		orange – non significant risk or only in selective groups	
		purple – increased risk	

Aim of this thesis

With the goal of reducing the burden of anastomotic leakage, this thesis aims to increase knowledge of both normal and disturbed bowel anastomotic healing, and more specifically to understand how the NSAID diclofenac disturbs this healing process.

Outline of this thesis

In order to take measures to prevent anastomotic healing disturbances and leakage, it is important to understand normal healing physiology. Chapter 2 presents a comprehensive overview of the cellular and biochemical processes relevant during anastomotic healing, based on a literature review in which knowledge from anastomotic healing studies was combined with insights from gastrointestinal, immunological, oncological and pharmacological research.

Most fundamental knowledge on anastomotic healing is derived from animal experiments. Thus far it has not been possible to adequately and sequentially study anastomoses in humans without the need for invasive or high-risk techniques. For this reason, prospective

clinical trials that study new techniques or risk factors are rare [73, 74]. Other strategies to study anastomotic healing, such as cell culture and migration assays, are rarely used and are likely to have little translational value. The process of anastomotic healing involves a versatile and multilevel system and is considered too complicated to be mimicked in vitro. Consequently, increasing knowledge on anastomotic healing depends on retrospective analyses of clinical data and prospective investigations in animal models.

Despite numerous animal studies, the contribution of animal research to clinical knowledge and modulation of intestinal AL is unknown. To continue performing animal research on anastomotic healing, and in view of the current societal call to 'replace, reduce and refine' animal experiments (the 3R principle), it is relevant to critically evaluate the animal research on this topic. In Chapter 3 the quality of animal research on AL is systematically reviewed by checking items from the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. The chapter also provides an overview of the main topics studied regarding anastomotic healing, with the aim of guiding future animal experiments and helping other research groups avoid unnecessary repetition of studies. We evaluate the various animal models for their differences and address the question of what improvements in models are needed.

In **Chapters 4–8** we aim to increase the understanding of how NSAIDs affect anastomotic healing by conducting randomised controlled blinded animal experiments. In each chapter we use the same validated experimental model in male Wistar rats [46, 47]. The model involves the creation of a complete small- and/or large-bowel anastomosis on day 0 and sacrifice on days 3–5 to assess anastomotic complications, the strength of the anastomosis and the anastomotic histopathology [46, 47]. Further details of the model are described in each chapter.

The effect of diclofenac is subjected to particular investigation because it is an NSAID used frequently in clinical practice and has been associated with leakage in previous human and rat studies. By choosing diclofenac we attempt to increase the translational value of the animal experiments. We study different routes of administration of diclofenac to understand the mechanisms by which the drug exerts its detrimental effects. More specifically, we study the pharmacologic action, the biliary excretion pattern, the degradation products of the drug within the gut and the potential of NSAIDs to alter gut microbiota composition.

Chapter 4 examines whether diclofenac affects anastomotic leak rate, leak severity and anastomotic strength differently depending on the location of the anastomosis in the colon. This may shed light on the relevance of faecal content (e.g. liquidity, microflora and bile acids) compared to the tissue morphology and the pharmacologic action of diclofenac in inducing leakage.

In **Chapter 5** we investigate whether the healing of an anastomosis can be improved by abrading the serosal surface of bowel ends that are invertedly anastomosed, based on the concept that serosal damage evokes an inflammatory response leading to scar tissue strongly connecting both bowel ends. We also study whether the expected increased inflammatory response can compensate for the anti-inflammatory effect of diclofenac. Outcome measures are anastomotic leak rate, leak severity, bursting pressure and breaking strength.

In **Chapter 6** the effects of bile and bile composition on intestinal anastomosis healing are determined. Diclofenac is largely excreted through bile and may thereby increase the toxicity of the bile and gut content. Based on reports showing the relevance of biliary metabolites in NSAID-associated enteropathy, the effects on anastomotic healing are studied by using a bile replacement technique involving the cannulation of bile duct and duodenum [75–78]. The type, concentration and kinetics of diclofenac metabolites are measured in bile and plasma, and differences in anastomotic healing are determined.

Chapter 7 investigates the relevance of the biliary metabolite diclofenac-acyl-glucuronide. Reactivation of this metabolite has been shown to be toxic and to cause enteropathy in rodents [77]. The relevance for intestinal anastomoses is studied by administration of Inh1 to inhibit the reactivation process by bacteria in the gut. Anastomotic leak rate, leak severity and anastomotic strength are compared between rats with and without Inh1, and the effect of Inh1 on diclofenac plasma levels is determined.

In **Chapter 8** the microbiota composition of different bowel segments is analysed to determine the influence of both anastomotic surgery and NSAID treatment, and to explore whether alterations in microbiota can explain the differences in leak rates. Faecal samples collected from the anastomotic site are analysed by sequencing of the 16S rRNA V4 gene region. Taxonomic composition, alpha diversity, beta diversity and microbial taxa are compared between rats with and without NSAIDs, before and after surgery, and among intestinal segments.

Chapters 9 and 10 present a general discussion, with future perspectives and a summary.

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2.

INTESTINAL ANASTOMOTIC HEALING – CELLULAR REACTIONS AND INFLAMMATORY MEDIATORS

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ABBREVIATIONS

AD – adenosine diphosphate
ASA – acetylsalicylic acid
CAM – cell adhesion molecules
CC – chemokine of CC family
CCL – CC-ligand
CD40L – CD40 ligand
CD40R – CD40 receptor
CGRP – calcitonin gene related peptide
COX – cyclooxygenase
CSF – colony stimulating factor
CTAP – connective tissue activating peptide
CXC – cysteine-aminoacid-cysteine type chemokine
ECM – extracellular matrix
EGF – epidermal growth factor
ELAM - endothelial-leukocyte adhesion molecule
EPO – erythropoietin
FAK – focal adhesion kinase
FCS – fetal calve serum
FGF – fibroblast growth factor
GH – growth hormone
GM – granulocyte macrophage
GP – glycoprotein
HB – heparin binding
HGF – hepatocyte growth factor
HMG – high mobility group
HSP – heat shock protein
ICAM - intercellular adhesion molecules
IEC – intestinal epithelial cell
IFNγ – interferon γ
IGF – insulin like growth factor
IL - interleukin
I-R – ischemia reperfusion
ITF – intestinal trefoil factor
ITGAM - integrin α M
KGF – keratinocyte growth factor
LPS – lipopolysaccharide
MBR – mannose binding receptor
MC – mast cell
MMP – matrix metalloproteinase
NADPH - nicotinamide adenine dinucleotide phosphate
NAP - neutrophil activating peptide
NGF – nerve growth factor
NO – nitric oxide
PAF – platelet activating factor
PAI – platelet aggregation inhibitor
PECAM – platelet-endothelial cell adhesion molecule
PEPI – pro-epithelin
PF – platelet factor
PG – prostaglandin
PRP – platelet rich plasma
PTK2 – FAK, focal adhesion kinase
RNI – reactive nitrogen intermediates
ROS – reactive oxygen species
SLPI – secretory leukocyte protease inhibitor
SMC – smooth muscle cells
SP – substance P
TER – transepithelial resistance
TGF – transforming growth factor
TF – tissue factor
TL – toll ligands
TLR – toll like receptor
TNF – tumor necrosis factor
TRAP – TNF related activation protein
TXA – thromboxane
VCAM - vascular cell adhesion molecule
VEGF – vascular endothelial growth factor
vWF – Von Willebrand factor

2.1 Introduction

Cellular reactions are the essence of tissue repair. Understanding of these reactions is fundamental to improve anastomotic healing. Research has identified the cells most important in anastomotic healing. However, their exact role and the involved biochemical processes are complex and less understood. Cytokine studies on healing of intestinal anastomoses are relatively scarce. Related knowledge derives from studies on healing of skin, bone, peritoneum and intestinal mucosa. Since the healing process of the intestinal wall follows the same basic phases as wound healing in other human tissues, main principles seem applicable to anastomotic healing, although differences are encountered on a cellular level[1, 2]. Also specific cellular architecture and physiological factors, such as peristalsis and intraluminal microbiome, make the intestine a unique organ.

The growing amount of knowledge on molecular mechanisms of cellular function has triggered physicians, pharmacists and scientists to modulate cellular processes in an attempt to enhance tissue repair. This is mainly done by using growth factors, such as 'transforming growth factor-β' (TGF-β), 'vascular endothelial growth factor' (VEGF)-A, 'epidermal growth factor' (EGF), 'insuline like growth factor' (IGF), 'fibroblast growth factors' (FGF)) and hormones ('growth hormone' (GH), erythropoietin (EPO)). Unfortunately many factors have not made their way into clinical practice, which may be due to the fact that each factor is part of a versatile and multi-level system in which simple and unilateral causality is rare. In this chapter we review current insights in cellular function and tissue regeneration and the modulation hereof regarding the healing of an intestinal anastomosis.

2.2 Phases in intestinal healing

As in other human tissues, intestinal anastomotic healing starts with the hemostatic and inflammatory phase. These precede the proliferative phase which in turn is followed by a long remodeling phase. All are descriptive names for overlapping activities, rather than confined time periods. Platelets, mast cells, granulocytes and macrophages are the main initiators of the inflammatory response and act primarily within this phase. They are the response of the body to deal with harmful stimuli, such as damaged cells and the pathogen rich content of the bowel lumen. Gradually proliferative activity increases as macrophages start to stimulate and assist smooth muscle cells and fibroblasts to restore local architecture of the intestinal wall. This requires the formation of a firm collagen network to connect the two bowel parts together and a functioning vascular and lymphatic system to provide nutrient transport. Mucosal repair is achieved by epithelial cells on the intraluminal side, and serosal sealing by mesothelial cells on the peritoneal side. Complete restoration of anatomical structures takes longer and is achieved in the remodeling phase.

2.3 Inflammation

Inflammation is the response of the body to tissue damage and infection, in order to protect it from injurious agents and to facilitate healing. It includes a large range of integrated processes and involves the vascular, immunologic and neurologic systems.

2.3.1 Initiation of inflammatory response

The inflammatory response starts immediately after incision and entry in the abdominal cavity, thus even before the intestinal wall has been manipulated. Abdominal entry elicits a release of inflammatory mediators into the peritoneal fluid [3, 4]. This response is the first to affect the subsequent healing process and an example of why tissue repair is not only a local or isolated process.

Incision of the intestinal wall means a disruption of normal tissue and triggers inflammation by several simultaneous pathways, here divided into four groups (see Fig. 1). Tissue damage is inevitably associated with damage of capillaries. The following coagulation cascade is the first important initiator of subsequent inflammatory reactions. Second, damaged cells expose intracellular proteins (e.g. 'heat-shock proteins'(HSP), and mitochondrial proteins[5, 6] and 'high mobility group 1'(HMGB1)[7]).Third, stimulated nerve cells release a number of peptides (e.g. 'calcitonin gene related protein'(CGRP) and substance P (SP) [8]). Finally, disruption of the mucosal barrier results in exposure to microbes which trigger innate and adaptive immune responses.

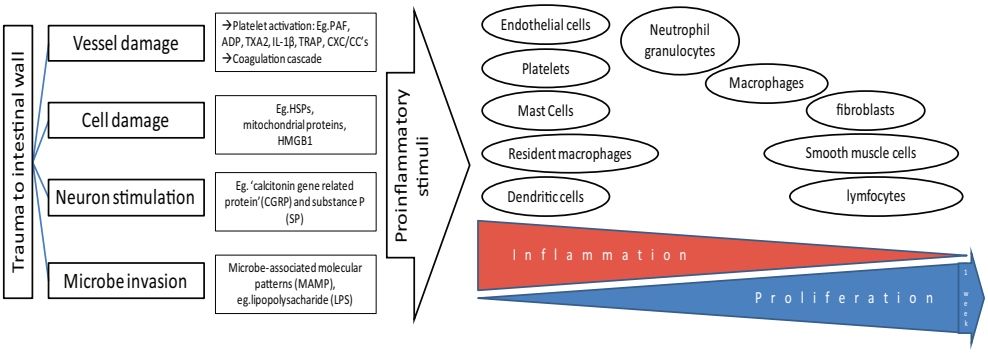


Figure 1 Schematic overview of inflammatory stimuli and cellular response.

2.3.2 Platelets

Vessel damage exposes the subendothelial collagen layer. Contact with exposed tissue factor (TF) protein in this layer causes adhesion and activation of passing platelets. Platelets are pivotal in primary hemostasis, but simultaneously also act as important initiators of the inflammatory process[9]. They synthesize many different cytokines and release and modify signaling molecules [9, 10]. By these actions platelets contribute to tissue repair.

Hemostasis and inflammatory signals

Primary hemostasis is achieved by adhesion of platelets to the different proteins of the vascular wall (e.g. collagen, fibronectin and laminin) and aggregation with other platelets by glycoprotein-1a (GP1a) and Von Willebrand Factor (vWF)[11]. The release of 'platelet activating factor' (PAF), 'adenosine diphosphate' (ADP), serotonin and thromboxane A2 (TXA2) attracts and activates other platelets. Activated platelets secrete a number of coagulation factors (V, XI, and XIII) and fibrinogen, which contribute to the completion of the coagulation cascade.

The release of PAF and TXA2 also contributes to the inflammatory activity of platelets, but more important is the release of the inflammatory cytokine Interleukin 1β (IL-1 β) [12-14]. An example of the many effects of IL-1β is the upregulation of the pro-inflammatory cyclo-oxygenase-2 (COX-2) expression in monocytes when these cells bind to activated platelets [15]. Other inflammatory mediators of the platelet secretome include 'TNF related activation protein' (TRAP), CD40L, platelet factor 4 (PF4) and a number of other CXC and CC chemokines[10]. All directly or indirectly attract and signal other cells, like neutrophil granulocytes, monocytes or lymphocytes [9, 16-18].

Proliferative activity

The influence of platelets reaches beyond the 'inflammatory phase'. Apart from the 'platelet derived growth factor'(PDGF), other growth factors like 'fibroblast growth factor-2'(FGF2), 'transforming growth factor α and β'(TGF-α/β), 'vascular endothelial growth factor' (VEGF), 'epidermal growth factor' (EGF), insulin-like growth factor (IGF-I) are found in the platelet secretome and contribute to proliferative actions like angiogenesis and collagen synthesis[13, 19-21].

Potential clinical relevance

Platelets contribute to tissue repair in many ways. Although unknown to what extent, intervening in platelet activity could influence the process of anastomotic healing. Many commonly used drugs in clinical practice influence platelet function such as platelet aggregation inhibitors and COX-inhibitors. Acetylsalicylic acid (ASA) is an inhibitor that primarily works via COX-1 inhibition. In platelets COX-1 is responsible for converting arachidonic acid to prostaglandin G2 and H2, which are precursors for TXA2. Thus, inhibition of COX-1 in platelets results in reduced TXA2 levels[11]. Hereby aggregation and activation of other platelets are prevented which in turn reduces inflammatory response and may impair wound healing. Indeed many studies reported impaired healing of multiple tissues (skin, bone, intestinal mucosa) caused by ASA in animals, but this effect has not drawn sufficient attention in clinical practice. NSAIDs with selective COX-2 inhibitory effect have been associated with anastomotic leakage in animal experiments and retrospective human studies [22-26]. From these experiments the relative contribution of disturbed platelet function on anastomotic healing, in comparison with other possible detrimental effects of COX-2 inhibition, is not known.

Paradoxically platelet inhibition has also been proposed as beneficial for wound healing, hypothesizing that decreased platelet aggregation and clotting improve capillary blood flow. Prostacyclin (also a product of COX-1 en 2) is secreted by endothelial cells and is a natural inhibitor of platelet aggregation. Analogs of prostacyclin (such as iloprost), have shown to improve anastomotic healing in rats that were subjected to ischemia, steroids or chemotherapy [27-30]. No publication was found reporting on the effects of ASA on anastomotic healing.

In contrast to improving healing by platelet inhibition is the strategy to apply platelet rich plasma as a means to deliver a relatively balanced cocktail rich of growth factors to a site of tissue repair. Various tissues have been subjected to this seemingly safe treatment, with

success in some experimental rat and porcine intestinal anastomosis studies [31-34]. No clinical studies have been started to confirm its beneficial effects.

2.3.3 Mast cells

Mast cells (MC's) belong to the earliest actors in the inflammatory phase as most are already at the site in the peritoneal cavity[35]. MC's are known to play a role in cutaneous wound healing by generating inflammatory mediators that cause vasodilatation, vascular permeability and leukocyte infiltration (heparin, histamine, tryptase, chymase, serotonin, TNFα, C3a, C5a, leukotriene(LT)-B4, LTC4, PAF), and proliferative mediators that induce neoangiogenesis, fibrinogenesis and re-epitheliazation (VEGF, FGF2, PDGF, TGFβ, nerve growth factor(NGF), interleukin(IL)-4, IL8)[36].

Potential clinical relevance

Mast cells have proved their importance in surviving bacterial peritonitis in a mast-cell-deficient mouse model, but were rarely studied in the context of intestinal anastomoses [37, 38]. In one rat study, ketotifen, a suppressor of mast cell function, decreased the strength of both normal and ischemic colonic anastomoses [39]. A promoter of mast cell degranulation (compound 48/80) increased anastomotic strength in this same study [39]. In contrast, pentoxifylline, a suppressor of TNFα release from monocytes and mast cells, protected intestinal anastomoses in situations in which excessive inflammation occurs, such as ischemia reperfusion injury and obstructive jaundice[40, 41]. Other actions of the drug, such as increasing capillary blood flow, may be responsible for this favorable finding.

2.3.4 Neutrophil granulocytes

In the first 24-48 hours the cellular influx is dominated by neutrophil granulocytes [42]. Their involvement in the healing process can be categorized into two major activities: the degradation and phagocytosis of cell fragments and microbes, and the signaling of other cells (e.g. other neutrophils, monocytes and lymphocytes). Several studies have shown the relevance of a balanced neutrophil action in healing. When neutrophil infiltration or activity is too abundant or prolonged, anastomotic strength appears to be decreased [43-46]. Ischemia reperfusion injury resulting in decreased anastomotic strength was associated with an 18-fold increase of neutrophil infiltration[47]. The same accounts for foreign body reactions in which neutrophil and lymphocyte activity persist[48]. On the contrary, a reduced neutrophil number or activity due to cytostatic or immunosuppressive agents was also associated with impaired anastomotic healing[49].

Neutrophil activity, measured by myeloperoxidase (MPO) is very frequently used to assess anastomotic healing, or as general measure for inflammation during healing [50-55]. However, it is not clear how to interpret MPO values or changes with respect to anastomotic healing [53].

Extravasation and infiltration

Extravasation of neutrophil granulocytes is partly a matter of efflux from leaking capillaries. After hemostasis there is a coordinated cascade of events[56]. Briefly, the capturing by and rolling along the endothelium are primarily facilitated by E-, L- and P-selectins found on activated endothelial cells and platelets[57]. Neutrophil activation is done by multiple chemokines, also deriving from platelets and resident inflammatory cells (e.g. CCL5, CXCL4+5). Next, a firm steady adhesion to the endothelium is achieved by integrin β 1 and 2 , Intercellular Adhesion Molecules (ICAMs) and Vascular Cell Adhesion Molecule-1 (VCAM-1)[58]. Finally, transmigration through the vessel wall relies on the aforementioned integrins as well as 'integrin α M' (ITGAM), 'platelet-endothelial cell adhesion molecule-1' (PECAM-1), ICAM1 [59](reviewed by ref[56, 60, 61]. The inactivation of any of these adhesion molecules, such as in steroid treatment (reduction of ELAM and ICAM1), reduces neutrophil infiltration, thus impairing host defense and healing [56, 62-64]. Also the interaction with platelets underlines how intervening in platelet function may alter subsequent steps in the healing process. 'Connective tissue activating peptide-3' (CTAP-3), which is secreted by platelets, is converted by neutrophils to 'neutrophil activating peptide-2'(NAP-2) to attract other neutrophils in an exponential increase[65].

Other early attractors of neutrophils include a broad spectrum of cytokines, cellular proteins and bacterial components (see fig.1). Bacterial components for example, cause a short lasting upregulation of the complement receptor C5a on neutrophils, which also contributes to early neutrophil influx[65]. The complexity of pathways allowing neutrophil infiltration is illustrative of the profoundness of the healing process on a biochemical level.

Phagocytosis and collagenolysis

When on site the neutrophil granulocyte's primary objective is to defend the host against bacteria emerging from the gut lumen. Elimination of this threat is crucial to prevent abscesses, peritonitis and anastomotic failure. However, the same mechanisms by which neutrophils kill bacteria potentially threat healthy tissue and the healing anastomosis. Knowledge of this balance would be of great value, but mechanisms are still incompletely understood.

It has long been thought reactive oxygen species (ROS), like peroxides and oxygen ions are the molecules that kill bacteria. The current supported theory states that bacteria are degraded by enzymes in the phagocytic vacuole, and the ROS are produced by intracellular NADPH oxidase only to induce and enhance the enzymatic process. ROS induce an electron and K+ transport across the vacuole membrane to achieve the specific potential, pH and tonicity needed for enzyme rich granules to enter the vacuole and for the enzymes (e.g. neutrophil elastase, cathepsin G) to function[66]. Since ROS can also be harmful, it is not clear if ROS or neutrophil enzymes cause most tissue damage during excessive neutrophil infiltration. In the case of I-R injury when neutrophil infiltration abounds, ROS production was associated with a decrease in anastomotic strength. More precise, inhibition of ROS production by aprotinin (a protease inhibitor) reduced the negative effects of I-R on colonic anastomotic strength [47, 67]. Also several other studies have demonstrated a beneficial effect of strategies to reduce oxidative stress [68, 69].

A physiological mechanism to minimize tissue damage from proteolytic enzymes is the loss of enzymatic activity in the interstitial environment as a result of different extravacuolar pH and tonicity. However, not every proteolytic activity is ceased outside the cell. Neutrophils produce elastase, protease-3 and a number of matrix metalloproteinases (e.g. MMPs 8, 9, 25) meant for extracellular activity. This initially enables them to migrate through the interstitial collagen network and later shapes the surrounding tissue in such a way that it traps microbes and prevents bacterial invasion of the lymphatics or bloodstream[70, 71]. This collagenolytic activity results in a net decrease in collagen content during the first few days.

MMPs are produced by many different cells and can be categorized into different collagenases and gelatinases that cleave specific types of matrix structures [72-75]. 'Neutrophil collagenase' (MMP-8) cleaves collagen types I, II and III but favors type I[70, 76]. This matches with the findings that the collagen type I/III ratio in the anastomotic margins is decreased in first 3 days, when neutrophil infiltration is most prominent[77]. Overall collagenolytic activity is at its most 12 hours after surgery, followed by a steady decline to normal levels[78]. The anastomotic collagen content is lowest after 48 hours[42]. Type I collagen is most prominent in unaffected intestine (68%) and is the main contributor to mechanical strength. It has been found that a decreased type I/III ratio is associated with anastomotic leakage (human:[79], diabetic rats:[80]). MMP-8 is the main MMP accounted for a significant decrease in collagen at the anastomotic wound margins and is also elevated in the peritoneal fluid of patients who develop anastomotic leakage[3, 81]. Other MMPs shown to be active during anastomotic healing are MMP 1, 2, 9 and 13[79]. Activity of specific MMPs appears to vary depending on the location within the gut (i.e. ileum or colon)[82].

An example of beneficial MMP activity is that of MMP-7 in intestinal epithelial ulcer healing, which was delayed when MMP-7 was blocked [83]. On the contrary, when over expressed under the influence of IL-1β, wound healing was also delayed.

Many papers have described the influences of MMP's on anastomotic healing and some investigators have successfully tried to improve healing by blocking MMP activity [84-86], but many questions remain. Which cytokines are primarily responsible for the stimulation of MMP synthesis, which MMPs contribute most and how are they stopped at the right moment? Blocking certain MMPs for example, might result in less collagenolysis, but inevitably means jeopardizing cellular tools in the process of moving around, killing bacteria and shaping the interstitial matrix.

Stopping neutrophil activity

To cease neutrophil activity and presence, several mechanisms come into action around the second day. Macrophages, endothelial cells, but also neutrophils produce mediators that suppress proteolytic activity, such as 'secretory leukocyte protease inhibitor' (SLPI) [87] and 'pro-epithelin' (PEPI). Granulocytes also initiate a switch from prostaglandin production to lipoxin production. Both are formed by enzymatic transformation of arachidonic acid, but lipoxin initiates the resolution of inflammation[88]. Finally, neutrophils undergo a complex programmed cell death and attract macrophages that recognize and phagocytose aging neutrophils [71, 89].

Conclusion

In conclusion, neutrophils are essential for host defense, modification of extracellular matrix and stimulation of numerous cellular processes during anastomotic repair. Excessive activity involves increased tissue destruction and jeopardizes healing. Current knowledge of neutrophil biochemical activity during anastomotic healing is insufficient to use MPO-assays as measure for anastomotic inflammation and healing, let alone to use neutrophil modulation to enhance healing.

2.3.5 Macrophages - Suppression of inflammation and transition to proliferative phase

The intestinal tract is the richest source of resident macrophages in the human body[90]. Also in the process of intestinal repair these cells are key players [91]. Supported by new monocytes coming from the bloodstream, macrophages will outnumber the neutrophils around the second to third day[42]. They aid in host defense, cease inflammation and guide the process to the proliferative phase by stimulating angiogenesis and collagen synthesis. The macrophage is characterized by many executive and directive functions. Because macrophages play such a major role in tissue repair, several attempts have already been made to modify their function in order to promote wound healing [92, 93].

Timely infiltration

Prior to definitive activation, monocytes have to be attracted from the bloodstream. The right combination of inflammatory mediators is needed to coordinate a cascade of events that allow for monocytes to arrive just after the neutrophils. This is partly achieved by neutrophils properties promoting monocyte extravasation. Neutrophils release numerous granule proteins when they leave the bloodstream [94]. These proteins (e.g. azurocidin and proteinase-3) directly mediate activation and adhesion of monocytes, but also signal endothelial cells to express more adhesion molecules such as VCAM-1, ICAM-1CCL2 and IL-8[94]. General inflammatory cytokines and agents, such as TNFα, IL-1β and LPS, are also considered important in the orderly process of monocyte extravasation by promoting the synthesis and expression of cell adhesion molecules[95, 96].

Macrophage subtypes and activity

To understand macrophage function in wound healing, the heterogeneity of this cell type is explained. Macrophage properties and behavior are shown to be different in various parts of the body, where they are influenced by specific signals of surrounding tissue(review by ref[97]). Furthermore resident macrophages behave differently when compared to 'inflammatory' macrophages. For example, the resident macrophages in the lamina propria of the intestinal wall, being close to the 'hostile' environment of the gut lumen, are very potent regarding phagocytosis and bactericidal activity, but lack the capacity of large cytokine production[98].

By using a simplified categorization, infiltrating macrophages are divided into two subtypes. The macrophage M1 is 'classically activated' by mediators like LPS and IFNγ, and is an immune effector cell with strong microbicidal behavior and inflammatory cytokine production. The other 'alternatively activated' subtypes can be put under the name of M2 and include those involved in tissue repair and those halting inflammation[97]. Polarization towards M2 is very important in the second stage of wound healing and is partly promoted by neutrophils [99]. Without neutrophil activity, macrophages produce more pro-inflammatory TNFα and IL-6 and less TGFβ [99]. The 'tissue repair' macrophage is 'alternatively activated' by IL-4 and IL-13 but also IL-6, and more anti-inflammatory macrophage behavior is stimulated by TGFβ, IL-10 or ligands for CD200r and CD172a[97]. In a mouse skin wound model, macrophages produce more TNFα and IL-6 on day 1 (primarily resident macrophages [98]), but more TGFβ on day 7[100]. In turn the IL-6 produced in the early phase enhances expression of the M-CSF-receptor on monocytes[101], which directs the macrophage towards an M2 subtype by autocrine mechanisms [102]. Another example of macrophage polarization is that classically activated macrophages release relatively many antifibrinogenic or fibrinolytic factors (e.g. MMP-7), whereas alternatively activated macrophages enhance fibrinogenesis by factors like TGFβ, IGF-1, PDGF-AA and PDGF-BB [103, 104]. The M2 subtype also upregulates arginase activity, which results in polyamine and proline biosynthesis and thereby in cell growth and collagen formation [105, 106]. Other growth factors produced by the macrophage are TGFα[107], 'heparin binding'(HB)-EGF[108] and FGF1,2,4 [109]. Most of these proliferative mediators contribute to collagen synthesis. Another important proliferative feature of the macrophage is their role in angiogenesis, a crucial element for tissue repair. They produce a battery of pro-angiogenic factors of which most affect endothelial cell and fibroblast behavior[110, 111]. In case of hypoxia and lactate acidosis, and stimulated by factors like PGE2, LPS, TGFβ and CD40L, macrophages produce IL-8[112], TNFα[113] and most important, different forms of VEGF[114-117],

Potential clinical relevance

Because macrophage activity appears mostly beneficial for wound healing, some investigators aimed at improving anastomotic healing by macrophage function enhancement. In animal experiments modulation of macrophages by glucan phosphate increased collagen production and anastomotic strength [92, 93].

Another rather broad way of cellular modulation is achieved by the administration of 'granulocyte-macrophage colony stimulating factor' (GM-CSF). GM-CSF is particularly found in states of inflammation and enhances the proliferation, differentiation and activity of all myeloid cells[118]. Local or systemic administration of GM-CSF has shown to improve healing in experimental anastomoses that were subjected to radiation[119], intraperitoneal chemotherapy[120], peritonitis[121], corticosteroids[122], icterus[123] or ischemia[124]. No beneficial effect was seen in non-compromised experimental anastomoses [125, 126].

Conclusion

In conclusion, the inflammatory stage attracts the 'alternatively activated macrophage' to play a large role in halting neutrophil activity and stimulating the proliferative processes required for tissue reconstruction.

2.3.6 Lymphocytes

Lymphocytes are relatively underexposed in literature when it comes to anastomotic healing. In cutaneous wounds T-lymphocyte concentrations start to rise from day 5 to peak for 1-4 weeks, after which B-lymphocytes emerge [127, 128]. This implies that these immune cells primarily act in the remodeling phase of skin repair when contamination and inflammation are overcome and closure is already completed [129]. Few have reported on lymphocyte concentrations during intestinal anastomotic healing. They are mostly related to foreign body reactions in novel stapling or sealing devices (e.g. [130]). In contrast to findings in skin wounds, a study in rats reported a normal to slight increased number of lymphocytes during the first 3 days, after which their number rapidly declined[131].

Although their exact contribution is unknown, lymphocytes are found to be essential in general wound healing[132]. One of the reasons for their importance could be the large amount of TGF-β released by activated T-lymphocytes[133]. TGF- β helps in damping inflammatory activity and promotes collagen production (see also paragraph 2.2.5 and 2.3.2).

Lymphocytes are also linked to the negative effects of blood transfusion on anastomotic healing[134]. Transfusion suppresses cellular immunity and is considered a risk factor for anastomotic leakage [23, 135-139].

2.4 Proliferation

When damaged cellular components have been removed and any associated microbial threat has been eliminated, the anastomotic wound is ready for tissue reconstruction. Proliferative activity increases exponentially over the first 3 days, is most prominent within the first week, but extends until the second week. It is in this short period where the barrier against the intraluminal content is restored and the new connection is strengthened to withstand peristaltic movement and passage of stools [140, 141]. If not successful, it is also this period when most leaks occur[142], [143-145].

From inflammation to proliferation

Important for proper tissue repair is the timely and coordinated conversion from to proliferative processes, from proteolytic to proteogenic[146]. As mentioned earlier, a presence of neutrophils or a delayed influx of fibroblasts are associated with anastomotic failure [44, 45, 67]. When the number of microbes and damaged cellular components declines, the level of pro-inflammatory stimuli will also diminish. Anti-inflammatory products released by macrophages and other cells (e.g. SLPI, PEPI, TGF-β1) will additionally decrease neutrophil activity and number[88].

A relevant phenomenon in the progress from inflammation to proliferation is the shifting effects of different cytokines. Because cytokines execute their function depending on molecules, some will switch from proinflammatory to anti-inflammatory signaling (such as TNFα, IFNγ, TGF-β, PGE2, ROS, 'reactive nitrogen intermediates' (RNI))[146]. For example,

when levels of the inflammatory cytokine PGE2 become high, COX2 and 5-lipoxygenase enzymes in neutrophils are inhibited and PGE2 and leukotriene B4 production (both from arachidonic acid) is consequently reduced [147]. Another delayed effect of PGE2 is the induction of 15-lipoxygenase in neutrophils, which uses arachidonic acid to produce anti-inflammatory lipoxins [147].

Tissue repair

The multilayer anatomy of the intestine makes perfect anatomical repair unachievable. Scarless healing is possible for isolated mucosal or peritoneal wounds, due to sufficient proliferative capacity of the involved cells. In the case of an anastomosis however, multiple layers of smooth muscle layers and connective tissue are involved. Moreover, the current standard in anastomotic technique, either sutured or staples, involves an inverting anastomosis in which intestinal layers are unlikely to precisely oppose each other. Thus anastomotic healing relies on proper scar formation. The creation of secure scar tissue in anastomotic healing can be categorized in three main elements essential for a firm new connection. Restoration of the interrupted mucosal barrier to protect the site from intraluminal threats, a new strong collagen network to provide strength and a vascular plexus to restore tissue oxygenation. For the intraperitoneal parts this also includes mesothelial sealing of the extraluminal side. The more specific reconstruction of lymphatics and a neuronal plexus across the anastomotic line are described as part of the remodeling phase.

2.4.1 The Mucosa – Intestinal Epithelial Cells (and lamina propria)

Mucosa related threats

With a mucosal surface area of up 300m2, the gut is an enormous and important immunological barrier preventing the translocation of commensal flora into the body(148). In comparison, this barrier is about 2m2 for the skin and 80m2 for the lungs. The construction of an anastomosis inevitably involves a circular defect in this mucosal barrier. Thereby the subepithelial layers are exposed to many possibly harmful agents, such as bowel content, bile, pancreatic juices, yeasts and bacteria[149].

Aside from the surgical defect that is created, the mucosa is very sensitive to hypoxia. Macroscopically this may be seen by cyanotic appearance during suturing. When transient ischemia is induced and perianastomotic tissue has a vital appearance, the mucosa can still be affected[150]. The ischemic effects include detachment of the epithelial layer from the lamina propria, decrease of villi height and occurrence of denuded areas, which consequently hampers submucosal healing[150]. Moreover, hypoxic epithelial cells produces factors (like 'hypoxia inducible factor' (HIF)-1alpha) which can augment microbial virulence (e.g. that of Pseudomonas Aeruginosa) and thereby effectuate an additional threat [151-153].

Because the mucosa consists of rapidly proliferating cells, it is also very sensitive to the (neo-) adjuvant cancer therapies often involved in intestinal anastomoses, such as radiation or cytostatics. Both have shown to significantly impair anastomotic healing, especially when

given too close in time to the operation [139, 142, 154-157]. Both chemotherapy and radiation can cause severe mucositis [158, 159]. By Nf-κB pathways increased apoptosis causes decreased villous height, a reduced number of goblet cells and a lowered mucus production[160]. In turn, this means increased permeability and excessive inflammation which both threaten tissue repair.

Mucosal defense

Normal mucosal defense consists of several elements. The lamina mucosa is covered with a single layer of epithelial cells which are sealed together with tight junctions regulating passive movement of fluids, proteins and lipids and preventing the passage of microbes. The epithelial cells express multiple receptors (Toll-like receptors (TLRs), mannose-binding receptor (MBR), scavenger receptors) that recognize microbes[161]. TLRs are situated on the basolateral side, under the tight junction level, under normal conditions. When activated by microbial molecules TLRs induce the expression of multiple inflammatory, cytoprotective and angiogenic factors (e.g. COX2, keratinocyte growth factor(KGF)-1 and 2, hepatocyte growth factor(HGF), TGFb1 and VEGF), influencing processes in the underlying lamina propria mucosae(reviewed in[161]).

Mucosal repair

When it comes to mucosal healing, most knowledge derives from research on inflammatory bowel diseases and gastric ulcer healing[162, 163]. Isolated healing of superficial defects in the intestinal epithelial cell layer is relatively efficient and fast since these cells have strong proliferative and migratory capacity. However, in the case of an anastomosis the subepithelial layers part and the solid base for epithelial reunion is disturbed. Nevertheless, restitution of the epithelial barrier across the anastomotic line is rapidly achieved and almost scarless after two weeks[164].

The process of epithelial healing in general can be divided in four steps (Figure 2). First, villous contraction (flattening) reduces the surface area for repair (this does not account for the colon where villi are not present). Second, intestinal epithelial cells (IEC) migrate and, third, proliferate to completely cover the defect. The final step is paracellular closure of mucosal cells.

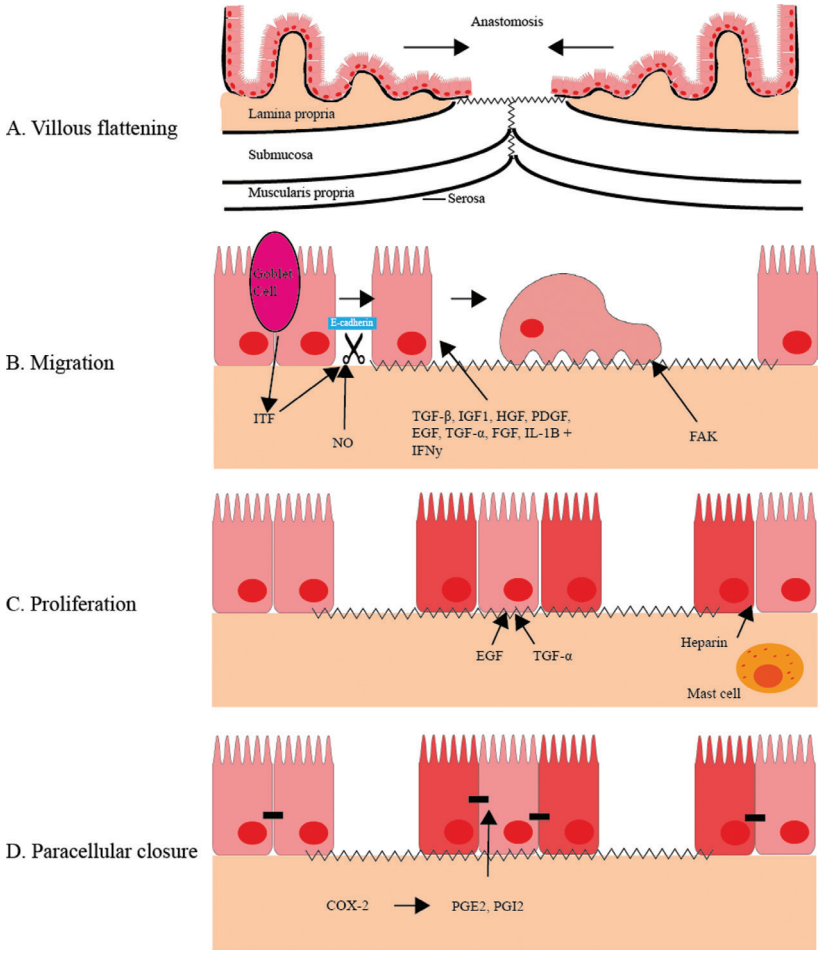


Figure 2 Schematic overview of mucosal healing.

A. Villous flattening. B. Migration by lamellipodal extensions, regulated by focal adhesion kinase (FAK or PTK2) and stimulated by numerous growth factors and cytokines, including (TGF-β, IGF1, HGF, PDGF, EGF, TGF-α, FGF, IL-1B + IFNγ)[148, 165, 166]. Subepithelial myofibroblasts supply TGFβ1, which inhibits proliferation but enhances migration of IECs [167, 168]. Nitric oxide (NO) permits numerous growth factors to stimulate migration[169-171], by preventing adhesive behavior of cells[172]. Trefoil peptides, such as 'intestinal trefoil factor' (ITF), are secreted by goblet cells throughout the intestine and act similarly by keeping adhesion molecules like E-Cadherin away from adherens junctions to allow migration [162, 173, 174]. C. Proliferation of IECs through EGF-receptor pathway [175-177]. TGFα and EGF are two of four factors activating the EGF-receptor to promote proliferation and to halt apoptosis (partly through COX2 expression) [178-180]. Heparin deriving from subepithelial mast cells act with heparin-binding growth factors to induce epithelial crypt cell proliferation[181]. D. Paracellular closure by formation of tight junctions. COX products PGE2 and PGI2 restore 'transepithelial electrical resistance' (TER), a measure for intestinal permeability [182-184].

Potential clinical relevance

The importance of the mucosal barrier is reflected in the many attempts that have been done, and are being done, to protect the anastomosis by intraluminal sealants or tubes[185]. Also on a biochemical level people have aimed to improve anastomotic healing by enhancing mucosal repair. An increased anastomotic strength was observed when rats were treated with intraperitoneal administration of KGF [186]. Later it was seen that intravenous KGF resulted in an increased number of Goblet cells and a thicker mucin layer around the anastomosis resulting in higher strength [187].

2.4.2 The Submucosa

Since the 1887 study of Halstedt on intestinal anastomoses, the submucosal layer is considered to be the most important for providing strength to the anastomoses[188]. It primarily consists of collagen with a vascular, lymphatic and neurologic plexus running through it.

Smooth muscle cells and fibroblasts

When it comes to submucosal reconstruction, the layer is very much depending on its surroundings. The submucosal layer of the intestinal wall is enclosed by the thin muscularis musocae on the inner and the muscularis propria on the outer side. Thus, the strength providing collagen layer is packed between intestinal smooth muscle cells (SMC) which are found to be most important in producing and maintaining the submucosa of uninjured intestine. In the case of anastomotic healing their involvement begins with landscaping the wounded area. In the early inflammatory phase, IL-1β stimulates proliferation of intestinal SMCs, it simultaneously down-regulates their collagen synthesis and augments collagenase expression[189]. The collagenolytic activity is gradually replaced by collagen synthesis, marking the transition to the proliferation phase. Intestinal SMC's synthesize type I, III and V collagen[190]. This is different in other tissues (e.g. skin) where fibroblasts are accounted for most collagen production[190]. Moreover, colonic fibroblasts behave quite differently from cutaneous fibroblasts. The latter show a considerable increase in collagen synthesis when stimulated with fetal calve serum (FCS) while their colonic relatives do not[191]. Also, collagen production can be inhibited by IL-1β or corticosteroids in skin but not in colonic fibroblasts[191].

TGF-β and other growth factors

Relatively few growth factors have been identified as direct stimulators of collagen formation. The best studied growth factor involving collagen synthesis is TGF-β, which is primarily produced by inflammatory cells like T-lymphocytes and macrophages[133]. Both collagen synthesis and TGF-β levels in anastomotic tissue gradually increase from day 0 to day 7[192, 193]. In comparison, other growth factors like EGF and PDGF-B do not increase over the first week[192] and do not contribute to collagen formation[133]. Moreover, EGF, which is considered more important within the mucosa, even antagonists collagen formation[133]. TGF-β inhibits the proliferation of intestinal SMCs, but has shown to increase their collagen production[194].

In rats given postoperative chemotherapy, reduced levels of TGF-β were associated with decreased collagen synthesis and less anastomotic strength [91, 195]. TGF-β is not solely a promoter of collagen production but, like the other cytokines, a dynamic player within the entire process. An example of its versatile role in extracellular matrix (ECM) formation is the promotion and prolongation of MMP-2 activity resulting in collagen breakdown [196]. Furthermore, it is a major (indirect) contributor to angiogenesis[133].

Another growth factor that is thought to be involved in submucosal healing is 'insulin like growth factor' (IGF) [50, 197]. Intravenous administration has shown to increase collagen formation and anastomotic strength in rats [198]. Other compounds known to be essential in collagen synthesis are Vitamin C and iron. If these are deficient wound healing is disturbed {Kumar, 2014 #859}.

Neovascularisation

New capillary growth is essential for proliferating cells and for the new anastomosis to survive as it provides the necessary nutrient and oxygen support and serves as the main infrastructure for cellular transportation. Insufficient vascularisation is the most notorious risk factor for a failing anastomosis and is often used as a method to compromise healing in animal experiments [153, 199, 200]. Patterns and mechanisms of vascularisation have been investigated in several studies on intestinal anastomoses. Studies in mice, rats, dogs, pigs and horses have shown that the first new miniature vessels are visible starting from day 2[201], the first vessels cross the anastomotic line from day 3[202], cross-anastomotic networks appear between day 7 and 14, and a mature plexus is formed after 3 to 6 weeks[203-206]. The basic steps of angiogenesis include vasodilatation, increased vascular permeability, endothelial proliferation and migration, lumen formation and further maturation[207]. Each step relies on a multitude of factors of which many derive from platelets, macrophages and fibroblasts[113]. The VEGF-receptor pathways are the most studied, but numerous other pathways are involved in angiogenesis[208]. For example, proteolytic activity of multiple MMP's is required to facilitate the migration of endothelial cells, whereas the recruitment, migration and proliferation of vascular smooth muscle cells also relies on other cytokines(angiopoietin 2(Ang2), PDGF-BB, FGF-1, TGF-β, IGF-1, IL-8, TNFα)[207]. Many factors involved in the stimulation of collagen synthesis also cause pro-angiogenic action (e.g. IL-1A and B, TNFα, PDGF and FGFs) [209]. Since many factors (e.g. TGF-β) can effectuate agonist and antagonist activities depending on cofactors present, it remains challenging to intervene in this process. Exogenous administration of angiogenic factors has evoked effects different from endogenous factors[207]. Hence, VEGF-A and FGF-2 DNA-mediated gene therapy was claimed to improve both mechanical and histological healing parameters of ischemic anastomoses [210]. The same was found for local injection of VEGF into the lamina propria of rabbit colon anastomoses. Surprisingly, anti-angiogenic therapy with the monoclonal VEGF-antibody bevacizumab was considered to be safe for use when an anastomosis is made since no difference in histology, strength or clinical symptoms was noted between groups in both animal and human studies[211, 212]. In contrast continuous treatment with angiostatin, a fragment of plasminogen and endogenous inhibitor of vascular growth, significantly impaired the healing of colonic anastomoses in mice[213].

Lymphangiogenesis

Relatively underexposed compared to vascular regeneration is the formation of a new lymphatic network. Functional lymph vessels provide an important route to transport cell debris from the anastomotic area. Incomplete or blocked lymph drainage causes insufficient interstitial transport and edema and has shown to increase leak rates in animal studies[214]. Also, enlargement of lymph folliculae has been reported as a cause of anastomotic leakage [215]. When inflammatory activity is high, the production of VEGF-C and VEGF-D, which are the main factors in lymphangiogenesis, is stimulated[216]. One Japanese group has studied the lymphatic regeneration following intestinal anastomoses in the late 1970's and was able to visualize the formation of lymph vessels along the muscularis mucosa and across the anastomosis starting from the 7th day until completion around the 21st[217].

2.4.3 Mesothelial cells

The outermost layer of the intestinal wall, the serosa, consists of a squamous epithelium (mesothelial cells) and a thin layer of loose connective tissue underneath. There are two interesting aspects about the serosa. The first is that the extraperitoneal parts of the gut which do not have a serosal layer (e.g. the rectum or esophagus), tend to leak more often than the intraperitoneal parts. The second aspect is that in the case of inverting anastomoses, the serosal layers oppose each other. Ironically the original physiological purpose of a mesothelial cell layer is to provide a lubricious surface to allow intestinal movement and not to adhere. It is the field of research on adhesion formation that has provided a substantial amount of knowledge on peritoneal healing[218, 219]. However, since most research concerns the healing of superficial serosal defects, it is hard to translate this to anastomotic healing. Additionally, no histological studies have yet assessed the serosal role in anastomotic healing.

Normal mesothelial healing

A remarkable aspect of mesothelial healing in general is that when a defect is made, the entire surface becomes epithelialized simultaneously and not just gradually from the border as in skin epidermalization. New mesothelial cells derive from differentiating fibroblasts or primitive mesenchymal cells from subserosal layers[220]. Meanwhile migration from wound edges also contributes significantly to closure[221]. Mesothelial defects are rapidly covered with macrophages and fibrin. Proliferating mesothelial cells use these fibrin strands and the serosal fluid when migrating to cover the defect[220]. For small defects the covering process takes place within 3 days and, in optimal conditions, scarless healing can be achieved within a week[221].

The growth factors involved in mesothelial repair differ slightly from submucosal repair. Whereas EGF does not promote submucosal healing, it is a known contributor to proliferation of mesothelial cells and peritoneal fibroblasts [222, 223]. Others include FGF2, PDGF and hepatocyte growth factor/scatter factor (HGF/SF). HGF/SF is produced by mesothelial cells and stimulates their own proliferation and motility[224]. Similar to the submucosa, TGF-β and IL-1α again contribute to collagen synthesis[225].

Where the submucosa is mostly infiltrated with cells and growth factors from the blood stream, the serosa is relatively dependent on cells and cytokines deriving from the peritoneal fluid. Post-operative levels of inflammatory cytokines like TNFα, IL-6 and IL-10 are higher within the peritoneal fluid than in peripheral blood[4]. A relative lack of cytokines may account for impaired healing of extraperitoneal anastomoses.

2.5 Remodeling phase

Since the first few days of anastomotic healing are most important and failure is most likely to occur within the first week, relatively few have looked at the later phases of healing. Longer follow-up periods have been used in studies on effects of radiotherapy, degradation profiles of absorbable material, and new anastomotic devices (e.g. staplers). In general, remodeling concerns all systems within anastomotic healing and thus cells from every system continue to optimize tissue structure. When mucosal defects are closed, new villi arise and a proper functioning mucosa is formed. The remaining inflammatory cells around sutures disappear from the fourth to the eighth week[226]. Macrophages, lymphocytes, smooth muscle cells and fibroblasts strengthen collagen networks and the new vascular plexus turns into a more mature and efficient web of vessels [203-206, 227]. Few have studied neuronal repair. In dog anastomoses, new nerve fibers are seen within 3 weeks[228]. It takes neurons about 6 months to cross the anastomotic line, but this is not required for adequate intestinal motility [229, 230]. Finally, within 6 to 10 months the multilayer anatomy is grossly restored though the orientation of muscular fibers remains disturbed [227, 231].

2.6 Conclusion

Wound healing is one of the most complicated processes within the human body. Anastomotic healing requires the collaboration of many different cell types to complete the course of an adequate inflammatory response combined with the reconstruction of specialized tissue. Biological influences and mechanical forces acting on an intestinal anastomosis greatly challenge the healing process, resulting in failure rates of 3-14%.

In this chapter an overview is given of the main cellular and molecular processes that cooperate to achieve repair. Infiltrating platelets, mast cells, granulocytes, macrophages, dendritic cells, lymphocytes, fibroblasts work together with their resident relatives and others like mesenchymal stem cells, neurons, and endothelial, epithelial, mesothelial and smooth muscle cells. The intended full thickness intestinal repair requires an orderly, four dimensional system in which alternate cellular and balanced cytokine activity gradually reconstruct the mucosal barrier, the submucosal basis, the circulatory infrastructure and the serosal covering.

The relevance of different steps and cellular interactions within the process becomes apparent when certain drugs or risk factors disturb one or more steps in the healing process. Cellular and molecular mechanisms might serve as potential therapeutic targets, but the complexity and interaction of the different systems impedes the development of such therapeutic strategies. Scarcity of studies on the molecular biology of anastomotic

healing makes one rely on knowledge extrapolated from related research fields. Though these have provided substantial insight into biochemical mechanisms of tissue repair, many fundamental elements of the system are still unknown. The exact role of cells and cytokines concerning their amount, location and timing remains unsolved. Other topics relatively underexposed in anastomotic research include the essence of peritoneal cytokines and the influence of intraluminal factors such as bile, nutrients and microflora on the healing process. Complementation of our current knowledge of anastomotic healing by future research should improve our understanding of leakage etiology and also provide better suited therapies when it comes to cellular or molecular acting agents.

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3.

SYSTEMATIC REVIEW OF EXPERIMENTAL STUDIES ON INTESTINAL ANASTOMOSIS

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Abstract

Background

The contribution of animal research to a reduction in clinical intestinal anastomotic leakage is unknown, despite numerous experimental studies. In view of the current societal call to replace, reduce and refine animal experiments, this study examined the quality of animal research related to anastomotic healing and leakage.

Methods

Animal studies on intestinal anastomotic healing were retrieved systematically from PubMed and Embase. Study objective, conclusion and animal model were recorded. Reporting quality and internal validity (reporting of randomization and blinding) were assessed.

Results

A total of 1342 studies were identified, with a rising publication rate. The objectives of most studies were therapeutic interventions (64·8 per cent) and identification of risk factors (27·5 per cent). Of 350 articles studying experimental therapies, 298 (85·1 per cent) reported a positive effect on anastomotic healing. On average, 44·7 per cent of relevant study characteristics were not reported, in particular details on anastomotic complications (31·6 per cent), use of antibiotics (75·7 per cent), sterile surgery (83·4 per cent) and postoperative analgesia (91·4 per cent). The proportion of studies with randomization, blinding of surgery and blinding of primary outcome assessment has increased in the past two decades but remains insufficient, being included in only 62·4, 4·9 and 8·5 per cent of publications respectively. Animal models varied widely in terms of species, method to compromise healing, intestinal segment and outcome measures used.

Conclusion

Animal research on anastomotic leakage is of poor quality and still increasing, contrary to societal aims. Reporting and study quality must improve if results are to impact on patients.

Introduction

Worldwide, intestinal resection and anastomosis are performed annually in over a million people with colorectal cancer, inflammatory bowel disease, complicated diverticulitis and morbid obesity[1–3]. Despite improvements in technique, anastomotic devices and perioperative care, an intestinal anastomosis has a 3–14 per cent risk of leakage[4,5]. This postoperative complication often results in severe morbidity, with a consequent mortality rate of up to 18 per cent[6–8]. Many risk factors have been identified from clinical studies[4,9]. Male sex, low colorectal anastomosis, preoperative chemoradiation, advanced tumour stage, immunosuppression, perioperative bleeding and multiple staple firings have all been associated with increased leak rates[4,9,10].

Animal models have been used extensively to study causality and underlying pathophysiological mechanisms[11,12], as well as the assessment of techniques and products that reinforce the anastomosis or improve healing[13,14]. The study of anastomotic failure in animals involves moderate to severe animal discomfort, dedicated human effort and considerable cost. This has led to a European directive that animal experiments need to be replaced, reduced and refined whenever possible[15,16]. Owing to its complex physiology, the process of anastomotic healing is presently considered too complicated to mimic in vitro. To reduce and refine animal experiments, it is necessary to perform a systematic review of the existing literature regarding methodology and quality, to see how experimental models and designs might be improved or standardized. The aim of the present systematic review was to investigate study characteristics and the quality of animal research on intestinal anastomotic healing.

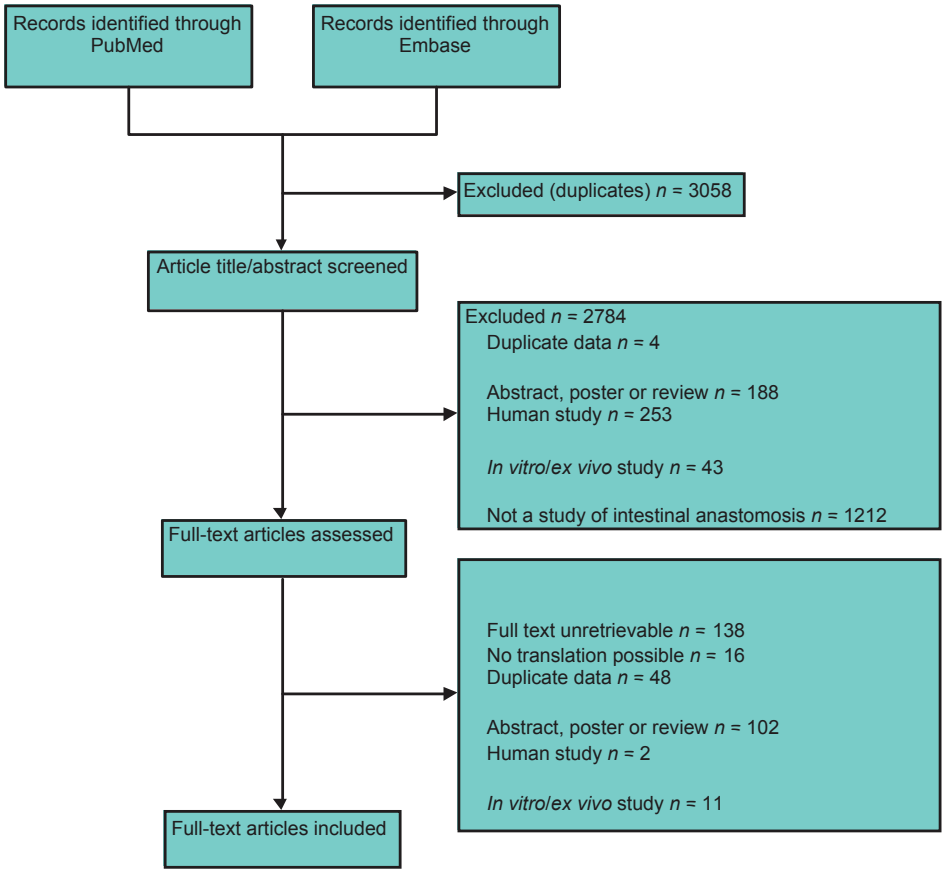
Methods

Search and study inclusion

This review is reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guideline17. A prespecified protocol was used for the study selection and extraction of study characteristics (available through https://www.radboudumc.nl/syrcle). A systematic search was conducted in the PubMed version of MEDLINE and the OvidSP version of Embase on 4 April 2014, by using different synonyms, Medical subject headings (MesH) and Emtree terms (for PubMed and Embase respectively) to cover any study on intestinal anastomoses (Table S1, supporting information)[18]. To limit the results to animal studies, a specifically designed and validated filter was used for both databases (Table S1, supporting information)[19,20]. Selection was made by screening the title and abstract of all results. Every title or abstract was screened by two authors. Disagreement was resolved by discussion. Experimental studies in which the healing of an anastomosis of the small bowel or colon was investigated in an in vivo animal model were included. All duplicates, abstracts, posters and review articles, all studies in human, in vitro or ex vivo studies, all studies on other types of anastomosis (for example vascular, urological, biliary, gastrojejunal, oesophagojejunal), intestinal transplants, studies reporting outcomes not related to anastomotic healing (such as cancer recurrence) and those in which enterotomy was performed rather than anastomosis were excluded. Articles selected through title and

abstract screening were then subjected to full-text screening (using the same inclusion criteria) and simultaneous data extraction by one of the authors. Full-text retrieval was attempted through online searching, by consulting Dutch and German university libraries, and by e-mail to the corresponding author. Articles in all languages except Japanese were included.

Figure 1 PRISMA flow diagram of study selection



Data extraction

Study objectives

The primary objective of each study was interpreted from the hypothesis or research question stated in the article. Study objectives were categorized as: therapeutic trial (for example 'Does growth hormone improve anastomotic healing?'), risk factor identification (for example 'Does chemotherapy cause leakage?'), study of physiology (for example 'What is the role of neutrophils during healing?') or animal model presentation (for example 'a leakage model is developed in the pig').

Study conclusions

Study conclusions on experimental therapeutics and risk factors were categorized as having a positive (for example 'growth hormone improves healing'), neutral (for example 'hyaluronic acid does not affect leakage rate') or negative (for example 'chemotherapy impairs anastomotic strength') effect on anastomotic healing according to the authors' conclusions. Only studies on experimental therapeutics and risk factors were used to categorize study conclusions, because these involved control groups given placebo or no treatment. In comparison, studies on surgical technique often compared two existing techniques (such as single versus double-layer suturing), making any unilateral conclusion debatable. If multiple interventions were studied with different outcomes, they were scored separately. Reporting of study details.

The reporting quality of each article was scored according to a reporting score, which was designed specifically for this review according to items listed in the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines21 (such as animal species, strain and age) and items relevant to anastomosis studies (such as suture material and technique).

Assessment of internal validity

Three determinants of internal validity were evaluated. Risk of selection bias was addressed by scoring randomized treatment allocation in every controlled study. Randomization was scored when reported, even if the method of randomization or allocation concealment was not described. Risk of performance bias was determined by scoring the blinding of the surgical procedures. Risk of detection bias was estimated by assessing the use of blinded outcome assessment. This was categorized in blinding of the primary or secondary outcome (for example if only 'histological analysis' but not 'macroscopic examination of leakage' was blinded). If blinding was reported, this was scored accordingly, independent of further specification of the method used.

Description of animal models used

Details were recorded on animal species, method of compromising healing, intestinal segment and primary outcome measure used.

Statistical analysis

Differences in mean values between groups were assessed using ANOVA and Tukey's post hoc test. The $\chi 2$ test was used to compare proportional values between groups. Linear regression analysis was employed to determine changes over time in relation to the number of studies, animal species, reporting score, randomization and blinding. SPSS® version 20 (IBM, Armonk, New York, USA) was used for statistical analysis. $P < 0.050$ was considered statistically significant.

Results

After screening title, abstract and full text (as applicable) of 4527 citations, 1342 individual articles were included for data extraction and analysis (Fig. 1). There was considerable experimental repetition for some topics: 88 studies on adverse effects of chemotherapy, 42 studies on fibrin glue and 18 studies on the use of growth hormone.

General characteristics and study objectives

The number of articles per year on intestinal anastomosis increased steadily over 60 years ($P < 0.001$). A mean of 36 articles were published each year from 2000 to 2009, and 53 articles each year from 2010 to 2014 (Fig. 2a). A total of 73 304 animals were used with a mean(s.d.) of 55(49) animals per study. The mean number of animals per study remained constant over time ($P = 0.455$).

There were 68 studies (5.1 per cent) with multiple objectives, giving a total of 1410 objectives. Of all studies, 914 (64.8 per cent) were therapeutic trials, 388 (27.5 per cent) were risk factor identification studies, 89 (6.3 per cent) investigated anastomotic healing physiology, and 19 (1.3 per cent) were on the presentation or evaluation of animal models. Over time there was a slight increase ($P < 0.001$) in the proportion of risk factor identification studies (Fig. 2b). Most therapeutic trials studied experimental therapies designed to improve healing (Fig. 2c), and most risk factor identification studies focused on chemotherapy (Fig. 2d). The variety of therapies and risk factors studied is detailed in Tables S2 and S3.

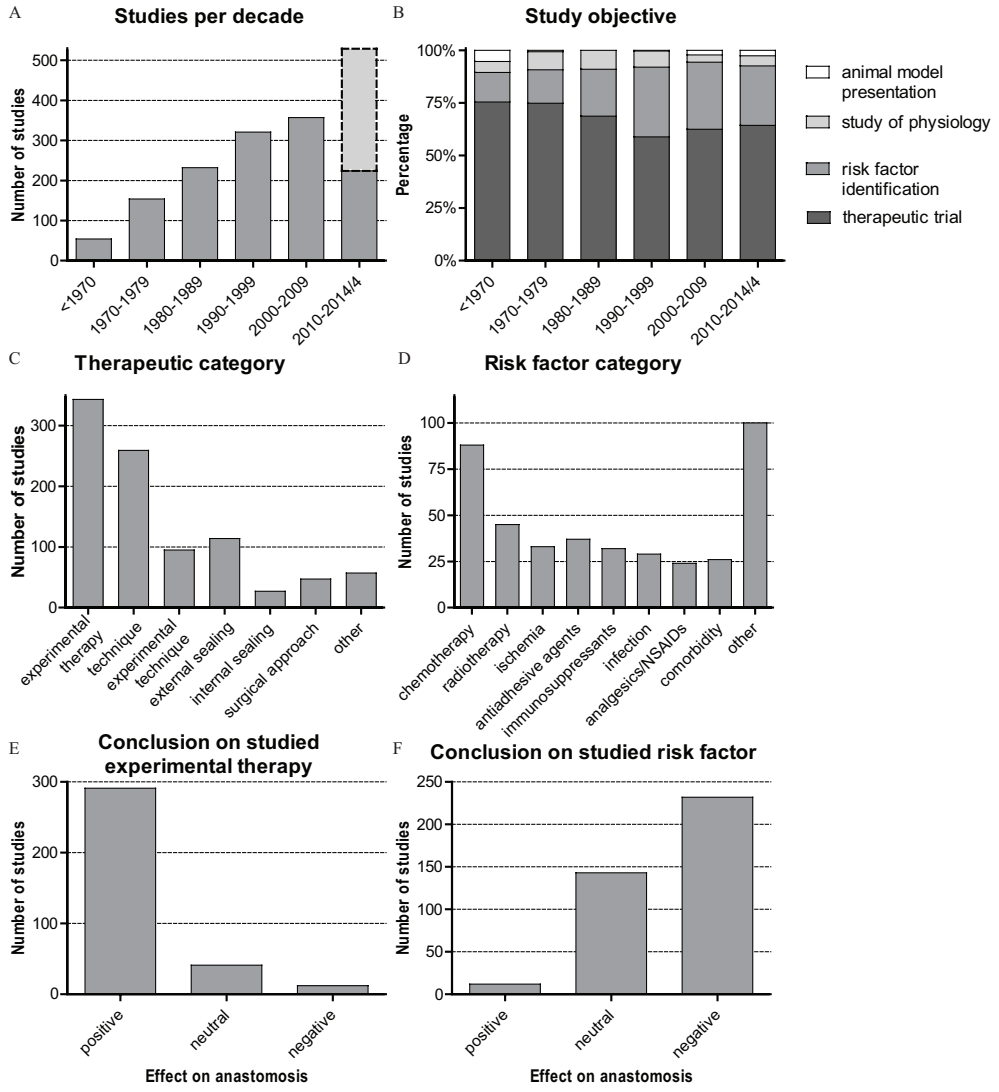


Figure 2 Number of studies, study objectives and study conclusions. a Number of studies per decade. The dashed line indicates the projected number of studies in the decade 2010–2019, based on the mean number of studies per month. b Study objective/character per decade. c Classification of studied strategies in the following therapeutic categories (with examples in parentheses): experimental therapy (growth factors, hormones, herbs, antioxidants), technique (comparing suture techniques or suture material), experimental technique (magnetic or glued anastomoses), external sealing (omental wrap, meshes, glue), internal sealing (tubes, glue), surgical approach (laparoscopy, natural-orifice surgery, single-incision surgery), other (nutritional management, ostomies, oximetry). d Classification of research topics of risk factor identification in the following categories (with examples in parentheses): chemotherapy (any pharmacological (neo)adjuvant therapy), radiotherapy, ischaemia (any form of ischaemia and/or reperfusion injury), antiadhesives, immunosuppressants (corticosteroids), infection (peritonitis, bacterial contamination, sepsis), analgesics,

co-morbidity (hypertension, diabetes, hypercholesterolaemia), other (blood transfusion, drains, malnutrition, trauma). NSAID, non-steroidal anti-inflammatory drug. e,f Effect of studied therapy (e) or risk factor (f) on anastomotic healing according to the conclusion stated in the article. c,d For further specification of therapeutic and risk factor categories, see Tables S2 and S3.

Study conclusions

Of 350 articles on experimental therapies, in 298 (85.1 per cent), the authors concluded that the treatment under investigation (approximately 200 different treatments) had a beneficial effect on anastomotic healing. Treatment was reported to have no effect in 41 studies (11.7 per cent) and only 12 (3.4 per cent) reported a negative effect on healing (Fig. 2e). Of 388 risk factor identification studies, it was concluded that the factor under investigation was a risk factor for anastomotic leakage in 235 (60.6 per cent) (Fig. 2f). The studied factor did not affect healing in 142 studies (36.6 per cent) and improved healing in 11 (2.8 per cent). A relatively large proportion of the latter two categories were articles studying the safety of antiadhesives: 28 (19.6 per cent) of 143 and four (36 per cent) of 11 articles respectively.

Quality of reporting study details.

A mean(s.d.) of 10.0(3.1) of 18 criteria (55 per cent) of the reporting score were met. The reporting score increased gradually, from 44 to 62 per cent, over six decades ($P < 0.001$) (Fig. 3a). Items that were most frequently unreported were the use of postoperative analgesics (91.4 per cent), sterile surgery (83.4 per cent) and antibiotics (75.7 per cent). Important data specific to an experiment were frequently omitted, such as the number of sutures used in models investigating anastomotic strength, reported in only 110 (56.4 per cent) of 195 studies. The occurrence of anastomotic complications, or their absence, was not reported in 424 studies (31.6 per cent) of the 1342. They were reported in 652 studies (48.6 per cent) and used for comparative outcome in 266 (19.8 per cent).

Assessment of internal validity

Measures to reduce selection bias increased over time, with randomization being reported in only 7.0 per cent of studies before 1970, compared with 62.4 per cent in the past decade ($P = 0.001$) (Fig. 3b). Measures to reduce performance bias were seldom used. Of 914 studies in which blinding of surgery was considered possible, this was reported in only 21 articles (2.3 per cent of the total; 4.9 per cent of studies since 2000), all published in the past three decades. With respect to detection bias, blinding of primary outcome assessment was reported in 4.9 per cent and blinding of secondary outcomes in 12.4 per cent. The reporting of blinding of primary outcome assessment has increased slowly since the 1990s ($P = 0.035$), but was still absent in 91.5 per cent of articles published since 2000 (Fig. 3c).

Animal models

Rats were used in most studies (792), followed by dogs (290), pigs (134), rabbits (100), mice (20), cats (11), horses (11), guinea-pigs (6), sheep (5), monkeys (3) and cows (1). Before 1980, dogs were used in most experiments. Since the beginning of the 1990s there has been a marked increase in the use of rats ($P = 0.002$) (Fig. 4a).

A factor to compromise healing or induce leakage was used in 32.0 per cent of therapeutic studies. In the 1970s and 1980s, the most commonly employed compromising factor was intestinal ischaemia (respectively 24.2 and 20.5 per cent of studies using a compromising factor). The induction of peritonitis was most popular in the past two decades (27.9 and 27.5 per cent) (Fig. 4b). The use of chemotherapeutic agents to impair healing varied between 15.1 and 28.4 per cent (mean 18.8 per cent) over time. The exact methods of inducing ischaemia, peritonitis, cytotoxic injury, radiation injury, obstruction, suture defects or immunosuppression varied greatly among studies, impeding further categorization. For example, the chemotherapeutic drug 5-fluorouracil was used regularly to compromise healing, but the route, timing and dose of administration varied greatly between studies, and different segments of the intestinal tract were used (Fig. 4c). Strength measurements were used most often, and increasingly, as the primary outcome (720 studies, 53.9 per cent of 1336 studies), followed by anastomotic complications (266, 19.9 per cent) and histological analyses (184, 13.8 per cent) (Fig. 4d).

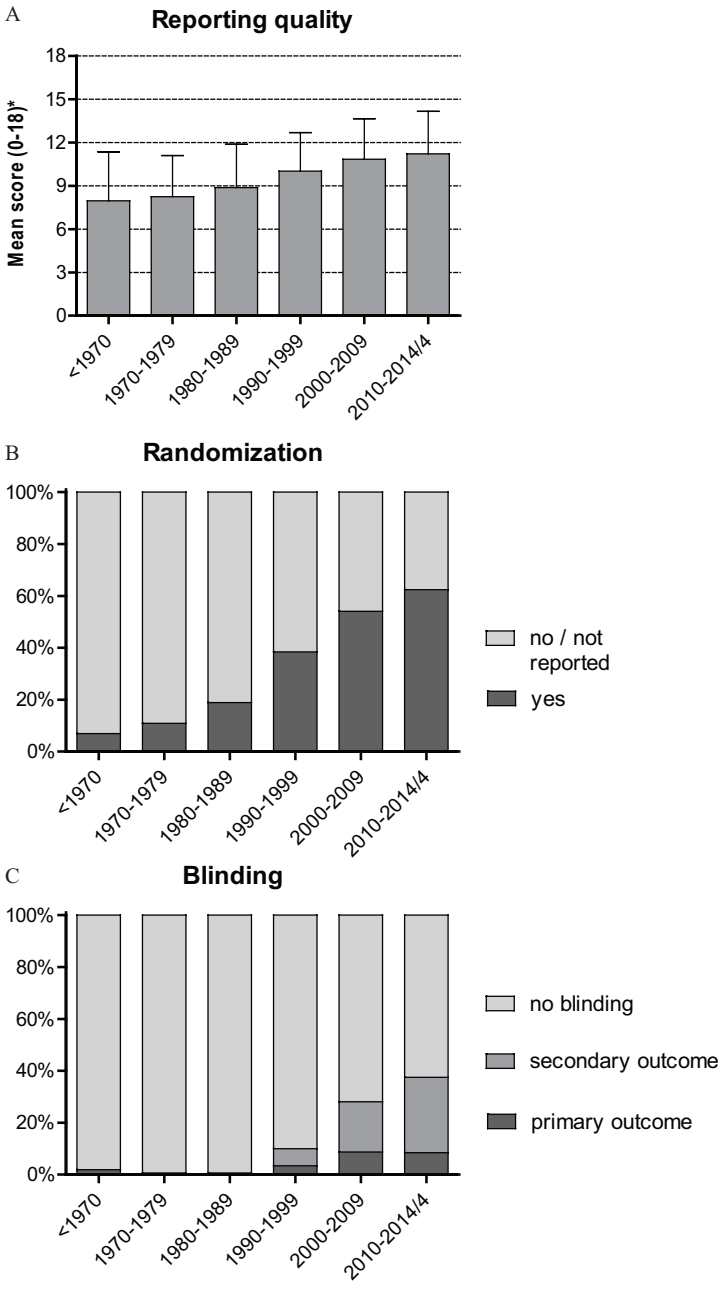

A
Reporting quality
Mean score (0-18)*
18
15
12
9
6
3
0
<1970
1970-1979
1980-1989
1990-1999
2000-2009
2010-2014/4
B
Randomization
100%
80%
60%
40%
20%
0%
no / not reported
yes
C
Blinding
no blinding
secondary outcome
primary outcome

Figure 3 Reporting quality, randomization and blinding. a Mean(s.d.) reporting score (maximum 18 points). Points (in parentheses) scored for reporting animal characteristics: strain (1), sex (1), age or weight (1); perioperative management: any remark on housing (1), fasting/bowel preparation (1), type of anaesthesia (1), use of antibiotics (1) and postoperative analgesia (only if used, 1 point); surgery: reporting of strict aseptic techniques (2 points, 1 point for semisterile technique), method of bowel apposition (for example inverted or Gambee) (1), suture material (if type and thickness reported, 1 point), suture layers (1), suture pattern (1), suture distance (such as number of sutures or 'every 3 mm') (1); outcome assessment: anastomotic complications (as comparative outcome, 2 points; if only reported (for example if absent), 1 point), animal welfare (for example weight loss) (1). b Use of randomized treatment allocation if possible. c Blinding of primary or secondary outcome assessment.

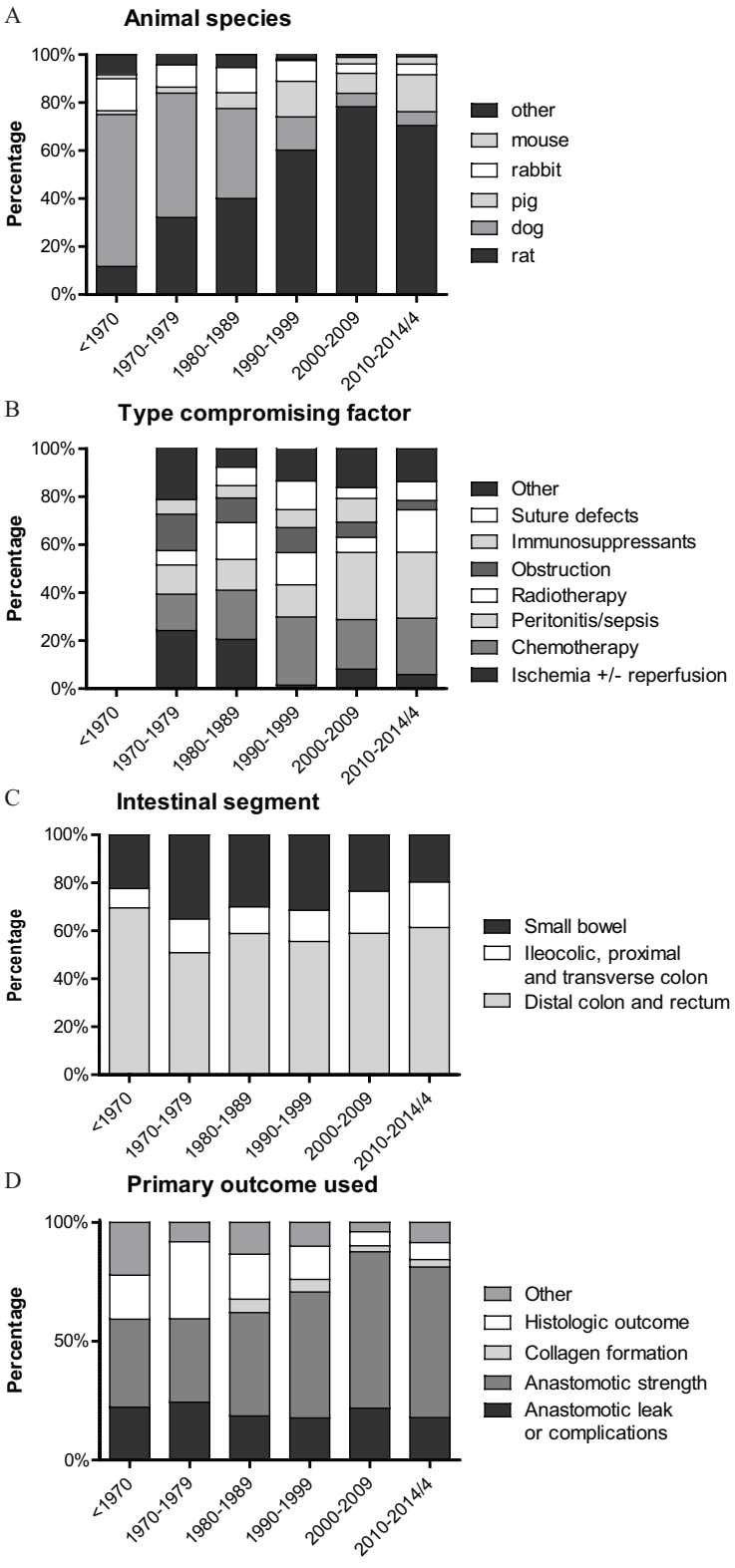

A
Animal species
Percentage
100%
80%
60%
40%
20%
0%
<1970
1970-1979
1980-1989
1990-1999
2000-2009
2010-2014/4
other
mouse
rabbit
pig
dog
rat
B
Type compromising factor
Percentage
Other
Suture defects
Immunosuppressants
Obstruction
Radiotherapy
Peritonitis/sepsis
Chemotherapy
Ischemia +/- reperfusion
C
Intestinal segment
Percentage
Small bowel
Ileocolic, proximal and transverse colon
Distal colon and rectum
D
Primary outcome used
Percentage
50%
Other
Histologic outcome
Collagen formation
Anastomotic strength
Anastomotic leak or complications

Figure 4 Animal models. a Animal species used. 'Other' included horses (11), cats (11), guinea-pigs (6), sheep (5), monkeys (3) and cows (1). b Categories of compromising methods (if used, with examples in parentheses): ischaemia (marginal artery clamping, mesenteric artery clamping, ischaemia and reperfusion protocols), chemotherapy (includes preoperative, intraoperative and postoperative regimens, and intravenous and intraperitoneal administrations), peritonitis (caecal ligation puncture, exogenous bacterial contamination), radiotherapy (various regimens), obstruction, immunosuppressants (corticosteroids) and suture defects. c Segments of the intestine in which the anastomosis was constructed. D Categories of primary outcome: anastomotic complications (leakage rate or anastomotic complications), strength (any form of bursting pressure or breaking strength testing), collagen (any form of collagen quantification or qualification other than histological, such as hydroxyproline assays), histology (any histological evaluation), other (microangiography, barium enema). If multiple outcomes were used and it was unclear which was used as primary outcome, the categorization was used in this order with 'anastomotic complications' first and 'other' last.

Discussion

The number of publications on animal research on intestinal anastomosis continues to increase. Reporting quality is poor and frequently insufficient. Internal validity was poor and external validity indicated wide variability in animal models. Although study quality has improved slightly over time, the use of important methodological strategies, such as blinding of primary outcome, is still inadequate.

The range of therapies and risk factors was diverse. The unnecessary repetition for some topics (adverse effects of chemotherapy, fibrin glue, growth hormone) highlights the importance of properly reviewing animal studies before commencing new projects[22].
The true translational value of animal studies to clinical practice was not assessed in this review, but it seems very limited, bearing in mind that a high proportion of positively judged experimental treatments have no current application in patients. Reporting quality, internal validity and external validity of the model used are all contributing factors[23,24].
This poor-quality reporting also makes it impossible to assess the internal validity of the studies and the reliability of the results[25]. For instance, breaking or tensile strength measurements determine the suture-holding capacity of the anastomosis by pulling it apart, and are highly dependent on the type and number of sutures used. Of 195 studies reporting breaking strength for sutured anastomosis, the number of sutures was not adequately reported in 110 (56.4 per cent). Anastomotic leakage is an outcome with infectious consequences. Consequently, signs of leakage such as abscess formation are influenced by antibiotic use and aseptic techniques, but these items were not reported in 75.7 and 83.4 per cent respectively of relevant studies. Postoperative analgesia was not reported in 91.4 per cent of studies. Apart from the fact that this raises concerns regarding animal welfare, there is increasing evidence that analgesia provided by non-steroidal anti-inflammatory drugs influences anastomotic healing and should therefore be reported[26–29].

A trend was seen in improved reporting of randomization measures to reduce selection bias, but even in the most recent decade this information was absent in nearly 40 per cent of studies. For example, the creation of an intestinal anastomosis in the rat is influenced by a technical learning curve, supporting the need for proper randomization. The influence of surgical technique also emphasizes the relevance of blinding the surgeon to reduce performance bias. Blinding of surgery was reported in only 2.3 per cent of studies. Although of considerable importance when assessing leakage by macroscopic inspection, blinding of primary outcomes assessment was reported only occasionally (4.9 per cent). As the definition of leakage is a frequent subject of debate, and the severity of leakage may vary, macroscopic assessment is a measure easily subject to detection bias. Overall, the internal validity of studies was poor.

External validity is highly dependent on the model used[23]. Animal models used for anastomotic healing varied widely in terms of the animal species, factors that compromised healing, the intestinal segment used and the outcome measures[30]. Although a narrative review[31] in 2011 recommended mouse and porcine models, the present review shows that rat models have dominated the field since the 1990s.
The increase in porcine studies may reflect their use in minimally invasive surgery techniques and anastomotic devices, as porcine models were used heavily in articles on surgical approach, use of staplers, anastomotic rings and stents.

Concerning the choice of outcome measure, signs of anastomotic leakage would in theory be most valuable as primary outcome, because it is most relevant in clinical practice. Nevertheless, anastomotic complications were not reported in 424 articles (31.6 per cent) and were used to compare groups in only 266 papers (19.8 per cent). Although heterogeneity of animal models and experimental protocols may be useful to assess treatment efficacy under different circumstances, greater similarity between studies would allow for better assessment of external validity and better interpretation, comparison and meta-analysis[32].
The present review has a number of limitations. Only full-text articles were included. The exclusion of abstracts, conference presentations and unpublished experiments leads to an underestimation of the number of animal studies on intestinal anastomosis actually undertaken. The scoring system to assess the quality of reporting of study methodology was designed specifically for anastomosis studies. Items such as details of housing, drug doses, sample size calculation and statistical analyses were not collected. The inclusion of such items might have revealed even more reporting deficiencies. The score should therefore be interpreted as a summary of included items, rather than a conclusive judgement. Reported randomization in less than half of articles and negligible reporting of blinding suggest a high overall risk of bias. True risk of bias assessment, however, was considered impossible because of insufficient reporting of study methodology. Complete descriptions of sequence generation, allocation concealment and blinding methods were hardly reported. The inclusion of such items in the present scoring system would only have suggested a greater risk of bias. The same is likely to be true for other items commonly included in risk of bias assessments, such as attrition bias and selective outcome reporting[33].

To improve the quality and applicability of future studies on intestinal anastomosis, these experiments should be properly randomized and blinded whenever possible. Details on analgesia, anaesthesia, antibiotics, antiseptic measures, intestinal segment involved,

surgical technique and anastomotic complications should be essential data items. Choice of species and leakage model based on the research question is a prerequisite. Rats, for instance, are useful to study leakage, but not to study sealants or anastomotic technique, owing to small diameter.

Delphi consensus meetings and evidence appraisal of studies that might inform a clinical trial, as well as international platforms for multicentre preclinical studies, could all be used to maximize the likelihood of translating this work from bench to bedside[34–36]. Study of reproducibility and translational ability of different animal models is essential to select the strategies most likely to have an effect in patients, and to minimize unnecessary animal experimentation.

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Table S1 Search strategy

Pubmed search command (intestinal anastomosis + customized animal filter)
((anastomo*[tiab] OR surgical anastomosis[MeSH:noexp]) AND (bowel[tiab] OR intestine[tiab] OR intestinal[tiab] OR intestines[tiab] OR enteral[tiab] OR ileum[tiab] OR colon[tiab] OR colonic[tiab] OR colorectal[tiab] OR rectum[tiab] OR rectal[tiab] OR "Colon"[Mesh] OR "Intestines"[Mesh:noexp] OR "Ileum"[Mesh] OR "Rectum"[Mesh]) OR (ileoileost*[tiab] OR ileo-ileost*[tiab] OR ileoascend*[tiab] OR ileo-ascend*[tiab] OR ileotransvers*[tiab] OR ileo-transvers*[tiab] OR ileo-colostom*[tiab] OR ileocolostom*[tiab] OR colocolost*[tiab] OR colo-colost*[tiab] OR colorectostom*[tiab] OR colo-rectostom*[tiab]) OR "anastomotic leak"[MeSH]) **AND** (("animal experimentation"[MeSH Terms] OR "models, animal"[MeSH Terms] OR "invertebrates"[MeSH Terms] OR "Animals"[Mesh:noexp] OR "animal population groups"[MeSH Terms] OR "chordata"[MeSH Terms:noexp] OR "chordata, nonvertebrate"[MeSH Terms] OR "vertebrates"[MeSH Terms:noexp] OR "amphibians"[MeSH Terms] OR "birds"[MeSH Terms] OR "fishes"[MeSH Terms] OR "reptiles"[MeSH Terms] OR "mammals"[MeSH Terms:noexp] OR "primates"[MeSH Terms:noexp] OR "artiodactyla"[MeSH Terms] OR "carnivora"[MeSH Terms] OR "cetacea"[MeSH Terms] OR "chiroptera"[MeSH Terms] OR "elephants"[MeSH Terms] OR "hyraxes"[MeSH Terms] OR "insectivora"[MeSH Terms] OR "lagomorpha"[MeSH Terms] OR "marsupialia"[MeSH Terms] OR "monotremata"[MeSH Terms] OR "perissodactyla"[MeSH Terms] OR "rodentia"[MeSH Terms] OR "scandentia"[MeSH Terms] OR "sirenia"[MeSH Terms] OR "xenarthra"[MeSH Terms] OR "haplorhini"[MeSH Terms:noexp] OR "strepsirhini"[MeSH Terms] OR "platyrrhini"[MeSH Terms] OR "tarsii"[MeSH Terms] OR "catarrhini"[MeSH Terms:noexp] OR "cercopithecidae"[MeSH Terms] OR "hylobatidae"[MeSH Terms] OR "hominidae"[MeSH Terms:noexp] OR "gorilla gorilla"[MeSH Terms] OR "pan paniscus"[MeSH Terms] OR "pan troglodytes"[MeSH Terms] OR "pongo pygmaeus"[MeSH Terms]) OR OR ((animals[tiab] OR animal[tiab] OR mice[Tiab] OR mus[Tiab] OR mouse[Tiab] OR murine[Tiab] OR woodmouse[tiab] OR rats[Tiab] OR rat[Tiab] OR murinae[Tiab] OR muridae[Tiab] OR cottonrat[tiab] OR cottonrats[tiab] OR hamster[tiab] OR hamsters[tiab] OR cricetinae[tiab] OR rodentia[Tiab] OR rodent[Tiab] OR rodents[Tiab] OR pigs[Tiab] OR pig[Tiab] OR swine[tiab] OR swines[tiab] OR piglets[tiab] OR piglet[tiab] OR boar[tiab] OR boars[tiab] OR "sus scrofa"[tiab] OR ferrets[tiab] OR ferret[tiab] OR polecat[tiab] OR polecats[tiab] OR "mustela putorius"[tiab] OR "guinea pigs"[Tiab] OR "guinea pig"[Tiab] OR cavia[Tiab] OR callithrix[Tiab] OR marmoset[Tiab] OR marmosets[Tiab] OR cebuella[Tiab] OR hapale[Tiab] OR octodon[Tiab] OR chinchilla[Tiab] OR chinchillas[Tiab] OR gerbillinae[Tiab] OR gerbil[Tiab] OR gerbils[Tiab] OR jird[Tiab] OR jirds[Tiab] OR merione[Tiab] OR meriones[Tiab] OR rabbits[Tiab] OR rabbit[Tiab] OR hares[Tiab] OR hare[Tiab] OR diptera[Tiab] OR flies[Tiab] OR fly[Tiab] OR dipteral[Tiab] OR drosphila[Tiab] OR drosophilidae[Tiab] OR cats[Tiab] OR cat[Tiab] OR carus[Tiab] OR felis[Tiab] OR 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gorilla[Tiab] OR gorillas[Tiab] OR pongo[Tiab] OR pygmaeus[Tiab] OR "pongo pygmaeus"[Tiab] OR orangutans[Tiab] OR pygmaeus[Tiab] OR lemur[Tiab] OR lemurs[Tiab] OR lemuridae[Tiab] OR horse[Tiab] OR horses[Tiab] OR pongo[Tiab] OR equus[Tiab] OR cow[Tiab] OR calf[Tiab] OR bull[Tiab] OR chicken[Tiab] OR chickens[Tiab] OR gallus[Tiab] OR quail[Tiab] OR bird[Tiab] OR birds[Tiab] OR quails[Tiab] OR poultry[Tiab] OR poultries[Tiab] OR fowl[Tiab] OR fowls[Tiab] OR reptile[Tiab] OR reptilia[Tiab] OR reptiles[Tiab] OR snakes[Tiab] OR snake[Tiab] OR lizard[Tiab] OR lizards[Tiab] OR alligator[Tiab] OR alligators[Tiab] OR crocodile[Tiab] OR crocodiles[Tiab] OR turtle[Tiab] OR turtles[Tiab] OR amphibian[Tiab] OR amphibians[Tiab] OR amphibia[Tiab] OR frog[Tiab] OR frogs[Tiab] OR bombina[Tiab] OR salientia[Tiab] OR toad[Tiab] OR toads[Tiab] OR "epidalea calamita"[Tiab] OR salamander[Tiab] OR salamanders[Tiab] OR eel[Tiab] OR eels[Tiab] OR fish[Tiab] OR fishes[Tiab] OR pisces[Tiab] OR catfish[Tiab] OR catfishes[Tiab] OR siluriformes[Tiab] OR arius[Tiab] OR heteropneustes[Tiab] OR sheatfish[Tiab] OR perch[Tiab] OR perches[Tiab] OR percidae[Tiab] OR perca[Tiab] OR trout[Tiab] OR trouts[Tiab] OR char[Tiab] OR chars[Tiab] OR salvelinus[Tiab] OR "fathead minnow"[Tiab]

OR minnow[Tiab] OR cyprinidae[Tiab] OR carps[Tiab] OR carp[Tiab] OR zebrafish[Tiab] OR zebrafishes[Tiab] OR goldfish[Tiab] OR goldfishes[Tiab] OR guppy[Tiab] OR guppies[Tiab] OR chub[Tiab] OR chubs[Tiab] OR tinca[Tiab] OR barbels[Tiab] OR barbus[Tiab] OR pimephales[Tiab] OR promelas[Tiab] OR "poecilia reticulata"[Tiab] OR mullet[Tiab] OR mullets[Tiab] OR seahorse[Tiab] OR seahorses[Tiab] OR mugil curema[Tiab] OR atlantic cod[Tiab] OR shark[Tiab] OR sharks[Tiab] OR catshark[Tiab] OR anguilla[Tiab] OR salmonid[Tiab] OR salmonids[Tiab] OR whitefish[Tiab] OR whitefishes[Tiab] OR salmon[Tiab] OR salmons[Tiab] OR sole[Tiab] OR solea[Tiab] OR "sea lamprey"[Tiab] OR lamprey[Tiab] OR lampreys[Tiab] OR pumpkinseed[Tiab] OR sunfish[Tiab] OR sunfishes[Tiab] OR tilapia[Tiab] OR tilapias[Tiab] OR turbot[Tiab] OR turbots[Tiab] OR flatfish[Tiab] OR flatfishes[Tiab] OR sciuridae[Tiab] OR squirrel[Tiab] OR squirrels[Tiab] OR chipmunk[Tiab] OR chipmunks[Tiab] OR suslik[Tiab] OR susliks[Tiab] OR vole[Tiab] OR voles[Tiab] OR lemming[Tiab] OR lemmings[Tiab] OR muskrat[Tiab] OR muskrats[Tiab] OR lemmus[Tiab] OR otter[Tiab] OR otters[Tiab] OR marten[Tiab] OR martens[Tiab] OR martes[Tiab] OR weasel[Tiab] OR badger[Tiab] OR badgers[Tiab] OR ermine[Tiab] OR mink[Tiab] OR minks[Tiab] OR sable[Tiab] OR sables[Tiab] OR gulo[Tiab] OR gulos[Tiab] OR wolverine[Tiab] OR wolverines[Tiab] OR minks[Tiab] OR mustela[Tiab] OR llama[Tiab] OR llamas[Tiab] OR alpaca[Tiab] OR alpacas[Tiab] OR camelid[Tiab] OR camelids[Tiab] OR guanaco[Tiab] OR guanacos[Tiab] OR chiroptera[Tiab] OR chiropteras[Tiab] OR bat[Tiab] OR bats[Tiab] OR fox[Tiab] OR foxes[Tiab] OR iguana[Tiab] OR iguanas[Tiab] OR xenopus laevis[Tiab] OR parakeet[Tiab] OR parakeets[Tiab] OR parrot[Tiab] OR parrots[Tiab] OR donkey[Tiab] OR donkeys[Tiab] OR mule[Tiab] OR mules[Tiab] OR zebra[Tiab] OR zebras[Tiab] OR shrew[Tiab] OR shrews[Tiab] OR bison[Tiab] OR bisons[Tiab] OR buffalo[Tiab] OR buffaloes[Tiab] OR deer[Tiab] OR deers[Tiab] OR bear[Tiab] OR bears[Tiab] OR panda[Tiab] OR pandas[Tiab] OR "wild hog"[Tiab] OR "wild boar"[Tiab] OR fitchew[Tiab] OR fitch[Tiab] OR beaver[Tiab] OR beavers[Tiab] OR jerboa[Tiab] OR jerboas[Tiab] OR capybara[Tiab] OR capybaras[Tiab]) NOT medline[subset])

Ovid Embase search command (intestinal anastomosis + customized animal filter)

((anastomo*.ab,ti. AND ((bowel OR intestine OR intestinal OR intestines OR enteral OR ileum OR colon OR colonic OR colorectal OR rectum OR rectal).ab,ti. OR exp intestine/su)) OR (ileoileost* OR ileo-ileost* OR ileoascend* OR ileo-ascend* OR ileo-transvers* OR ileotransvers* OR ileocolostom* OR ileo-colostom* OR colo-colost* OR colocolostom* OR colo-rectostom* OR colorectostom*).ab,ti. OR exp Anastomosis leakage/ OR exp colon anastomosis/ OR exp colorectal anastomosis/ OR exp ileorectal anastomosis/)

AND

anser OR harrier OR circus pygargus OR red knot OR great knot OR calidris OR canutus OR godwit OR limosa OR lapponica OR meleagris OR gallopavo OR jackdaw OR corvus OR monedula OR ruff OR philomachus OR pugnax OR lapwing OR peewit OR plover OR vanellus OR swan OR cygnus OR columbianus OR bewickii OR gull OR chroicocephalus OR ridibundus OR albifrons OR great tit OR parus OR aythya OR fuligula OR streptopelia OR risoria OR spoonbill OR platalea OR leucorodia OR blackbird OR turdus OR merula OR blue tit OR cyanistes OR pigeon OR pigeons OR columba OR pintail OR anas OR starling OR sturnus OR owl OR athene noctua OR pochard OR ferina OR cockatiel OR nymphicus OR hollandicus OR skylark OR alauda OR tern OR sterna OR teal OR crecca OR oystercatcher OR haematopus OR ostralegus OR shrew OR shrews OR sorex OR araneus OR crocidura OR russula OR european mole OR talpa OR chiroptera OR bat OR bats OR eptesicus OR serotinus OR myotis OR dasycneme OR daubentonii OR pipistrelle OR pipistrellus OR cat OR cats OR felis OR catus OR feline OR dog OR dogs OR canis OR canine OR canines OR otter OR otters OR lutra OR badger OR badgers OR meles OR fitchew OR fitch OR foumart or foulmart OR ferrets OR ferret OR polecat OR polecats OR mustela OR putorius OR weasel OR weasels OR fox OR foxes OR vulpes OR common seal OR phoca OR vitulina OR grey seal OR halichoerus OR horse OR horses OR equus OR equine OR equidae OR donkey OR donkeys OR mule OR mules OR pig OR pigs OR swine OR swines OR hog OR hogs OR boar OR boars OR porcine OR piglet OR piglets OR sus OR scrofa OR llama OR llamas OR lama OR glama OR deer OR deers OR cervus OR elaphus OR cow OR cows OR bos taurus OR bos indicus OR bovine OR bull OR bulls OR cattle OR bison OR bisons OR sheep OR sheeps OR ovis aries OR ovine OR lamb OR lambs OR mouflon OR mouflons OR goat OR goats OR capra OR caprine OR chamois OR rupicapra OR leporidae OR lagomorpha OR lagomorph OR rabbit OR rabbits OR oryctolagus OR cuniculus OR laprine OR hares OR lepus OR rodentia OR rodent OR rodents OR murinae OR mouse OR mice OR mus OR musculus OR murine OR woodmouse OR apodemus OR rat OR rats OR rattus OR norvegicus OR guinea pig OR guinea pigs OR cavia OR porcellus OR hamster OR hamsters OR mesocricetus OR cricetulus OR cricetus OR gerbil OR gerbils OR jird OR jirds OR meriones OR unguiculatus OR jerboa OR jerboas OR jaculus OR chinchilla OR chinchillas OR beaver OR beavers OR castor fiber OR castor canadensis OR sciuridae OR squirrel OR squirrels OR sciurus OR chipmunk OR chipmunks OR marmot OR marmots OR marmota OR suslik OR susliks OR spermophilus OR

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Table S2 Specification of research topics of therapeutic trials

Therapeutic category	No. of articles*	Studied therapies (no. of studies addressing subject*)
Pharmacologically active peptides, cytokines, etc.	61	Activated protein C (2), adenosine triphosphate (1), adrenomedulline (1), amelogenin (1), aminoguanidine (1), antithrombin III (2), β-aminopropionitrile intraperitoneally or orally (1), β-D-glucan (5), caffeic acid phenethyl ester (3), CGRP (2), carnitine (1), catalase (1), coagulation factor XIII (1), enterosan (2), flavonoids (1), glucagon-like peptides (1), MMP inhibitors (AG3340 (1), BB-94 (1), AZD3342 (1), BB-1101 (1), BB-16627 (1), GM6001 (1), kallikrein–plasmin inhibitor S-2441 (1), nafamostat mesylate (2), soybean trypsin inhibitor (2), TIMP (2), tiopronin (1), trasylol (2)), neurokinin 1 receptor antagonist (1), neurotensin (1), pancreatic polypeptides (1), parthenolide (1), pentadecapeptide BPC 157 (3), poly-L-lysine and poly-L-glutamate (2), prostaglandin E1 (2) and E2 (1), RGTA11 (1), ribose–cysteine (1), S-methylisothiourea hemisulphate (2), substance P (1), taurolidine (1)
Antioxidants	17	Allopurinol (6), aprotinin (4), diosmine–hesperidine (1), N-(3-(aminomethyl)benzyl)acetamidine (1400 W) (1), superoxide dismutase (6)
Vasodilators	18	Bosentan (2), nitroglycerine (1), papaverine (1), perflurane (1), prostacyclin analogues (iloprost, 6; other, 2), sildenafil (3), talafadil (1), trapidil (1)
Other drugs	32	Acetylcysteine (2), amifostine (1), famotidine (1), flurbiprofen (1), LMWH (1), ketotifen (1), lanreotide (1), lisinopril (1), mesalamine (1), montelukast (1), neostigmine (2), nifedipine (2), octreotide (2), pentoxifylline (6), phenytoin (1), piroxicam (2), propolis (2), ranitidine (2), simvastatin (2), tacrolimus (1), tranexamic acid (1), vinpocetine (1)

Growth factors	33	EGF (enterally, 1; in gelatine sponge, 1; topically, 1), FGF-2 (1), ghrelin (1), G-CSF systemically (1), GM-CSF local (8), IGF-1 systemically (2), IGF intraperitoneally (3), IGF coated sutures (2), KGF intraperitoneally (2), KGF systemically (1), leptin intraperitoneally (1), PDGF (1), platelet-rich plasma (4), TGF-β adenovirus-mediated gene transfer (1), VEGF locally (1), VEGF/FGF-mediated gene therapy (1)
Hormones	33	Anabolic steroids (2), dopamine (1), erythropoietin (6), oestrogen (1), growth hormone (18), melatonin (3), placental lactogenic hormone (1), progesterone (1), somatomammogenic chorionic hormone (1), thyroid stimulating hormone (1)
Antibiotic strategies	36	Amikacin (1), antibacterial sutures (4), antibiotic lavage (3), cephalosporins intramuscularly (1) or intraperitoneally (1), chlorhexidine intraperitoneally (1), doxycycline (1), imipenem (1), iodine irrigation (4), iodine topically (1), gentamicin (sponge, 1; topically/with fibrin glue, 4; systemically, 2), kanamycin + chloramphenicol (1), kanamycin + cephalotin intramuscularly (1), levamisole (2), metronidazole (2), neomycin orally (2), noxythiolin topically (2), penicillin/streptomycin/chlorostrep (chloramphenicol and dihydrostreptomycin) (1), penicillin orally (1)
Fatty acids	12	Short-chain fatty acids (mostly butyrate) (enema, 6; orally/intravenously, 9)
Amino acids	19	Arginine (6), branched-chain amino acid mix (1), glutamine (orally, intravenously or lavage) (14), glycine (1), methionine (1), other (1)
Vitamins, minerals	15	Vitamin A/retinoic acid (6), vitamin B5 (1), vitamin C (5), zinc (4)
Nutrition	14	Glucose (1), kefir (1), lactulose (1), low-residue diet (2), probiotics (3), whey (1), hyperalimentation (4), ketone bodies (1)
Organics	21	Algae (1), aloe vera (1), Aroeira do sertäo (timber tree) (2), Brazilian pepper tree extract (Schinus terebinthifolius Raddi) (1), castor oil (1), copaiba oil (1), ethyl pyruvaat (2), gingko bilboa extract Egb 761 (2), guar gum (2), honey (3), Jatropha gossypiifolia L. (bellyache bush) (1), Passiflora edulis extract (1), proanthocyanidins (Pycnogenol®; Horphag Research, Geneva, Switzerland) (1), reversatrol (2), shark cartilage orally (1), oxidized cellulose (1)
Non-solids	31	Electromagnetic field stimulation (8), extracorporeal shockwave therapy (1), hyperbaric oxygen (10), hyperoxygenated solution lavage (1), laser stimulation (7), positively charged diethylaminoethyl cross-linked dextran bead particles (1), other oxygen supplementation (2), ozone gas (1)

Other	27	Adipose stem cells (local injection, 1; suture coating, 2), Ankaferd BloodStopper® (Ankaferd Drug Inc., Istanbul, Turkey) (1), bone marrow-derived mesenchymal stem cells (2), bupivacaine intraperitoneally (1), cartilage bone marrow extract intramuscularly (1), chitosan (1), cholera toxin (1), interleukin 2 (1), intraoperative lavage (2), ischaemic preconditioning (locally, 3; remotely, 2), methylene blue (1), pancreatic islet transplantation (2), peritoneal lavage (1), pyrrolidine dithiocarbamate (2), taurocholate (1), TNF immunization (1), WR-2721 and RibCys radioprotective pretreatment (1)
		Technique
Suture technique	88	Number of suture layers (e.g. single versus double) (52), suture distance (3), suture pattern (e.g. interrupted versus continuous) (8), suture tension (2), suture traject (e.g. Gambee versus Lembert) (19), other (7)
Staplers	69	Stapling devices and techniques (39), staples versus sutures (30)
Apposition	73	Inversion versus eversion (28), invagination (24), opposition (6), orientation (e.g. end–end versus side–side) (16)
Suture material	34	e.g. cotton, silk, (chromic) catgut, polyglactin, polyglycolic acid, polydioxanone, polypropylene, ophthalmic tendons, collagen, stainless steel, Teflon
Other	17	Cutting with knife versus diathermy (4), laser (6) or LigaSure™ (Covidien, Mansfield, Massachusetts, USA) (1), microsurgical techniques (3), early suture removal (2), donut technique (1)
		Experimental technique
Anastomotic devices	41	Biofragmentable ring (14), compression ring (17), magnetic rings (8), other (2)
Glued anastomosis	35	Cyanoacrylate (17), fibrin glue (11), other (8)
Laser anastomosis	13	Carbon dioxide laser (5), KTP laser (1), Nd : YAG laser (7), other (3)
Other	7	Facilitated by prosthesis (e.g. sucrose) (2), jejunal interposition (1), radiofrequency welding (2), other sutureless technique (2)
		External sealing
Biological sealant	35	Amniotic membrane (3), albumin sealant (2), pericardium (2), collagen fleece (6), dura mater (1), gastric seromuscular flap (1), ileal patch (1), intestinal seromuscular flap (1), omentum (10), peritoneum (8), small intestinal submucosa (3), pericardial/ collagen staple buttress (2)
Glue	65	Acrylates (13), cyanoacrylate-coated sutures (1), fibrin glue (42), formaldehyde (1), gelatine adhesive (2), glycerinaldehyde (1), glycolaldehyde (1), polyethylene glycol sealant (1), polyurethane adhesive(2), other (5)

Other	21	Absorbable tape (e.g. Tissuepatch™; Tissuemed, Leeds, UK) (2), AlloDerm® buttress (LifeCell, Bridgewater, New Jersey, USA) (3), Dacron mesh (1), fibrin/thrombin patch (e.g. TachoSil®; Baxter, Deerfield, Illinois, USA) (6), photopolymerized hydrogel (1), polyglactin mesh (4), polyglycolic acid mesh (2), polypropylene mesh (2), polyurethane foam (1), other plastic (2)
		Internal sealing
All	27	Casein prosthesis (1), collagen (2), decalcified bone tube (2), fibrin glue (2), latex tube (5), polychlorovinyl (1), polyethylene glycol-based (2), polyester stent (1), polyglycolic acid (2), polyurethane (1), porcine submucosa (1), silicone (3), sucrose (1), vinyl (1), other degradable stents (8)
		Surgical approach
Laparoscopy	22	Laparoscopic feasibility studies (22)
NOTES	24	Transanal (20), transgastric (6), transvaginal (1) (includes pure NOTES or combined with laparoscopy)
Other	7	Single-incision laparoscopic surgery (5), single-incision robotic surgery (1), other robotic surgery (2)
		Other
Feeding regimens	19	Early postoperative enteral feeding (7), fasting (1) , liquid diet (2), parenteral (versus enteral) feeding regimens (9), other (1)
Ostomies	12	Diverting colostomy (12)
Viability assessment before anastomosis	10	Bioimpedance measurement (1), Doppler ultrasonography (3), electromyography (3), fluorometry (2), intramural pH (1), pulse oximetry (1), visible light spectroscopy (1)
Perioperative fluid management	8	e.g. colloids versus crystalloids (2), autologous blood (1), human albumin (1), hydroxyethyl starch (4), hypertonic saline (1), lactated Ringer's solution (1), volumes (2)
Other	8	Bowel acidification (1), externalization of anastomosis (1), mechanical bowel preparation (4), splenectomy (1), subcutaneous anastomosis (1)

CGRP, calcitonin gene-related peptide; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; RGTA11, regenerating agent 11 (dextran derivative with heparin-like properties); LMWH, low molecular weight heparin; EGF, epidermal growth factor; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating growth factor; GM-CSF;– granulocyte–macrophage colony stimulating growth factor; IGF, insulin-like growth factor; KGF, keratinocyte growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; TNF, tumour necrosis factor; KTP, potassium titanyl phosphate; Nd : YAG, neodymium : yttrium–aluminium–garnet; NOTES, natural-orifice transluminal endoscopic surgery.

Table S3 Specification of research topics of risk factor identification studies

Risk factor category	No. of articles	Studied risk factors (no. of studies addressing subject*)
Chemotherapy, hormonal therapy and other adjuvants	88	5-FU (monotherapy,25; with bleomycin + cisplatin, 5; folinic acid, 3; interferon α, 4; irinotecan, 1; levamisole, 1; leucovorin, 2; mitomycin C, 1; oxaliplatin, 2), angiogenesis inhibitor FR-118487 (1), angiostatin (2), bevacizumab (2), capecitabine (1), carboplatinum (1), cisplatin (6), HIPEC (3), local hyperthermia (1), mitomycin/doxorubicin (7), methothrexate (1), MMP inhibitor BB-94 (1), mycophenolate mofetil (2), oxaliplatin (4), paclitaxel (intravenously, 3; lavage, 1), photodynamic therapy (4), propranolol + etodolac (1), sirolimus (1), suramin (1), tamoxifen (1), thalidomide (1), tegafur (1), TNP-470 (1)
Radiotherapy	45	Chemoradiation (6), radiotherapy (preop., 34; intraop., 6; postop., 1)
Ischaemia	33	Ischaemia/hypoperfusion (18), transient ischaemia/ischaemia–reperfusion injury (9), hypovolaemia (6)
Antiadhesives	37	Acetylcysteine (2), ancrod (1), bioactive polypeptides (2), chitosan and/or dextran (4), gelatine film (1), hyaluronic acid and/or carboxymethylcellulose (10), icodextrin (3), lornoxicam (1), mitogen-activated protein kinase-activated protein kinase 2 inhibitor (1), phosphatidylcholine (1), polyvinyl alcohol (2), PXL01 (1), Seprafilm® (Sanofi-Aventis, Bridgewater, New Jersey, USA) (1), sunitinib (1), tetrachlorodecaoxide (1), tissue plasminogen activator (3), trasylol (3)
Immuno-suppressants	32	Adalimumab (1), azathioprine (2), corticosteroids (18), everolimus/tacrolimus (9), macrophage inhibitor (1), montelukast (1), promethazine (1), thalidomide (1)
Infection	29	Diverticulitis (1), peritonitis or bacterial contamination (22), Pseudomonas aeruginosa (with enhanced virulence) (1), Staphylococcus aureus contamination (1), sepsis (4)
NSAIDs	18	Carprofen (2), celecoxib (1), diclofenac (6), etofenamate (1), flurbiprofen (1), naproxen (1), piroxicam (2), rofecoxib (1), tenoxicam (1), valdecoxib (2)
Other analgesics	7	Epidural (5), metamizole (1), paracetamol (1)
Co-morbidity and patient factors	26	Colitis (2), diabetes and/or hyperglycaemia (8), ethanol (2), hypercholesterolaemia (1), hypertension (1), hypothyroidism (2), liver cirrhosis (1), renal failure and/or uraemia (2), old age (7)
Prokinetics	17	Bromopride (4), cisapride (1), CPSI-2364 (1), metoclopramide (4), neostigmine or pyridostigmine (3), other cholinergic drugs (3), sennatin (1)
Nutrition	13	Essential fatty acid deficiency (1), malnutrition/protein deficiency (9), mineral/zinc deficiency (2), vitamin A deficiency (1)

Other	70	ACTH suppletion (1), anastomotic location (high/low) (1), barium (1), blood presence (2), blood transfusion (2), buscopan (1), colostomy (3), colostomy closure (2), dehydration (1), extraperitoneal placement (1), faecal loading and obstruction (5), foreign material and drains (6), haemodilution + crystalloid overload (3), hyperbilirubinaemia (4), humidity (1), hypothermia (2), LMWH or prothrombin reduction (acenocoumarol) (4), laparoscopy (1), lymphostasis (2), malignant cells on anastomotic line (2), mechanical bowel preparation (1), microflora disturbance (1), negative-pressure therapy (1), octreotide (4), omentectomy (1), pinealectomy (1), pneumoperitoneum (9), polyethylene glycol/butyrate enema (1), portal triad occlusion (1), postoperative coloscopy (1), rectal manipulation (1), repeated laparotomy (1), simultaneous liver resection (2), splenectomy (2), statins (1), synchronous anastomosis (1), total colectomy (1), trauma (1)

5-FU, 5-fluorouracil; HIPEC, hyperthermic intraperitoneal chemotherapy; MMP, matrix metalloproteinase; NSAID, non-steroidal anti-inflammatory drug; ACTH, adrenocorticotrophic hormone; LMWH, low molecular weight heparin.

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4.

DICLOFENAC CAUSES ANASTOMOTIC LEAKAGE IN THE PROXIMAL COLON BUT NOT IN THE DISTAL COLON OF THE RAT

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Abstract

Background

Nonsteroidal anti-inflammatory drugs have been associated with anastomotic leakage. It was studied if diclofenac affects anastomoses differently depending on the location in the gut.

Methods

Ninety-five rats were randomized to 6 groups with an anastomosis in either ileum(IL), proximal colon (PC) or distal colon (DC). Groups IL+ (n=10), PC+ (n=30) and DC+ (n=10) received diclofenac (3mg/kg/day) from day 0 until sacrifice on day 3. Group PC- (n=15) did not receive diclofenac. Groups PC1+ and PC2+ (n=15 each) were given diclofenac from day 1-4 and from day 2-5.

Results

Leak rates were 10/10 in group IL+, 22/30 in PC+, 1/10 in DC+ and 1/15 in PC-. Delayed administration of diclofenac by one or two days (6/15, p=0.05) resulted in reduced leakage rates. Mechanical strength results corresponded with leak rates.

Conclusion

Diclofenac causes leakage of anastomoses in rat ileum and proximal colon, but not in the distal colon. This suggests a role for the ileal and proximal colonic content in diclofenac induced leakage.

Introduction

Leakage is the most serious complication of surgeries that include the construction of intestinal anastomoses and is associated with a mortality between 6% and 15%. [1] Leakage occurs in 3% to 14% of all intestinal anastomoses, a percentage that has not significantly changed in the past 2 decades [1-3]. Although the highest rates have been reported for the most distal rectal anastomoses, ileocolonic anastomoses also may leak in up to 10 percent of cases [4]. The persistence of high leakage rates is indicative of insufficient understanding of leakage etiology and urges for improving knowledge of factors influencing anastomotic healing.

In 3 retrospective studies and a recent review of randomized clinical trials the use of nonsteroidal anti-inflammatory drugs (NSAIDs) was associated with increased rates of anastomotic leakage[4-7]. In rat studies, NSAIDs with selectivity for COX-2 (carprofen, diclofenac and celecoxib) have repeatedly shown to cause leakage of ileal, but not of distal colonic anastomoses[8-10]. Despite these data NSAIDs are listed as perioperative analgesia for gastrointestinal surgery in present international guidelines and are recommended in fast track surgery[11]. More evidence is necessary to better understand the pathological mechanisms involved and, possibly, to adjust postoperative pain treatment recommendations [12, 13].
Two hypotheses for a detrimental effect of NSAIDs have been raised. First is the general suppressive effect of COX-2 inhibition on the principal inflammatory and proliferative activity in the early phase of wound healing[4, 8]. Second is the formation of microthrombi by COX-2 inhibition inducing ischemia[5]. So far, evidence supporting either hypothesis and at the same time explaining the difference between ileum (IL) and distal colon (DC) is lacking. In fact, other mechanisms than a direct effect of COX-2 inhibition on wound healing may play a role.
Previous studies have shown that COX-2 inhibitors cause leakage of ileal but not distal colonic anastomosis in rats[9, 10]. A possible explanation is that the rat DC is thicker and stronger than the IL and thus more resistant to leakage. However, IL and DC also differ in fecal content with respect to liquidity, microflora and bile acids. A good way to study the relevance of the gut content is to investigate the effect of NSAID on anastomotic healing of the proximal colon (PC), where gut morphology is more similar to the DC, but intraluminal content corresponds to the distal IL. This study investigates the effects of diclofenac on early anastomotic healing in the PC of the rat.

Material and methods

Ethics

This experiment was conducted according to the Dutch “Experiments on Animals Act” and European Federation of Laboratory Animal Science Associations (FELASA) guidelines and was approved by the institutional Animal Ethics Committee of the Radboud University. After the start of the experiment, the animals were inspected twice daily for signs of reduced wellbeing, including dirty nose, dirty eyes, piloerection, aberrant behavior, distended abdomen or diarrhea. Animals were weighed daily. Humane endpoints were defined to avoid unnecessary suffering of animals during the study period.

Animals

Adult male Wistar rats (Harlan, Horst, The Netherlands) were accustomed to laboratory conditions for 1 week and weighed 309 (SD±24) g at the start of the experiment. The rats were housed 2 per cage at 22-23°C with a 12 hour day cycle and had free access to standard rodent chow (Ssniff R/M-H, Bio Services BV, Uden, Netherlands) and acidified tap water. The cages were enriched with a shelter and nesting material and were randomly placed on the shelves.

Groups

Ninety-five rats were randomly assigned to one of the 6 groups (Figure 1). Rats in group PC- (n=15) served as control group and were subjected to a PC anastomosis without receiving diclofenac. An anastomosis was made in the IL in group IL+ (positive control; n=10), in the DC in group DC+ (negative control; n=10), and in the PC in group PC+ (n=30), all receiving diclofenac for 3 days with the first dose given 15 minutes before operation. Rats in groups PC1+ (n=15) and PC2+ (n=15) had a PC anastomosis and also received diclofenac for three days but with the first dose delayed one or two days after operation, respectively.

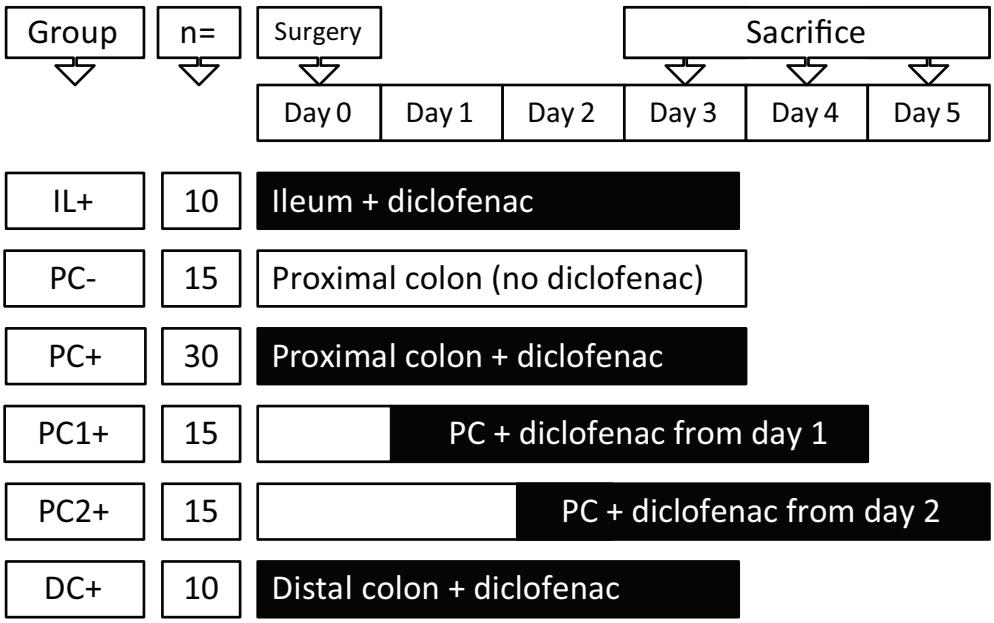


Figure 1 Experimental setup. PC = proximal colon.

Intervention and surgical technique

The rats were anesthetized by inhalation of 3% isoflurane (Abbott, Hoofddorp, The Netherlands) mixed with pressurized air and oxygen. They were shaved and operated under sterile conditions with the aid of an operation microscope at 10x magnification.

Through a 3 cm midline laparotomy, a 1 cm resection was performed of either the IL (15cm proximal to the cecum), the PC (2cm from the ileocecal transition) or the DC (3 cm proximal to the peritoneal reflection) and continuity was restored with an inverted single layer anastomosis made by 8 interrupted 8-0 Ethilon (Ethicon, Norderstedt, Germany) sutures. Precautions were taken preventing leakage of intestinal content into the abdominal cavity. During the operation the intestine was covered with gauzes soaked in 0.9%NaCl to minimize desiccation. Body temperature was kept at 38°C with a heating pad and lamp. The abdominal wall was closed with a running suture (Vircryl 3-0; Ethicon, Norderstedt, Germany) and the skin with staples. Immediately postoperatively 10 ml of 0.9% NaCl was administered subcutaneously for rehydration. For analgesia buprenorphine (Schering Plough, Houten, The Netherlands) 0.02mg/kg was given subcutaneously every 12 hours, starting 15 minutes before the operation and ending 48 hours later. Diclofenac sodium (Cayman Chemical Company Ann Arbor, MI, USA) was dissolved in 0.1% polysorbate in saline and was given in a dose of 3 mg/kg/day (in 2 equal doses) by oral gavage for 3 days.

Outcome assessment

On postoperative day 3, animals from groups PC+, PC-, IL+ and DC+ were euthanized by CO2 asphyxiation. For groups PC1+ and PC2+ this was on day 4 and 5, respectively. A relaparotomy was performed and the anastomosis and abdomen were inspected for signs of anastomotic leakage. The inspection was done by 2 researchers (S.Y. and R.L.) independently and any differences in scores were solved by consensus. The severity of macroscopic signs was scored as “0” for no signs of leakage, “1” for anastomotic abscesses, “2” for free pus or large abscesses and “3” for fecal peritonitis or visible dehiscence.

Mechanical strength

Anastomoses of all surviving rats were tested for mechanical strength, except for 3 animals from the PC-, PC+, PC1+ and PC2+ groups, which were used for immunohistological analysis. The anastomotic bowel parts were carefully resected with 2 cm of bowel on each side and any scar tissue or adhesions covering the anastomosis were left in place. These segments were infused with water containing methylene blue at 2 mL/min to determine the strength of the weakest spot within the anastomosis. The maximum pressure (mmHg) just before the sudden loss of pressure in case of failure was recorded as “bursting pressure”. Dehiscent anastomoses were scored as “0 mmHg”.

After bursting pressure measurements the segments were attached to a tensiometer and pulled apart at 3 cm/minute to determine the maximal suture holding capacity or breaking strength (Newton)14.

Immunohistochemistry

Anastomotic segments of approximately 1 cm were opened at the mesenterial side. After gentle washing with saline the samples were fixed in 4% buffered formaldehyde. Samples were embedded in paraffin and subsequently cut in 4 µm sections. The samples were dewaxed and endogenous peroxidase activity was blocked with 3% H2O2 in phosphate buffered saline. Antigens were retrieved by boiling the sections in a citrate-buffered solution (pH=6). After preincubation with 20% normal goat serum, the sections were incubated overnight with a 1:1,000 dilution of COX-2 antibody (rabbit polyclonal antiserum against murine COX-2, Cayman Chemical, An Arbor, MI, USA). Subsequently, sections were incubated with a 1:200 dilution of goat anti-rabbit biotynilated secondary antibody. Following incubation with avidin–biotin complex, protein was visualized with diaminobenzidine. Sections were counterstained with hematoxylin.

Statistics

The required sample size was determined to detect a difference of 50% in leakage rate, compared with expected leakage percentages of 70% in the positive (group IL+) and 10% in the negative control group (group DC+)9. Anticipating an analysis with Fisher's exact test for leakage rates and one-way Analysis of variance with post-hoc Tukey's for strength measures, this resulted in 12 animals per group when using an α of 0.025 (Bonferoni correction for 3 groups) and a β of 0.8. Because 3 animals of the PC groups were used for histology, total numbers for groups PC-, PC1+ and PC2+ were 15 each. Because the PC+ group was used to compare in terms of both anastomotic location (with groups IL+ and DC+) and timing of administration (with groups PC-, PC1+, PC2+) we chose to double the number of animals in this group to 30 to provide sufficient proportional data. Aiming at reducing animals for experiments in general we chose to lower the number of animals in groups DC+ and IL+ to 10, because previous experiments within our department showed very consistent leak rates for these anastomoses9. The spread of data is presented as standard errors of the mean. A P-value less than 0.05 was considered statistically significant.

Results

Animal welfare and mortality

One animal in the PC- group died on day 2 from an unknown cause, without signs of anastomotic leakage. Two animals in the IL+ group died on day 2 from generalized peritonitis caused by leakage. These rats were not used for strength testing. Fourteen rats (IL+: 6, PC+: 2, PC1+: 5, PC2+: 1) were sick on day 3 as reflected by a dirty nose, dirty eyes, pillow erection, distended abdomen or diarrhea. None of these rats reached the humane endpoint necessitating premature removal from the experiment. All other rats recovered well. The overall average weight loss on day 3 was 9.3% (± 3.7%) without differences between groups.

Anastomotic leakage

One abscess was observed in the PC- group (7%). Macroscopic signs of leakage were present in 22 of the 30 (73%) rats with a proximal colonic anastomosis and administration of diclofenac from day 0 (PC+). This rate was significantly (p=0.001) higher than the 1/10 (10%) leak rate in the DC+ group (Figure 2). All 10 anastomoses in the IL+ group leaked. This leak rate did not differ from the PC+ group (p=0.165), but the severity of the leak signs was significantly higher in the IL+ group (2.8±0.6 versus 1.7±0.5; p=0.004). Delay of the first administration of diclofenac by one or two days resulted in a gradual reduction in leak rates, 10/15 (66 percent) and 6/15 (40 percent) in the PC1+ and PC2+ group, respectively. Also the leak severity score declined from 1.7±0.5 to 1.3±0.3 and 0.5±0.2, respectively (Figure 2).

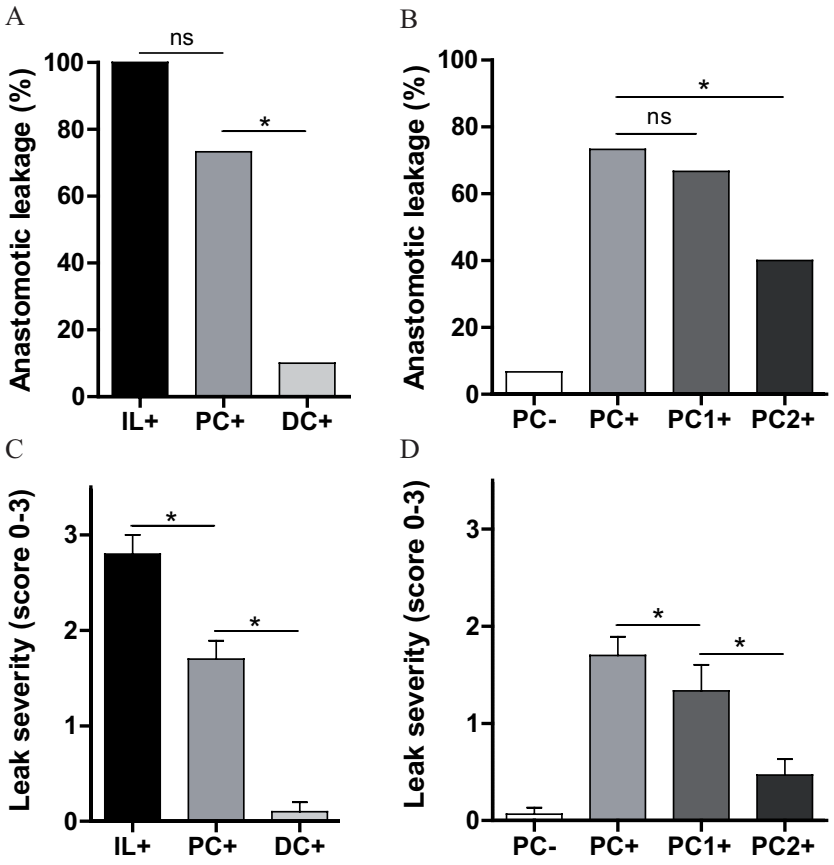


Figure 2 Anastomotic leakage.

Bars represent average leakage rates (A and B) or leak severity score (C and D). Anastomoses were constructed in ileum (IL), proximal colon (PC) or distal colon (DC) and animals were sacrificed on day 3. Animals in the 1+/2+ groups received diclofenac from day 1 or 2 and were sacrificed at day 4 or 5, respectively. * = p<0.05. , – = without diclofenac, + = with diclofenac.

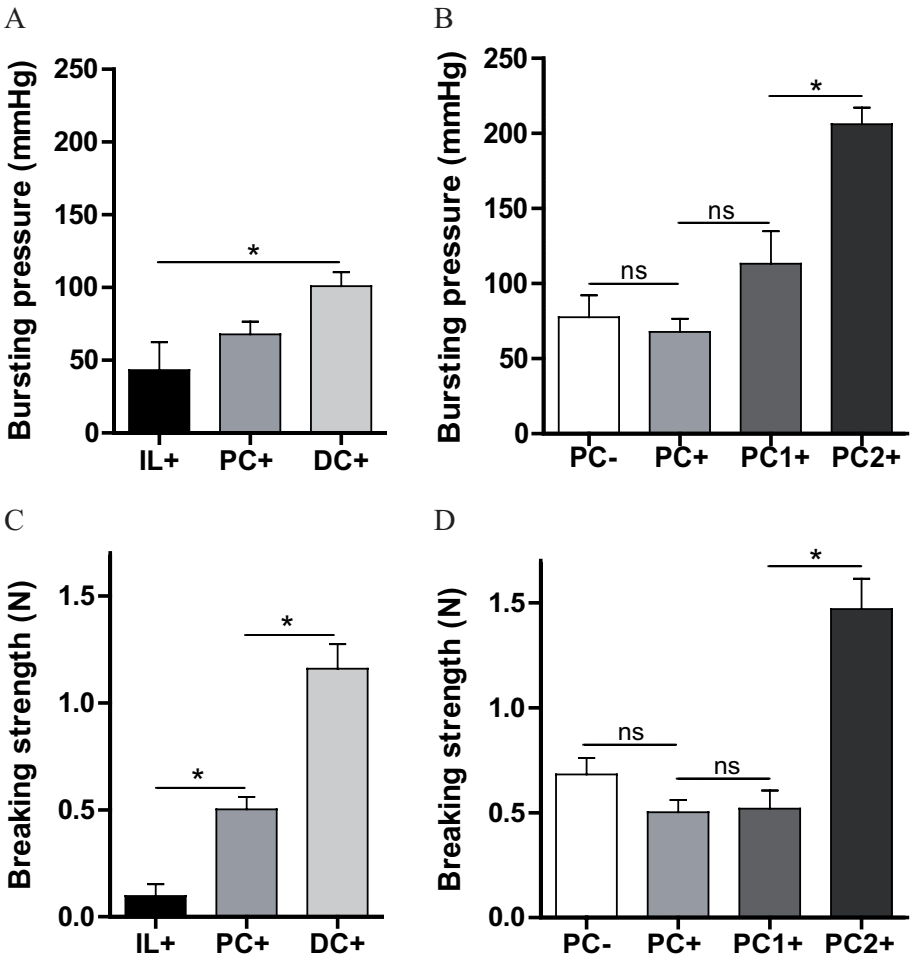


Figure 3 Bursting pressure (A+B) and breaking strength (C+D) per group. Error bars represent SEM. *= p<0.05. 1+/2+ = diclofenac from day 1 or 2. * = p<0.05. , – = without diclofenac, + = with diclofenac. ns = nonsignificant; SEM = standard error of the mean.

Anastomotic strength

In one rat of the PC- group and the PC+ group it was impossible to excise the anastomotic segment because of extensive adhesions and peritonitis; neither was used for strength assessment. The bursting pressure in the PC+ group was 68±9 mmHg, in the IL+ group 43±19 mmHg (p=0.356) and in the DC+ group 101±31 mmHg (Figure 3). Only the difference between the IL+ and DC+ groups was significant (p=0.023). There was no difference between the PC+ and PC- groups. Bursting pressure in group PC1+, sacrificed at day 4 was 113±22 mmHg (PC+ 68±9 mmHg; p=0.065). Bursting pressure in group PC2+, sacrificed at day 5 was

206±11mmHg (compared with PC+: p=0.000).
In line with the bursting pressures, the breaking strength of the PC+ group (0.50±0.06 N) was higher than of the IL+ group (0.10±0.05 N; p=0.004), but lower than of the DC+ group (1.16±0.11 N; p=0.000) (Figure 3). The breaking strength of the PC+ group was not significantly different from the PC- group (0.68±0.08 N; p=0.459). Breaking strength of group PC1+ was 0.52±0.09 N (p=0.999) and of group PC2+ 1.47±0.14 (p=0.000).

Immunohistochemistry for COX-2

COX-2 immunoreactivity was clearly present at the anastomotic sites in the PC, independent of the administration of diclofenac (Figure 4). Because of the many leaking anastomoses in group PC+, there were insufficient representative samples to allow quantitative analyses.

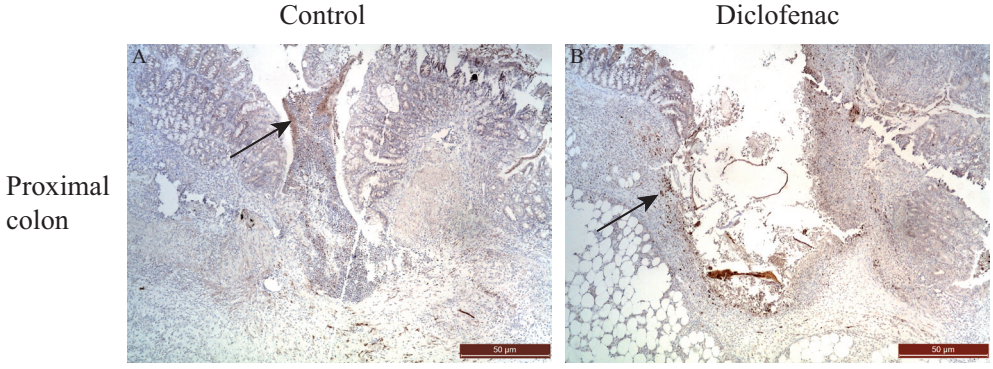


Figure 4 Immunohistochemical staining for COX2 presence (dark brown; arrows) at an unaffected anastomosis (A; group PC-) and an ulcerative anastomosis with fecal fibers and necrotic debris in it (B; group PC+).

Discussion

Diclofenac compromises anastomotic healing of the PC resulting in leakage with a severity approximating that observed in the ileal anastomosis. The high leakage rates in the IL and the low rates in the DC are comparable with those found previously[9, 10]. Incidence of leakage in the PC and leak severity are lower when administration of diclofenac is delayed. The results from this experiment show that, in the large bowel, diclofenac only affects the healing of the PC anastomosis, despite the morphological and functional similarities between the proximal and distal colon wall. This finding, together with the similar leakage rates observed in proximal colonic and ileal anastomoses, suggests that the fecal content in these bowel parts is relevant in the mechanism leading to leakage caused by diclofenac. Two hypotheses may explain the role of fecal content in diclofenac-associated leakage. First, diclofenac affects healing similarly in proximal and distal bowel segments (eg. by COX-2 inhibition), but this only leads to leakage in the proximal part because of the fluidity of the fecal content. However, the fluidity of feces as a denominator of leakage is not in line with human data

where the small bowel is at a lower risk of leakage than large bowel[15]. Second hypothesis is that diclofenac directly alters the composition of the bowel content leading to a more hostile intraluminal environment. Two such mechanisms have been described and include an increase in bile toxicity and an increase in microbial virulence caused by NSAIDs[16, 17].
Because most bile products are reabsorbed or converted before reaching the DC, the IL and PC are exposed to different bile products compared with the DC18. Like many NSAIDs, diclofenac is largely excreted through bile[19]. Both the changed bile salt composition and the metabolites of NSAIDs in bile may cause small intestinal damage[16, 20-22]. Likewise they may jeopardize anastomotic healing. The difference in metabolism between celecoxib and carprofen on one hand, and naproxen on the other hand supports the hypothesis that altered bile affects healing. The first 2 drugs, which cause about 85% leakage of experimental ileal anastomoses, are predominantly excreted through bile [23, 24]. Naproxen, causing less than 15% leakage, is for 98% excreted via the urine in both rat and human[9, 25, 26]. Future studies should further elucidate the role of bile in NSAID induced leakage, possibly by using bile binding agents or bile exchange techniques.

The terminal IL and PC host a different set of microbes than the DC.[27] It has been shown that minor changes in microbial composition and virulence may result in leakage.[28, 29] NSAID treatment can affect the gut's microflora by reducing the number of lactobacilli and diclofenac was shown to increase the total number of enteral gram negative bacteria resulting in increased mucosal permeability.[17, 30] Such alterations may differ along the gut and could play a role in the etiology of NSAID-induced leakage31.

Reduced leakage rates after delayed administration of diclofenac indicate that the detrimental effects of NSAIDs become evident already in the early stage of healing, on the first and second day after operation, in the inflammatory phase of the repair sequence. This finding supports both the hypotheses that healing is affected by COX-2 inhibition and that healing is affected by altered intraluminal content, because both COX-2 inhibition and change in intraluminal content virulence occur very rapidly after injury[17, 19]. The increased strength of anastomoses in group PC1+ and PC2+ is explained by the later sacrifice on day 4 and 5 respectively. In time, bursting pressure and breaking strength values increase and become stronger than the strengths of the intact bowel, reflected by bursting outside the anastomosis at day 5 and thereafter.

We studied diclofenac in a valid and reproducible animal model showing differences in a relevant clinical outcome, anastomotic leak. The relevance of mechanical strength tests in our study is debatable. Bursting pressure and breaking strength tests are the best available proxies for assessing healing alterations in intact anastomoses, but are confounded when an anastomosis leaks. Bursting pressures of an anastomosis with a moderate leak can be relatively high because of the surrounding adhesion formation as response to the leak. In our study mechanical strength data varied substantially and showed fewer differences between groups in comparison with the leak rate data. Because the intrinsic strength of intact IL and colon is different, a direct comparison of strength between various bowel parts is of limited value. Groups with an IL or DC anastomosis without diclofenac were not included. Thus, within the current experiment, the data available are insufficient to determine the specific quantitative effect of diclofenac on the strength of anastomoses in IL and DC.

The majority of animal studies on anastomotic healing address healing of the DC. In this segment no or only limited effects of NSAIDs were found[32]. In this study 2 colon locations were used for comparison of anastomotic leakage with completely different outcomes. Because the choice of the intestinal segment can significantly confound experimental outcomes, future experiments investigating the effect of drugs or biomaterials on anastomotic healing should preferably encompass both the PC and DC and not automatically focus on the latter which is most at risk in the human situation. Moreover, the advantage of the PC is that it is easier to induce leakage compared with the DC, where one has to rely on strength tests.

COX-2 immunoreactivity was found in the PC anastomoses irrespective of diclofenac administration. In previous experiments we also found COX-2 presence in the ileal and distal colonic anastomoses10. This supports a role for COX-2 during healing. COX-2 is a rapidly upregulated enzyme that persists for several days after injury and is not downregulated, but blocked by diclofenac[33]. Therefore, it is not possible to determine local differences in COX-2 inhibition on the basis of analysis of COX-2 immunoreactivity. Neither can it be used to confirm adequate COX-2 inhibition.

Clinical implications

Two large retrospective studies have demonstrated that the use of NSAIDs increases the rate of anastomotic leakage[4, 5]. Subgroup analysis of the data from the study by Gorissen et al, involving primary anastomoses in 311 patients after right hemicolectomy and 322 after left hemicolectomy revealed that NSAID use within the first 5 days increased the leak rate of ileocolonic anastomoses after right hemicolectomy (6.9% to 14.8%; odds ratio 2.3(1.1 to 5.0); p=0.027) but not of colo-colonic anastomosis after left hemicolectomy (8.5% to 8.9%; odds ratio 1.0 (0.5 to 2.3); p=0.917)(unpublished data)[4]. Our findings in PC and IL anastomoses support an early detrimental effect of diclofenac. This adds to increasing evidence that caution should be taken with diclofenac as early postoperative painkiller in gastrointestinal surgery.

In conclusion, it was shown that diclofenac causes evident leakage of anastomoses made in the rat ileum and proximal colon, but not in the distal colon. This suggests a role for the intestinal content in the pathology of anastomotic leakage caused by diclofenac.

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5.

SEROSAL ABRASION OF BOWEL ENDS DOES NOT ENHANCE ANASTOMOTIC HEALING

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Abstract

Background

Anastomotic leakage rates remain unacceptably high, warranting reconsideration of current anastomotic technique. Anastomotic healing may improve by abrading the serosal surface of bowel ends that are invertedly anastomosed, based on the concept that serosal damage evokes inflammatory adherent processes. It is studied if local abrasion leads to stronger anastomoses and reduces leakage.

Methods

Ninety-eight Wistar rats were allocated to six groups. Either a regular anastomosis (RA) or abraded anastomosis (AA) was constructed in the proximal colon. Animals were sacrificed at day 3 (groups RA3 and AA3, n = 2 x 17) or day 5 (groups RA5 and AA5, n = 2 x 17). Groups RA-Dic and AA-Dic (n = 2 x 15) received diclofenac from day 0 until sacrifice on day 3 to impair anastomotic healing. Outcomes were leakage, bursting pressure, breaking strength, adhesions and histologic appearance.

Results

Both in abraded (AA3 and AA5) and control (RA3 and RA5) groups without diclofenac 1 of 17 anastomoses leaked (6%). Leak rate was 9 of 15 (60%) in group AA-Dic and 8 of 15 (53%) in RA-Dic (p=1.0). The bursting pressure in group RA3 (127±44 mm Hg) was higher (p = 0,006) compared with group AA3 (82±34 mm Hg), breaking strength was comparable (p = 0.331). Mechanical strength was similar between groups RA5 and AA5. Abrasion did not increase mechanical strength in the diclofenac groups. Adhesion formation was not different between groups. Histology showed dense interserosal scar formation in abraded groups, compared with loose connective tissue in control anastomoses.

Conclusion

Abrasion of serosal edges of large bowel ends invertedly anastomosed does not improve anastomotic strength, neither does it reduce leakage in anastomoses compromised by diclofenac.

Introduction

Despite increased knowledge of anastomotic healing, leakage rates have not declined in the past decades and remain between 3 and 14%[1-3]. Leakage is a significant cause of increased morbidity and mortality after visceral surgery[4]. Attempts to reduce leakage by mechanical stapling, external sealants (e.g. fibrin glue), biological stimulants (e.g. growth factors), internal conduits or various suture techniques have failed[3, 5, 6].

The current standard for constructing an end-to-end or side-to-side handsewn or stapled anastomosis is an inverting anastomosis[7]. This means that the largest contact area of the two bowel parts is formed by the opposing serosal surfaces. However, the physiological function of the thin epithelial layer of mesothelial cells covering the serosa, is to provide a lubricant surface and not to adhere. Injury of the relatively large sero-serosal contact area might provide a way to optimize anastomotic healing. When serosa is damaged, fibrous attachments may form between viscera or the abdominal wall because of the inflammatory process[8]. Serosal abrasion is the most common method to induce adhesion formation in experimental adhesion research[9]. Also for other mesothelial tissue layers, like the parietal and visceral pleura, chemical injury and promotion of the inflammatory process is used to achieve proper adherence (e.g. treatment of relapsing pneumothorax). Several methods to achieve serosal abrasion and adhesion have been described. It is mostly done by sterile gauze rubbing, but dental brushes are also used[10]. A study in dogs showed that complete removal of the mesothelium before making an inverted anastomosis accelerated and improved the healing process without an increased risk of stenosis[11]. Based on pathophysiological principles of inflammation and wound healing, it is hypothesized that isolated injury to the serosal edges of connecting bowel parts may increase anastomotic strength and reduce leak rates by stimulating fibrous adhesions between bowel ends. In the first experiment of the present study the effect of serosal abrasion on anastomotic strength as the primary outcome was assessed. In the second experiment, it was studied if abrasion can reduce leakage of anastomoses compromised by diclofenac administration. A rat anastomosis model was used because of extensive experience with this model in our laboratory and the consistent findings of leak rates and strength over the years[12, 13]. Previous research showed that the administration of diclofenac provides a reliable model to study leakage of ileal and proximal colon anastomoses[13-15]. Diclofenac causes 60% to 100% leakage in the ileum and proximal colon when given from day 0 until sacrifice on day 3[13, 15].

MATERIAL & METHODS

Ethics

This experiment was conducted according to the Dutch “Experiments on Animals Act” and European Federation of Laboratory Animal Science Associations guidelines and was approved by the Institutional Animal Ethics Committee of the Central Animal Laboratory of the Radboud University Nijmegen (AEC-number 2012-290). Humane end points were defined to avoid unnecessary suffering of animals during the study.

Animals

Adult male Wistar rats (Harlan, Horst, The Netherlands) were accustomed to laboratory conditions for 1 wk and weighed 307 grams (standard deviation ± 19) at the start of the experiment. The rats were housed two per cage at 22 C – 23 C with a 12 h day cycle and had free access to standard rodent chow (Ssniff R/M-H, Bio Services BV, Uden, Netherlands) and acidified tap water throughout the experiment. The cages were enriched with a shelter and nesting material and were randomly placed on the shelves.

Groups

Ninety-eight male Wistar rats were randomly allocated to one of the six groups. Either a regular anastomosis (RA) or an abraded anastomosis (AA) was constructed in the proximal colon.

In experiment 1, animals were sacrificed at day 3 (group RA3, n=17 and group AA3, n=17) or at day 5 (group RA5, n=17 and group AA5, n=17). On day 3 anastomotic strength is at its lowest and thus most important to improve [22, 24]. Day 5 was chosen as an additional sacrifice day to study if the effect of abrasion needs additional time for wound healing. Postponing sacrifice to day 7 or longer would not be useful when assessing bursting strength because anastomotic strength exceeds that of normal intestine after this period[12, 13]. Three animals per group were used for histologic analysis and fourteen animals for mechanical strength testing.

In experiment 2, two groups of rats were given diclofenac (3mg/kg/day by oral gavage; Cayman Chemical Company Ann Arbor, MI, USA) from day 0 until sacrifice on day 3 to induce leakage (group RA-Dic, n=15 and group AA-Dic, n=15)[13, 15]. All animals in experiment 2 were sacrificed at day 3 because diclofenac induced leakage occurs mostly before day 3 and postponing sacrifice would increase animal discomfort[13, 15]. Twelve rats per group were used for anastomotic strength measurements and three for histologic analysis.

Intervention and surgical technique

The rats were anesthetized by inhalation of 3% isoflurane (Abott, Hoofddorp, The Netherlands) mixed with pressurized air and oxygen. They were shaved, disinfected and operated under sterile conditions using an operation microscope. By a 3 cm midline laparotomy the cecum was visualized and carefully placed outside the abdomen in wet gauzes. Two centimeters distal from the cecum the place for anastomosis was determined. In the abrasion groups, the complete circumference of the colon was abraded over a length of 2 cm with 10 soft strokes of a dental brush (Oral-B 1 2 3 Indicator Medium, Kruidvat, Nijmegen, The Netherlands) to create a precise and superficial damage of the serosal surface (Figure 1)[10]. The middle 10 mm segment was resected, leaving 5 mm of abraded colon on both sides. In the control animals group a 10 mm segment was removed at the same location in the colon without abrasion. The end-to-end anastomoses were all constructed under a microscope (Wild M650, Heerbrugg, Switzerland, at x10 magnification) by a trained researcher (S.T.K.Y.) using a single layer of eight interrupted, inverting sutures (Ethilon 8-0, Ethicon, Norderstedt, Germany). A monofilament synthetic suture was chosen because it causes little inflammatory reaction,

it is available in 8-0 size, it has produced consistent results in previous experiments and rats are sacrificed before absorption is expected to play a role in de healing process[13, 16]. The abdominal wall was closed with a running suture (Vicryl 3–0; Ethicon, Norderstedt, Germany) and the skin was closed with staples. During the operation body temperature was kept at 38 C using a heating pad and a lamp. To prevent postoperative dehydration, 10 mL of 0.9% normal saline was administered subcutaneously. For analgesia in all groups buprenorphine (Temgesic, Schering Plough, Houten, the Netherlands), 0.02 mg/kg was administered subcutaneously every 12 h, starting at least 15 min before the operation until 48 h postoperatively. All animals were weighed once daily and inspected twice daily for signs of reduced wellbeing, including dirty nose, dirty eyes, piloerection, aberrant behavior, distended abdomen, increased respiration activity and diarrhea.

Outcome assessment

The rats were euthanized by CO2 asphyxiation. A relaparotomy was performed to inspect for signs of anastomotic leakage, defined as anastomotic abscess, pus, fecal peritonitis or visible dehiscence. The inspection was done by two blinded researchers (S.T.K.Y. and R.M.L.M.L.) and only if both agreed on the leakage aspect, this was scored accordingly. The leakage severity was scored as “0” for no signs of leakage, “1” for anastomotic abscesses, “2” for free pus or large abscesses and “3” for fecal peritonitis or visible dehiscence, as previously reported[15]. Adhesions between the anastomosis and other viscera were scored as “0” if absent, as “1” if detachment was possible by light traction, as “2” if blunt dissection was needed or as “3” if sharp dissection was needed to detach the adhesive organ.

Mechanical strength

Except for the samples used for histologic analysis (n=3 and n=2 per group in experiment 1 and 2 respectively), all other anastomoses were subjected to mechanical strength testing. The anastomotic bowel parts were carefully resected en bloc with 2 cm of bowel on each side and any scar tissue or adhesions covering the anastomosis was left in place. To measure bursting pressure(BP), the segments were infused (2 mL/min) with water containing methylene blue, determining the strength of the weakest spot within the anastomosis(13) (17). Dehiscent anastomoses were scored as “0 mm Hg”. The site of rupture was noted as at the anastomosis or outside the anastomosis. To determine the maximal suture holding capacity, the segments were attached to a tensiometer (Aikoh 500, Aikoh Engineering CO. LTD. Tokyo, Japan) and pulled apart at 3 cm/min(13, 18). The highest force measured before rupture was recorded as breaking strength (BS).

Histology

The segments of 1 cm of normal bowel, resected at the initial operation, were collected to check if proper abrasion was performed. After sacrifice, 1 cm long segments containing the anastomosis were collected. All samples were opened at the mesenterial side. After gentle washing with saline the samples were fixed in 4% buffered formaldehyde before paraffin embedding. From these paraffin embedded samples, 4 µm sections were prepared and stained with hematoxylin and eosin. Sections were analyzed using a binocular light microscope.

Statistics

The required sample size for experiment 1 was determined to detect an absolute reduction of 30mm Hg in BP as the primary outcome. With an estimated standard deviation of 25 mm Hg, an α of 0.05 and β of 0.80, anticipating analysis of two groups (regular versus abraded) with an independent t-test, the group size was determined at 14. Adding three animals per group for histologic analysis of the anastomosis, group size in experiment 1 was 17. In experiment 2, sample size was calculated with leak rate as primary outcome. With an expected leak rate in the positive control between 70% and 80% and anticipating analysis with Fisher exact test 15 animals per group were needed to detect a risk reduction to 25%-35%. As leakage assessment does not interfere with histologic analysis, no extra animals were added to these groups.

Fisher exact test was used for analyzing leak percentages and adhesion percentages. The two-tailed unpaired T-test was used for analysis of BP, BS, leak severity and adhesion severity. One-way analysis of variance was used for comparing weight loss among all groups. Results were considered statistically significant at $P < 0.05$.

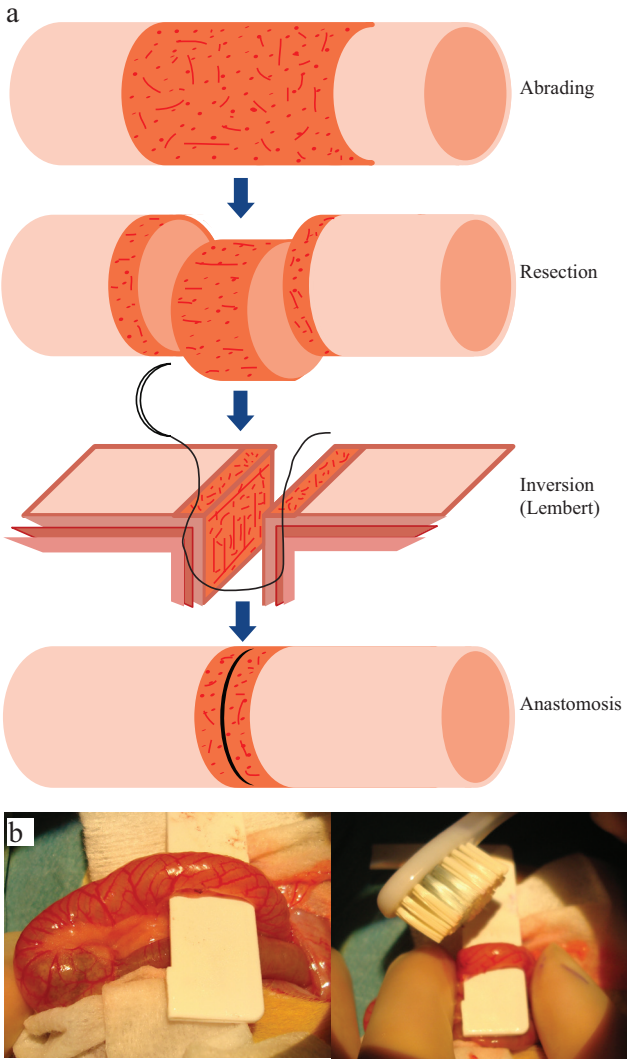


Figure 1 Anastomotic abrasion technique. Serosa is abraded by dental brush. A 10 mm segment is resected, leaving approximately 5mm abraded tissue on each side. These were then invertedly anastomosed by eight interrupted sutures. (a) schematic drawing, (b) intra-operative view.

Results

Animal welfare and mortality

All rats showed minor signs of discomfort during the first 2 days after surgery visible by a dirty nose, dirty eyes, pillow erection, distended abdomen or diarrhea. One animal in the AA5 group died from an unknown cause and one from the same group reached the humane end point and was taken out of the experiment showing severe inactivity, low body temperature and aberrant shape. No signs of leakage were observed in these two animals; they were not used for strength analysis. All rats had weight loss in the first 2 to 3 days after surgery, but rapidly regained normal weight thereafter. The percentage of weight loss in group AA-Dic (6.2±2.9%) was significantly less than in the groups AA3 (10.4±2.0%; p=0.010), AA5 (10.9±2.4%; p=0.047) and RA5 (9.9±3.5%; p=0.035), probably due to diclofenac administration and slight tissue edema. Differences among other groups were not significantly different.

Anastomotic leakage

In experiment 1, 1 rat in each group of 17 rats without diclofenac administration (AA3, RA3, AA5, and RA5) showed macroscopic signs of leakage (6%) (Table 1). In experiment 2, signs of leakage were present in 9 of 15 rats (60%) in the AA-Dic group and 8 of 15 rats (53%) in the RA-Dic group. The overall leak incidence in experiment 2 (57%) was significantly higher than in experiment 1 (6%; p=0.000) (Table 1). The mean leakage severity score was not significantly different between the AA-Dic group (1.0±0.9) and the RA-Dic group (1.2±1.2; p=0.740(Table 1).

Table 1 Anastomic leak, leakage severity and premature death in the six groups.

Outcome	Group					
	RA3	AA3	RA5	AA5	RA-Dic	AA-Dic
Rats per Group, n	17	17	17	17	15	15
Anastomotic leak, n(%)	1(6)	1(6)	1(6)	1(6)	8(53) *	9(60)*,**
Leakage severity Score 0-3 (SEM)	0,1(0,1)	0,1(0,1)	0,1(0,1)	0,1(0,1)	1,2(0,3)	1,1(0,2)
Premature death, n	0	0	0	2	0	0

SEM = standard error of the mean.
Groups: RA3/RA5: regular anastomosis, sacrifice at day 3 or 5. AA3/AA5: abraded anastomosis, sacrifice at day 3 or 5. RA-Dic/AA-Dic: regular or abraded anastomosis, compromised by diclofenac administration from day 0 until sacrifice on day 3.
* $P < 0.01$ compared with groups without diclofenac.
** $P > 0.05$ compared with RA-Dic.

Anastomotic strength

The BP in the RA3 group (127±44 mm Hg) was significantly higher (p=0,006) compared to the AA3 group (82±34 mm Hg, Figure 2a). There were no differences in BP between the groups sacrificed at day 5 (AA5, 207±71 mm Hg; RA5, 205±64 mm Hg; p=0.941). Both in group AA5 and RA5 5 out of 14 anastomoses burst outside the anastomotic line, all other anastomoses burst within this line. There were no differences in BP between the AA-Dic (85±28 mm Hg) and the RA-Dic (83±51 mm Hg) groups (Figure 2a).

Abrasion did not affect BS (AA3 group, 1.0±0.3 N; RA3 group, 1.1±0.3 N; p=0.331). No differences were found between groups sacrificed at day 5 (AA5 group, 1.6±0.4 N; RA5 group, 1.8±0.4; p=0.157) and between both groups of rats treated with diclofenac (AA-Dic group, 0.9±0.3 N; RA-Dic, 0.8±0.3 N; p=0.540) (Figure 2b).

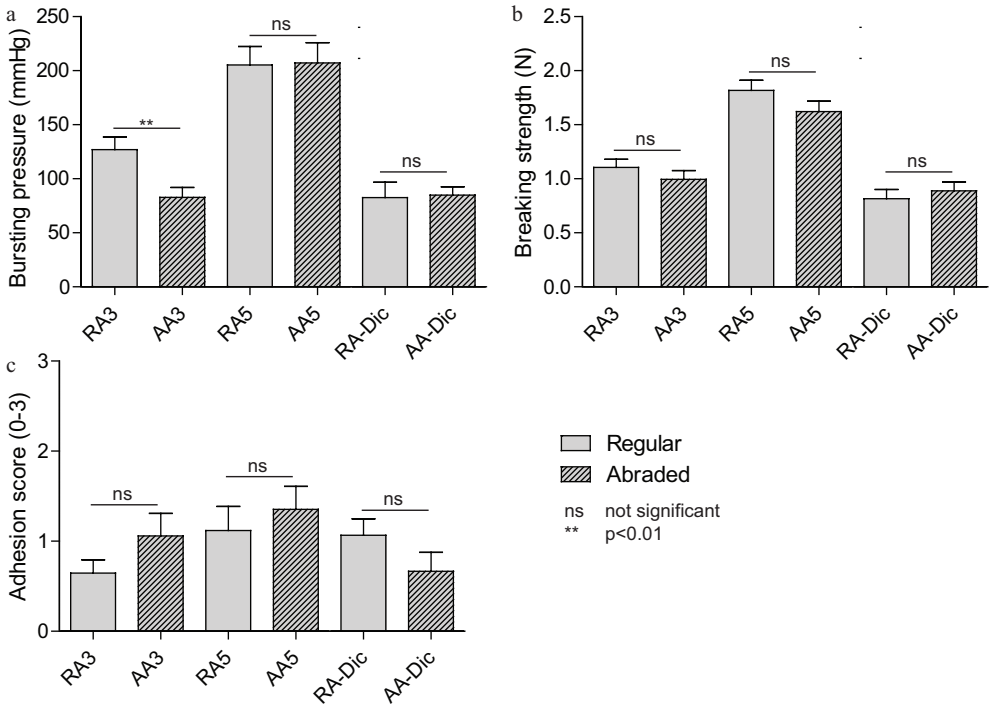


Figure 2 Bursting pressure (a), breaking strength (b) and adhesion score (c) per group. Groups: RA3 / RA5: regular anastomosis, sacrifice at day 3 or 5. AA3 / AA5: abraded anastomosis, sacrifice at day 3 or 5. RA-Dic / AA-Dic: regular or abraded anastomosis, compromised by diclofenac administration from day 0 until sacrifice on day 3.

Adhesions

Adhesion scores were low and not significantly different between groups AA3 and RA3 (1.1±0.2 and 0.7±0.2; p=0.165), and between groups AA5 and RA5 (1.4±0.3 and 1.1±0.3; p=0.532), and groups AA-Dic and RA-Dic (0.7±0.2 and 1.1±0.2; p=0.162, Figure 2c).

Histology

Because of one technical failure (group AA3), two dehiscent anastomoses (groups RA-Dic and AA-Dic) and two premature deaths (group AA5), a reduced number of anastomotic samples could be analyzed. Histologic analysis of the bowel pieces resected at the initial operation showed a moderate degree of mesothelial damage in all samples of both abraded (n=7) and non abraded (n=7) groups (Figure 3a-c). An intact mesothelial segment was only seen in one control sample (Figure 3a). The connective tissue layer beneath the mesothelium was more frequently injured in the abraded groups, as visible by microscopic hematomas (Figure 3c).

In 6 out of 7 anastomotic control samples (RA3 n=3, RA5 n=3 and RA-dic n=1) the connection of the opposing serosal layers of both bowel parts appeared incomplete (Figure 3d, f, and h), whereas dense connective tissue was seen between the serosal layers in 3 out of 4 anastomoses in the abraded groups (AA3 n=2, AA5 n=1, and AA-Dic n=1) (Figure 3e, g, i).

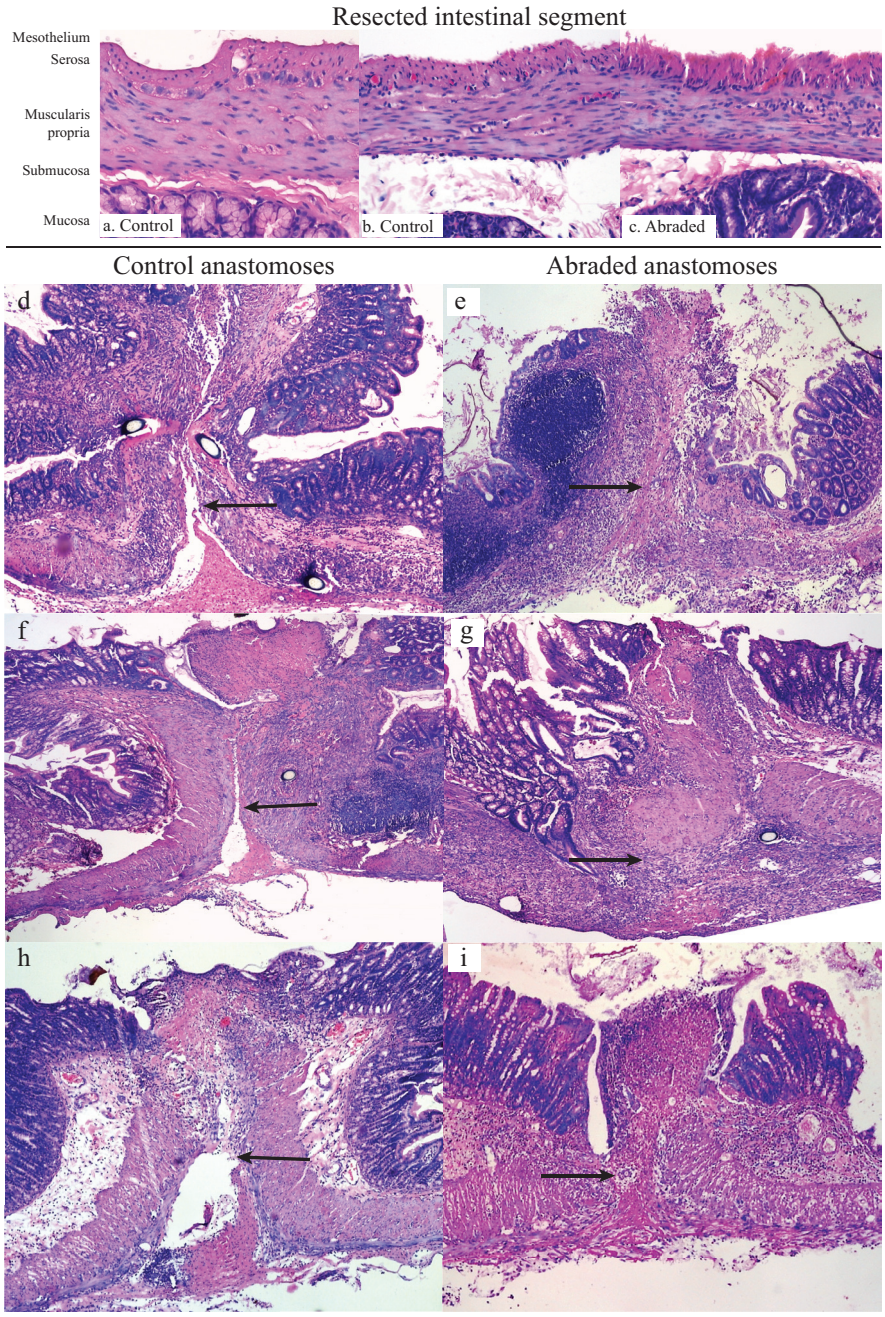


Figure 3 (next page) - Hematoxylin and eosin stained samples of resected intestinal segments (a-c) and anastomoses (d-i). Examples of intact mesothelium in control group (a), damaged mesothelium and serosa in control groups (b) and more severely damaged serosa in abrasion groups (c). Examples of incomplete connection of the opposing serosal layers in control anastomoses (d, f, h) and dense connective tissue in abraded anastomoses (e, g, i). Arrows indicate interserosal area.

Discussion

The results from the present study show that surgical abrasion of the bowel serosal layer does not result in stronger anastomoses and does not reduce leakage rate of compromised anastomoses in a rat model despite histologic evidence of more dense interconnective tissue. The assumed benefit of serosal abrasion of bowel ends would be an increased adhesive and thus fibrous reaction between the two inverted serosal edges resulting in a stronger anastomosis. An increased response on abrasion was suggested by analysis of the histologic samples, in which loose connective tissue was seen between the two opposing serosal layers in most nonabraded anastomoses, compared with marked scar tissue in most abraded anastomoses. Apparently such increase in fibrous connections does not lead to a stronger anastomosis at two relevant time points in the process of anastomotic wound healing of rats[13, 15].

Possible explanation for the findings is that the healing process of normal intestinal anastomoses in the rat is near optimal, and improvement is difficult to achieve and to assess with additional interventions. Notably, it is difficult to induce leakage in a normal rat anastomosis and even incomplete rat anastomoses can heal well in less than a week[19]. On the opposite, the impairment of the healing process by administration of diclofenac might have been too strong to find a beneficial effect of abrasion on the leak rate in a compromised anastomosis. In addition, the relative contribution of scar tissue formation because of serosal abrasion might have been low compared with the scar formation by the sutures or the strength provided by the repair of the submucosal layer, which is mostly responsible for the anastomotic strength[20-22]. Abrasion may even have delayed healing as indicated by lower bursting pressures after 3 days. An excessive inflammatory response and edema formation on injury both negatively interfere with normal healing[23, 24]. Histology, however, did not show edema whereas fibrosis, as a product of an excessive inflammatory response, was present in the specimens of abraded anastomosis already at day 3.

It was expected that adhesions to other surrounding structures and organs would be more extensive in the intervention groups, but no difference in adhesion severity was observed. Notably, assessment of adhesion formation and severity is typically done after one week or even longer, and not after 3 or 5 days[10]. Particularly adhesions at day 3 may be fibrinous of nature and are still susceptible for fibrinolysis by plasminogen activator in the abdominal cavity[25]. Day 3 and 5 were chosen in this study because at these times, anastomotic healing disturbances are most likely to occur. Comparable adhesion formation further indicates that the consequences of abrasion seem limited when considering the healing processes evoked by the surgical resection or the suturing. From several animal studies it has been suggested that nonsteroidal anti-inflammatory drugs reduce abdominal adhesion formation[26, 27]. Intraperitoneal administration of cyclooxygenase-2 inhibitors showed anti-adhesive effects in several animal studies[28-30]. In our study we did not find a reduction of adhesions after diclofenac administration in the second experiment. The frequent occurrence of anastomotic leakage in these groups is a confounding factor when assessing adhesion formation because intraperitoneal infection strongly induces adhesion formation[31, 32].

A validated and frequently used animal model with a sufficient number of animals was used to assess the effects of abrasion on anastomotic strength and leakage ruling out a type 2 error. The number of histologic samples however, was not sufficient to allow for conclusive interpretation.

Potential disadvantage of the rat model is the small size of the intestine, which may have hampered adequate abrasion. A larger bowel size may be the reason why a beneficial effect of abrasion was reported in a dog study[11]. Therefore, the negative results of the present study do not exclude a beneficial effect in humans. Another disadvantage of the small bowel size is the relative damage done by handling the bowel ends while creating the anastomosis. Though trying to avoid damage, swabs and forceps may have already induced serosal injury comparable with that in abraded groups, as was also observed in some histologic samples (Figure 3b). A possible concern regarding outcome assessment involves the diagnosis of leakage. The clinical definition often involves the need for reintervention, which is not applicable to animals[33]. Taking differences in leakage definitions into account, macroscopic signs were scored according to severity to allow for a more accurate discrimination of the adverse effect on the animal. The severity score was used in a previous study in which mean severity scores corresponded with leak rates and strength. However, the score was not statistically validated and thus should be interpreted with caution [15]. To minimize observer bias, scoring was done by two observers in a blinded fashion. The compromised anastomosis model that was used is more appropriate to study healing processes compared with models using extensive ischemia or large suture defects, which are unrealistic in the clinical situation[19]. Leakage was successfully induced by diclofenac administration with rates comparable with previously obtained results, making it a consistent model of anastomotic leakage[15]. The increased leak rate after diclofenac administration in proximal rat colon adds to the increasing evidence that nonsteroidal anti-inflammatory drugs impair anastomotic healing[34].

Conclusion

Abrasion of the serosal layers of large bowel ends that are invertedly anastomosed does not improve strength and does not reduce leak rate in a rat model of normal and compromised anastomotic healing.

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6.

EXPERIMENTAL STUDY OF DICLOFENAC AND ITS BILIARY METABOLITES ON ANASTOMOTIC HEALING

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Abstract

Background

Diclofenac increases the risk of anastomotic leakage, but the underlying mechanism is unknown. As diclofenac is excreted largely as biliary metabolites, the aim of this study was to determine the effect of these metabolites on intestinal anastomoses.

Methods

This was a randomized controlled blinded experiment using 210 male Wistar rats to assess the effect of 'diclofenac bile' on the anastomotic complication score, leak rate and anastomotic strength following oral and parenteral administration of diclofenac. Bile duct and duodenal catheterization techniques were used for diversion and replacement of bile, and biliary diclofenac metabolites were determined.

Results

Replacement of control bile with diclofenac bile resulted in higher anastomotic complication scores (P = 0.006) and leakage in five of 18 animals, compared with one of 18 controls (P = 0.089). In turn, following oral diclofenac administration, replacement of diclofenac bile with control bile reduced anastomotic complications (P = 0.016). Leak rate was seven of 15 versus 13 of 17 without replacement (P = 0.127). After intramuscular administration of diclofenac, the reduction in anastomotic complications was not significant when bile was replaced with control bile (P = 0.283), but it was significant when bile was drained without replacement (P = 0.025). Diclofenac metabolites in bile peaked within 2 h after administration. Administration of diclofenac bile resulted in nearly undetectable plasma levels of diclofenac (mean(s.d.) 0.01(0.01) μg/ml) after 120 min. Following oral diclofenac, bile replacement with control bile did not affect the plasma concentration of diclofenac (0.12(0.08) μg/ml versus 0.10(0.05) μg/ml with diclofenac bile; P = 0.869).

Conclusion

Altered bile composition as a result of diclofenac administration increases the ileal anastomotic complication rate in rats.

Introduction

Anastomotic leakage is the leading cause of morbidity and mortality after intestinal surgery, with leak rates varying from 3 to 14 per cent1. Non-steroidal anti-inflammatory drugs (NSAIDs) have been related to increased leak rates in both animal and retrospective human studies2–4. This is worrisome because NSAIDs are recommended as postoperative analgesics in current guidelines5. Clinical evidence is not considered sufficient to ban postoperative use of NSAIDs, as some studies do not report adverse effects[5,6]. Further research is required to delineate the role of NSAIDs in clinical anastomotic leakage[4,7].

The main mechanism by which NSAIDs are believed to jeopardize anastomotic healing is the cyclo-oxygenase (COX) 2 inhibitory action, which may cause suppression of inflammatory, proliferative or proangiogenic pathways relevant for normal wound healing[2,8]. An alternative explanation could be direct toxicity of intraluminal factors resulting from NSAID treatment (such as drug metabolites and bile products). This is based on a similar relationship found between NSAIDs and small intestinal mucosal damage[9–11].

It is hypothesized that the NSAID diclofenac may display its detrimental effect on bowel anastomoses through altered and toxic bile composition. At least three observations support this hypothesis. First, NSAID-induced intestinal mucosal damage has been attributed to increased bile toxicity[12–15]. Biliary excretion of toxic drug metabolites and reduced availability of protective phosphatidylcholine in bile due to competitive binding by NSAID metabolites appear to be responsible for the bile toxicity[9,12,13,16]. Second, diclofenac causes leakage of anastomoses in the ileum and proximal colon, but not in the distal colon of the rat, which can be explained by the fact that most bile components are reabsorbed or converted to non-toxic substances before reaching the distal colon[17]. Third, NSAIDs that are predominantly excreted through bile (such as diclofenac, celecoxib and carprofen) cause 80–100 per cent leakage of experimental ileal anastomoses, whereas naproxen, which is excreted almost entirely through urine in the rat and human, results in less than 15 per cent leakage[18–20]. In clinical practice, bile toxicity could pose a threat to a large number of patients, as it could be harmful for both small and large bowel anastomoses, considering the altered physiology following partial colectomy.

The aim of this study was to investigate the effect of altered bile composition induced by diclofenac on small bowel anastomotic healing in rats using bile drainage and bile replacement techniques.

Methods

The study was carried out according to the ARRIVE guidelines[21]. It was approved by the Animal Ethics Committee (AEC number 2013-171) and performed in the Central Animal Laboratory of Radboud University.

Animals

In total, 210 adult male Wistar rats (mean(s.d.) weight 313(25) g; Harlan, Horst, The Netherlands) were used (138 to assess anastomotic healing and 72 to donate bile). The animals were acclimatized to laboratory conditions and initially housed, two per cage, at 22–23°C with a 12-h day cycle, and free access to standard rodent chow (Ssniff® R/M-H; Bio Services, Uden, The Netherlands) and acidified tap water throughout the experiment. The cages were enriched with a rat retreat and nesting material, and were placed randomly on the shelves. All animals were checked at least twice daily. Humane endpoints were defined; animals were killed if they showed signs of severe discomfort (such as weight loss greater than 20 per cent, distended abdomen or severely reduced activity).

Materials

Diclofenac sodium (DCF) (Cayman Chemical, Ann Arbor, Michigan, USA) dissolved in 0.1 per cent polysorbate in saline (sodium chloride 0.9 per cent) was used for administration by oral gavage, and as a standard for high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS) analyses. DCF injection fluid (25 mg/ml; Centrafarm, Etten-Leur, The Netherlands) diluted with saline to 3.33 mg/ml was used for intramuscular injections. 4-Hydroxydiclofenac (4OH-DCF) and 5-hydroxydiclofenac (5OH-DCF) (Toronto Research Chemicals, Toronto, Ontario, Canada), and diclofenac acyl-β-glucuronide (DAG) (LGC Standards, Wesel, Germany) were used as standards for LC–MS analyses, and diclofenac-d_4 (phenyl-d_4-acetic) (J. H. Ritmeester, Nieuwegein, The Netherlands) was used as an internal standard for bile and plasma.

Study design

The study consisted of three experiments (Table 1). A distal ileum anastomosis model was used in all experiments and rats had surgery on day 0; anastomotic healing was assessed after killing on day 3[17]. This interval was used because most diclofenac-induced leakage occurs before day 3, when the anastomosis is at its weakest, and postponing killing would increase suffering[20]. Rats received 1.5 mg/kg diclofenac twice daily either orally (O+) or intramuscularly (IM+), or they received no oral or intramuscular diclofenac (O− or IM−). Cannulation of the common bile duct (CBD) and the duodenum was performed to drain and infuse bile respectively (Fig. 1). Allocation to groups was done randomly by using the online tool at www.randomization.com.

In experiment 1, 72 rats were allocated to four groups of 18 each, and 72 rats were used as matched bile donors. In group O−B−, no diclofenac was administered and bile was drained and replaced by control bile from matched donor rats. In group O−B+ rats, the bile was replaced by ‘diclofenac bile’ from donor rats that had also received 1.5 mg/kg diclofenac orally twice daily for 3 days. Rats in group O+B− were administered diclofenac orally, and their bile was replaced with control bile[17]. Rats in group O+B+ were given diclofenac orally and received diclofenac bile from donors.

In experiments 2 and 3, a parenteral route of diclofenac administration was used to explore the effect of avoiding exposure of the gut to drug molecules as a direct result of oral admin-

istration[13,14]. In experiment 2, 42 rats were allocated to three groups. Rats in group IM+B= (n = 18) received diclofenac intramuscularly, and no cannulations of the bile duct or duodenum were done. In groups IM+B0 and IM−B0 (n = 12 animals each), bile was drained completely without replacing it to prevent diclofenac metabolites and bile salts from entering the gut. In experiment 3, rats were given diclofenac intramuscularly and either received their own diclofenac bile (group IM+B+; n = 12) or bile was replaced with control bile from donor rats (group IM+B−; n = 12).

Table 1 Experimental setup and groups

Group name	No. in group*	Diclofenac administration	Bile drainage and replacement
Experiment 1			
O−B−	18	None	Bile drained and replaced by 'control bile' from donor rats
O−B+	18	None	Bile drained and replaced by 'diclofenac bile' from donor rats
O+B−	15[1,2,3]	Oral	Bile drained and replaced by 'control bile' from donor rats
O+B+	17[3]	Oral	Bile drained and replaced by 'diclofenac bile' from donor rats
Experiment 2			
IM+B=	18	Intramuscular	No bile drainage or reinfusion
IM+B0	12	Intramuscular	Bile drained; no bile reinfused
IM−B0	11[4]	None	Bile drained; no bile reinfused
Experiment 3			
IM+B+	12	Intramuscular	Bile drained and reinfused instantly (rat receives its own bile)
IM+B−	10[4,5]	Intramuscular	Bile replaced with 'control bile' from donors

*Each superscript number denotes an animal that died during or just after operation. These were not included in the statistical analysis. Causes of death were: intraoperative bleeding of portal vein; [2]anaesthetic overdose; [3]catheter displacement; [4]unknown cause; [5]failure of bile duct cannulation. O, oral; IM, intramuscular; +, with diclofenac; −, without diclofenac; 0, bile drained and not returned.

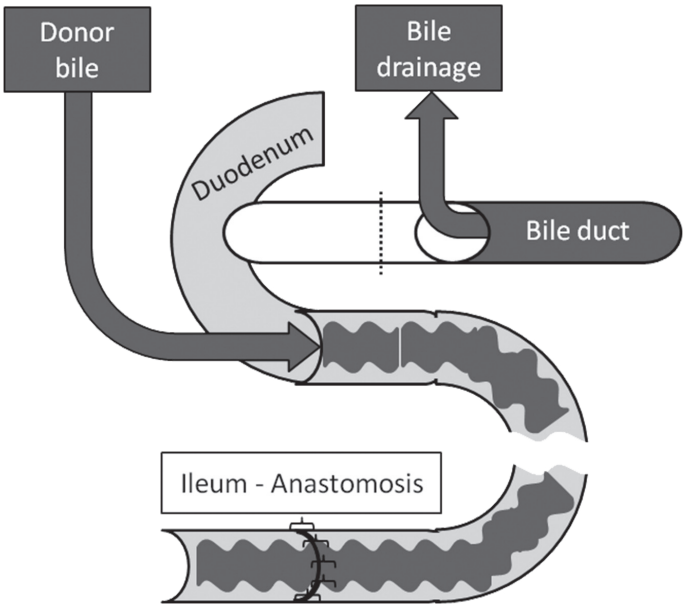


Figure 1 Schematic illustration of the experimental model used in experiments 1 and 3. In experiment 2, in groups IM+B0 and IM−B0, no bile was reinfused. IM, intramuscular; +, with diclofenac; −, without diclofenac; B0, bile drained and not returned

Operative procedures

All operations were done by two experienced researchers. For analgesia, 0.02 mg/kg buprenorphine (Temgesic®; Schering-Plough, Houten, The Netherlands) was given subcutaneously every 12 h, starting 1 h before the operation until 48 h afterwards. No antibiotics were given and animals were not fasted. Anaesthesia was with isoflurane (Abbott, Hoofddrop, The Netherlands), 5 per cent for induction and 2.5–3.5 per cent for maintenance, in a 1 : 1 mixture of oxygen and pressurized air. Rats were prepared by shaving, skin disinfection with iodine and sterile covers, and then operated on through a 4–5-cm midline laparotomy using strict aseptic technique under a microscope (Wild M650; Wild Heerbrugg, Gais, Switzerland) at 10–16× magnification). Body temperature was kept at 38°C with a heating pad and lamp.

The procedures started with cannulation of the CBD to drain bile and placement of a catheter in the duodenum to readminister bile, if applicable. This was followed by construction of the bowel anastomosis. The laparotomy wound was closed with a running suture, 3/0 Vicryl® (Ethicon, Amersfoort, The Netherlands) for the fascia and staples for the skin.

Bile drainage and infusion techniques

The CBD was ligated with 7/0 silk (Pearsalls, Taunton, UK) just before it enters the tail of the pancreas, and a V-shaped hole was made in the duct with iridectomy scissors

proximal to the ligation. A custom-made 3-Fr polyurethane cannula (BTPU-040; Instech Laboratories, Plymouth Meeting, Pennsylvania, USA), 20 cm in length, and a silicone ring (BTSIL-047; Instech Laboratories) at 7 and 50 mm from the sharpened tip, was inserted into the bile duct and fixed with 7-0 silk proximal and distal to the silicon ring at 7 mm. A similar, but blunt, cannula was inserted through a purse-string suture (6/0 Vicryl®; Ethicon, Norderstedt, Germany) in the duodenum. Cannulas were fixed to the abdominal wall (6/0 Vicryl®), tunnelled through the rectus muscle and subcutaneously towards the back between the scapulas, and then connected to a harness (VAHD95AB or VAHD115AB; Instech Laboratories). In turn, the harness was connected to a tether and swivel system (VAH95T+375/22PS or VAHD115T+375/D/22 with BTCOEX-22 tubing; Instech Laboratories), through which the bile was transferred to 50-ml tubes in a cooled (0–10°C) polystyrene box. When attached to the swivel, rats were still able to move within the entire cage and use nesting material.

In experiment 1, the tube with donor bile was changed twice daily and kept at 4°C until reinfusion the next day. Because the donor rats in experiment 1 also provided the control bile for experiment 3, and this experiment was conducted later, the bile was kept at −80°C until reinfusion. Because it involved control bile, there was no concern for loss of toxicity. Bile was infused into the duodenal cannula at room temperature (22–23°C) using a 20-ml syringe and an infusion pump (IVAC Medical Systems, San Diego, California, USA) at a rate of 0.7–1.0 ml/h.

Intestinal anastomosis

Anastomoses were created as described previously[17]. Briefly, 1 cm of ileum was resected at 15 cm proximal to the caecum. An inverted anastomosis was made with a single layer of eight interrupted (Lembert) sutures (8/0 Ethilon®; Ethicon, Norderstedt, Germany). After operation, rats were housed individually because they were attached to the swivel system.

Assessment of outcome

Animals were killed by asphyxiation with a mixture of 95 per cent carbon dioxide and 5 per cent oxygen, and thereafter 100 per cent carbon dioxide (donor rats), or by cardiac or inferior vena cava puncture combined with cervical dislocation under general anaesthesia with isoflurane (experimental rats). A relaparotomy was done and the anastomosis was inspected macroscopically for anastomotic complications by a researcher blinded to the group randomization.

Signs were graded according to the anastomotic complication score: 0, no abnormality; 1, small (less than 2 mm) anastomotic abscess; 2, free pus or large abscess; 3, faecal peritonitis or visible dehiscence17. Grade 2 or 3 was considered an overt anastomotic leak.

Bursting pressure and breaking strength were assessed as described previously[17]. Briefly, bursting pressure (mmHg) was determined by infusing anastomotic segments with blue fluid and measuring the maximum pressure sustained before leakage occurred. Subsequently, segments were pulled apart with a tensiometer and the highest force measured before rupture was recorded as breaking strength (newtons).

Analysis of diclofenac excretion profile in bile

To determine whether and when diclofenac products are found in bile, bile samples from four donor rats were obtained fractionally every hour after a single dose of diclofenac 1.5 mg/kg orally and analysed by HPLC; for methods used see Appendix S1 (supporting information).

Quantitation of diclofenac metabolites in bile and plasma

Bile samples were obtained as described above and analysed by LC–MS/MS to determine which diclofenac metabolites were most abundant, and when. Plasma samples were collected in experiment 1 to assess whether bile replacement with either diclofenac bile or control bile influenced plasma concentrations through the enterohepatic circulation. Samples of 1 ml were drawn from the tail vein on postoperative day 2, either at 60, 90 or 120 min after diclofenac administration. Any change in plasma level was expected to occur after 60 min, based on biliary excretion time. For a description of methods used, see Appendix S1 (supporting information).

Statistical analysis

Sample size was determined with expected leak rates of 70–80 per cent in groups receiving diclofenac bile and 10–20 per cent in groups receiving control bile or no bile17. Anticipating analysis of leak rates with Fisher's exact test, and anticipating a larger loss to follow-up in the first experiment, 18 animals per group were used in experiment 1 and in group IM+B= of experiment 2, and 12 animals in other groups. Absolute leak rates, and not anastomotic complication scores, were used because this facilitates better sample size calculations. Mann–Whitney U test was used to compare complication scores, and an independent t test to compare strength. Spread of data is presented as the standard deviation. $P < 0.050$ was considered statistically significant. Statistical analysis was with IBM SPSS® Statistics version 22 (IBM, Armonk, New York, USA).

Results

Animal mortality and welfare

One rat in group O+B+ and two in IM+B+ were killed before day 3 because of severe illness due to peritonitis (humane endpoint). Other rats had a normal postoperative recovery or had signs of illness starting on day 3, just before killing. Six rats (3 in group O−B−, 2 in group O+B−, and 1 in group O+B+) had to be anaesthetized to relieve cannula obstruction by manipulating the subcutaneous part of the catheter (leakage occurred in the 2 rats in group O+B−). Two rats (1 in group O−B− and 1 donor rat) required a relaparotomy to relieve intra-abdominal cannula kinking.

Postoperative weight loss in experiment 1 was comparable between groups (mean(s.d.) 9.8(2.8) per cent). In experiment 2, weight loss was significantly higher in groups where bile was not replaced (IM+B0: 13.7(4.4) per cent; IM-B0: 14.8(3.0) per cent) compared with

loss in group IM+B=, where bile was replaced (10.0(2.6) per cent; P = 0.007 and P < 0.001 respectively). In experiment 3, weight loss was higher in group IM+B− than in group IM+B+ (14.6(2.7) versus 8.3(3.0) per cent respectively; P < 0.001).

Anastomotic complication score and leak rate

In experiments 1, 2 and 3, anastomotic complication scores were the highest in 'diclofenac bile' groups (O−B+ versus O−B−, P = 0.006; O+B+ versus O+B−, P = 0.016; IM+B= versus IM+B0, P = 0.025; IM+B+ versus IM+B−, P = 0.283) (Fig. 2a). Grades 2 and 3, which were considered leakage, occurred in five of 18 animals (28 per cent) after administration of diclofenac bile (O−B+), compared with one of 18 (6 per cent) with control bile (O−B−) (P = 0.089). Most leakage occurred in groups receiving oral or intramuscular diclofenac combined with diclofenac bile: 13 of 17 animals (76 per cent) in group O+B+ versus seven of 15 (47 per cent) in group O+B− (P = 0.127); 12 of 18 (67 per cent) in group IM+B= versus three of 12 (25 per cent) in group IM+B0 (P = 0.060); and six of 12 (50 per cent) in group IM+B+ versus two of ten (20 per cent) in group IM+B− (P = 0.117) (Fig. 2b). No leakage occurred in group IM−B0 (P = 0.122 versus IM+B0; P = 0.001 versus IM+B=).

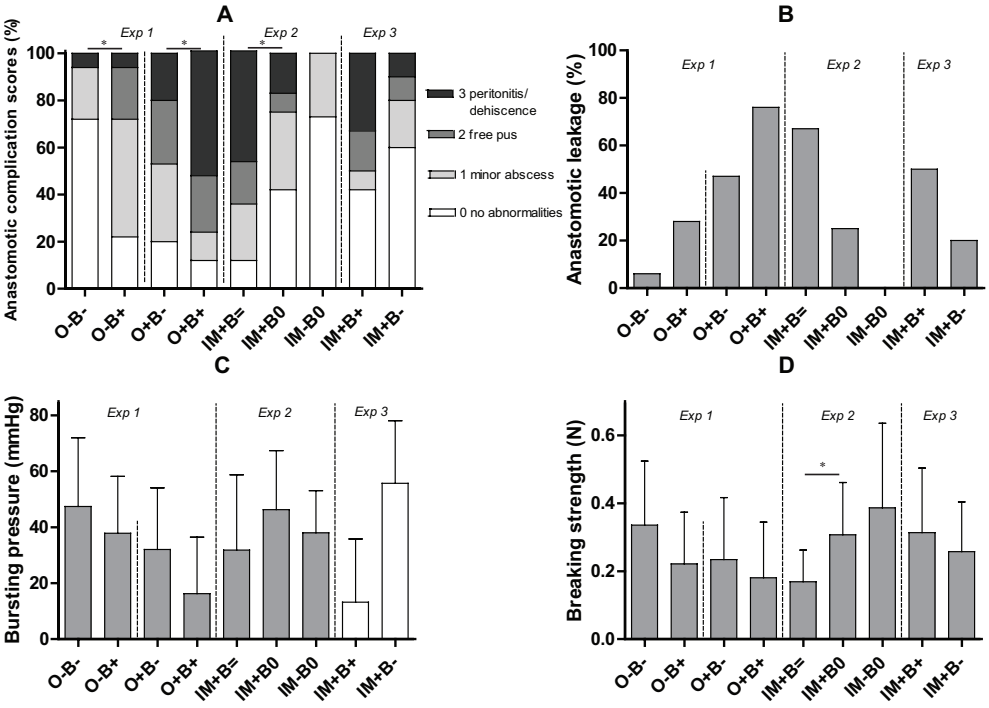


Figure 2 Anastomotic healing assessed by: a anastomotic complication scores, b anastomotic leakage, c bursting pressure (n = 5 in group IM+B+, n = 4 in group IM+B−, due to sensor failure) and d breaking strength. O, oral; IM, intramuscular; +, with diclofenac; −, without diclofenac; 0, bile drained and not returned. *P < 0.050 (Mann–Whitney U test); †P < 0.050 (independent t test). [Ileum anastomosis without diclofenac administration or bile duct cannulation have normal healing without signs of leakage]

Anastomotic strength

Bursting pressures corresponded with leak rates; values tended to be lower in groups with higher leak rates in experiments 1 and 3 (Fig. 2c). In experiments 1 and 2, breaking strength was the highest in groups that received neither oral or intramuscular diclofenac nor diclofenac bile, with no significant differences (Fig. 2d). A significant difference was observed between group IM+B= and group IM+B0 (mean(s.d.) 0.17(0.09) versus 0.31(0.15) N respectively; P = 0.006).

Diclofenac excretion profile in bile

HPLC analysis revealed that diclofenac was detected in bile 1 h after oral administration, that levels peaked within 2 h, and that diclofenac remained detectable in bile at least 6 h after administration (Fig. 3a).

Bile production and analysis of diclofenac metabolites in bile

In the donor groups, bile production was comparable at a mean(s.d.) rate of 14.9(2.4) ml/day in control rats and 14.6(2.0) ml/day in diclofenac rats (P = 0.317). DAG was the most abundant metabolite in bile pooled over 2–8 h after oral administration (Fig. 3b). Diclofenac metabolites were detected almost immediately after intramuscular administration, and levels of DAG, DCF and 4OH-DCF peaked around 2 h after administration (Fig. 3c).

Analysis of diclofenac metabolites in plasma

Administration of 'diclofenac bile' in group O−B+ resulted in low mean(s.d.) plasma levels of DCF (0.01(0.01) μg/ml), 4OH-DCF (0.01(0.01) μg/ml) and 5OH-DCF (0.02(0.02) μg/ml) after 120 min. 5OH-DCF was the most prominent metabolite found in plasma samples, with levels of 0.35(0.05) μg/ml in groups O+B− and O+B+ at 90 min, followed by DCF (0.26(0.02) μg/ml) and 4OH-DCF (0.10(0.01) μg/ml). DAG was not detected.

The plasma DCF level in group O+B− at 120 min was comparable to that of group O+B+ (0.12(0.08) versus 0.10(0.05) μg/ml respectively; P = 0.869). The total concentration of DCF and metabolites combined is displayed in Fig. 3d.

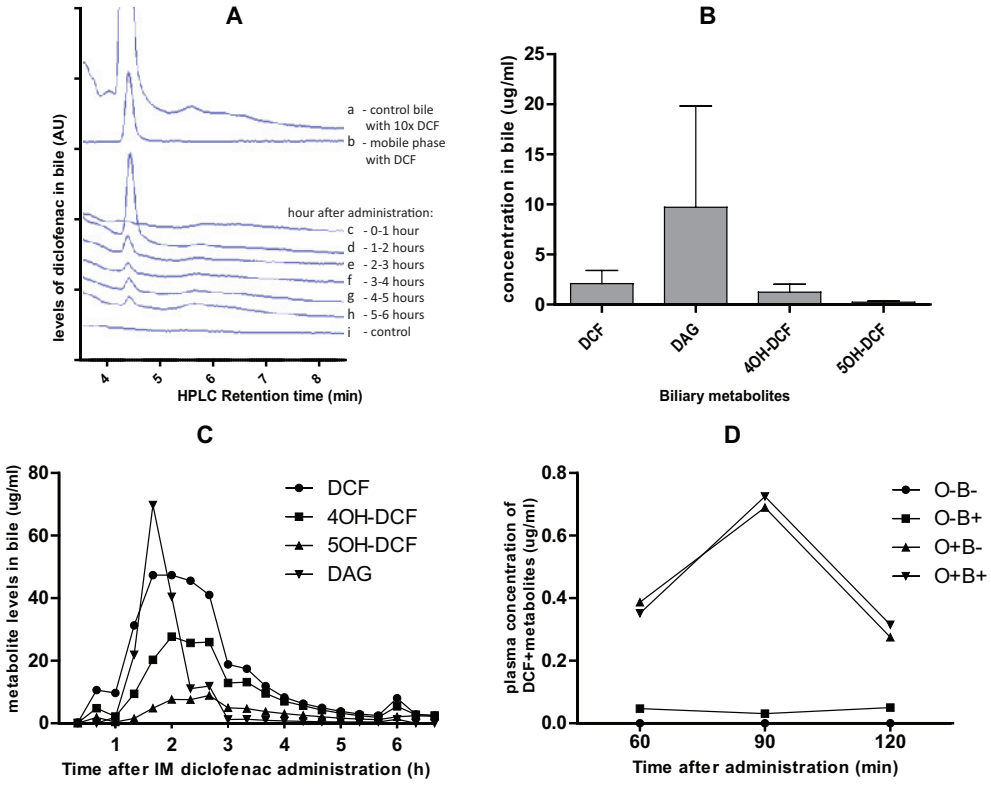


Figure 3a High-performance liquid chromatogram (HPLC) of hourly bile samples showing peak diclofenac levels within 1–2 h after oral administration (AU – arbitrary units): a, control bile with diclofenac sodium (DCF) 0.5mg/ml added; b, mobile phase with DCF 50ug/ml; c, 0–1 h after administration; d, 1–2 h; e, 2–3 h; f, 3–4 h; g, 4–5 h; h, 5–6 h; i, control. b Liquid chromatography–mass spectrometry (LC–MS) or MS analysis of diclofenac metabolites in 'diclofenac bile' pooled over 2–8 h after oral administration (n = 9), showing diclofenac acyl-β-glucuronide (DAG) to be most abundant. c Quantitative LC–MS/MS analysis of metabolites in bile after intramuscular (i.m.) administration, showing DCF and DAG to be most abundant, and concentrations peaking at around 2 h (n = 2–6 per time point). d LC–MS/MS-detected plasma levels of DCF, 4-hydroxydiclofenac (4OH-DCF) and 5-hydroxydiclofenac (5OH-DCF) combined, in samples from groups in experiment 1, showing that administration of diclofenac bile results in minimal plasma concentrations in group O−B+ and no discernible difference between O+B− and O+B+, with concentrations again dropping after 90 min; DAG could not be detected in plasma (n = 1–6)

Discussion

This study has demonstrated that increased bile toxicity due to diclofenac administration results in more anastomotic complications in rats. Replacing normal bile with 'diclofenac bile' disturbed anastomotic healing and, in turn, replacement of diclofenac bile with control bile reduced signs of anastomotic leakage following administration of diclofenac. It is suggested that the increase in bile toxicity is caused by biliary excretion of diclofenac metabolites and the subsequent increase of enteral drug and metabolite concentrations.

The composition of bile was shown to be altered rapidly by the presence of diclofenac metabolites after both oral and intramuscular administration. The metabolites, DAG and unconjugated DCF in particular, rapidly enter the gut via biliary excretion, regardless of the route of administration. It is hypothesized that these intraluminal molecules disturb anastomotic healing. In the present study, this was supported by the slightly higher leak rates in oral diclofenac groups compared with those in groups with intramuscular administration. That altered bile composition and intraluminal drug molecules are detrimental in small bowel anastomosis corresponds with earlier findings[17], where diclofenac caused leakage of anastomosis in the rat ileum and proximal colon, but not in the distal colon. Bile and drug concentrations are considerably lower in the distal colon and are known to cause most mucosal damage in the terminal ileum13. Furthermore, sustained release forms of diclofenac with absorption in more distal intestinal segments appear to divert the mucosal damage from the small to the large intestine[22]. Other studies[10,12,13] of NSAID-induced enteropathy have also attributed the damage to topical effects of drug molecules. Possible underlying mechanisms involve reuptake of active diclofenac molecules, initially causing mitochondrial injury and cell death, and, subsequently, initiation of an excessive inflammatory response by activation of Toll-like receptor 4 on macrophages[11,14,23]. Another possibility is that the diclofenac molecules suppress secretion of mucus, making the anastomosis more vulnerable to leakage, as was recently suggested by Bosmans and colleagues[24]. Although diclofenac is excreted largely as DAG, this metabolite is reactivated by bacterial glucuronidase in the terminal ileum, where it can cause mucosal damage[12,25]. In the present study, the acyl-glucuronide metabolite (DAG) was also abundant in bile. Reactivation of this metabolite in the gut together with biliary excretion of unconjugated forms of diclofenac may lead to high enteral accumulation of potentially harmful drug molecules, which may jeopardize healing from the luminal side of the anastomosis[17,25].

The finding that healing was still disturbed when diclofenac was administered via the parenteral route and bile was either diverted or replaced by control bile may suggest a systemic role for diclofenac in causing anastomotic leakage. COX-2 inhibition is considered the main feature of systemic diclofenac activity, and could affect essential inflammatory and proliferative pathways[8,26]. Administration of 'diclofenac bile' had little impact on plasma levels of diclofenac through the enterohepatic circulation; thus the effect of diclofenac bile could not be explained by more potent systemic activity.

A valid model was used to study comprehensively the effect of diclofenac and bile composition on the most clinically relevant outcome: anastomotic complications. Comparison of leak severity showed significant differences; comparison of absolute leak rates did not. However, leak rate trends pointed in the same direction. Scoring leakage according to severity and

clinical consequences is currently advised in literature[27]. The lack of significance may have been the result of a slightly smaller effect size than expected from previous studies; the greatest difference was 42 per cent, compared with 50 per cent expected.

The daily amount of bile produced in rats of about 12–16 ml is comparable to the amount in humans of 250–1000 ml per day when adjusted for body surface area. Biliary excretion of diclofenac is poorly studied in humans and depends on genetic polymorphisms in drug metabolism, but is reported to be somewhat lower in humans than in rats[28]. After oral administration (the preferred prescription form of diclofenac) and excretion in bile, total diclofenac concentrations could reach comparable levels in the human gut for anastomotic injury.

Bile toxicity would have most effect on anastomoses that comprise the terminal ileum, where bile is reabsorbed and concentrations are higher, compared with large bowel anastomoses. However, considering the physiological changes that occur following partial or subtotal colectomy, bile toxicity could be comparably relevant for colorectal anastomoses. Clinical cohort studies[2–4,8] reporting an increased risk of leakage following NSAID administration involved both ileocolic and colorectal anastomoses.

The relevance of the properties of bile and possibly intraluminal drug concentrations in relation to anastomotic healing is a novel perspective on leakage pathophysiology, and underlying mechanisms should be further elucidated. Current evidence provided by retrospective clinical studies and animal models makes it ethically controversial to study NSAIDs in a prospective randomized trial[29], and experimental studies are suggested to clarify further the role of intraluminal diclofenac metabolite concentrations, the influence of bile salts, and the role of other drug metabolites, such as those of ketorolac and ibuprofen, in leakage pathophysiology.

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7.

MICROBIAL GLUCURONIDASE INHIBITION REDUCES SEVERITY OF DICLOFENAC–INDUCED ANASTOMOTIC LEAK IN RATS

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Abstract

Background

The nonsteroidal anti-inflammatory drug diclofenac has been associated with intestinal anastomotic leakage, although the underlying pathophysiology is unclear. Previous data suggest that reactivation of biliary diclofenac metabolites by microbial β-glucuronidases in the gut plays a role in harming the intestinal mucosa, and that microbiome-targeted glucuronidase inhibition prevents this damage. Here, the microbial glucuronidase inhibitor 'Inh1' was examined for its ability to reduce diclofenac-induced anastomotic leakage in rats.

Methods

90 male Wistar rats were allocated to five groups. In the two diclofenac groups, group DCF received diclofenac (3mg/kg/day) and group DCF-Inh1 additionally received 800ug/kg/d of glucuronidase inhibitor Inh1 solution orally. In non-diclofenac groups, animals received either Inh1 (800ug/kg/d; group Inh1) solution, the vehicle (methylcellulose; group Veh) or no solution (group Ctrl). All solutions were provided from the day of surgery until sacrifice on day three. Plasma levels of diclofenac were determined. Outcomes were anastomotic leakage, leak severity and anastomotic strength.

Results

Anastomotic leak rates were 89% in group DCF and 44% in group DCF-Inh1 (p=0.006). Leak severity was reduced in group DCFic-Inh1 (p=0.029). In non-diclofenac cohorts mostly minor leakage signs were observed in 25% in group Ctrl, 39% in group Inh1 (0.477), and 24% in group Veh (p=1.000). Bursting pressure and breaking strength were not significantly different. Plasma levels of diclofenac were not changed by Inh1.

Conclusion

Microbial glucuronidase inhibitor reduces diclofenac-induced anastomotic leakage severity, which suggests a harmful effect of diclofenac metabolite reactivation in the gut. This finding improves the understanding of the pathogenesis of anastomotic leakage.

Introduction

The main cause of severe morbidity and increased mortality following intestinal and colorectal surgery is leakage of the anastomosis.[1] Perioperative use of nonsteroidal anti-inflammatory drugs (NSAIDs) has been identified as a risk factor for leakage in multiple preclinical and retrospective clinical studies.[2-4] Current evidence, however, is considered insufficient or too ambiguous to discourage postoperative NSAID use.[5-7] To better understand the role of NSAIDs and possibly other drugs in surgical outcomes, more fundamental research is needed to understand how NSAIDs, such as diclofenac, affect anastomotic healing.[6, 8]

Diclofenac is largely excreted through bile and we previously demonstrated that alterations in bile composition disturb healing of anastomoses (unpublished data).[9] This finding shows similarities with results of studies on NSAID induced enteropathy.[9-15] A wide range of compounds, including NSAIDs such as diclofenac, are detoxified by addition of glucuronic acid moieties by liver-resident UDP-glucuronosyltransferase (UGT) enzymes, and excreted through bile. In the gut the glucuronic acids are enzymatically cleaved from drug conjugates by microbial ß-glucuronidase. These reactivated previously detoxified conjugates damage the intestinal epithelium, and, in the case of NSAIDs, results in enteropathy and mucosal damage. [16-18] We have previously demonstrated that inhibiting bacterial ß-glucuronidase using potent, bacteria-selective, non-lethal inhibitors protects the intestinal mucosa from damage resulting from the anticancer drug CPT-11, which is also excreted as a glucuronide.[19] In subsequent studies, we have applied this inhibitor, termed Inhibitor 1 (Inh1), in a model of NSAID-induced enteropathy.[18] By co-adminstering Inh1 with an ulcerogenic dose of diclofenac, we found that Inh[1] reduces the multiplicity and area of small intestinal ulcers, reduces epithelial permeability and decreases systemic levels of serum alkaline phosphatase, a widely-accepted biomarker of enteropathy.[20] Notably, pharmacokinetics of diclofenac remained unchanged, indicating the analgesic benefit of the compound is not affected by Inh1 treatment. Similar results were observed with ketoprofen and indomethacin.[16, 17]

We hypothesize that toxic aglycones released by reactivation of drug conjugates, such as diclofenac-acyl-glucuronide in the gut, impairs anastomotic integrity (Figure 1). In the present study this is investigated by inhibiting β-glucuronidase in a validated rat model for diclofenac-induced anastomotic leakage (AL).[10]

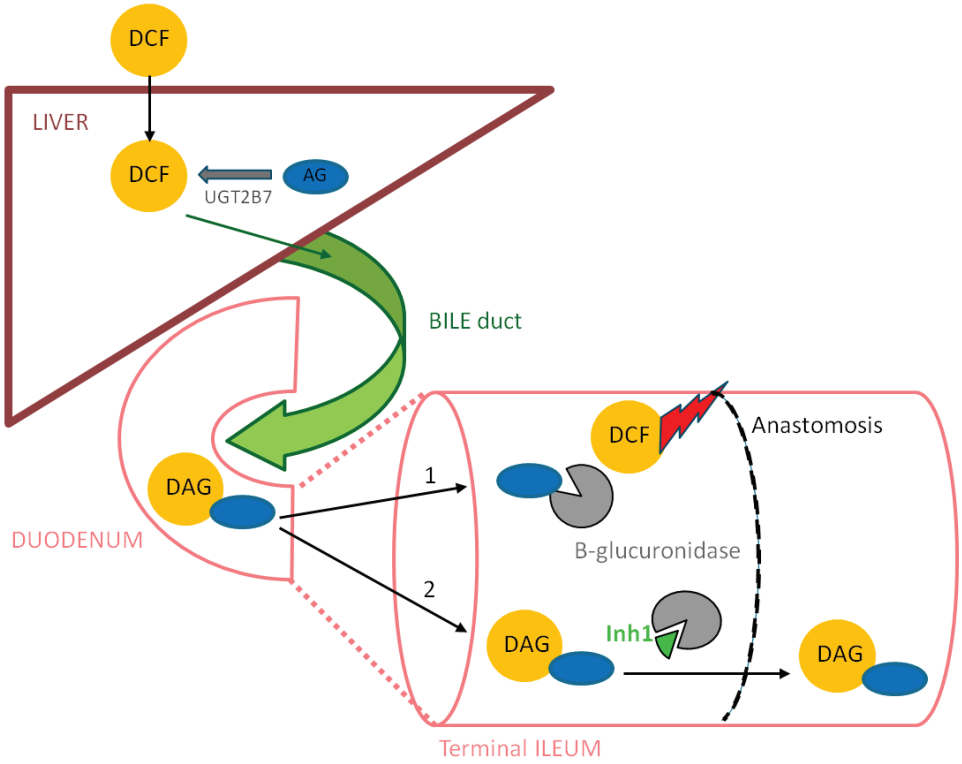


Figure 1 Following glucuronidation of DCF in the liver, DAG enters the gut through biliary excretion. In the gut (terminal ileum) DAG is either cleaved by bacterial glucuronidase, resulting in reactivation of the metabolite which is thought to injure the bowel wall and anastomosis (arrow 1). Or, inhibition of enzymatic hydrolysis by Inh-1 preserves the DAG metabolite which can be safely excreted through the feces (arrow 2). DCF, diclofenac; DAG, diclofenac-acyl-glucuronide; UGT2B7, uridine diphosphate glucuronosyltransferase 2B7; AG, acyl-glucuronide (UDPGA, uridine diphosphate glucuronic acid); Inh1, inhibitor 1.

Methods

Ethics

This experiment was done according to the EU Directive 2010/63/EU and European Federation of Laboratory Animal Science Associations guidelines, and was approved by the Animal Ethics Committee, and executed in the Central Animal Laboratory, of the Radboud university medical center (AEC-number 2013-200).

Animals and perioperative care

Ninety adult male Wistar rats (weight 299 ± 24 gram; Harlan, Horst, Netherlands) were used. The rats were accustomed to laboratory conditions and housed two per cage at 22-23°C with a 12-hour day cycle. They had free access to standard rodent chow (Ssniff Bio Services, Uden, Netherlands) and water throughout the experiment. The cages were enriched with a rat retreat and sizzle nest and were randomly placed on the shelves. All animals were checked at least twice daily. Humane endpoints were defined; animals were killed if they showed signs of severe discomfort (e.g. weight loss > 20% or severely reduced activity).

Materials

Diclofenac sodium injection fluid (Centrafarm, Etten-Leur, The Netherlands) dissolved in saline to 3.33mg/ml was used for intramuscular injections. The β-glucuronidase-inhibitor Inh1 was obtained as previously described.[19] It was first dissolved in DMSO (20mg/ml) and further in 0.5% methylcellulose (SigmaAldrich, St.Louis, Missouri, USA) in saline to a final concentration of 200ug/ml. Inh1 was administered 800ug/kg/d by oral gavage in two doses. Solution vehicle was methylcellulose (SigmaAldrich, St.Louis, Missouri, USA). Diclofenac sodium (Cayman Chemical Company Ann Arbor, MI, USA) and 4-hydoxydiclofenac and 5-hydroxydiclofenac (Toronto Research Chemicals, Canada), and diclofenac-acyl-β-glucuronide (LGC standards GMBH, Wesel, Germany) were used as standards for liquid chromatography mass spectrometry (LC-MS/MS) analyses and diclofenac-d_4 (phenyl-d_4-acetic) (JH Ritmeester, Nieuwegein, The Netherlands) was used as an internal standard of bile and plasma.

Study design

Animals were randomly allocated to two diclofenac groups and three non-diclofenac groups of 18 animals each by using www.randomization.com. Group DCF received diclofenac (1.5 mg/kg twice daily) intramuscularly. The dose was based on the recommended daily dose in human (3mg/kg/day) and on previous experiments.[10] Group DCF-Inh1 additionally received Inh1 (800ug/kg/d). In the non-diclofenac groups, animals received only Inh1 (800ug/kg/d) in group Inh1, vehicle (methylcellulose, 0.5ml orally twice daily) in group Veh or no solution in group Ctrl. All solutions were given orally twice daily from the day of surgery until sacrifice on day three. This period was used because diclofenac induced leakage mostly occurs before day 3, when the anastomosis is at its weakest, and postponing sacrifice would increase suffering.[10, 21]

Operative procedures

In random order, all animals were subjected to the following operation, which was done by experienced researchers (S.Y. or R.L.) blinded to group allocation. For analgesia 0.02mg/kg buprenorphine (Temgesic; Schering Plough, Houten, the Netherlands) was given subcutaneously every 12 hours, starting approximately 1 hour before the operation until 48 hours post-operatively. No antibiotics were given and animals were not fasted. Anesthesia was 5% (induction) or 2,5-3,5% (maintenance) with isoflurane (Abott, Hoofddrop, The Netherlands) in a 1:1 mixture of oxygen and pressurized air. Rats were shaved, iodized, draped with sterile covers and then operated through a 4-5 cm midline laparotomy using strict aseptic techniques under a microscope (Wild M650; Heerbrugg, Switzerland, at 10-16x magnification). Body temperature was kept at 38°C with heating pad and lamp. One centimeter of ileum was resected at 15cm proximal to the cecum. Subsequently an inverted anastomosis was made with a single layer of 8 interrupted inverted (Lembert) Ethilon 8-0 sutures (Ethilon 8-0; Ethicon, Norderstedt, Germany). The abdominal wall was closed with a running suture (Vircryl 3-0; Ethicon, Norderstedt, Germany) and the skin with staples. Postoperatively 10ml of 0.9%NaCl at 37°C was administered subcutaneously for rehydration, and subsequently rats were housed two per cage.

Outcome assessment

Animals were euthanized by cardiac or inferior vena cava puncture combined with cervical dislocation under general anesthesia with isoflurane. A relaparotomy was done and the anastomosis was inspected macroscopically for signs of leakage by researchers blinded to the group randomization. The signs were graded according to an Anastomotic Complication Score: '0' for no abnormalities, '1' for small anastomotic abscesses, '2' for free pus or large abscesses and '3' for fecal peritonitis or visible dehiscence (Figure 2). AL was defined as having a grade 1, 2 or 3 ACS. The final score was confirmed by agreement between the two researchers.

Mechanical strength

All anastomoses were carefully resected and any scar tissue or adhesions covering the anastomosis were left in place. These segments were infused with 0.9%NaCl containing methylene blue at 2ml/min to determine the strength of the weakest spot within the anastomoses. The segments were clamped at 2cm from the anastomosis. The maximum pressure (mmHg) just before the sudden loss of pressure in case of failure was recorded as 'bursting pressure'(BP).

To determine the maximal suture holding capacity or scar strength, the segments were attached to a tensiometer (Single Column Testing Machine with 20N load cell, Zwick/Roell, Ulm, Germany) and pulled apart at 3 cm/min. The highest force measured before rupture was recorded as breaking strength (BS) and is noted as Newton.

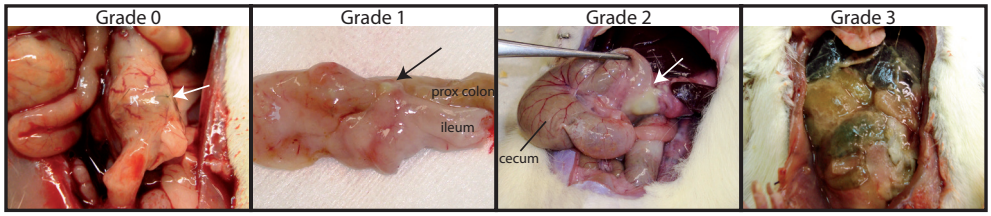


Figure 2 Anastomotic Complication Score examples. Grade 0 = no abnormalities; Grade 1 = minor abscess; Grade 2 = free pus; Grade 3 = generalized peritonitis or visible dehiscence.

Analysis of diclofenac plasma levels by LC-MS/MS

After removing proteins of the plasma samples diclofenac concentrations were determined with LC-MS/MS, using an Acquity UPLC (Waters, Milford, MA, USA) coupled to a Xevo TQ-S (Waters) triple quadrupole mass spectrometer. The compounds were separated on an HSS T3 analytical column (1.8 µm; 100 × 2.1 mm, Acquity UPLC®, Waters, Ireland). As internal standard we used deuterated diclofenac: Diclofenac-d4 (phenyl-d4-acetic) (JH Ritmeester, Nieuwegein, The Netherlands). The elution gradient was as follows: 0 min, 40% B; 7-10 min, 80% B; and 11-14 min, 40% B. Solvent A consisted of 0.1% formic acid in H2O and Solvent B consisted of 0.1% formic acid in Methanol. The column temperature was set at 40°C, and the flow rate was 300µl/min. The effluent from the UPLC was passed directly into the electrospray ion source. Positive electrospray ionization was achieved using nitrogen as a desolvation gas with ionization voltage at 1000 Volt. The source temperature was set at 550°C and argon was used as collision gas. Detection of diclofenac and the internal standard was based on isolation of the protonated molecular ion, [M + H]+ and subsequent MS/MS fragmentations and a multi reaction monitoring (MRM) were carried out. The following MRM transitions were used: for diclofenac m/z 295.9(parent ion) to m/z 215.0 and 250.0 (both product ions), for diclofenac-d4 m/z 300.0 (parent ion) to m/z 219.0 and 254.0.

Sample size calculation and statistics

Sample size was determined with expected leak rates of 70-80% in the positive control group (group DCF) and 0-20% in the control group (group Ctrl).[10] Group DCF-Inh1 was compared with group DCF. In the non-diclofenac cohort groups Inh1 and Veh were compared with group Ctrl. Anticipating analysis of leak rates with Fisher exact test and detection of an absolute difference of 50% in either direction, 18 animals per group were needed. A Mann-Whitney U test was used for the graded anastomotic complication score and an independent t-test for continuous variables (strength tests). In the non-diclofenac cohort, ANOVA and Kruskal-Wallis were used to compare the three groups. The spread of data is presented as standard deviation. P-values <0.05 were considered statistically significant.

Results

Animal welfare and mortality

All animals experienced minor discomfort on the first postoperative day indicated by dirty eyes or nose, and reduced activity. One animal in group DCF died on day 2 due to leakage, and two animals in group DCF-Inh1 were killed on day 2 because of leakage related illness (Table 1). Rats that died prematurely of leakage were included in analysis of leak rates and severity, but were not used for strength tests. Four rats (three in group DCF; one in group DCF-Inh1) showed considerable signs of illness on day 3. In the non-diclofenac groups three animals died prematurely of other causes: one peroperative isoflurane overdose (group Ctrl), and two because of ileus and diarrhea without signs of leakage at autopsy (one in group Ctrl; one in group Veh). These rats were excluded from statistical analysis and were not used for strength tests. Weight loss from day 0 to day 3 was 10.0±2.6 percent in group DCF, 9.6±2.6 percent in group DCF-Inh1 ($p>0.05$), 7.7±2.7 percent in group Veh, 7.7±2.5 percent in group Inh1 ($p>0.05$) and 7.4±2.7 percent in group Ctrl ($p>0.05$).

Table 1

Group name	Size	Weight loss %(SD)	Illness on day 3	Non-AL mortality, n	AL-related mortality n
DCF	18	10.0±2.6	3	-	1
DCF-Inh1	18	9.6±2.6	1	-	2*
Veh	[18]	7.7±2.7	-	-	-
Inh-low	[18]	7.7±2.5	-	1†	-
Ctrl	18	7.4±2.7	-	2†,‡	-

Animal welfare and mortality. Weight loss is comparable among groups. AL, anastomotic leakage; ACS, Anastomotic Complication Score (score 0, no abnormalities; score 1, minor abscess; score 2, free pus; score 3, generalized peritonitis or visible dehiscence); * killed on day 2 because reaching humane endpoint; †died on day 2 due to ileus and diarrhea without signs of leakage; ‡ died of peroperative isoflurane overdose

Anastomotic leakage

Anastomotic leak rates were 89% in group DCF and 44% in group DCF-Inh1 ($p=0.006$) (Figure 3A). Leak severity according to anastomotic complication scores was reduced by the inhibitor ($p=0.029$) (Figure 3B). In the non-diclofenac groups leak rates were 25% in group Ctrl, 39% in group Inh1 ($p=0.477$), and 24% in group Veh ($p=1.000$). Leak severity was comparable for the non-diclofenac groups.

Anastomotic strength

All bursts and breakings occurred at the anastomotic line. Mean burst pressure values and breaking strength were not statistically different between diclofenac groups (Figure 3C and 2D).

Mean bursting pressure was 32±27 mmHg in group DCF and 42±29 mmHg in group DCF-Inh1 (p=0.245) (Figure 3C). In the non-diclofenac cohort bursting pressures were slightly higher than in the diclofenac groups but without significant difference between groups (52±14 mmHg in group Veh, 43±21 mmHg in group Inh1, and 48±23 mmHg in group Ctrl; p=0.329)). Breaking strength (Figure 3D) was .17±.09 N in group DCF, .16±.12 N in group DCF-Inh1 (p=0.757); .23±.17 N in group Veh, .22±.10 N in group Inh1, and .25±.12 N in group Ctrl (p=0.742).

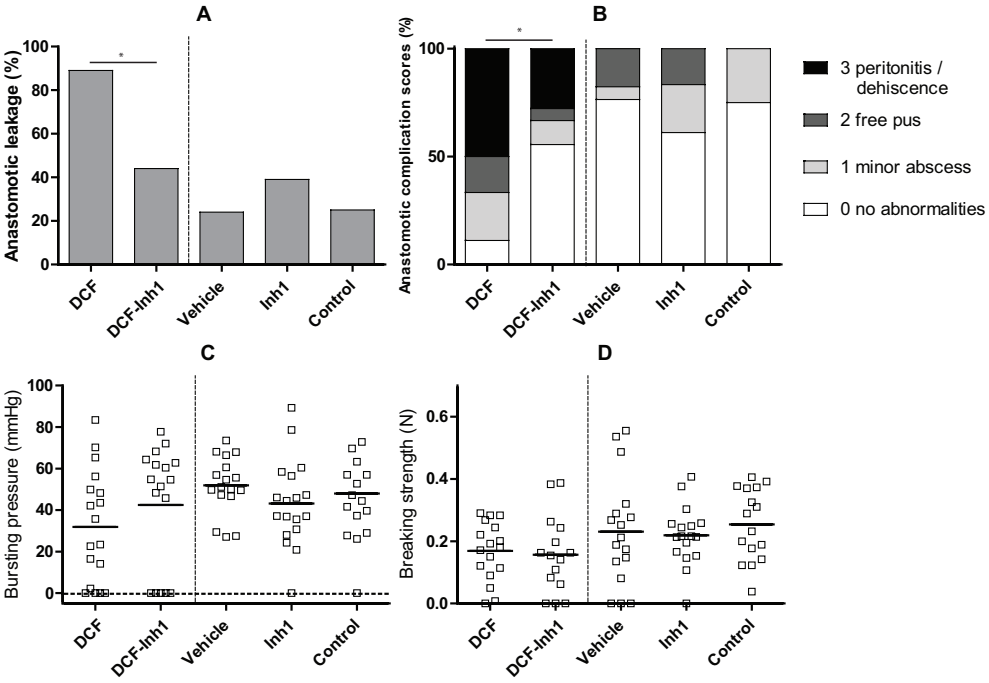


Figure 3 Anastomotic healing assessed by: A – anastomotic leakage; B – Anastomotic complication scores C – Bursting pressure; D – Breaking strength. * = P<0.05.

LCMS/MS analysis of diclofenac levels in plasma

Plasma concentrations of diclofenac dropped 60 minutes after intramuscular administration in all diclofenac groups (Figure 4). There was no significant difference between groups at any timepoint.

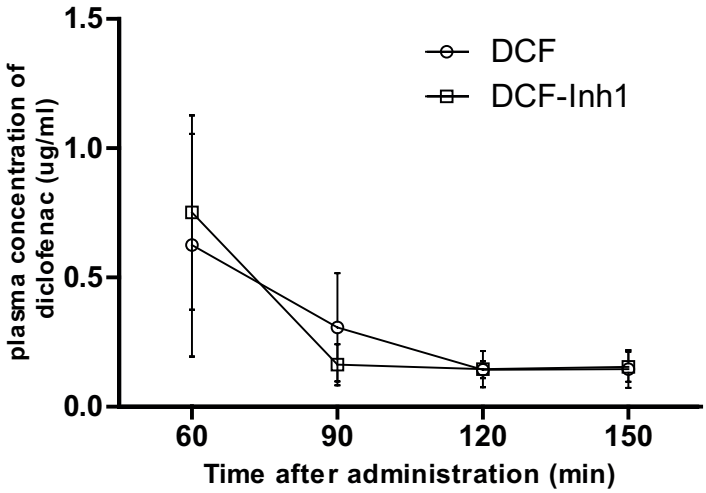


Figure 4 LCMS analysis showing no significant difference in plasma levels of diclofenac two hours after intramuscular administration.

Discussion

Oral administration of Inh1 significantly reduced anastomotic leak rate and severity in diclofenac treated rats. In groups receiving Inh1 or vehicle, minor signs of anastomotic healing disturbance were seen.

The finding that administration of a glucuronidase inhibitor reduces signs of leakage suggests that, without inhibition, cleavage of diclofenac-acyl-glucuronide metabolites to diclofenac aglycones disturbs ileal anastomotic healing. To assess if administration of Inh1 could lead to weaker systemic action by preventing reuptake of diclofenac molecules, we determined plasma levels of diclofenac at different time points. These showed similar pattern as without inhibition making a systemic action unlikely.[15, 22] The harmful effect of reactivated diclofenac metabolites is in line with other studies demonstrating that drug aglycones cause mucosal damage in the terminal ileum.[18, 19, 23] Findings also corresponds with results of former anastomotic healing experiments, in which it was demonstrated that diverting or replacing bile, and thereby preventing the entrance of drug metabolites into the gut, reduces severity of diclofenac induced leakage (yet unpublished data).

In the present study, a certain amount of leakage (33%) still occurred despite Inh1 treatment. Several explanations are possible. First, diclofenac is excreted through bile in multiple forms, including unconjugated diclofenac.[24, 25] This means that even if β-glucuronidase is blocked, other active diclofenac metabolites might reach and harm anastomotic tissue.[26] Second, inhibition of β-glucuronidase by Inh1 was insufficient. In contrast to previous studies, in which a single high dose of diclofenac was given to mice, in the present study diclofenac was administered twice daily for three days.[18] This results in a longer exposure to drug

metabolites. The amount of inhibitor we administered was increased accordingly, but may have been insufficient still. Efforts were made to administer higher doses of Inh1 (4000ug/kg/d) but preparation of the highly concentrated solution was troublesome and included severe clumping. Third, the remaining leak rate in group DCF-Inh1 could theoretically be a result of counteraction caused by intrinsic toxicity of Inh1 as a relatively high leak rate was observed in the inhibitor-only group. However, it mostly concerned low grade healing disturbances without other remarkable signs of toxicity and previous toxicity studies have shown no adverse effects.[19] Another plausible explanation for remaining leakage in group DCF-Inh1 is the pharmacological action of diclofenac, which involves COX1/2 inhibition. Whereas pharmacological COX1/2 inhibition is thought to be less relevant in NSAID induced enteropathy, the associated inhibition of prostaglandin E synthesis may play a role by interfering with inflammatory and proliferative processes in the healing cascade.[15, 27, 28]

The mechanism of topical damage and healing disturbance by NSAID molecules is thought to be a multistage process in which reuptake of diclofenac aglycones initially causes mitochondrial injury and cell death.[29, 30] Subsequently, activation of TLR4 on macrophages initiates an excessive inflammatory response.[30-32] This disturbs the macrophage-mediated balance of inflammatory and proliferative processes during wound healing. Excessive inflammation involves increased collagen breakdown and may thereby cause weak anastomotic scar tissue and leakage.[33, 34] Alternative mechanisms by which hepatic clearance of diclofenac can cause bowel damage are the formation of more toxic bile acid micelles and an increase in harmful enteric gram-negative bacteria.[14, 15]

A valid and reproducible rat model was used to study the influence of Inh1 on clinically relevant anastomotic leakage. In contrast to clinic though, animals were not fasted and no prophylactic antibiotics were given, in accordance to previous studies with this model. Fasting is considered to cause major discomfort to rats and both fasting and antibiotics could disturb the mucosa and microbial glucuronidase activity and thereby the objective of this study.

In the control group absolute leak rate was more than twenty percent, but it only concerned minor signs of healing disturbance. It is thought that such disturbances would also be found in clinic if every anastomosis would be checked and clinical relevance of such disturbances is hard to predict. The vehicle methylcellulose can have a laxative effect and thereby influence bowel physiology. Though no diarrhea was observed, this may have slightly confounded findings as three grade 2 complications were noticed in the vehicle group.

The mechanical strength results show that bursting pressure only moderately corresponds to leakage signs and that much larger sample sizes would be needed to detect differences. In accordance to previous studies breaking strength poorly correlated with leakage. As leakage is a much more relevant outcome the additional value of strength test is arguable.

Conform previous Inh1 studies, we attempted to measure mucosal permeability and ulceration with immunohistochemistry to have supportive evidence for leak reduction. However, quantitative analysis was not possible because destruction by peritonitis and ileus did not reveal sufficient samples.

The detrimental effect of reactivated diclofenac metabolites in the gut, as suggested in the present study, is thought to be similarly relevant in human. In humans, diclofenac metabolism also involves glucuronidation in the liver.[24, 35-37] This, however, also depends on individual pharmacokinetics, as disposition of drugs that are predominantly eliminated via acylglucuronidation can significantly vary among humans.[37] Differences in drug metabolism may explain why NSAIDs have not been convincingly identified as a cause of leakage. Possibly NSAIDs only harm anastomoses in a particular group of patients.[38]

In the current study an anastomosis of the distal ileum was used to assess the effect of Inh1 as previous studies found that both diclofenac-induced enteropathy and diclofenac-induced leakage are most severe in this particular segment. However, also for colonic and colorectal anastomoses this mechanism may be relevant. Altered bowel content and physiology following partial or subtotal colectomy may lead to comparably high levels of diclofenac metabolites and bile salts, and thus comparably high risk.[39]

In the current experiment Inh1 was used to evaluate the relevance of diclofenac-acyl-glucuronide reactivation in leakage pathophysiology and not as a potential therapeutic agent for preventing leakage in humans. Though this does not exclude its therapeutic potential in certain situations, it is simply easier to avoid diclofenac use because alternative analgesics are available.[40]

The present study suggests that reactivation of diclofenac metabolites in the gut leads to anastomotic healing disturbances. Administration of Inh1 to block this process reduces signs of leakage, but could not prevent a leak in all cases. In subsequent experiments a stronger effect could be aimed for by increasing frequency of Inh1 administration, instead of increasing Inh1 concentration.

In general, the present study contributes to the evidence that intraluminal compounds are relevant in leakage pathophysiology.[41] This means that, in order to reduce the clinical burden of anastomotic leakage, more research should be done beyond the scope of known partially non-correctable risk factors and etiologies (e.g. ischemia). It should aim to clarify the role of potential harmful intraluminal factors, which may also include specific bacterial virulence factors and some enteral nutrients.[41, 42, 23],

Administration of glucuronidase inhibitor Inh1, aiming at preventing reactivation of diclofenac-acyl-glucuronid metabolites in the gut, reduces diclofenac-induced anastomotic leakage.

Author disclosure statement

Co-author MR is co-founder of Symberix, Inc., a pharmaceutical company developing microbiome-targeted therapeutics. All other authors declare to have no conflict of interest concerning the current paper.

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THE SHIFT IN MICROBIAL COMPOSITION AND DIVERSITY IN A RAT MODEL OF NSAID INDUCED ANASTOMOTIC LEAK CAN BE ATTRIBUTED TO SURGICAL STRESS

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Abstract

Objective

To explore the influence of anastomotic surgery and NSAID treatment on the microbiome composition of different bowel segments and the association with leak rates.

Summary background data

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

Methods

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

Results

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

Conclusion

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

Introduction

Anastomotic leak (AL) is the most feared complication following intestinal surgery. It is associated with high morbidity and mortality and occurs in 6-12% of patients after colorectal resection [1, 2]. Leak rates have failed to decline over the past decades despite intensive research in this field, emphasizing the need for a better understanding of leak pathogenesis. Several recent studies have reinforced the hypothesis that gut bacteria play a central role in the pathogenesis of AL, by demonstrating a protective effect of antibiotics, and oral antibiotics in particular, against anastomotic leak[3, 4]. It has been shown that multiple surgery-related factors, such as fasting, bowel preparation, antibiotics, surgical trauma and analgesics, can influence the microbiome[5], and in turn, that microbial changes and increased bacterial virulence can disturb anastomotic healing[6, 7].

Since the past decade, nonsteroidal anti-inflammatory drug (NSAID) use has been associated with an increased risk of developing AL in both human and experimental studies[8, 9]. Although the main hypotheses are that this is caused by COX2-inhibition or topical toxicity of drug metabolites, several studies also support a role for the microbiome in NSAID-induced leakage[10-13]. Ueijima et al. demonstrated that the presence of specific microbes is a prerequisite for the induction of small intestinal ulcers in rats treated with NSAIDs[10]. Another study provided evidence that NSAIDs also have a direct influence on the microbiota composition. Following injection with indomethacin, an overgrowth of E. faecalis was observed in the rat small intestine which resulted in the relative diminution of commensal microbes such as segmented filamentous bacteria [14]. Interestingly, high collagenase producing strains of E. faecalis have also been associated with AL in both experimental and clinical studies [15]. We have previously demonstrated that the use of the NSAID diclofenac causes leakage of anastomoses in the ileum and proximal colon in rats, but not in the distal colon [16]. These observed differences in AL may be explained by differences in microbiota, as this is an important discriminating factor between these segments [17, 18].

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

Materials and methods

Ethics

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

Animals

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

Study design

The experiment was conducted as previously described [16]. 50 rats underwent a 1 cm bowel resection followed by the construction of an anastomosis. Depending on the group randomization, resection and anastomosis construction occurred either in the ileum (IL), proximal colon (PC) or distal colon (DC), under strict aseptic conditions. No antibiotics were administered. Groups IL+ (n=10), PC+ (n=15) and DC+ (n= 10) received diclofenac (3mg/kg/day) until sacrifice on post-operative day 3 (POD3), with the first dose administered 15 minutes prior to the operation. Rats in group PC- (n=15) did not receive diclofenac.

On POD3 animals were euthanized by CO2 asphyxiation. After euthanization, a relaparotomy was performed and the anastomosis and abdomen were inspected for macroscopic signs of anastomotic leakage. Two investigators independently assessed the presence and extent of leak by means of an anastomotic leak severity score. Any differences in scores were resolved by consensus. A score of '0' constituted no signs of leakage, a score of '1' constituted anastomotic abscesses, a score of '2' represented free pus or large abscesses, and a score of '3' stood for fecal peritonitis or visible dehiscence[19].

Feces sample collection

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

DNA isolation

DNA isolation was performed using a combination of repeated bead-beating and column-based purification as described previously [20]. Briefly, PSP lysis buffer (Stratec Molecular, Berlin, Germany) and approximately 50 mg of frozen stool aliquots were added to a sterile vial containing 0.5 g of 0.1 mm zirconia/silica beads and 4 3.0–3.5 mm glass beads (BioSpec, Bartlesville, USA). The samples were homogenized in a MagNA Lyser instrument (Roche, Basel, Switzerland) in three cycles of 1 minute at a speed of 5500 rpm, interspersed with cooling on ice for one minute in between cycles. DNA isolation was continued using the PSP Spin Stool Kit (Stratec Molecular, Berlin, Germany) according to the manufacturer's instructions. DNA was finally eluted in 200 µl elution buffer.

Sequencing

Amplicon libraries and sequencing were performed according to previously published protocols [21]. Briefly, the V4 region of the 16S rRNA gene was PCR amplified from each DNA sample in triplicate using the 515f/806r primer pair described previously [21]. Pooled amplicons from the triplicate reactions were purified using AMPure XP purification (Agencourt, Massachusetts, USA) according to the manufacturer's instructions and eluted in 25µl 1× low TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Quantification of amplicons was subsequently performed by the Quant-iT PicoGreen dsDNA reagent kit (Invitrogen, New York, USA) using a Victor3 Multilabel Counter (Perkin Elmer, Waltham, USA). Amplicons were mixed in equimolar concentrations to ensure equal representation of each sample, and sequenced on an Illumina MiSeq instrument.

Data analysis and statistics

Filtering, denoising, removing of chimeric sequences and clustering of sequences in Operational Taxonomic Units (OTUs) at 97% similarity was conducted using the LotuS pipeline (version 1.39) [22]. First, sequences with an average quality below 27, a read length below 170 bases, with one or more ambiguous bases, or containing homopolymer stretches of over 8 bases were discarded. Retained sequences were chimera filtered and clustered into OTUs using UPARSE[23]. Taxonomic annotation of OTUs was derived from RDP naïve Bayes classifier annotations (minimum acceptance confidence set at 0.8). OTUs representing less than 0.01% of the total number of observations (sequences) and OTUs that were only observed in a single sample were discarded. Downstream analyses were conducted using QIIME version 1.8.1 and R version 3.1.3[24].

Taxonomy, alpha and beta diversity, microbial core

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

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

Enterotyping

Enterotype analyses were performed as described previously by Arummougham and colleagues[26]. We used the R[27] package "vegan: Community ecology", version 2.2-1 by Oksanen et al. from 2011 for calculating the Bray-Curtis distance on the genus-level relative abundance profiles. To cluster the samples, we used the Partitioning Around Medoids (PAM) clustering algorithm in the R[27] package "Cluster analysis basics and extensions", version 2.0.1 ed. by Maechler et al. from 2012. The optimal number of clusters was chosen based on Calinski-Harabasz (CH) index and validated by the silhouette index (57) using the R[27] package 'clusterSim'. The optimal number of 2 clusters was identified based on the CH index, which was confirmed by the silhouette index although clustering was moderate (SI 0.47).
Between-class analysis (BCA) was performed to plot the samples using the R[27] package "Analysis of Ecological Data: Exploratory and Euclidean Methods in Environmental Sciences" version 1.7.2. by Dray et al. from 2015. The similarity percentage analysis (SIMPER)[28] was used to identify taxa contributing to similarity within- and dissimilarity between groups.

Results

Anastomotic leakage

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

Sequencing

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

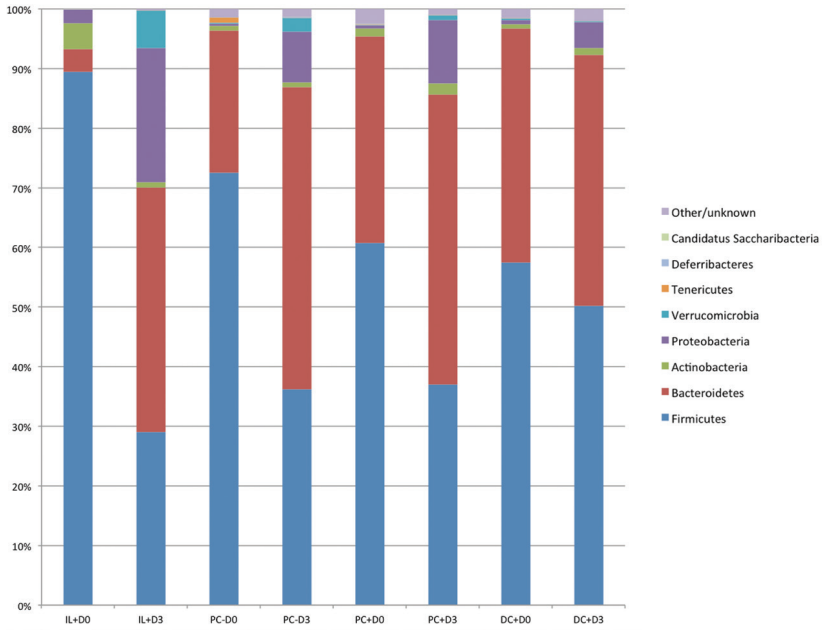


Figure 1A Phylum abundance in different anatomical regions (IL = ileum, PC = proximal colon, DC = distal colon), pre- (D0) and post-surgery (D3) and with or without diclofenac (+ or – respectively).

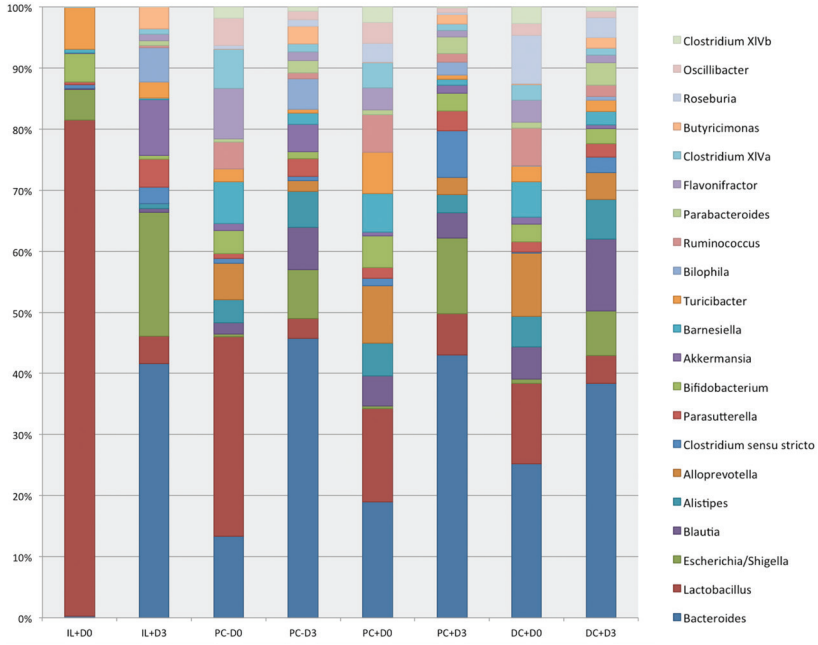


Figure 1B Genus abundance of 21 most abundant genera in different anatomical regions (IL = ileum, PC = proximal colon, DC = distal colon), pre- (D0) and postsurgery (D3) and with or without diclofenac (+ or – respectively).

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

Phylogenetic classification of the microbiota in the different anatomical locations identified 21 different families belonging to 9 bacterial phyla. The most abundant phyla in the fecal samples obtained from the ileum were Firmicutes, followed by Bacteriodetes and Actinobacteria. The most abundant genera were Bacteroides, Lactobacillus, Escherichia-Shigella, Turicibacter and Bifidobacterium (relative abundance of 23.91, 8.92, 7.17, 2.72 and 2.05% respectively). The genera that formed major constituents of the microbiota in the colon were Lactobacillus, Bacteroides, Alloprevotella, Blautia and Alistipes. More distally along the intestinal tract, the abundance of Firmicutes decreased, whereas Bacteriodetes showed an increase as compared to the ileum (Fig. 2).

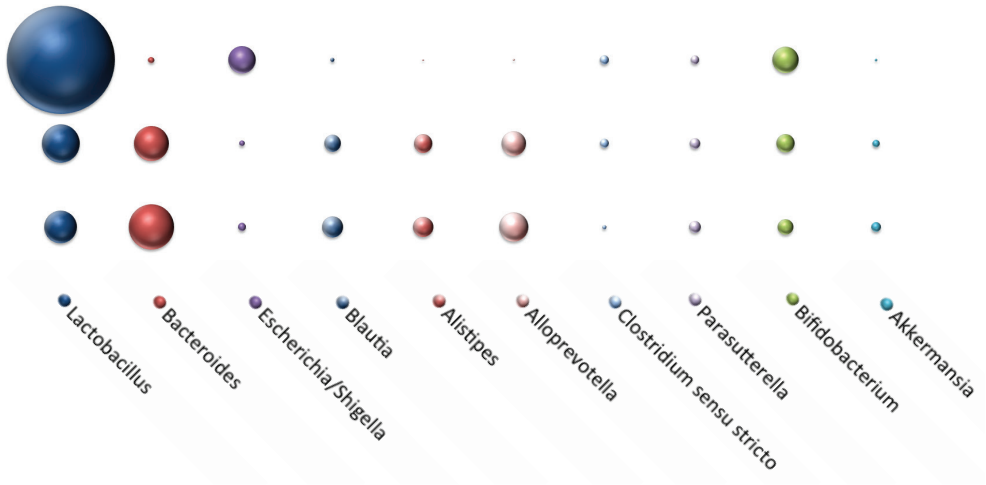


Figure 2 Bubblechart depicting the relative abundance of the ten most common genera in the ileum (IL), proximal colon (PC) and distal colon (DC).

Microbial diversity and community structure

Microbial richness and diversity of feces samples collected before anastomosis construction was expressed as the number of observed species, Chao1 index and Shannon diversity index respectively. The analysis revealed baseline differences in diversity between the various segments of the intestine with a gradual increase in diversity from ileum towards the distal colon; see figure 3 for a comparison of the different alpha diversity indexes. Preoperative fecal samples retrieved from the ileum demonstrated a significantly lower estimated microbial richness than samples retrieved from the proximal (PC- and PC+ combined) and distal colon ($p = 0.002$ and $p = 0.001$ respectively, as calculated from Chao1 data).

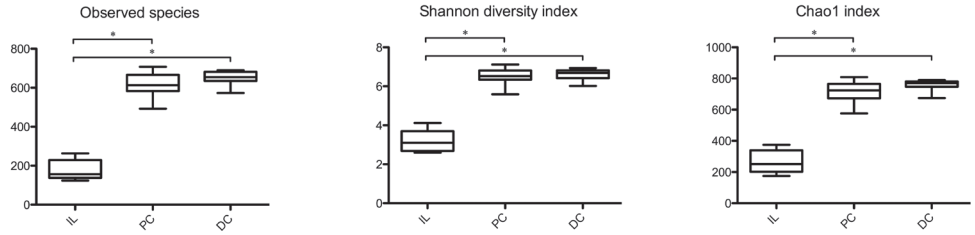


Figure 3 comparison of alpha diversity indexes of pre-operative samples of the different anatomical locations: IL (ileum), PC (proximal colon), and DC (distal colon).

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

The influence of surgery on microbial diversity and community structure

Surgery significantly decreased microbial richness and diversity in proximal (only PC+) and distal colon samples (Figure 4). A similar trend could not be observed in ileal samples, likely due to the limited number of paired pre- and post-surgery ileal samples.

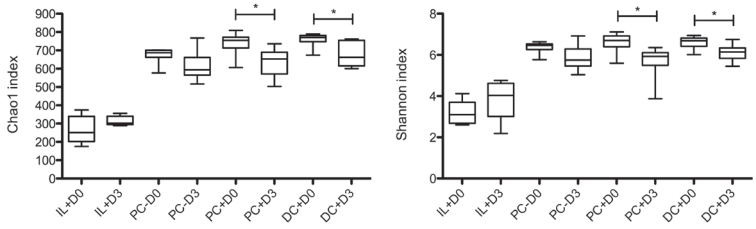


Figure 4 Microbial richness (Chao 1 and Shannon index) for all groups measured pre- and post-surgery. Asterisk indicates significance <0.05.

To further examine the effect of surgery on the microbial community structure in specific intestinal segments, a PCoA plot was created for each segment individually. A clear separation between pre- and post-operative proximal colon samples was observed along the first principal coordinate in PCoA plot based on the Bray-Curtis distance (Fig. 5B). This separation was partially, but not completely, driven by a reduced microbial richness in post-operative samples (see supplementary data for a colored PCoA plot according to the Chao1 index). This surgery-effect was also observed for both ileum and distal colon samples (see figure 5A and 5C respectively). In all intestinal segments, the same trend could be observed: all pre-operative samples clustered together, while the majority of the post-operative samples form a separate cluster with the exception of a few post-operative samples that remained clustered with the pre-operative samples.



Figure 5A Bray-Curtis: the first principal coordinate shows the effect of surgery on ileum samples: 75% of the variation could be attributed to PC1.

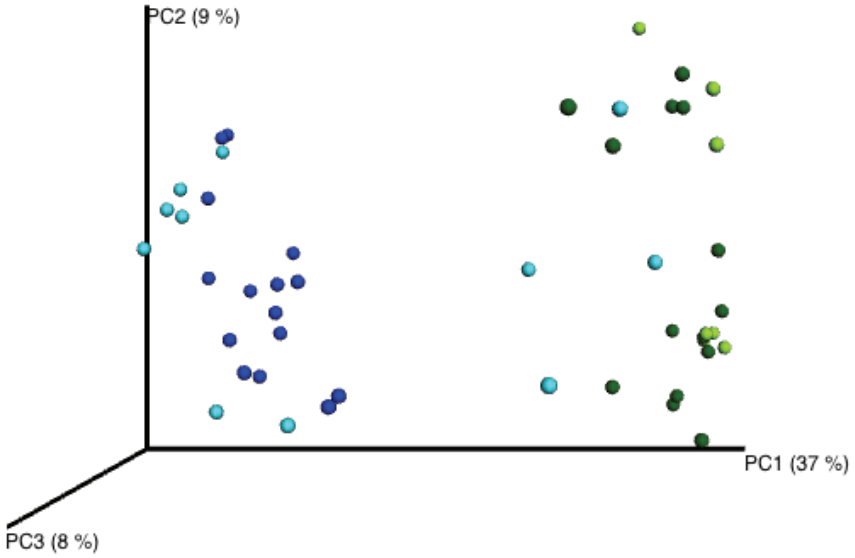


Figure 5B Bray-Curtis: the first principal coordinate shows the effect of surgery on PC+ and PC- proximal colon samples. No obvious effect of diclofenac can be observed in this separation.

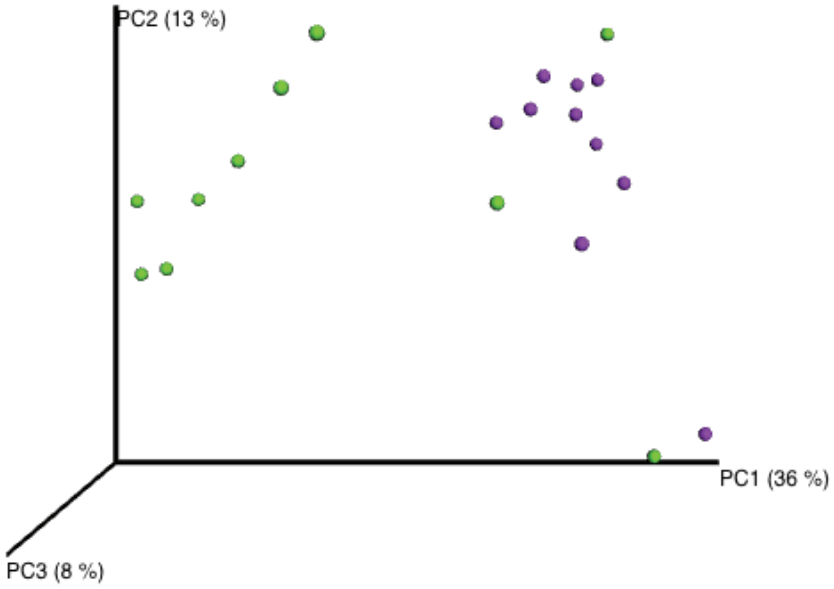


Figure 5C Bray-Curtis: the first principal component shows the effect of surgery on distal colon samples.

Enterotyping

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

Subsequently, paired analyses were performed to examine the differences in microbiome composition between pre- and post-operative proximal colon (PC+ and PC-) samples at phylum and genus levels (figure 6A). These analyses revealed a significantly lower relative abundance of the Firmicutes phylum on D3 compared to D0 ($p<0,001$), whilst the decrease in the abundance of Lactobacillus (the most important genus within this phylum) was not significant ($p=0.26$). An overall increase in the phyla Bacteroidetes ($p=0.001$) and the genera Bacteroides ($p<0.001$) and Alistipes ($p<0.014$) was observed, as well as an increase in the Proteobacteria phylum ($p<0.001$), which confirmed the findings in the enterotyping analyses. The most important genera within the Proteobacteria phylum showed the expected postoperative increase (Escherichia-Shigella $p<0.001$, Bilophila $p<0.001$ and Parasutterella $p<0.001$). A postoperative decrease in overall relative abundance was found for the phylum of Candidatus Saccharibacteria ($p=0.001$).

As was the case for the PC samples, paired analysis of the distal colon samples revealed a significantly higher abundance of several genera within the Firmicutes phylum, including the Blautia (p=0.005) and Clostridium sensu stricto (p=0.022) genera preoperatively, as well as a bloom of Proteobacteria genera such as Escherichia-Shigella (p=0.007) and Bilophila (p=0.009) post-operatively (Figure 6B).

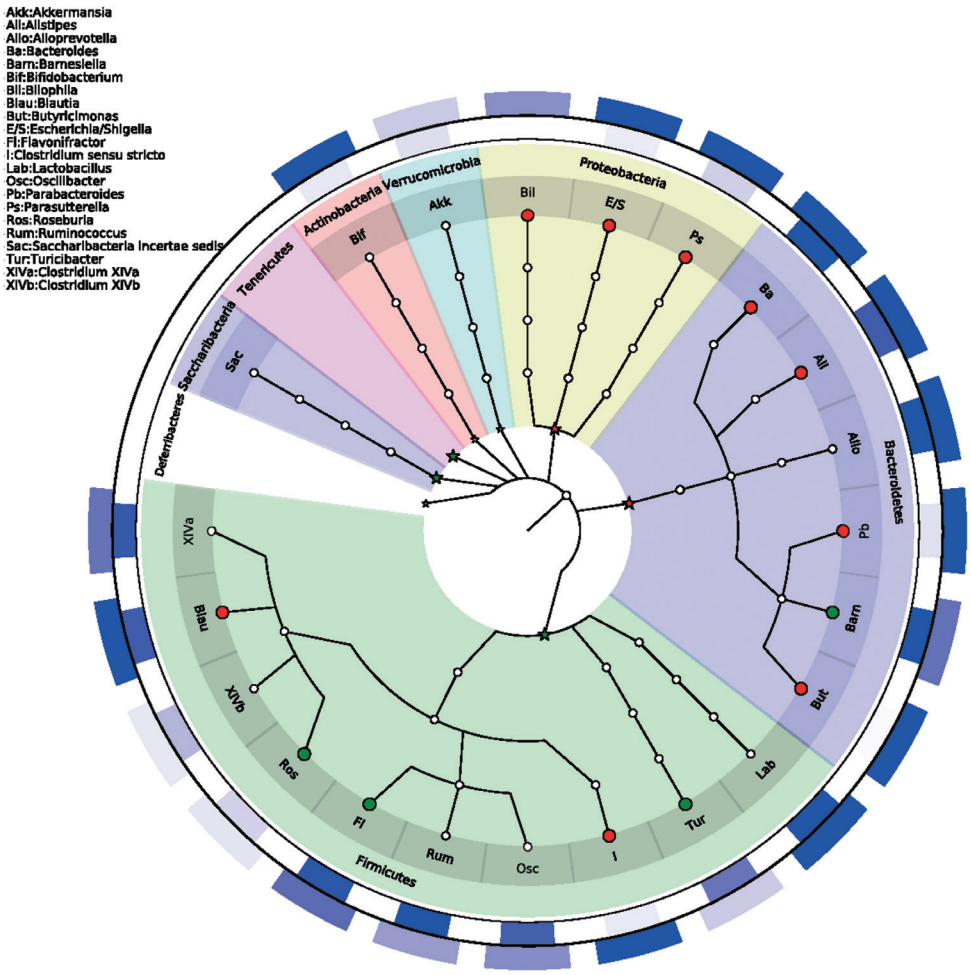


Figure 6A Cladogram representation of microbiota composition of proximal colon samples on different taxonomical levels. Green indicates significant higher relative abundancy in pre-operative samples, higher relative abundancy of post-operative samples is depicted in red.

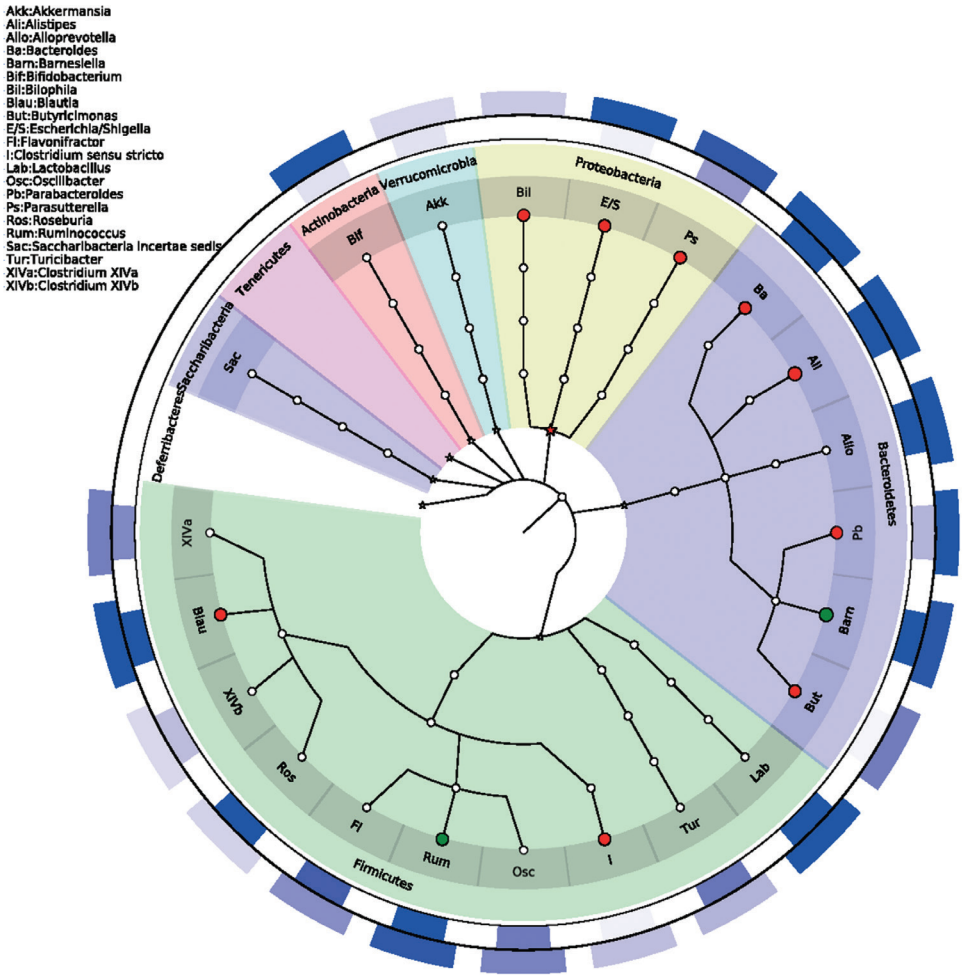


Figure 6B Cladogram representation of microbiota composition of distal colon samples on different taxonomical levels. Green indicates significant higher relative abundancy in pre-operative samples, higher relative abundancy of post-operative samples is depicted in red.

The effect of diclofenac administration on microbial diversity and composition

Alpha diversity

As stated before, in the PC+ group a decrease in alpha diversity was observed in samples obtained at postoperative day 3 compared to the postoperative samples (p = 0,001 for both alpha diversity measures). In the proximal colon group that did not receive diclofenac, microbial richness was also lower post-operatively, but this difference did not reach statistical significance (Chao1 index PC-D0: Mdn. 687,59, PC-D3: Mdn. 593,82, z = -1,483 p

= 0,138, Shannon index PC-D0: Mdn. 6,46, PC-D3: Mdn. 5,75, z = -2,023, p > 0,05 after FDR correction). Surprisingly, significant baseline differences in microbial richness were found between PC-D0 and PC+D0 samples collected before anastomosis construction (Chao1 index p = 0,035), but not in microbial diversity, (Shannon index p = 0,292).

Despite these baseline differences, the diversity in the PC+D3 group and PC-D3 group was comparable (Chao 1 index PC+D3: Mdn. 652,97, PC-D3: Mdn. 593, 82, p = 0,694, Shannon index PC+D3: Mdn. 5,93, PC-D3: Mdn. 5,75, p = 0,823). The difference in diversity in the PC+ group between D0 and D3 was not significantly greater than that in the PC- group (p = 0,123 and p = 0,956 for Chao1 and Shannon index respectively). See figure 7 for the associations between the different time-points for the paired samples.

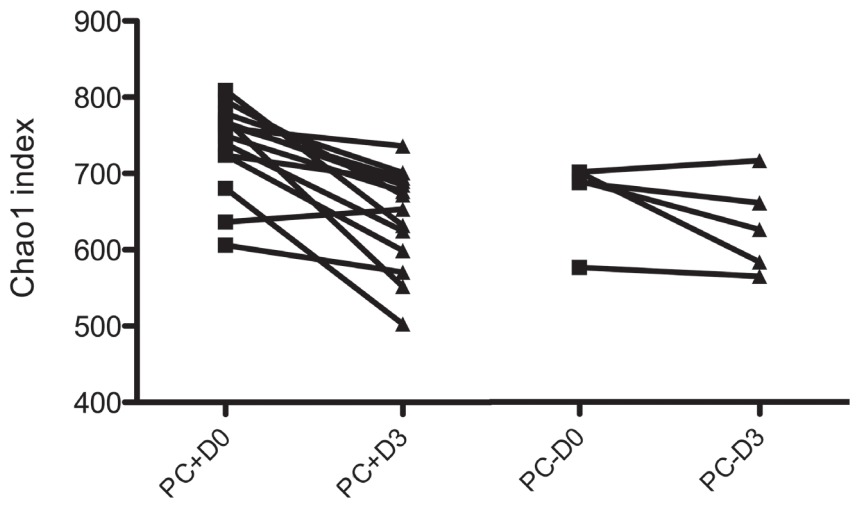


Figure 7 Difference in Chao1 index for paired samples on different timepoints between groups PC- and PC+.

Beta diversity

To examine whether the microbial community structure within the PC+D3 and PC-D3 groups was more similar than the microbial community structure between the groups, the average within- and between-group Bray Curtis dissimilarity was examined. Samples within the PC+D3 group showed the strongest homogeneity, as indicated by significantly lower within-group Bray Curtis dissimilarity compared to the PC-D3 group (p = <0.001) (Fig. 8). When comparing OTUs between PC+D3 and PC-D3 group, only one out of >1000 OTU's was found to be significantly different: the OTU belonging to Anaerostipes from the family of Lachnospiraceae (p = 0,046 after FDR correction). Together with the similar post-operative decrease in alpha-diversity in the PC+D3 and PC-D3, these results provide little evidence for an effect of diclofenac administration on microbiota composition.

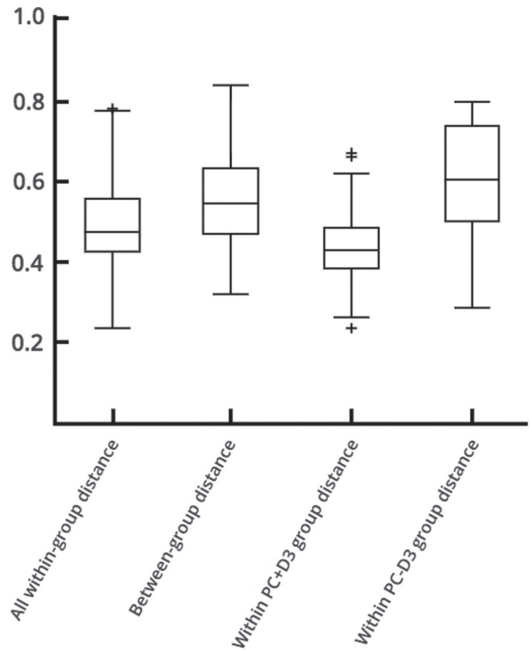


Figure 8 Within-group Bray-Curtis dissimilarity of proximal colon samples. Samples within the PC+D3 group showed the strongest homogeneity.

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

Anastomotic leakage was observed in ileal and proximal colon (PC+) samples, but only sporadically in PC- or distal colon samples. For the analysis of the association between AL and microbial composition and diversity, only paired samples were taken into account: in the PC+ group, 10/14 rats (71,4%) showed signs of AL, whereas only 1 of 10 rats in the distal colon (10%) leaked. In order to investigate the relation between the microbial composition and the occurrence of leakage in the proximal colon and distal colon samples, we compared alpha diversity scores between leakage and non-leakage samples. Alpha diversity indices were not significantly different between postoperative leakage and non-leakage samples from the proximal colon (Chao1 index: Mdn. 642,40 vs. 682,91 respectively, z=-0.990, p=0.37, Shannon index: Mdn. 5,66 vs. 6,07 respectively, z=-1,414, p=0.19), or the distal colon (Chao1 index: Mdn. 606,00 vs. 665,53 respectively, z=-1,219, p=0.40, Shannon index: Mdn. 6.01 vs. 6.20, respectively, z=-0,522, p=0.80).

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

Discussion

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

A possible explanation is that the current study was not able to detect such influence, as surgery alone has proven to be a strong factor to alter the microbiota, and may have subsequently camouflaged influences exerted by the administration of diclofenac[7]. Other studies have shown that NSAID treatment lead to an obvious decrease in the number of gram-positive bacteria[13, 29]. Another study found increased numbers of Enterobacteriaceae and Acidominococcaceae in the feces of NSAID-users compared to controls[30].

The trauma of undergoing surgery alone may suffice in explaining the alteration of the normal microbial composition[7]. Lactobacilli and short chain fatty acids, both considered to have a cytoprotective effect, decrease by more than 90% within 6 hours after major trauma[31]. Our results are also in concordance with a study by Shogan et al. in which a decrease in the relative abundance of the Firmicutes phylum and an increase in Proteobacteria genera such as Escherichia-Shigella was observed post-operatively[5]. Shogan et al., however, found a more dramatic increase in the relative abundance of Enterococcus. Because of the short time frame in which this alteration takes place, it is considered to be a result from chemosignalling rather than a depletion of nutrients in the gut[32]. Another possible explanation is the exposure of the gut lumen to atmospheric air, which will affect the obligate anaerobic species[33, 34].

Several features of the luminal content as well as the structure of the intestine have been suggested to play a role in the observed segmental differences[17, 18]. The small intestinal content is known to have a lower pH, higher oxygen and bile acid levels as compared to the colon, which facilitates colonization by facultative anaerobes, mainly Lactobacillaceae and Enterobacteriaceae (belonging to the Firmicutes and Proteobacteria phyla respectively). The colon on the other hand, characterized by low oxygen levels, slow transit and high nutrient levels, is mainly inhabited by Bacteriodetes or Firmicutes phyla. The microbiome changes observed in this study were greater in the proximal colon compared to the distal colon. The anastomotic leak rate was also higher in the proximal colon.

It is possible that the microbial shift in the proximal colon increased its susceptibility to damage caused by diclofenac. Other studies have shown that certain antibiotics and probiotics reduce the severity of NSAID-induced enteropathy, especially when they target gram-negative bacteria[35, 36]. Another important clue for the interaction between NSAIDs

and bacteria is the finding that NSAID enteropathy does not occur in germ free mice, but, when Eubacterium limosum or E. coli is introduced, intestinal ulceration is observed [10, 37].

However, there may be other explanations than an altered microbiota composition for the increased leakage rates in the ileum and proximal colon in this study. Inhibition of cyclo-oxygenase (COX) consequently leads to inhibition of its products: the prostaglandins. By inhibiting the prostaglandins E2 (PGE2) and I2 (PGI2), two important inflammatory mediators in the gut, several hallmarks of inflammation are reduced as well [38]. As they also play an important role as mediators of mucosal defense and repair, NSAID administration leaves the gut lining more susceptible to cytotoxic components of the luminal content [39].

Another property of this group of drugs that may explain the observed alterations is their metabolism. Many NSAIDs (including diclofenac) are excreted through bile after being absorbed in the distal intestine and being glucuronidated in the liver. After their excretion together with the bile (free or glucuronidated), they exert secondary effects on the intestinal wall, contributing to epithelial damage [40].

The decrease in the relative abundance of Lachnospiraceae observed in the colonic segments may have detrimental consequences for epithelial permeability as many of the intestinal butyrate producers belong to the Lachnospiraceae family [41]. Aside from facilitating tight junction assembly, butyrate is associated with many other beneficial properties in the colon. Butyrate acts as an energy source for intestinal epithelial cells, reduces oxidative stress in the mucosa and by extension inflammation, and is an anti-carcinogenic [42]. Interestingly, in inflammatory bowel disease the most consistent change that is observed is also a marked decrease in the representation of the Firmicutes phylum (including Lachnospiraceae) [43].

No clear association between microbial composition and the occurrence of anastomotic leakage was found in this study. This could be explained by the relatively low numbers of leakage, as well as the strong influence of the surgical intervention on the microbiota. A recently conducted clinical study did find a positive correlation between the occurrence of CAL and low microbial diversity, as well as with a high abundance of Bacteroidaceae and Lachnospiraceae families[44]. It has been further suggested that aside from microbial composition and diversity, microbial virulence also plays an important role in leakage pathogenesis. Bacteria have been shown to demonstrate an upregulation of virulence factors such as collagenase activity, swarming motility and epithelial adhesion in response to changes in their direct environment such as phosphate depletion[45], release of inflammatory mediators[46], and the release of endogenous opioids[47]. Even symbiotic microbes can express virulence as a reaction to cues coming from their host. This increase in bacterial virulence can promote the emergence of a pathobiome capable of penetrating the intestinal barrier and causing anastomotic leak [48, 32]. In order to investigate this hypothesis, analyses cannot remain limited to taxonomic composition and diversity quantification; analysis of the functional context of microbial species is vital in order to determine the clinical relevance of the presence of specific bacterial species.

The strength of this study is the simplicity of the model used; results are not tainted by the administration of antibiotics, bowel preparation or radiotherapy – interventions that are commonly used in other animal experiments and, most importantly, in clinical practice. This allowed us to isolate and identify changes on the microbiota composition that were

induced by surgery or NSAIDs alone. We included the proximal colon anastomosis in our experiments, a type of anastomosis that is often neglected in experimental CAL research, despite the fact that right-sided (ileocolic) anastomosis are common and show significant leak rates in recent series[49]. The most important limitation of this study is the low leak rate of the distal colon anastomoses, which can largely be attributed to the type of model used. Several additional interventions can be employed in order to increase the leakage rate of distal anastomoses (e.g. ischemia, radiotherapy, morphine), but this would increase the complexity of this specific model and would hamper interpretation of single intervention effects.

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

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9.
GENERAL DISCUSSION AND FUTURE PERSPECTIVES

9.1 Anastomotic healing (patho)physiology

We showed in our overview of anastomotic healing that studies on the molecular biology of anastomotic healing are scarce and many pathways and fundamental elements of the healing process remain unknown. To follow healing over time, researchers used animal studies in which sacrifice and immunohistochemistry analysis was conducted in different animals at consecutive times [1–4]. In human research, only surrogate markers of healing processes can be determined, via drains in the operative field near the anastomosis or by faecal content analyses. In particular, the (disturbance of) cellular and molecular processes of healing in time in one individual is difficult to assess in both animals and humans, due to a lack of non-invasive techniques.

Several recent techniques used in human cancer and inflammatory bowel disease research have the potential to shed light on the dynamic process of anastomotic healing. Two promising techniques are confocal endomicroscopy, which provides visualisation of mucosal micro-architecture, and endocytoscopy, which uses ultra-high magnification (450x) to visualise mucosal cells [5]. Both techniques yield on-site tissue information and allow researchers to visualise cellular processes within the anastomotic wound in real time [5]. This is a step forward compared with traditional histology, which has to be done on biopsies or resected specimens. Endoscopic microscopy can be used in translational research, but also in clinical research. Using endomicroscopy could involve the risk of trauma to the fresh anastomosis by the scope. However, intra- and postoperative endoscopy has been proven to be safe, and some researchers even suggest that it can lower anastomotic leak rates [6–8].

A technique which holds promise to increase knowledge of the molecular processes of healing is mass spectrometry [9]. Variants of this technique, such as matrix-assisted laser desorption/ionisation mass spectrometry and imaging mass spectrometry, can identify specific proteins such as collagen in deeper layers of anastomotic tissue. Such analyses could reveal which set of proteins (the proteome) in the bowel wall reflects a firm and healthy scar and which proteome develops towards a fragile and leaking scar. Similar approaches determining the proteome of the bowel wall have been used to predict the progress of inflammatory bowel disease [9]. Histological collagen analyses have been conducted on anastomotic tissue, but modern proteomics have yet to be incorporated into anastomosis research [1, 2].

Real-time non-invasive monitoring of cellular and molecular processes may reveal temporal clues for enforcement of healing and prevention or reversal of disturbed healing. Targeted therapies may then be developed to modulate inflammatory and proliferative processes in a specific direction for each patient and at the right moment [10–12].

9.2 Review of published animal research in this thesis

In Chapter 3 we concluded that animal research on AL is of poor quality and still increasing in magnitude, which is contrary to societal aims regarding the use of animals for research. The main points of criticism were the large variety of animal models, the insufficient quality of reporting, and the failure to use randomisation, blinding and relevant outcome measures. In all studies in this thesis a rat model was used, which is in line with the recommendation

of a recent Delphi analysis on anastomosis research in animals. The authors of this analysis advised against using rabbits, dogs, guinea pigs and monkeys for this topic, but rather mice, rats and pigs, depending on the research question [13]. The authors aimed to provide further recommendations on the best anastomosis model but were unable to reach a consensus.

The rat experiments in the current thesis have been well validated by previous researchers within our department [1, 14, 15]. By standardising the rat strain, gender and age, as well as the anastomotic technique, perioperative care and strength measurements, the risk of confounding was markedly reduced. Randomisation and a proper perioperative analgesic regimen were also used in the experiments [16]. As discussed, many studies still do not use or report these measures, which are important for the internal validity of study results.

In our experiments we improved the operative procedure by introducing a complete sterile approach, mimicking the clinical situation, as routine. Prior to this we had used an aseptic technique which only involved shaving and disinfecting the rats, as many research groups still do. This runs the risk of bacterial contamination in the peritoneal cavity, with potential to disturb anastomotic healing processes. Now, rats were also draped with sterile covers, instruments were autoclaved in sterile packages, sterilised between procedures and prepared on sterile tables, and sterile gloves and gowns were used during surgery (see Figure 1).



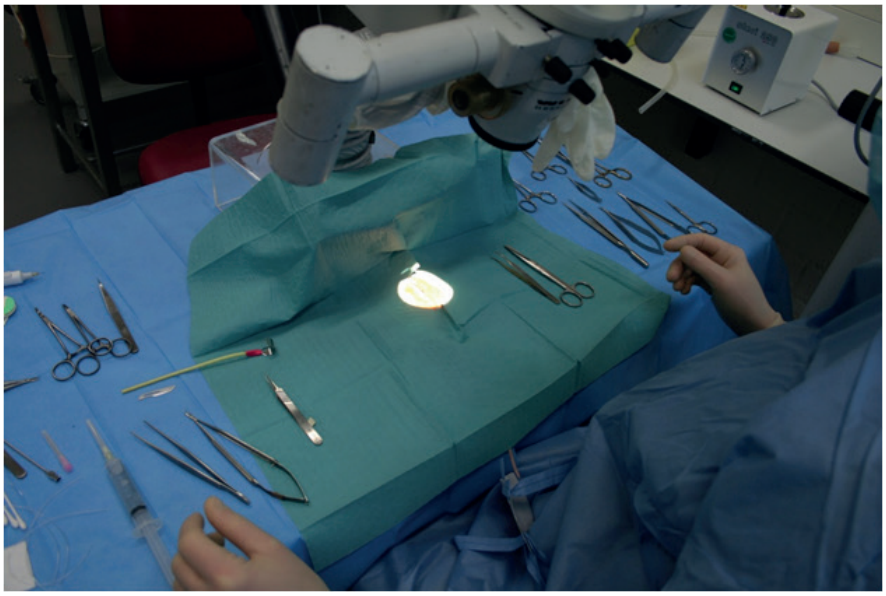


Figure 1 Pictures of the surgery, including sterile drapes, instruments, gowns and gloves.

We introduced blinding of the researcher performing the surgery and blinding of the outcome assessor to improve validity in all our experiments. This was facilitated by a second researcher who randomised the order and covered the tail markings before surgery and before sacrifice. Blinding of the surgeon and blinding of the outcome assessor was reported only in 5% and 8% of experimental studies on anastomotic healing, respectively (17).

An important insight from the review was that many research groups failed to adequately describe and report the occurrence of AL in their studies. We addressed this topic by using a detailed scoring system for reporting disturbed anastomotic healing. It was first termed the Leak Severity Score and later the Anastomotic Complication Score (ACS). This four-point scale distinguishes between no abnormalities, minor abscesses, free pus, or generalised peritonitis, which are relevant entities for clinical outcomes (Figure 2). Scoring leakage according to severity and 'clinical' consequences is more in line with current advice in the literature and allows for a better comparison among groups [18, 19]. It also matches the leak categories of national audits and databases [20].

The ACS is easy to apply and shows good interobserver agreement, but it has not been formally validated. One drawback is the need for sacrifice of the animal or the use of an invasive technique (laparoscopy or laparotomy). Recently we started a project to frequently scope the proximal and distal bowel anastomosis in rats to map the macroscopic healing of an anastomosis under normal and compromised conditions from the luminal side. In this project we aim to develop an endoscopic healing score which we will compare with the ACS. A validated endoscopic healing score in animals holds more promise compared to the intraperitoneal ACS for use in clinical studies.

Some researchers are attempting to mathematically approach anastomotic healing with computer modelling, in the hope of avoiding animal experiments in future. Animal data of molecular and cellular processes can fuel these models [21]. Although some processes, for example collagen formation, might be modelled accurately, we believe that experimental animals are still needed to unravel the complex healing processes of an anastomosis. However, the number of animals can certainly be reduced in the coming years by rigorously following the guidelines for animal research, sharing study designs and methodologies more freely among research groups and reporting negative results. Several collaborative initiatives to optimise animal research in general are being undertaken by SYRCLE (SYstematic Review Center for Laboratory animal Experimentation) and the CAMARADES group (Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies) [22, 23].

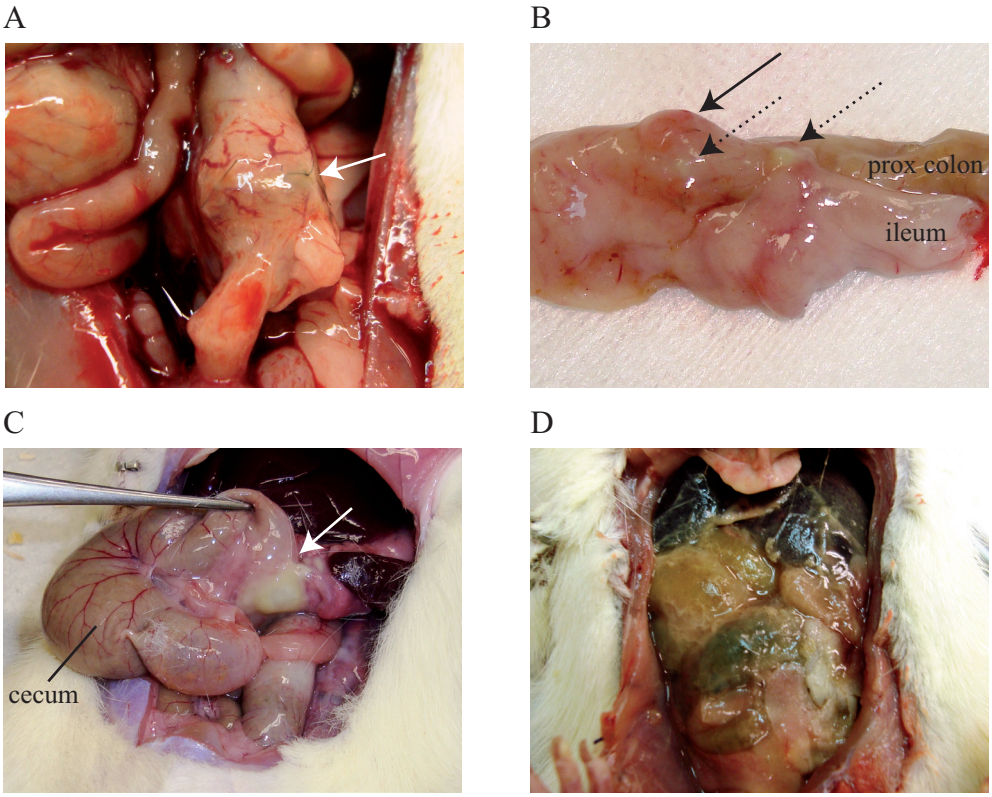


Figure 2 Anastomotic Complication Score with examples of each score. Signs were scored as 0 for uncomplicated anastomoses (A; distal colon), 1 for minor healing disturbances such as small abscesses (B; proximal colon), 2 for free pus or large abscesses (C; proximal colon) or 3 for dehiscent anastomoses or generalized (fecal) peritonitis (D; ileum). Solid arrows: anastomosis. Dotted arrows: small abscesses.

9.3 Pathophysiology of NSAID-induced anastomotic leakage

The reported risk of leakage following NSAID use varies from 0% in some clinical studies to 100% in some rat studies and in the current thesis [24, 25]. The risk seems to depend on NSAID subtype, but evidence is equivocal as to which NSAIDs are most harmful [15, 26, 27]. The subtype diclofenac is associated with AL in almost all reported studies [15, 27–32]. Risk of leakage increases with repeated administration of diclofenac starting at surgery, compared with occasional and postponed (repeated) administration [25, 27, 29]. The association between delay in administration and leak occurrence suggests a detrimental effect during the early inflammatory and vulnerable phase of wound healing[25].

Intestinal segment

Clinical cohort studies on NSAID-related leak risk mostly involve colon and rectum surgery [24, 27, 30]. We demonstrated that diclofenac causes leakage in the ileum and the proximal colon of rats, and not in the distal colon. The proximal colon leakage model was also adapted by the Maastricht group in mice, in which a Muc2-deficiency (Muc2 is a structural mucus protein) caused leakage in this segment [33]. With ex vivo studies the Maastricht group also showed that administration of intraperitoneal prostaglandin E2 (PGE2) increases mucous secretion in the small intestine and proximal colon, but not in the distal colon. NSAIDs like diclofenac limit the formation of PGE2 by inhibition of cyclooxygenase-2 (COX-2). This suggests a mucous modulating action of NSAIDs on leakage in the small intestine and proximal colon.

In patients, leak rates of ileocolic anastomosis are often higher than those of colo-colonic anastomosis following left hemicolectomies or proximal sigmoid resections [27]. The differences in leak susceptibility may underlie differences in leak aetiology along the gut [27, 34–36]. An intestinal-part-dependent leak aetiology has not been described earlier and should be further explored clinically because it has important consequences for measures to prevent leakage. Available animal data indicate that NSAIDs should not be prescribed during or soon after small-bowel and right-colonic surgery.

Damaging mechanisms

There are several hypotheses for the detrimental effect of NSAIDs. Some concern the suppressive effects of COX-2 inhibition on the principal inflammatory and proliferative activity in the early phase of wound healing [25, 27, 37]. These could include impaired vascularisation, reduced host defence, delayed mucosa regeneration and weakened mucosal secretion [33, 38]. Other hypotheses concern NSAID-related enteropathy and direct toxicity from the gut lumen. This may imply toxicity of drug molecules, changed bile or an altered microbiome [39–46].

In our study, anastomotic complications seemed the greatest when diclofenac was given orally, when bile contained diclofenac metabolites and when reactivation of metabolites in the gut was not inhibited. Enteral drug molecules could ‘simply’ cause direct cytotoxic damage. This is supported by studies with sustained-release forms of NSAIDs, by case reports of colonic perforations caused by unsolved diclofenac pills and by studies on NSAID-induced

enteropathy [39–49]. Alternatively or additionally, high enteral concentration of active drug molecules may exert high pharmacologic activity, e.g. COX-2 inhibition locally at the site of the anastomosis, and impair neovascularisation and reduce mucus secretion [33, 38]. Such an underlying mechanism is supported by studies with COX-2 knockout mice and studies in which prostaglandin E2 (PGE2) is used to promote healing. It is difficult to point out which action of COX-2 is most relevant, because the enzyme is involved in multiple other cellular reactions, such as paracellular closure by tight junction formation and migration of epithelial cells (Chapter 2). As previously mentioned, proteomics and microscopic endoscopies could shed light on these events in anastomotic healing.

Microbiota composition

An estimated 1013 bacteria reside in the gut to facilitate tissue homeostasis. It is attractive to ascribe leakage to gut bacteria in general and when induced by diclofenac [50, 51]. Factors such as fasting, bowel preparation, antibiotics, surgical trauma and medication potentially disturb the microbiome and are negatively or positively correlated with anastomotic healing [52–56]. Several studies support a role for the microbiome in NSAID-induced leakage by demonstrating that NSAID treatment can alter the microbiome or make the gut more vulnerable to specific microbes [40, 57–59].

We demonstrated that anastomotic surgery drastically altered the microbiome in different segments of the gut. A short period of postoperative diclofenac treatment did not lead to detectable changes. It is probably that large alterations caused by the surgery itself masked a potential diclofenac effect. Although our hypothesis that diclofenac exerts (part of) its actions through microbial changes could not be confirmed, significant changes in microbiome composition were observed. There is growing evidence from our study and others that gut bacteria play an important role in leak pathogenesis. In a recent study the mucosal diversity of anastomotic tissue doughnuts of patients who developed AL were compared with those who did not develop leakage. The authors found a correlation between AL and a low microbial diversity. The authors argued that the reduction in diversity was associated with an overabundance of members from the mucin-degrading families Lachnospiraceae and Bacteroidaceae [60]. Other studies showed a benefit from adding oral antibiotics (selective digestive decontamination, SDD) to the standard intravenous prophylactic antibiotic regimen to reduce anastomotic leak [56, 61–63]. With SDD more-pathogenic gram-negative bacteria and yeasts are eliminated in favour of less-pathogenic gram-positive bacteria. Recent experimental studies performed by a Chicago group in collaboration with our group emphasised the detrimental role of changed virulence of the normal microbiome by demonstrating that more virulent gram-negative phenotypes are present at the site of a leaking anastomosis [64–66]. It has been suggested that these bacteria become more virulent in a phosphate-depleted environment, and that this status can be reversed by phosphate administration [64].

Though evidence on the relation between gut microbes and AL is growing and becoming more detailed, this research is still in its infancy. This is understandable because techniques to analyse microbiota composition (e.g. 16S rRNA analysis and sequencing) have only recently become more efficient, readily available and affordable. We believe that future experimental studies addressing anastomotic healing should incorporate microbial

analyses and ideally also aim to analyse the gut metabolome. In metabolomics, nuclear magnetic resonance spectroscopy or mass spectrometry is used for a more comprehensive assessment of all endogenous metabolites within a biological system, such as the gut [67]. Analyses of intestinal tissue or faeces can provide the complete set of metabolites that is present, which involves proteins, fatty acids and bile salt [68, 69]. Detection of favourable and harmful metabolomes could set the stage for proper measures to prevent leakage.

Interacting intraluminal factors

In the search for the cause of leakage, emphasis has been placed on the condition of the bowel wall, e.g. anastomotic technique and vascularisation. A watertight, well-vascularised anastomosis is obviously favourable to maintaining integrity. Recent evidence, however, shows that the relevance of ischaemia in causing AL is limited [70]. This is in line with some animal models in which extensive ischaemia must be induced to cause only a limited amount of leakage.
Intraluminal factors have been largely neglected in anastomosis research. The gut is filled with many factors that may be relevant for anastomotic integrity, such as bacteria, bile products, nutrients, enzymes and metabolites [71]. Our research demonstrates that intraluminal factors can induce anastomotic complications. In concordance with the literature on drug-induced enteropathy, we suggest that luminal drug metabolites, biliary products and gut bacteria play a role in jeopardising the anastomotic wound. Moreover, we suggest that the interaction among these factors induces a more hostile luminal environment (Figure 2).

By definition, drugs which are hepatically cleared affect the bile composition. Bile salts and drug metabolites are changed by gut bacteria. In turn, the supply of bile products and the administration of drugs can affect the microbial composition. Drug metabolites, bile products and certain bacteria may all be toxic for the intestinal wall, and each factor may affect the other [47–49, 72, 73]. This interplay of intraluminal factors should be further investigated to gain more detailed knowledge of leak pathology. The bile exchange model we developed provides a means to study such interactions. Not only bile, but also nutrients, bacteria and drugs can be delivered directly into the gut to intervene with intraluminal factors and study the effect on anastomotic healing and other bowel diseases. Taking into account the elaborate execution of the bile diversion and exchange experiments, concerted action by multiple research laboratories might be necessary to obtain results in a timely manner.

9.4 Clinical use of NSAIDs

This thesis adds to the cumulative evidence that NSAIDs increase the risk of AL, particularly for right-sided anastomoses. The animal findings concur with some clinical studies showing higher leak rates in patients using NSAIDs. However, several large cohort studies report no significant increase in the risk of leakage [74]. Kverneng et al. recently showed a leak rate of 11% in the NSAID group and 14% in the non-NSAID group [74]. Notably, ibuprofen, a non-selective COX-2 inhibitor, was the most-used NSAID (70%) in this study. When comparing leakage between the selective and non-selective COX-2 inhibitor groups there was a trend of higher leak rates in the selective COX-2 inhibitor group (17%, compared with 10% in the non-selective group). There were also significantly more ileostomies constructed in the NSAID group and patients suffered less blood loss, two factors that are known to affect leak

rate. Furthermore, leakage which occurred in the first 48 hours was excluded from analysis because the authors concluded that technical failure must have been the cause. This shows that results of clinical studies should be interpreted with caution and that the type of NSAID should be taken into account when claiming an effect or no effect on anastomotic leakage.
A recent review concluded that available data did not permit a formal recommendation regarding clinical use of NSAIDs because of heterogeneous patient populations and multiple biases [75]. The principal conclusion was that the balance of benefit versus risk (i.e. analgesic effect versus risk of anastomotic disruption) is acceptable. Hence, the authors advised avoiding post-operative NSAID use in the presence of other risk factors for leakage.

Based on the current thesis and available literature, and because good analgesic alternatives are available, we call for a withdrawal of at least diclofenac from current guidelines in gastrointestinal surgery [76, 77]. If deemed necessary, we advise starting an NSAID no earlier than 48 hours after anastomosis and only for a limited time, but not using an NSAID in right-sided or small-bowel anastomoses.

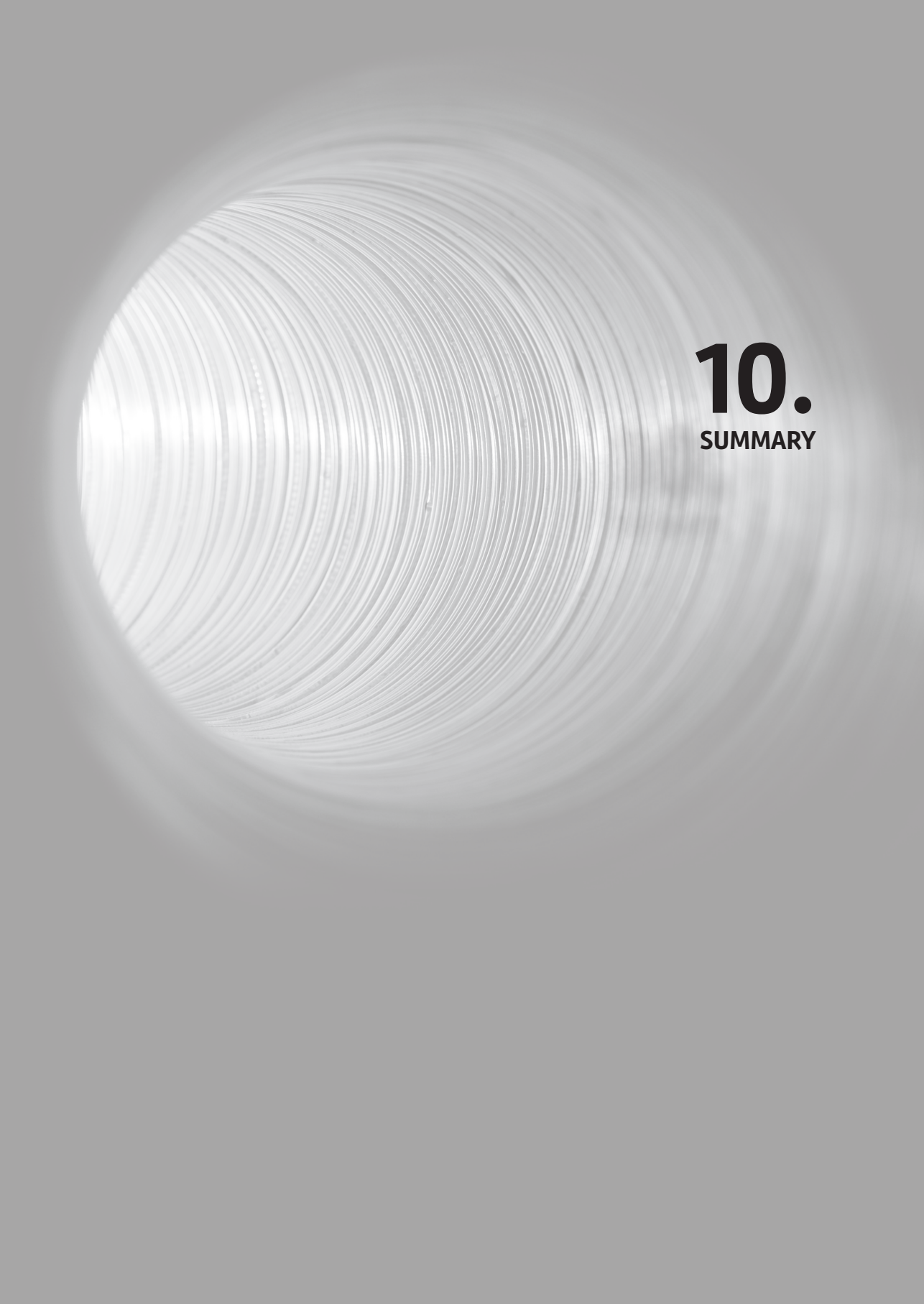
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10.
SUMMARY

The burden of anastomotic leakage (AL) has been well described in literature and includes higher morbidity and mortality, greater recurrence of cancer and increased costs [1, 2]. This burden has not decreased significantly in the past decades [3, 4]. An important reason is that leakage aetiology is still poorly understood [3]. In this thesis we aimed to increase knowledge of (disturbed) healing of small- and large-bowel anastomoses. We investigated the detrimental effects of NSAIDs—common painkillers used in gastro-intestinal surgery—on ileal and right-colonic anastomoses.

Anastomotic healing physiology: a complex cascade of cellular reactions and inflammatory mediators – Chapter 2

A literature review was conducted to provide an overview of relevant cellular interactions occurring during the anastomotic healing cascade. Knowledge of anastomotic healing was combined with knowledge of other types of wound healing (e.g. skin, bone and intestinal ulcers) and general cellular physiology.

The healing of multilayer intestinal tissue is highly complex and requires an interplay among infiltrating platelets, mast cells, granulocytes, macrophages, dendritic cells, lymphocytes, fibroblasts with resident inflammatory cells, mesenchymal stem cells, neurons, endothelial, epithelial, mesothelial and smooth muscle cells. It is essential for optimal tissue repair that all cell types exert the right action at the right time. Inflammatory activity, involving proteolytic processes, is necessary for host defence and tissue reorganisation. The subsequent cessation of inflammation and timely transition to proliferation is a physiologically challenging process. Biological substances, e.g. bile, bacteria and inflammatory products, and mechanical forces such as peristalsis and intraluminal pressure interact with the fresh anastomosis from the beginning and greatly challenge the balance and order of cellular reactions. When certain drugs or risk factors additionally disturb cellular and tissue processes, the anastomosis becomes at risk for leakage.

Cellular and molecular mechanisms might be potential therapeutic targets, e.g. growth factors or hormones, but the complexity and interaction of the different systems impedes the development of such therapeutic strategies.

Review of experimental research on anastomotic leakage: reporting and quality of animal studies must improve – Chapter 3

Numerous animal studies on intestinal anastomosis have been conducted with regard to different aspects of healing and leakage. However, the clinical impact of animal data on diagnosis, prevention and treatment of AL is unknown. Therefore, and in view of the current societal call to replace, reduce and refine animal experiments, we conducted a systematic review to examine the quality of animal research related to anastomotic healing and leakage. A total of 1,342 animal studies on AL were identified, with exponential publication rates in recent years. Of 350 articles studying experimental therapies, 298 (85%) reported a positive effect on anastomotic healing. Given the very small number of these therapies are studied or used clinically, the translatability and quality of these studies is questionable. On average, 44.7% of relevant study characteristics were not reported, in particular details on

anastomotic complications (31.6%), use of antibiotics (75.7%), sterile surgery (83.4%) and postoperative analgesia (91.4%). Anastomotic complications were only reported as a binary outcome (leak or no leak), despite current advice to grade AL according to severity and clinical consequences [3, 5].

The proportion of studies with randomisation, blinding of surgery and blinding of primary outcome assessment has increased in the past two decades but remains insufficient, being included in only 62.4%, 4.9% and 8.5% of publications respectively.

This systematic animal review demonstrates that animal models vary widely in terms of species, method to compromise healing, intestinal segment and outcome measure used. Animal research on AL is of poor quality and is increasing in volume each year, which contradicts societal aims. Both reporting and study quality must improve for better transfer of animal data to the clinic.

Diclofenac causes leakage in the ileum and proximal colon, but not in the distal colon of rats – Chapter 4

Multiple studies have reported an association between NSAID use and AL, which raises questions about the causal role of NSAIDs in leakage [6–11]. Important questions include the proportion of leak rate attributable to NSAIDs, how relevant the NSAID subtype, dose and time of administration are, and which bowel segment is most susceptible for NSAID-induced AL.

Clinical cohort studies on NSAID-related leak risk mostly address colorectal surgery [7, 8, 12]. Rat experiments by our group showed that NSAIDs disturb healing of the ileal, and not the distal, colonic anastomosis [6, 13]. We were interested in the proximal colon, whose morphology and susceptibility to pharmacologic NSAID activity is thought to be more similar to the distal colon, but intraluminal content, i.e. liquidity, microflora and bile acids, corresponds more to the terminal ileum.

In **Chapter 4** we demonstrated that diclofenac causes leakage in the proximal colon (73%) and not in the distal colon of rats. Similar to ileum anastomoses, healing of proximal colon anastomoses is most disturbed when diclofenac is administered from the day of surgery. Leakage rates were lower if administration was postponed to postoperative day 1 or day 2. Disturbed healing of the ileal and proximal colonic anastomoses and not the distal anastomosis suggests a role for bowel content in diclofenac-induced leakage [14].

Initiation of inflammation and adhesion by serosal edge abrasion before anastomosing does not attenuate leakage by diclofenac – Chapter 5

Several studies report that NSAIDs suppress intraperitoneal adhesion formation, a phenomenon that theoretically weakens intestinal anastomoses [15–18]. We designed a rat model of proximal colon anastomosis enhancing the inflammatory response and promoting anastomotic scar tissue to study if increased fibrosis can prevent diclofenac-induced leakage.

In **Chapter 5** we evoked an additional inflammatory and adherent process by surgically abrading the serosal edges of the anastomosis before restoring continuity. Explorative histologic analysis showed that serosal abrasion leads to dense scar formation at the serosal edge, compared to loose connective tissue in control anastomoses. However, this dense scar formation did not result in a stronger anastomosis, nor did it reverse diclofenac-induced leakage. This finding suggests that anastomotic healing and strength does not depend on adhesion formation on the outside of the anastomosis. It seems that the detrimental effect of diclofenac on anastomotic healing cannot be reversed by fibrosis-inducing measures, possibly due to the magnitude of this effect.

Biliary excretion of diclofenac affects healing of ileum anastomosis in rats – Chapter 6

NSAIDs that are excreted by the hepatic route (diclofenac, carprofen, celecoxib) cause more leakage than those excreted by the renal route (naproxen) [6, 13, 19]. NSAID-induced intestinal mucosal damage has been attributed to biliary excretion of NSAIDs [20–27]. We hypothesised that altered bile from NSAIDs is associated with leakage.

In **Chapter 6** the relevance of bile composition in diclofenac-induced leakage was studied in 138 rats and 72 rats as bile donors. By cannulating the bile duct and duodenum of rats, bile was drained and replaced by bile from donor rats. The leak rate was 28% after replacement of bile with 'diclofenac bile' from donors, and 6% (p=0.089) after replacement with control bile. After oral diclofenac administration, leakage was 76% with 'diclofenac bile' and 47% (p=0.127) when bile was replaced with control bile. After intramuscular diclofenac administration 67% leaked, and 25% (p=0.060) leaked when bile was drained. After intramuscular diclofenac, 50% leaked when bile was first diverted and then returned, and 20% leaked (p=0.117) if bile was replaced with control bile. Taken altogether, leakage was significantly more severe in 'diclofenac bile' groups. HPLC and LCMS analyses demonstrated that diclofenac metabolite levels in bile peak within two hours, indicating that altered bile reaches the intestine shortly after diclofenac administration. The results demonstrated that altered bile composition by diclofenac administration disturbs the healing of ileal anastomoses in rats. It was not determined whether metabolites or diclofenac itself are responsible for the effects observed.

Microbial glucuronidase inhibition, aimed at preventing reactivation of diclofenac metabolites in the gut, reduces diclofenac-induced anastomotic complications in rats – Chapter 7

In experimental studies on diclofenac-induced small-intestinal mucosal damage it has been shown that reactivation of the biliary metabolite diclofenac-acyl-glucuronide by bacterial glucuronidase in the gut harms intestinal mucosa. The beta-glucuronidase inhibitor Inh1, developed by Wallace et al., prevented this toxic process. We aimed to test the glucuronidase inhibiting effect on anastomotic healing. In **Chapter 7**, 90 rats were used to study whether Inh1 reduces AL in diclofenac-treated rats. Administration of Inh1 resulted in a drop in the leak rate from 89% to 44% (p=0.094) and significantly reduced leak severity (p=0.029). Analysis of plasma levels of diclofenac revealed no changes in groups receiving Inh1. The finding that administration of a glucuronidase inhibitor reduces signs of leakage indicates

that cleavage of diclofenac-acyl-glucuronide metabolites to diclofenac aglycones disturbs ileal anastomotic healing. Next to the human glucuronidase located in the brush border of the gut, β-glucuronidase is mainly produced by bacteria. In this way gut microbes are involved in the pathology of diclofenac-induced leakage.

Surgical stress causes a marked shift in microbial composition and diversity, though effects of diclofenac were not observed – Chapter 8

We wanted to further investigate the gut bacteria in diclofenac-induced AL. For this reason we explored the extent to which the operation and diclofenac administration alter the microbiome. It appeared that the surgery alone drastically alters the microbiome in different segments of the gut. A short period of postoperative diclofenac administration did not lead to additional changes. Either diclofenac has no effect on the diversity of the microbiome, or the effect was masked by an overwhelming effect of the surgery. In this study we did not determine a change in virulence of the microbiome which has been associated with AL in other animal models. It was concluded that the microbiota composition significantly changes following bowel resection and anastomosis. This study did not further contribute to understanding the diclofenac effect on AL, such as mediation via microbiome changes.

Conclusions

This thesis provides an overview of relevant cellular interactions occurring during anastomotic healing, illustrating that this process is insufficiently understood. It demonstrates that experimental research on AL is of low to moderate quality. In particular there is a need for better reporting of study methods and outcomes and for more profound measures to reduce bias.

The thesis provides evidence that diclofenac affects anastomotic healing differently along the gut, and that biliary excretion of diclofenac is relevant in causing anastomotic complications. A luminal intervention targeted at microbial metabolism of diclofenac molecules reduces the effect of diclofenac. The finding that such luminal factors impact anastomotic healing is novel and provides directions for future research and clues for targeted treatment.

Surgical stress, such as bowel resection and anastomosis, significantly alters the gut microbiome. Specific changes in this major luminal factor may make anastomoses more susceptible to leakage in general, or specifically when diclofenac is used, although the latter suggestion could not be demonstrated in this thesis.

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11.
NEDERLANDSE SAMENVATTING

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Wereldwijd moeten jaarlijks miljoenen mensen een darmoperatie ondergaan vanwege darmkanker of andere darmaandoeningen zoals de ziekte van Crohn, colitis ulcerosa en diverticulitis. Na het verwijderen van het zieke darmdeel worden de overgebleven darmuiteinden vaak weer verbonden middels een darmnaad. Helaas treedt er gemiddeld in 6-14% van de gevallen een naadlekkage op. De gevolgen van deze gevreesde complicatie zijn enorm voor de patiënt omdat het kan resulteren in abcessen, een buikvliesontsteking en zelfs overlijden. Daarnaast is er een grotere kans op terugkeer van de kanker en is de kwaliteit van leven op de lange termijn verminderd. De maatschappelijke impact is groot door een langer ziekteverzuim en een verdubbeling van de kosten als gevolg van meer re-operaties, meer Intensive Care opnames, langere ziekenhuisopnames en een groter aantal heropnames [1, 2]. De ernstige gevolgen van naadlekkage zijn in de afgelopen decennia uitgebreid onderzocht, maar helaas heeft dit nog niet geleid tot een duidelijke afname van deze complicatie [3, 4]. Een belangrijke reden is dat er nog onvoldoende bekend is over de ontstaanswijze van naadlekkage [3]. Belangrijk doel van dit proefschrift is om de kennis van de normale en verstoorde darmnaadgenezing te vergroten om daarmee de incidentie en de gevolgen van naadlekkage te kunnen verminderen. We hebben specifiek gekeken naar de schadelijke effecten van een veel gebruikte perioperatieve pijnstiller (i.e. diclofenac) op dunne- en dikkedarmnaden.

Fysiologie van darmnaadgenezing: een complexe cascade van celreacties en ontstekings-mediatoren – Hoofdstuk 2

Om de relevante celreacties die optreden tijdens een naadgenezing in kaart te brengen hebben we een literatuuronderzoek verricht. Hierbij werd geput uit kennis van studies over darmnaadgenezing en over andere vormen van wondgenezing zoals huid, bot en darmslijmvlies.

De darmwand bestaat uit meerdere lagen en de genezing hiervan, na beschadiging en na het maken van een darmanastomose, is complex. Er is een juiste interactie nodig tussen de bloedplaatjes, mestcellen, granulocyten, macrophagen, lymphocyten en fibroblasten die het weefsel infiltreren, en de cellen die al in het weefsel aanwezig zijn, zoals gladde spiercellen, epitheelcellen en mesenchymale stamcellen, maar ook neuronen, endotheelcellen, mesotheelcellen en talrijke immunologische cellen. Ontstekingactiviteit en eiwitafbrekende processen door deze cellen zijn nodig ter bescherming tegen pathogenen en om de weefselstructuur te herorganiseren, maar teveel van deze activiteit kan het weefselherstel weer verstoren. Voor een optimale genezing is het dus belangrijk dat de ontstekingsactiviteit op het juiste moment wordt afgerond en wordt overgegaan op proliferatieve processen die zorgen voor een sterk litteken. Dit is in de darm uitdagend omdat het proces verstoord kan worden door biologische factoren (bijvoorbeeld gal, darmenzymen en darmbacteriën) en mechanische factoren (bijvoorbeeld darmperistaltiek en hoge intraluminale druk). Als bepaalde medicijnen of andere risicofactoren zorgen voor extra verstoring, dan kan dit het verschil maken tussen wel of geen naadlekkage.

De interacties tussen cellen kunnen aangrijpingspunten zijn voor behandelingen, bijvoorbeeld met groeifactoren of hormonen, maar deze interacties en systemen zijn nog onvoldoende begrepen om adequate en doelgerichte behandelstrategieën te ontwikkelen.

Review van experimenteel onderzoek op het gebied van naadlekkage: de kwaliteit en de rapportage van dierenstudies moet verbeteren – Hoofdstuk 3

Er worden veel dierenstudies uitgevoerd op het gebied van naadlekkage, maar het is niet bekend wat de invloed hiervan is op de preventie en behandeling van naadlekkage in de kliniek. Omdat we volgens het actuele '3V' principe dierexperimenteel onderzoek waar mogelijk willen vervangen, verminderen en verbeteren, hebben we een systematische review verricht om de kwaliteit van dierenstudies op het gebied van naadlekkage te evalueren.

Er werden in totaal 1.342 studies gevonden en het aantal publicaties nam vooral in het laatste decennium exponentieel toe. Van de 350 artikelen waarin experimentele therapieën werden bestudeerd, rapporteerden 298 (85%) een positief effect op de naadgenezing. Aangezien er slechts een beperkt aantal van deze therapieën wordt gebruikt of bestudeerd bij mensen, lijkt de vertaalbaarheid van uitkomsten van deze studies erg klein. Het viel op dat in gemiddeld 44.7% van de studies relevante studiemethoden en studieresultaten niet werden gerapporteerd. Het ging hierbij vooral om de beschrijving van de opgetreden naadcomplicaties (31.6%), perioperatief antibioticagebruik (75.7%), steriliteit aspecten van de operatieve procedure (83.4%) en toegepaste postoperatieve pijnstilling (91.4%). Naadlekkage werd alleen gerapporteerd als binaire uitkomstmaat (wel of geen lekkage), terwijl in de huidige literatuur wordt geadviseerd om lekkage ook te scoren aan de hand van de ernst en klinische consequenties [3, 5].

Het percentage studies waarin gebruik werd gemaakt van randomisatie, blindering van de chirurg en blindering van degene die de uitkomst onderzoekt, is de laatste twee decennia iets toegenomen maar is met respectievelijk 62.4%, 4.9% en 8.5% nog altijd ruim onvoldoende. Deze systematische review laat zien dat diermodellen voor naadlekkage erg verschillen wat betreft diersoort, darmsegment, uitkomstmaat en methode om lekkage te induceren. In tegenstelling tot maatschappelijke en politieke doelstellingen neemt het aantal dierstudies naar naadlekkage elk jaar toe terwijl de kwaliteit van het gepubliceerde onderzoek matig blijft. Zowel de kwaliteit als de rapportage van de studies moet worden verbeterd om de impact van het experimentele onderzoek op de kliniek te vergroten.

Diclofenac veroorzaakt lekkage in het ileum en de proximale dikke darm, maar niet in de distale dikke darm van de rat – Hoofdstuk 4

Meerdere studies hebben aangetoond dat er een verhoogd risico is op naadlekkage wanneer er rondom de operatie non-steroidal anti-inflammatory drugs (NSAIDs) gebruikt worden [6-11]. Daarbij is het nog onduidelijk in welke mate NSAIDs verantwoordelijk zijn voor het ontstaan van lekkage, welke subtypen NSAIDs schadelijk zijn, wat de relevantie is van de toegediende dosis en de timing hiervan, en welk darmsegment het meest kwetsbaar is voor de effecten van NSAIDs.

Klinische cohort studies over NSAID-gerelateerde naadlekkage hebben vooral groepen patiënten die colorectale chirurgie ondergingen onderzocht [7, 8, 12]. Uit eerdere experimenten van onze groep bij ratten bleek dat NSAIDs juist lekkage veroorzaken van ileumnaden en niet van distale colonnaden [6, 13]. Hieruit ontstond de vraag wat het effect zou zijn op naden in het proximale colon. In dit darmdeel lijkt de darmwand meer op het distale colon, terwijl de darminhoud (vloeibaarheid, darmbacteriën, galzouten) meer op het terminale ileum lijkt.

In Hoofdstuk 4 hebben we aangetoond dat diclofenac wel lekkage veroorzaakt in het proximale colon (73%), maar niet in het distale colon van ratten. Net als bij de ileumnaden ontstaan de meeste lekkages als diclofenac wordt toegediend vanaf de dag van operatie en neemt dit aantal af als de toediening wordt uitgesteld naar dag 1 en dag 2. De bevinding dat de genezing wel wordt verstoord in het ileum en proximale colon en niet in het distale colon suggereert dat de darminhoud in de proximale segmenten relevant is bij het ontstaan van een diclofenac-gerelateerde naadlekkage [14].

Het oppervlakkig beschadigen van de darmserosa, ten behoeve van het stimuleren van inflammatie en adhesievorming, vermindert niet de diclofenac-gerelateerde naadlekkage – Hoofdstuk 5

Enkele studies beschrijven dat NSAIDs de vorming van intraperitoneale adhesies onderdrukken [15-18]. Dit fenomeen zou in theorie ook de darmnaadgenezing kunnen verzwakken. We hebben een rattenmodel ontworpen om te bestuderen of het stimuleren van de inflammatoire respons en de ontwikkeling van littekenweefsel rondom een proximale colonnaad de aan de diclofenac gerelateerde lekkage kan voorkomen.

In Hoofdstuk 5 hebben we deze inflammatoire en adhesieve processen uitgelokt door een mechanische interventie, zoals ook wordt gedaan in experimentele studies naar adhesievorming. De interventie bestond uit het oppervlakkig opschuren van de serosa aan de randen van de naad, vlak voordat de naad wordt gehecht. Histologische analyse liet zien dat dit in veel gevallen leidde tot vast en uitgebreid littekenweefsel, terwijl het weefsel losmazig bleef bij controle naden zonder voorbewerking. Dit resulteerde echter niet in een sterkere naad en ook niet in vermindering van naadlekkages. Het lijkt alsof de genezing en sterkte van de darmnaad dus niet afhangen van de verklevingen aan de serosale zijde van de naad. De schadelijke effecten van diclofenac op de naad kunnen niet worden tegengegaan door de gebruikte fibrose-inducerende technieken.

De uitscheiding van diclofenac via gal heeft een nadelige invloed op de genezing van ileumnaden – Hoofdstuk 6

NSAIDs die worden geklaard door de lever (diclofenac, carprofen, celecoxib) veroorzaken meer lekkage dan de NSAIDs die voor het grootste deel worden uitgescheiden door de nieren (naproxen) [6, 13, 19]. Meerdere studies schrijven ook NSAID-geïnduceerde dunne darmschade, die optreedt zonder operatie, toe aan de hepatogene klaring en biliaire uitscheiding van NSAIDs [20-27]. Op grond hiervan vroegen we ons af of de verandering in de galsamenstelling door NSAID gebruik invloed heeft op het ontstaan van naadlekkage.

In Hoofdstuk 6 hebben we deze relevantie van de galsamenstelling onderzocht in 138 ratten en met gebruik van 72 ratten als galdonoren. Door het canuleren van de ductus choledochus werd de gal afgetapt en via een canule in het duodenum werd donorgal teruggegeven. Het lekkagepercentage was 28% als er 'diclofenac-gal' werd gegeven ten opzichte van 6% (p=0.089) wanneer er controlegal werd gegeven. Na orale toediening van diclofenac was er een 76% lekkage percentage als de ratten hun 'diclofenac-gal' terugkregen en dit percentage was 47% (p=0.127) als het werd vervangen door controle gal. Na intramusculaire toediening van diclofenac lekte 67% van de naden, er lekte 25% (p=0.060) als de gal volledig werd afgetapt. Als na intramusculaire toediening van diclofenac de gal eerst werd afgetapt en dan teruggeven was er 50% lekkage, en als het gal werd vervangen door controlegal dan was er 20% lekkage (p=0.117). Samenvattend was er een significant ernstigere lekkage waarneembaar in de groepen die 'diclofenac-gal' kregen in vergelijking met controle groepen. Met behulp van High Performance Liquid Chromatography en Liquid Chromatography Mass Spectrometry (LCMS) analyses toonden we aan dat de spiegels van diclofenac metabolieten in gal binnen twee uur na toediening pieken. Hieruit blijkt dat de veranderde galsamenstelling al kort na de diclofenac toediening de darm bereikt.

Deze studie laat zien dat een veranderde galsamenstelling door diclofenac toediening de genezing van ileumnaden bij ratten verstoort. Er kon niet worden vastgesteld of deze verstoring komt door een veranderde galzoutsamenstelling, door de gemetaboliseerde vorm van diclofenac, of door de niet omgezette diclofenac in de darm.

Remming van microbiële glucuronidase activiteit in de darm, om reactivatie van diclofenac metabolieten in de darm te voorkomen, vermindert diclofenac-geïnduceerde naadcomplicaties bij ratten – Hoofdstuk 7

Uit experimentele studies blijkt dat reactivatie van de biliaire metaboliet diclofenac-acyl-glucuronide door bacteriële glucuronidase activiteit in de darm leidt tot schade aan de dunne darmmucosa. De door Wallace et al. ontwikkelde Inh1, die glucuronidase activiteit blokkeert, voorkomt het ontstaan van de mucosale schade en ulcera [23, 28]. In Hoofdstuk 7 hebben we in 90 ratten onderzocht of Inh1 ook naadlekkage kan voorkomen na toediening van diclofenac. Toediening van Inh1 kon lekkage niet voorkomen, resulteerde in een niet significante daling van 89% naar 44% (p=0.094) maar verminderde wel significant de ernst van de lekkages (p=0.029). Uit de LCMS analyse bleek dat de plasmaspiegels van diclofenac onveranderd waren bij toediening van Inh1. De bevinding dat een glucuronidase remmer naadcomplicaties vermindert, suggereert dat reactivatie van diclofenac-acyl-glucuronide in de darm schadelijk is voor de genezing van ileumnaden. Bij mensen is er humaan glucuronidase aanwezig tussen de darmvilli, maar wordt het grootste deel van dit enzym geproduceerd door darmbacteriën. Dit suggereert een additionele rol van de darmflora in de pathophysiologie van naadlekkage door diclofenac.

Darmchirurgie veroorzaakt een duidelijke verandering in de samenstelling en diversiteit van het microbioom, terwijl toediening van diclofenac hier geen duidelijk effect op heeft – Hoofdstuk 8

Om de rol van de darmflora bij diclofenac-geïnduceerde naadlekkage verder te onderzoeken, hebben we in verschillende darmsegmenten onderzocht of en hoe het microbioom verandert door de operatie en door de toediening van diclofenac. Het blijkt dat alleen al de operatie zorgt voor significante veranderingen in het microbioom, zowel in het ileum, het proximale colon als het distale colon. Een korte kuur van diclofenac na de operatie leidde niet tot verdere veranderingen. In deze explorerende studie konden we geen effect van diclofenac aantonen op de samenstelling van de darmflora. Mogelijk maskeerde het grote effect van de operatie dat van de diclofenac. We hebben in deze studie niet onderzocht wat het effect is van diclofenac op de virulentie van darmbacteriën. Bacteriële virulentie is een factor die in andere onderzoeken is gerelateerd aan naadlekkage. Uit de studie in hoofdstuk 8 kan worden geconcludeerd dat de samenstelling van het microbioom significant verandert als gevolg van een darmresectie en het aanleggen van een darmnaad.

Conclusie

Dit proefschrift bevat een overzicht van de verschillende cellulaire interacties die relevant zijn tijdens darmnaadgenezing en beschrijft waarom het proces van naadgenezing nog onvoldoende begrepen wordt. Er wordt beschreven dat de kwaliteit van experimenteel onderzoek naar darmnaadgenezing matig is en dat er vooral behoefte is aan betere rapportage en gebruik van betere methodes om bias te voorkomen.

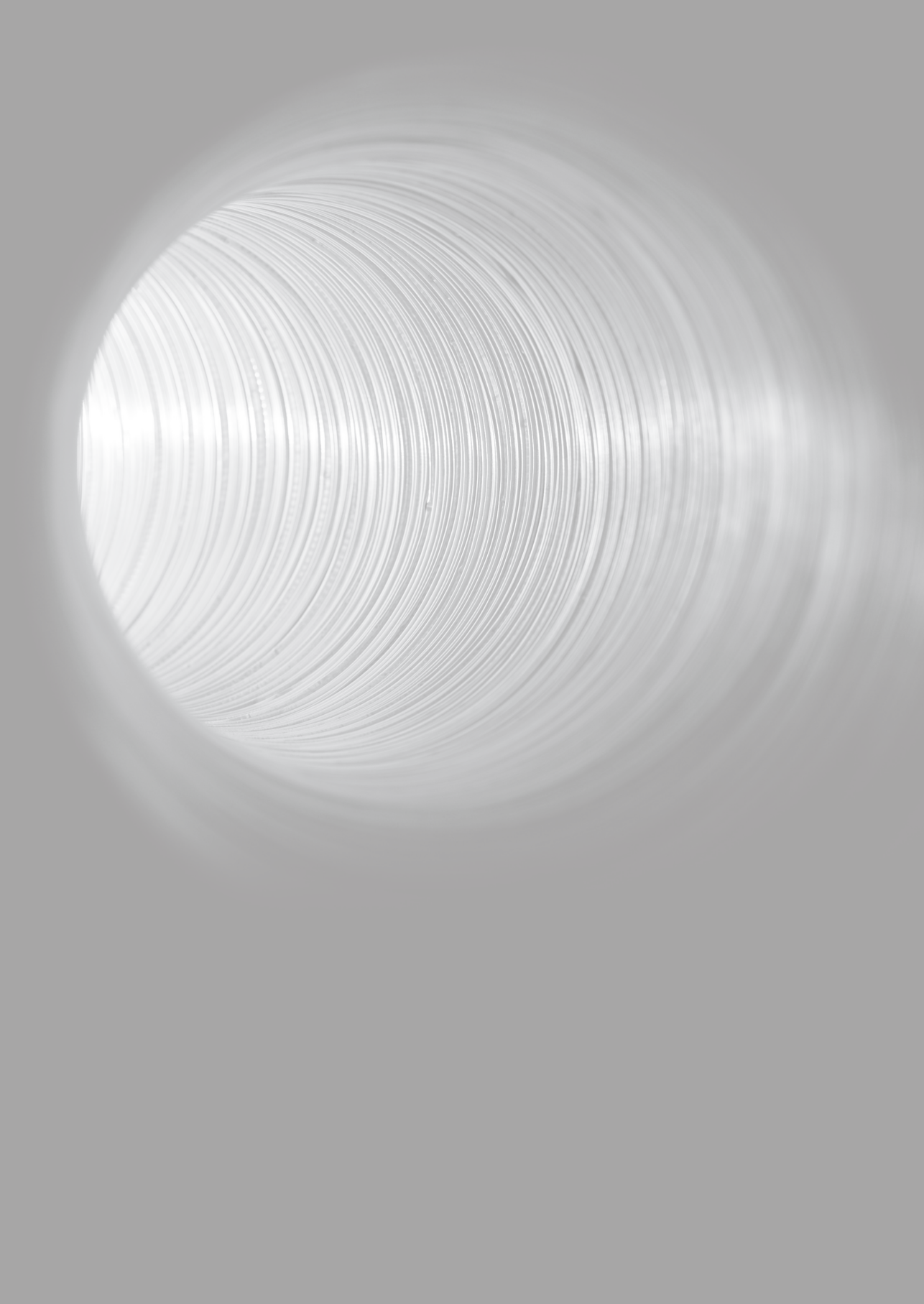
In dit proefschrift wordt aangetoond dat de invloed van diclofenac op darmnaadgenezing verschilt per locatie in de darm en dat de uitscheiding van diclofenac via gal relevant is bij het ontstaan van naadcomplicaties in de rat. Een enterale interventie, gericht op het metabolisme van diclofenac moleculen door darmbacteriën, vermindert de effecten van diclofenac.

Deze nieuwe bevindingen dat luminale factoren en bijpassende interventies invloed hebben op de experimentele naadgenezing opent de weg naar toekomstig naadlekkage onderzoek gericht op luminale factoren. Een luminale factor van omvang is het microbioom en in dit proefschrift wordt in detail beschreven dat darmchirurgie hierin significante veranderingen teweeg brengt. Een specifieke samenstelling van de darmflora, of veranderingen hierin, kunnen de darmnaad kwetsbaarder maken voor lekkage in het algemeen, of specifiek bij gebruik van diclofenac, hoewel dit laatste niet door ons kon worden aangetoond.

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PhD PORTFOLIO

PhD Training and courses

- Specialty skills in emergency surgery and trauma (2015), Royal College of Surgeons, London
- Basiscursus Regelgeving en organisatie voor Klinisch onderzoekers (BROK)(2015), Examenbureau Medisch-Wetenschappelijk Onderzoeker (EMWO), E-course
- Academic Writing (2014), PAO Heyendael, Nijmegen
- Scientific Integrity (2014), PAO Heyendael, Nijmegen
- Tips and Trics on grant writing (2014), Radboud Postdoc Initiative, Nijmegen
- Masterclass colorectal surgery (2014), Foundation of Surgery, St. Gallen, Switzerland
- Microsurgery – bile fistula in rats (2013), René Remie Surgical Skill Centre, The Netherlands
- Synthesis of evidence in animal experimentation (2013), ZonMw/SYRCLE, Nijmegen
- Course on Laboratory Animal Science (Artikel 9)(2013), PAO Heyendaeol, Nijmegen
- CE certification for medical devices and risk management (2013), Mikrocentrum, The Netherlands

Presentations

- Bile composition affects diclofenac induced leakage in rats, Annual Academic Surgical Congress 2015, Las Vegas, USA
- Need for better animal experiments on intestinal anastomosis, Annual Academic Surgical Congress 2015, Las Vegas, USA
- Half a century of animal experiments on intestinal anastomosis – a critical appraisal, ZonMw / NOW – Synthesis of evidence in animal studies 2014, The Hague, The Netherlands
- Diclofenac causes anastomotic leakage of the ileum and proximal colon, but not of the distal colon in the rat.
- European congress on Surgical Infection 2013, Vienna, Austria
- European Colorectal Congress 2013, St. Gallen, Switzerland (poster)
- Najaarsdag NVvH 2013, 's Hertogenbosch, The Netherlands
- Symposium Experimenteel Onderzoek Heelkundige Specialismen(SEOHS) 2014, Maastricht, The Netherlands
- Voorjaarsvergadering NVGE / NVGIC (Dutch Societies for Gastroenterology and Gastrointestinal Surgery) 2014, Veldhoven, The Netherlands
- De 'Hindfoot Arthrodesis Nail' – a one step solution in worst case ankle fractures? Assistentensymposium Traumachirurgie NVvT (Dutch Society for Traumasurgery) 2012, Soesterberg, The Netherlands

Prizes / Grants

- 'Best poster prize' at European Colorectal Congress 2013, St. Gallen, CH, €2000
- 'More Knowledge with less animals' publication grant 2014, ZonMW/NWO, €5.482,-

Tutoring, mentoring

- Arta Hoesseini, Science Internship 2013, The effect of abrasion on the anastomotic strength and healing in the proximal colon of Wistar rats, with or without diclofenac.
- Melissa Arron, Science Internship 2014-2015, Anastomotic Leakage – beta-glucuronidase inhibitor and the adverse effects of diclofenac on intestinal anastomoses
- Robin Klabbers, Science Internship and Honours Project 2014-2015, The effect of polyphosphate on microbial virulence expression and its role in intestinal anastomotic leak.

List of Publications

S.T.K. Yauw, R.M.L.M. Lomme, P. Van den Broek, R. Greupink, F. Russel, H. van Goor. Experimental study of diclofenac and its biliary metabolites on anastomotic healing. BJS Open, 2018 May e-pub ahead of print

S.T.K. Yauw, M. Arron, R.M.L.M. Lomme, P. van den Broek, R. Greupink, A.P. Bhatt, M.R. Redinbo, H. van Goor. Microbial Glucuronidase Inhibition Reduces Severity of Diclofenac-Induced Anastomotic Leak in Rats. Surgical Infections, 2018 May/Jun;19(4):417-423

A. Alken, J.M. Luursema, M. Weenk, S.T.K. Yauw, C. Fluit, H. van Goor. Integrating technical and non-technical skills coaching in an acute trauma surgery team training: Is it too much? Am J Surg 2017; [epub ahead of print]

A.A. Chaturvedi, S.T.K. Yauw, R.M.L.M. Lomme, T. Hendriks, H. van Goor. Safety and Efficacy of Alginate Adhesion Barrier Gel in Compromised Intestinal Anastomosis. Surg Inf 2017; Aug 18(6):670-675

STK Yauw, R.M.L.M. Lomme, R.J. vd Vijver, T. Hendriks, C.J. v Laarhoven, H. v Goor. Diclofenac causes anastomotic leakage of the ileum and proximal colon, but not of the distal colon in the rat. Am J Surg 2015 Aug:210(2);382-8

STK Yauw, K. Wever, A. Hoesseini, M. Ritskes-Hoitinga, H. van Goor. Systematic Review of experimental studies on intestinal anastomosis. Br J Surg 2015 Jun:102(7)726-34

STK Yauw, A. Hoesseini, R.M.L.M. Lomme, H. van Goor. Serosal edge abrasion does not enhance healing of inverted anastomoses in the rat colon. J Surg Res. 2015 Feb;193(2):634-41

P. Bouten, M. Zonjee, J. Bender, S.T.K. Yauw, H. van Goor, J. Van Hest, R. Hoogenboom. The chemistry of tissue adhesive materials. Progress in Polymer Science. 39 (2014) 1375-1405.

S.T.K. Yauw, B.A. Twigt, M.J.M. Segers. Behandeling van complexe enkelfracturen met de Hindfoot Arthrodesis Nail. Nederlands Tijdschrift voor Traumatologie. 2012; 6; 183-7

S.T.K. Yauw, W. Kramer. Eggs, cornflower and papayapulp. Medisch Contact 2010;65(15); 686-7

S.T.K. Yauw, Burns in Malawi, Medicus Tropicus, December 2010, volume 48;4; 7-9

List of Publications as collaborative researcher

Dutch Snapshot Research Group. Hospital Variation in temporary diversion after rectal cancer surgery; impact on long term permanent stoma rate. Submitted

Blok RD, Musters GD, Borstlap WAA, Buskens CJ, Bemelman WA, Tanis PJ; Collaborative Dutch Snapshot Research Group. Snapshot Study on the Value of Omentoplasty in Abdominoperineal Resection with Primary Perineal Closure for Rectal Cancer. Ann Surg Oncol. 2018 Mar;25(3):729-736

Jonker FHW, Hagemans JAW, Burger JWA, Verhoef C, Borstlap WAA, Tanis PJ; Dutch Snapshot Research Group; collaborative researcher. The influence of hospital volume on long-term oncological outcome after rectal cancersurgery. Int J Colorectal Dis 2017 Dec; 32(12):1741-1747

2015 European Society of Coloproctology collaborating group. Risk factors for unfavourable postoperative outcome in patients with Crohn's disease undergoing right hemicolectomy or ileocaecal resection An international audit by ESCP and S-ECCO. Colorectal Dis 2017, sep [epub ahead of print]

Borstlap WAA, Westerduin E, Aukema TS, Bemelman WA, Tanis PJ; Dutch Snapshot Research Group Anastomotic Leakage and Chronic Presacral Sinus Formation After Low Anterior Resection: Results From a Large Cross-sectional Study. Ann Surg. 2017 Jul 25

Dutch Snapshot Research Group. Benchmarking recent national practice in rectal cancer treatment with landmark randomized controlled trials. Colorectal Dis. 2017 Jun;19(6):O219-O231

2015 European Society of Coloproctology collaborating group. The relationship between method of anastomosis and anastomotic failure after right hemicolectomy and ileo-caecal resection: an international snapshot audit. Colorectal Dis. 2017 Mar 6

CURRICULUM VITAE

Simon Yauw was born on February 12, 1985 in Nijmegen, The Netherlands. After graduating from high school (Stedelijk Gymnasium Nijmegen) in 2003, he studied Medicine at the University Medical Center Utrecht. After finishing Medicine, he started working as surgical intern at the Department of Surgery of the St. Antonius Hospital, Nieuwegein. In 2013 he started as a PhD candidate at the Department of Surgery of the Radboud university medical center Nijmegen under supervision of prof. dr. Harry van Goor.

For his research on intestinal anastomoses he collaborated internationally with the Department of Soft Matter and Chemistry of ESPCI Paris, France, the Department of Chemistry, Biochemistry, Microbiology and Genomics of the University of North Carolina at Chapel Hill, USA, and the Department of Surgery of the University of Chicago Medical Center. Nationally he collaborated with the Department of Pharmacology and Toxicology of the Radboud Institute for Molecular Life Sciences, and the Departments of Surgery and Medical Microbiology of the Maastricht UMC. Together with the Department of Chemistry of the Radboud University and GATT-technologies he conducted research on the development of the General Adhesive Tissue Tape and Patch.

During his PhD he was awarded the best poster price at the European Colorectal Congress in St. Gallen, Switzerland, and received a publication grant from ZonMw.

In 2015 he started his surgical training at the Department of Surgery at the Radboud university medical center and in 2017 he continued his training as surgical resident in Ziekenhuis Gelderse Vallei in Ede.

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