MAJOR ABDOMINAL SURGICAL COMPLICATIONS
INNOVATIVE APPROACHES

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Major Abdominal Surgical Complications
Innovative Approaches

Abdominale chirurgische complicaties:
innovatieve benaderingen

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Chapter 1

General introduction, aim and thesis outline

Patients undergoing abdominal surgery run the risk of postoperative complications. The three most frequent major complications are represented by:

1) incisional hernia (IH);
2) postoperative ileus (POI);
3) colorectal anastomotic leakage (CAL).

The cause of developing a surgical complication is mostly multifactorial. Several patient-related risk factors for all three complications such as age, gender, prior abdominal surgery, comorbidities, immune deficiency, and emergency surgery cannot be controlled [1]. Risk factors that can be influenced are nutritional status, obesity, smoking, and experience of the surgeon among others. Nevertheless, surgical complications also frequently happen to patients without any obvious reason.

DEFINITION AND INCIDENCE

Incisional hernia (IH):
IH can be defined as any abdominal wall gap with or without bulging in the area of a postoperative scar perceptible or palpable by clinical examination or imaging [2]. IH is a very common complication after abdominal surgery, with an incidence ranging between 10-23% [3]. The highest rates of IH occur after midline abdominal incisions for conventional ('open') colorectal or vascular surgery. IH is associated with pain, discomfort, decreased quality of life, incarceration and even strangulation [3].

Prolonged postoperative ileus (PPOI):
POI, a transit cessation of bowel mobility occur in each patient undergoing abdominal surgery due to a normal physiologic response. But after major abdominal surgery PPOI has an incidence of up to 14.9-19% which contributes to postoperative morbidity [4,5]. Postoperative inhibition of motility of the gastro-intestinal tract leads to symptoms like nausea, vomiting, abdominal pain, delayed passage of flatus and stool, and the inability to tolerate solid food. Patients are considered to have PPOI if they do not tolerate solid food for at least 24 hours and experience absence of flatus over 24 hours, occurring on or after day 5 postoperatively [6].

Colorectal anastomotic leakage (CAL):
CAL is the most severe complication of abdominal surgery and represents a defect of the intestinal wall integrity at the anastomotic site. A pelvic abscess close to the anastomosis is also considered as CAL [7]. Because CAL results in 3-32% of the cases to mortality and

severe morbidity, including reoperation, intensive care admittance, abscess drainage and stoma construction [8-10], CAL is the most feared complication with an incidence of 5.9% (after colon resection) and 9.3% (after rectum resection) in The Netherlands in 2014 [8]. As a consequence, CAL also leads to reduced quality of life, a higher cancer recurrence and doubled hospital charges [9].

THE ROLE OF THE INFLAMMATORY RESPONSE

Surgical injury induces a hemodynamic, metabolic, and systemic inflammatory response with rise of systemic and local cytokine release. The initial pro-inflammatory immune response in the intraoperative and early postoperative periods is initiated by macrophages and monocytes at the initial site of injury. The pro-inflammatory cytokines including tumor necrosis factor (TNF)-α and IL (interleukin)-1β stimulate the production of IL-6. The postoperative inflammatory response plays a major role in all above-mentioned surgical complications. The crucial role of macrophages with regard to postoperative complications was not discovered until recent decades.

MACROPHAGES

Macrophages can derive from monocytes, one of the major groups of white blood cells. These are initially characterized by their ability to perform phagocytosis. When macrophages are stimulated for example by foreign substances, they will increase their capacity of phagocytosis and intracellular digestion and exhibit enhanced metabolic and lysosomal enzyme activity. Macrophages are remarkably plastic and can even change their functional phenotype depending on the environmental cues they receive. Macrophages are also secretory cells that produce cytokines that stimulate in defensive and reparative functions. Activation of macrophages is critical in the acute phase of wound healing [11, 12]. Macrophages can be roughly divided into pro-inflammatory macrophages, also called M1 macrophages, and anti-inflammatory macrophages, also called M2 macrophages or repair macrophages [13, 14]. M1 macrophages secrete high levels of pro-inflammatory cytokines e.g. TNF-α, IL-6, IL-1β and generate reactive oxygen and nitrogen species via activation of inducible nitric oxide synthase (iNOS). Conversely, M2 macrophages activate arginase 1 that blocks iNOS activity and therefore inhibits nitric oxide production. They also secrete anti-inflammatory cytokines e.g. IL-10, TGF-β, IL-4 essential for inflammatory response resolution. The balance between M1 and M2 plays a critical role in the phagocytosis of pathogens, the clearance of apoptotic cells and the healing and remodeling of injured tissues.

BIOMATERIALS

Biomaterials such as hernia meshes and tissue adhesives can be used to prevent surgical complications for example IH and CAL. A biomaterial is made of natural or synthetic material that is suitable for introduction into living tissue to replace or repair tissue. The surface properties and the material itself play an important role for the foreign body response. Host reactions following implantation of biomaterials include injury, blood-material interactions, provisional matrix formation, acute inflammation, chronic inflammation, granulation tissue development, foreign body reaction, and fibrosis/ fibrous capsule development [11]. The inflammatory response is very important following surgical tissue damage and material implantation or application, every biomaterial induces an inflammatory foreign body response.

PREVENTION AND DETECTION

IH can be prevented by using a small tissue bites suture technique of 5 mm instead of large bites of 1 cm every 1 cm, as shown in a randomized controlled trial [3]. For example obese patients or patients with an aorta aneurysm are at high risk to develop complications after surgery such as IH and wound infection. The mechanisms to explain an increased tendency for wound complications in obese patients are various. Vascular insufficiency and oxidative stress of the tissue causes decrease in collagen synthesis and limits an adequate inflammatory response [15]. Obese patients always have a low-grade inflammatory response in the obese tissue and therefore a more pro-inflammatory profile [15,16]. Because of the higher complication risk in this specific patient category it is desirable to prevent IH by primary mesh augmentation [17]. Although every individual patient has a unique response to biomaterials is would be ideal to investigate this response prior to surgery.

Different strategies have been explored to prevent POI. Surgical procedures trigger two different phases of POI: an early neurogenic phase and a late inflammatory phase. This inflammatory phase is considered to be a more relevant factor in gastrointestinal dismotility [18]. In this phase, local macrophages, activated by intestinal manipulation, induce an inflammatory response that results in muscle dysfunction, also inducing the infiltration of inflammatory cells, including neutrophils into the intestinal muscularis layer. Therefore, systemic inflammatory markers, such as interleukin-1 (IL-1), IL-6 , TNF-α, and CRP, might be valuable for the early detection of POI. Pharmacological management with μ-opioid receptor antagonists and serotonin receptor agonists appeared to significantly shorten the duration of ileus. Another strategy is stimulation of the

gastro-intestinal motility by chewing gum that induces cephalo-vagal stimulation and activating the cholinergic anti-inflammatory pathway by activating the vagal nerve [19, 20]. Early detection of PPOI prevents a high postoperative morbidity.

If a patient develops CAL, the cause is mostly multifactorial. Roughly, CAL can be due to technical failure or impaired anastomotic healing. Although the surgical technique is improved in the last decades, the incidence of CAL is not clearly decreased, aging of the patient population and the application of chemoradiotherapy for rectal carcinoma representing important factors. Some factors can be influenced, but most patient-related risk factors cannot be changed. Therefore there is more need to understand the pathophysiology of anastomotic healing and there should be more attention for the prevention of leakage by optimizing the surgical technique. Moreover, when CAL happens detection in an early phase is needed to prevent from worse.

AIM AND OUTLINE OF THIS THESIS

Even anno 2017 it is still necessary to explore new strategies to prevent complications in abdominal surgery. Also with new surgical techniques, for example the use of staplers for colorectal anastomosis, prevention of CAL is still an issue. In addition, early detection of a complication is necessary to prevent from worse. This also requires more attention and research since detection of leakage is still mainly based on clinical observation and the experience of the surgeon. Research on more advanced technical and/ or digital solutions to objectively assess and quantify leakage is strongly needed. The aim of this thesis was to develop different strategies that may facilitate prevention, prediction, and diagnosis of different important abdominal surgical complications such as CAL, POI, IH, and infectious complications.

In *Part 1*, **Chapters 2-6,** of this thesis, prediction and detection of complications are evaluated in patients who are not at risk to develop a surgical complication.

In **Chapter 2** it is investigated whether aortoiliac calcification is linked to CAL by measuring the calcified plaques in the various abdominal arteries.

In **Chapter 3** the perioperative bowel perfusion is evaluated with a miniaturized Dynamic Light Scattering device, to determine whether anastomotic perfusion correlates with the anastomotic healing process.

In **Chapter 4** a clinical study is presented in which it is examined whether it is possible to detect POI and infectious complications in an early stage.

In **Chapter 5** an overview of the current evidence regarding clinical endpoints, early detection, and differential diagnosis of POI will be presented.

In *Part 2,* **Chapters 6-8,** the focus is on the prevention of surgical complications.

In **Chapter 6** the hypothesis that perioperative nicotine administration may reduce postoperative opioid use and prevent postoperative nausea and vomiting is discussed.

In **Chapter 7** the focus is on the prevention of CAL. The possibility to use hyperbaric oxygen treatment to prevent CAL is evaluated.

In **Chapter 8** another innovative approach to prevent CAL and improve colorectal wound healing will be presented. Here, a study is presented with adipose tissue-derived stem cells (ACS) cultured in high density to compose an ASC-sheet. This sheet will be applied on the colorectal anastomosis in a CAL rat model.

In **Chapter 9** the prevention of CAL with cyanoacrylate tissue adhesive will be evaluated in a systematic review.

In *Part 3,* **Chapters 10-14,** prevention of surgical complications in patients at risk is investigated.

In **Chapter 10** different tissue adhesives in the prevention of CAL in a bacterially contaminated environment are investigated.

In **Chapter 11** the same tissue adhesives will be used in a new developed IBD-colectomy model in the rat. Here, the same adhesives are evaluated but then in a different high-risk situation.

In **Chapter 12** cyanoacrylate glue will be tested in an ischemic porcine and CAL model.

In **Chapter 13** the effect of common used biomaterials on polarization of macrophages in healthy subjects will be reviewed.

In **Chapter 14** the response of macrophages will be examined in more detail in a developed culture model with human primary macrophages from patients with or without

obesity to evaluate the acute response of macrophages to biomaterials in this specific patient group.

In **Chapter 15** the findings of this thesis will be discussed.

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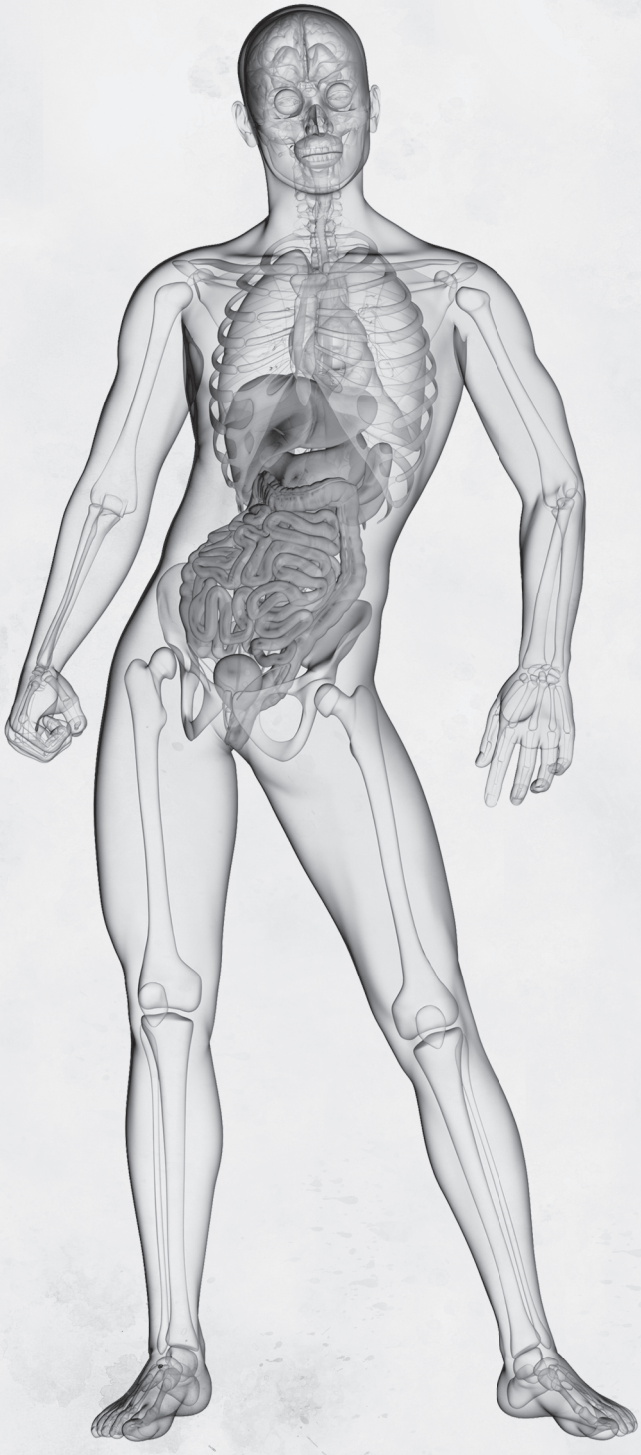

PART 1

Surgical complications: prediction and detection

Chapter 2

Is aortoiliac calcification linked to colorectal anastomotic leakage? A case-control study

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ABSTRACT

Background

Anastomotic leakage in bowel surgery remains a devastating complication. Various risk factors have been uncovered, however, high anastomotic leakage rates are still being reported. This study describes the use of calcification markers of the central abdominal arteries as a prognostic factor for colorectal anastomotic leakage.

Methods

This case-control study includes clinical data from three different hospitals. Calcium volume and calcium score of the aortoiliac tract were determined by CT-scan analysis. Cases were all patients with anastomotic leakage after a left-sided anastomosis (n=30). Three controls were randomly matched for each case. Only patients with a contrast-enhanced pre-operative CT-scan were included.

Results

The measurements of the calcium score and calcium volume of the different trajectories showed that there was one significant difference with regard to the right external iliac artery. Multiple regression analysis showed a significant different negative odds ratio of the presence of calcium in the right external iliac artery.

Conclusion

This study demonstrates that calcium volume and calcium score of the aortoiliac trajectory does not correlate with the risk of colorectal anastomotic leakage after a left-sided anastomosis.

INTRODUCTION

The occurrence of anastomotic leakage (AL) after colorectal surgery remains a severe complication leading to high morbidity and mortality. Literature has identified the main risk factors for colorectal anastomotic leakage: male gender, pre-operative radiotherapy, low anastomosis (<10cm from the anal verge), high BMI, high comorbidity, ligation above the left colonic artery, advanced age, and a history of vascular disease [1-8]. Despite the accumulation of knowledge and the improvements brought by novel surgical techniques, the incidence of AL remains high, approximately 8-18% [4, 9-14].

Sufficient tissue perfusion is an important factor for anastomotic healing. Several experimental and clinical studies showed a correlation between a higher risk of AL when the oxygenation rate of the cut anastomotic edges did not rise during operation [15-18]. In these studies the oxygen saturation of the anastomosis was measured with different tools like near-infrared reflection spectroscopy or by custom-made electrodes. These studies conclude that perioperative low oxygen values lead to a higher risk of anastomotic leakage. Karliczek et al. used visible light spectroscopy to measure the oxygen values during colorectal surgery, they investigated that leaking anastomoses showed less rising oxygen values during operation than normal anastomoses [15].

Smoking, high BMI, hypertension and hypercholesterolemia are risk factors for AL but also for atherosclerosis [19]. An atherosclerotic plaque is made up of fat, cholesterol, and calcium, hardening and narrowing the arteries with limitation of oxygen-rich blood flow. Atherosclerosis is correlated with tissue ischemia and anastomotic leakage caused by poor microcirculation [20-25]. The calcium in the atherosclerotic plaques in the arteries can be scored on computed tomography (CT) by dedicated scoring software. The Agatston calcium score, which is the most validated and most frequently used scoring system as a predictor in previous studies, is the product of the density factor and the area of the calcified plaques (mm^2) [26]. The calcium volume score represents an actual volume of calcium in the artery and reduces calcium measurement variability between scans [27]. The presence of calcium is considered to stabilise the plaque, which decreases the chance of plaque rupture and subsequent ischemic disease [28]. Nevertheless, several studies have shown a correlation between the calcium score and cardiac events [26, 29]. Patients with high Agatston scores have a higher frequency of cardiac adverse events whereas a calcium score of zero portrays a very low risk for short term and midterm cardiac events [30, 31]. A correlation has also been described between higher Agatston score, calcium mass, and calcium volume in various abdominal arteries and the risk of AL after colorectal surgery [32].

In this case-control study we investigated whether the calcium score using Agatston or volume score also can be used as a predictive factor for left-sided colorectal anastomotic leakage, based on enhanced CT measurements.

METHODS

Study population

During 2009 and 2011 all patients undergoing a primary left sided colorectal anastomosis, who preoperatively received a contrast enhanced abdominal CT-scan with a 5mm slice thickness, were included. Data was obtained from the following teaching hospitals; the Erasmus University Medical Center Rotterdam (EMC), Albert Schweitzer hospital Dordrecht (ASD), and the University Medical Center Groningen (UMCG). In this retrospective case-control study cases were patients who had radiographically confirmed anastomotic leakage within 30 days postoperatively. At least 3 controls per case were randomly selected and matched by sex, age, ASA classification, diverting stoma and operation, some cases having 4 controls.

Data collection included age, sex, body mass index (BMI), American Society of Anaesthesiologists (ASA) score, medication use, smoking, alcohol use, operative procedure, postoperative complications and postoperative course.

This study was approved by the Medical Ethical Committee of the Erasmus University Medical Center, Rotterdam, Netherlands, in accordance with the Dutch law on medical research in humans. Permit number MEC-2011-121.

Measurements of calcium score and calcium volume

Two different types of CT-scanner were used in this study. UMCG and EMC used Siemens Somatom Sensation 16 or 64 (Forcheim, Germany) and ASD used Philips Brilliance 40 or 64(Eindhoven, The Netherlands). The calcium scores and volumes were retrospectively determined by two researchers, with Siemens software (Syngo.via® 2009-2012, serial number 100106, Siemens AG, Germany). The score was determined for the total aortoiliac trajectory starting from the T12-L1 disk space (just above the superior mesenteric artery) up to the internal iliac arteries, with a convolution kernel (CK) of 30f, slice thickness of 5mm, and kV of 120 as described by Komen et al. [32, 33]. The calcium score and calcium volume were determined at a threshold level of 500 Hounsfield Units (HU) according to Komen et al. We used a higher threshold, than standard 130 HU, because of the intra-arterial contrast.

Calcium score and volume were determined on the CT-scan at 7 different segments in the aortoiliacal trajectory. The presence of atherosclerotic plaques were computed at several segments: in the abdominal aorta (starting from vertebra T12-L1 up to the aortic bifurcation), the left and right common iliac arteries, the left and right internal iliac arteries, and the left and right external iliac arteries. These segments are representative for the entire aortoiliac trajectory and are important for the vascularisation of the colon and rectum. The vascularisation of the descending colon, sigmoid colon and rectum, which have an important role in the perfusion of the left-side anastomosis, partly arise from the inferior mesenteric artery (IMA). The origin of the IMA is at the ventral side of the abdominal aorta. The IMA is branching into the left colic artery, the sigmoid arteries, and the superior rectal artery. Vascularisation of the rectum originates from the superior, middle and inferior rectal arteries. The latter originates from the internal iliac artery (hypogastric artery) [34].

Statistical analysis

The statistical analysis was carried out using the Statistical Product and Service Solutions (SPSS Inc., Chicago, USA, version 20.0 for Windows). Univariate analysis between the groups with or without AL was done by the median and mean values of the Mann-Whitney U test or chi-square test. Data in the tables on the calcium score and volume are displayed in the original scale of measurement. However, these data were normalized by a logarithmic transformation prior to formal analysis. To determine whether there were significant differences between calcium score and calcium volume in relation to AL or no AL we performed multiple regression by generalized linear models. All reported *p* values were two-sided; a p value < 0.05 was considered to indicate statistical significance.

RESULTS

In total 36 cases and 167 controls were included in the database, six cases and 62 controls were excluded from further analyses because they could not be matched properly, implicating 135 patients (30 cases and 105 controls) were included. Table 1 illustrates the demographic, clinical, and operative characteristics of the included patients.

Univariate analysis of the patient and operative characteristics showed significant differences in cardiac comorbidity and packed cell use during or after the operation between patients with or without AL. The mortality rate in the group with AL was not significantly different to the group without AL ($p = 0.533$; Table 2). Significant differences were found for postoperative bleeding, wound infection, postoperative ileus, intra-abdominal abscess, and urinary tract infection in the AL group, ($p < 0.05$; Table 2).

Table 1. Patient and operation characteristics

	(30 cases)	(105 controls)	
	Anastomotic leakage (%)	No anastomotic leakage (%)	*p value*
Patient characteristics			
Age (yrs)	64.50 ± 11.6	66.49 ± 11.4	0.412
Gender			0.508
Male	16 (53.3)	58 (55.2)	
Female	14 (46.7)	47 (44.8)	
BMI (kg/m2)	28.25 ± 6.7	26.88 ± 4.88	0.227
ASA score[a]			0.897
1	5 (19.2)	20 (20.6)	
2	16 (61.5)	62 (63.9)	
3	5 (19.2)	15 (15.5)	
4	0	0	
Comorbidity			
Cardiac comorbidity	9 (30.0)	14 (13.3)	**0.035**
Peripheral vascular disease	1 (3.3)	8 (7.6)	0.364
Diabetes Mellitus	3 (10)	12 (11.4)	0.563
Smoking (current)[a]	9 (33.3)	29 (30.9)	0.490
Medication			
Use of steroids	1 (3.3)	6 (5.7)	0.513
Use of statins[a]	5 (17.9)	13 (12.5)	0.324
Use of antihypertensiva[a]	10 (33.3)	39 (37.5)	0.424
Neoadjuvant radiotherapy	2 (6.9)	6 (5.7)	0.566
Operation characteristics			
Surgeon vs. resident	21 (70.0)	76 (73.1)	0.452
Type of operation			0.442
Sigmoid Resection	17 (56.7)	48 (45.7)	
Low Anterior Resection	7 (23.3)	32 (30.5)	
Rectosigmoid resection	3 (10)	15 (14.3)	
Hemicolectomy left	1 (3.3)	8 (7.6)	
Other	2 (6.7)	2 (1.9)	
Approach[a]			0.184
Laparotomy	22 (75.9)	68 (64.8)	
Laparoscopy	7 (24.1)	37 (35.2)	
Stapled vs. hand sutured[a]			0.098
Sutured	15 (51.7)	38 (36.2)	
Stapled	14 (48.3)	67 (63.8)	
Conversion[a]	5 (26)	14 (74)	0.205

Table 1. Patient and operation characteristics (continued)

	(30 cases)	(105 controls)	
	Anastomotic leakage (%)	No anastomotic leakage (%)	*p value*
Anastomotic configuration[a]			0.315
Side-end	5 (17.2)	31 (35.6)	
Side-side	5 (17.2)	10 (11.5)	
End-side	2 (6.9)	5 (5.7)	
End-end	17 (58.6)	41 (47.1)	
Stoma[a]	4 (14.3)	15 (17.9)	0.455
Packed cells[a]	25 (100)	36 (43.9)	**0.000**
Prophylactic drainage[a]	20 (69)	51 (49.5)	0.073

[a] The numbers do not add up to 135 because of occasional missing data

Table 2. Postoperative complications

	(30 cases)	(105 controls)	
	Anastomotic leakage (%)	No anastomotic leakage (%)	*p value*
Complications			
Postoperative ileus	7 (23.3)	9 (8.6)	0.035
Dehiscence of laparotomy wound	2 (6.7)	3 (2.9)	0.308
Wound infection	7 (23.3)	5 (4.8)	0.005
Intra-abdominal abscess*	12 (40)	2 (1.9)	0.000
Urinary tract infection	2 (6.7)	0 (0)	0.048
Pneumonia	3 (10)	3 (2.9)	0.123
Bleeding	3 (10)	1 (1)	0.034
Mortality**	1 (3.3)	2 (1.9)	0.533

* not close to anastomosis ** (<30days post-operative)

Table 3 presents the mean and standard deviation (SD) of the calcium score and calcium volume of the different trajectories. The measurements show that there is only one significant difference in one trajectory (right external iliac artery), indicating that patients without anastomotic leakage had a significant higher calcium volume in that specific trajectory.

Multiple regression analysis showed significant differences in the odds ratio for AL after higher calcium score in the right external iliac artery (Table 4). Negative odds ratio indicates a negative relationship between the probability of anastomotic leakage and the calcium score or calcium volume.

Table 3. Calcium volume and calcium score of the different trajectories 500 HU

		Calcium Volume			Calcium Score		
Trajectory	*n*	*Mean*	*SD*	*p value*	*Mean*	*SD*	*p value*
Total trajectory	30	4.98	2.77	0.712	5.50	2.88	0.707
	104	4.75	2.95		5.26	3.12	
Aorta abdominalis	30	4.42	2.85	0.561	4.93	2.93	0.713
	104	4.06	3.04		4.70	3.10	
Iliac artery							
Left common	30	2.25	2.34	0.898	2.75	2.55	0.863
	104	2.18	2.42		2.65	2.76	
Right common	30	2.86	2.35	0.112	3.36	2.62	0.697
	104	2.26	2.41		3.01	2.75	
Left internal	30	1.12	1.72	0.109	1.49	2.12	0.412
	104	1.85	2.33		1.66	2.14	
Left external	30	0.66	1.44	0.227	0.99	1.75	0.527
	104	1.32	2.09		1.34	2.17	
Right internal	30	0.81	1.70	0.372	1.06	1.97	0.279
	104	1.12	1.67		1.50	1.96	
Right external	30	0.69	1.67	***0.036***	0.93	1.91	0.159
	104	1.62	2.22		1.58	2.30	
Left and right common	30	2.55	0.43	0.610	3.06	2.59	0.615
	104	2.22	2.42		2.83	2.76	
Left and right internal	30	0.97	1.71	0.440	1.28	2.05	0.378
	104	1.49	1.00		1.58	2.05	

Table 4. Multiple regression by generalized linear models calcium volume and calcium score

500HU	Calcium Volume		Calcium Score	
Trajectory	odds ratio	*p value*	odds ratio	*p value*
Total trajectory	0.03	0.710	0.03	0.704
Aorta	0.04	0.558	0.03	0.710
Left common iliac artery	0.01	0.897	0.01	0.862
Left internal iliac artery	-0.17	0.116	-0.04	0.694
Left external iliac artery	-0.25	0.117	-0.09	0.410
Right common iliac artery	0.10	0.227	0.05	0.524
Right internal iliac artery	-0.12	0.371	-0.12	0.279
Right external iliac artery	-0.25	0.043	-0.15	0.163
Left and right common iliac arteries	0.04	0.607	0.04	0.162
Left and right internal iliac arteries	-0.09	0.437	-0.08	0.376

DISCUSSION

Anastomotic leakage remains one of the most feared complications after colorectal surgery. There are several well-known risk factors for colorectal anastomotic leakage, such as high BMI, advanced age, high level of comorbidity and a history of cardiovascular disease. All these risk factors are also correlated to atherosclerosis. However, atherosclerosis alone has not been identified as a risk factor for colorectal anastomotic leakage.

The results of our study suggest that the abdominal calcium score and calcium volume in aortoiliac trajectory are not correlated with anastomotic leakage in left-sided colon anastomoses. We did not find any indications that calcium measurements correlate to the onset of AL. Although, the collected data give a good reflection of reality because of the use of different CT-scanners in both local and university hospitals.

It is well known that left-sided anastomoses are associated with higher AL percentages when compared to more proximal anastomoses [35, 36]. However, in this study we showed that the calcium scores in the atherosclerotic plaques of patients with AL, are not higher than in patients without AL for the same type of surgery.

Our results are in line with previous research by Kornmann et al., who scored the stenosis caused by arteriosclerotic lesions in the visceral arteries. They studied 242 patients who underwent colorectal anastomosis. The authors suggested that asymptomatic visceral artery occlusive disease is not a risk factor for anastomotic leakage [37]. This is maybe caused by the presence of a sufficient collateral network [38].

Komen et al. investigated that patients with higher calcium scores in the common iliacal arteries had an increased leakage risk of colorectal anastomosis. This study also mentioned that the calcium score of the left internal iliac is significantly higher in patients with colorectal AL, however the reason for this finding remained unclear [32].

In this present study we used the same measurement method of the calcium score as described by Komen et al. [32]. In the current study we could not measure the calcium mass, due to the lack of software support on this value for the different types of CT-scanners. Although the calcium volume and calcium score correlate very well with the calcium mass, Komen et al. showed a better correlation between the calcium mass and AL than the calcium score and AL [32]. It is possibly due to the smaller study population in Komens' study.

Measuring the calcium volume and calcium score with a threshold of 500HU is higher than usual because of the contrast enhanced CT scan. Because of the intra-arterial contrast we probably missed some calcifications but this is the same in the AL group as in the non-AL group. This will not have any influence on the results. This will also explain the low scores of calcium mass and calcium volume compared to other studies.

From the results obtained in this study, we conclude that abdominal atherosclerotic calcifications expressed as calcium score and calcium volume is not useful for preoperative risk assessment for left-sided anastomoses. This study indicates that calcified atherosclerosis in the large abdominal arteries does not influence the perfusion of the anastomotic edges. It is known that coronary calcified plaques represents approximately 20% of the total plaque, the other 80% is lipid-rich plaque or fibrous plaque [27]. This may indicate that the calcified plaques are not representative enough for the total atherosclerosis in the abdominal arteries.

Future research regarding the relationship between abdominal atherosclerosis and the risk of colorectal anastomotic leakage should concentrate on the relationship between abdominal calcifications and the severity of abdominal artery lumen stenosis and the perfusion of the colon during surgery. Furthermore, visualization of the collateral network around the anastomosis may provide extra information on the onset of ischemia leading to AL.

CONCLUSION

This study demonstrates that the abdominal calcium score and calcium volume in the aortoiliac trajectory are not correlated with anastomotic leakage in left-sided colon anastomosis.

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Chapter 3

Postoperative hemodynamic index measurement with miniaturized dynamic light scattering predicts colorectal anastomotic healing

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ABSTRACT

Introduction

Perioperative bowel perfusion (local-hemodynamic index, LHI) was measured with a miniaturized Dynamic Light Scattering (mDLS) device, aiming to determine whether anastomotic perfusion correlates with the anastomotic healing process, and whether LHI measurement assists detection of anastomotic leakage (AL) in colorectal surgery.

Methods

A partial colectomy was performed in 21 male Wistar rats. Colonic and anastomotic LHI were recorded during operation. On postoperative day (POD)-3, the rats were examined for AL manifestations. Anastomotic LHI was recorded before determining anastomotic bursting pressure (ABP). The postoperative LHI measurements were repeated in another 15 rats with experimental colitis. Clinical manifestations and anastomotic LHI were also determined on POD3. Diagnostic value of LHI measurement was analyzed with the combined data from both experiments.

Results

Intraoperative LHI measurement showed no correlation with the ABP on POD3. Postoperative anastomotic LHI on POD3 was significantly correlated with ABP in the normal rats (R^2=0.52, p<0.001) and in the colitis rats (R^2=0.63, p=0.0012). Anastomotic LHI on POD3 had high accuracy for identifying ABP < 50 mmHg (AUC=0.86, SE=0.065, p<0.001). A cut-off point of 1236 yielded a sensitivity of 100% and a specificity of 65%. On POD3, rats with LHI<1236 had significantly higher dehiscence rate (40% vs. 0%), more weight loss, higher abscess severity and lower ABP (p<0.05 respectively); worse anastomotic inflammation and collagen deposition were also found in the histological examination.

Conclusion

Our data suggest that postoperative evaluation of anastomotic microcirculation with the mDLS device assists the detection of anastomotic leakage in gastrointestinal surgery.

INTRODUCTION

Despite successful resection of the primary disease, an uncomplicated healing process after construction of an anastomosis is crucial after colorectal surgery. Failure in anastomotic healing results in anastomotic leakage (AL), which is a serious short-term complication contributing to one-third of all postoperative mortality after colorectal surgery [1]. When AL strikes, nonsterile intraluminal content leaks into the abdominal or pelvic cavity, which causes infection or peritonitis and may lead to sepsis, multiple-organ failure, and even death if not detected and treated on time [2]. Because of these catastrophic outcomes, early detection of AL is the crucial intervention.

Conventionally, surgeons select patients suspicious for AL for radiological examinations based on abnormal postoperative manifestations. But those "abnormal" manifestations (e.g. fever, leukocytosis, tachycardia, tachypnea) are remarkably common after abdominal surgery, occurring in a substantial number of uncomplicated recoveries [3]. To date, AL is usually detected between day 5 and day 8 or even later after surgery, [4] and more than half of the detected leakages require reoperation [5,6]. Surgeons are of the opinion that many leakage cases are not detected until too late with the current strategy.

Similar to other types of wounds, one direct consequence of an anastomotic wound is tissue ischemia, which has been considered as a risk factor of AL [7-9]. In skin wounds, tissue perfusion can be conveniently revealed by repeated evaluation or even real-time monitoring with laser Doppler imaging device, flowmetry, or other techniques [10,11]. Unfortunately, evaluation of anastomotic perfusion is not a simple task, and to date, it is still confined to intraoperative measurement because of the large sizes of most devices [12-14]. Monitoring of postoperative anastomotic circulation is not feasible unless a much smaller device is available, which patients may carry during hospitalization or even afterward.

Recent developments in laser technology include a novel miniaturized dynamic light scattering (mDLS) device, which can be used to carry out postoperative evaluation of anastomotic microcirculation. However, whether the perfusion data correlate with anastomotic healing remains a fundamental question to be answered prior to further application of the device. To this end, this study was designed to determine whether blood flow measurement with the mDLS device may aid in the diagnosis of failed anastomotic healing. The perioperative local hemodynamic index (LHI) was recorded during rat colectomy and analyzed afterward to determine its diagnostic value.

METHODS

Male Wistar rats were purchased from a licensed breeder (Harlan Laboratories, Boxmeer, Netherlands). All rats were bred under specific pathogen-free conditions. Rat chow and water were supplied ad libitum. The Ethical Committee on Animal Experimentation of Erasmus University Rotterdam approved the protocol.

LHI monitoring device and data extraction

The LHIs were measured with a mDLS device (Elfor, Elfi-Tech Ltd., Rehovot, Israel). The device consists of 3 parts: an mDLS sensor, a data-collecting device, and a data-analyzing system (ie, real-time monitoring software). The mDLS sensor consists of a vertical-cavity surface-emitting laser (wavelength 852.4 ± 2 nm; continuous wave; peak energy 0.7 mW) and an optoelectronic detection system and is linked to a data-collecting device via flexible wires. When measuring, one can choose to send the recorded data directly to the computer program via Bluetooth or USB link for real-time monitoring. At the same time, the data are also saved on an SD card, which can be analyzed afterward (Figure 1).

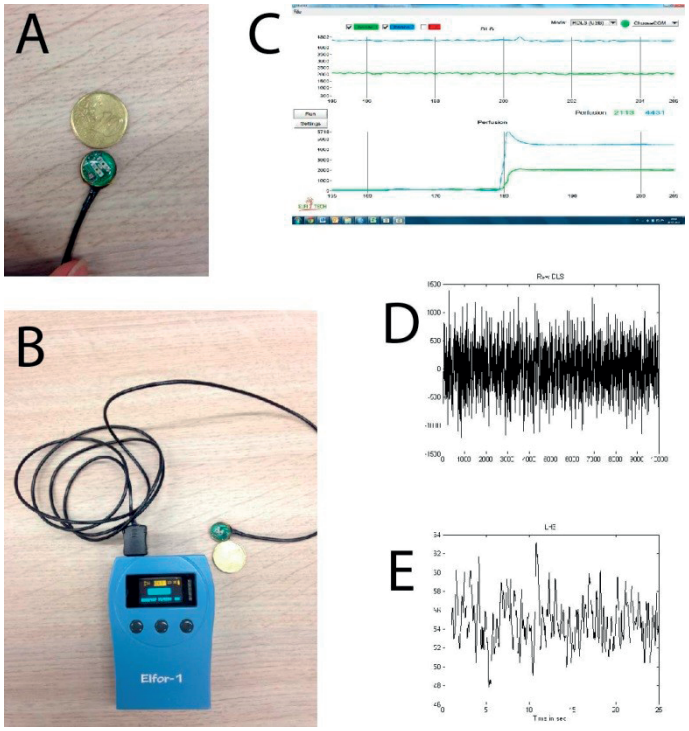

Figure 1. The miniaturized dynamic light scattering (mDLS) device, software interface and local hemodynamic index (LHI) data. A. The mDLS sensor: (1) 10-cent euro coin; (2) the sensor, size = 10 × 10 × 1 mm3 . B. The mDLS device, size: 8 × 5 × 1.5 cm3 . C. Interface of the real-time monitoring software. D. Raw LHI data recorded by the device. E. LHI data extracted from raw data, 1 to 5 kHz

This device measures LHI with laser-speckle analysis of moving red blood cells (RBCs) [15]. The measurement is based on time-dependent analysis of the scattered coherent light from an ensemble of moving RBCs and can be used for an assessment of local blood shear rate and blood flow [15]. Based on the estimated blood shear rate contributions to the overall signal, different blood flow components into the measured laser speckle signal can also be extracted. In this study, the data with a frequency between 1 and 5 kHz, which represent RBC movement in capillaries and are of the most relevance to tissue perfusion, were analyzed [16,17]. A detailed description of the algorithm of LHI measurement and calculation is provided in the supplementary data.

In this study, we measured LHI at different time points during the rat colectomy (Figure 2). Blinded measurements (ie, measuring without real-time monitoring) were used to ensure the highest level of objectiveness. During each measurement, the sensor was covered with a sterile transparent plastic bag to prevent contamination. Afterward, the raw data were uploaded and converted into LHIs (1-5 kHz) with the offline software. The median value of each LHI measurement was used for statistical analysis.

Evaluation of a correlation between LHI and anastomotic healing

The rats were anesthetized with an isoflurane/O2 mask. Partial colectomy was performed according to the methods described in our previous study [18]. The baseline LHI data of the ascending and descending colon were first recorded at the site of the intended cutting edges (Figure 2). Subsequently, the major part of the colon was resected, followed by a full-thickness end-to-end anastomosis (ie, continuous suture with 12 stitches) with Dafilon 8/0 (B. Braun, Germany) under the microscope. Then, the LHI of the upper and lower anastomotic edges were measured. After the LHI measurement on top of the anastomosis (Figure 2), the abdominal wall was closed.

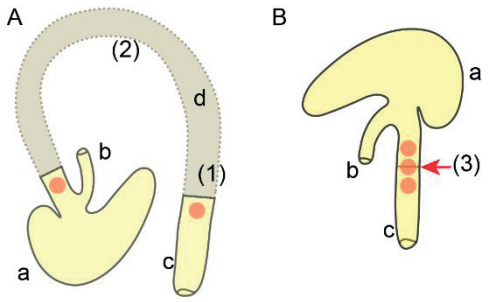

Figure 2. Schematic overview of the rat colon and the locations of perfusion measurement, anterior view. A. Before colectomy. B. After colectomy. Anatomy: (a) cecum, (b) terminal ileum, (c) rectum and anus. Procedure: (1) The baseline local hemodynamic index (LHI) is first measured before colectomy at the intended cutting edges (ie, indicated with orange dots); (2) a major part of the colon (ie, indicated in gray) is resected; (3) the anastomosis (red arrow) is constructed, and the LHI is measured again. Adapted from Wu et al (2015) with permission [19].

Because a standard colectomy yields a relatively low rate of AL, [18] we used an insufficiently sutured anastomosis in 3 rats to achieve a higher rate of AL, according to the leakage model developed. In these rats, 5 interrupted stitches were used instead of 12 stitches to construct a technically insufficient anastomosis, and the other procedures remained the same as in the aforementioned partial colectomy.

Postoperative LHI measurements in a colitis-colectomy model

To verify whether the findings from the previous experiment also exist in a colon disease model, postoperative anastomotic LHI measurements were repeated in rats with colitis. Colitis was induced by transanal injection of 0.25 mL 2,4,6-trinitrobenzenesulfonic acid (TNBS, 10 mg diluted in 25% ethanol) under anesthesia according to the previously published TNBS-colitis model [19]. One week later, colectomy was performed with the aforementioned technique, and the anastomosis was constructed with a continuous suture with 12 stitches.

Follow-up and postoperative evaluation

The rat abdomen was examined on postoperative day (POD) 3. We first checked for signs of AL, including abscess formation and anastomotic dehiscence. The abscess severity was determined by an abscess scoring system, [20,21] and adhesion formation was evaluated using the Zühlke score, [22] anastomotic LHI having been determined with the device. The anastomotic bursting pressure (ABP) was determined after the measurement using the methods described in our previous study [18]. The anastomotic segment was harvested and stained with hematoxylin and eosin. The slides were scored using the Ehrlich and Hunt numerical scale as modified by Phillips et al. [23].

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad Software, Inc, CA). Data are presented as mean ± standard deviation, unless stated otherwise. The Mann-Whitney test, t test, Pearson correlation test, and receiver operating characteristic (ROC) analysis were used. All reported *p* values were 2 sided; a *p* value <0.05 was considered to indicate statistical significance.

RESULTS

Perioperative LHI and anastomotic healing in normal rats

A total of 21 rats (450-550 g) were used. Weight loss and diarrhea after operation were observed in all rats. Two deaths occurred after surgery with no signs of AL during autopsy.

The baseline LHI was similar between the ascending and descending colon. Although LHI changes were observed during colectomy, the average LHI remained stable at both cutting edges. A LHI similar to the baseline was found at the anastomotic site after construction ($p > 0.05$, Pearson correlation test). None of the intraoperative LHI measurements showed a significant correlation with ABP on POD3 (Figure 3).

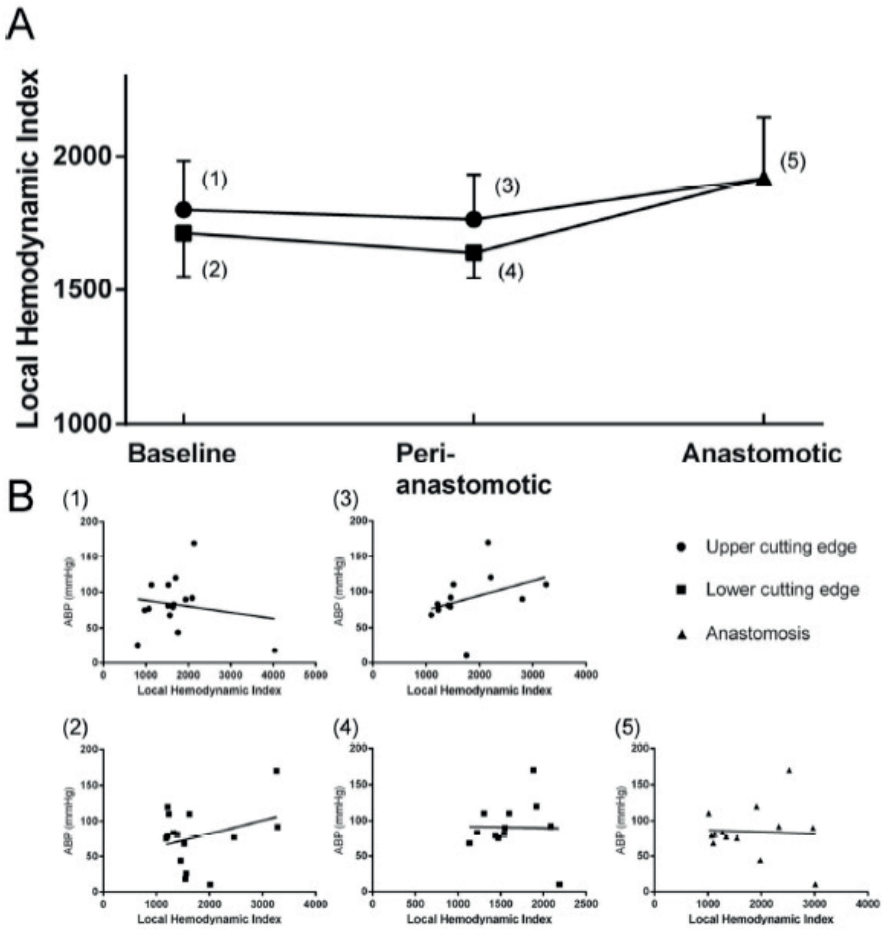

Figure 3. Local hemodynamic index (LHI) during colectomy and correlations between intraoperative LHI and the anastomotic bursting pressure (ABP) in normal rats. Only rats with the 12-suture anastomosis were included, n = 12-15; three rats with the 5-suture anastomosis were excluded for analysis. (A) The baseline LHI was similar between the intended upper and lower cutting edges, which remained stable during the operation; similar LHI to the baseline was found at the anastomotic site ($p > 0.05$ respectively), values are mean (± S.E.), paired Mann-Whitney test. (B) None of the intraoperative LHI showed a significant correlation with ABP ($p > 0.05$ respectively). Each numbered time point in Figure A corresponds to the average of the intraoperative LHI values in the numbered figures in Figure B respectively.

The correlation between postoperative LHI and ABP on POD3 was statistically significant ($R^2 = 0.52$; $p < .001$, Pearson correlation test). Further linear regression analysis showed that Y = 0.044X + 1.0 (Y is the ABP; X is the anastomotic LHI on POD3; Figure 4).

Validating the LHI measurements in a colitis-colectomy model

Based on the results of the previous experiment, postoperative LHI measurements were repeated in 15 rats with colitis after colectomy. One death occurred after surgery, and no sign of AL was observed during autopsy. Similarly, LHI on POD3 yielded a significant correlation with ABP in the colitic rats after colectomy ($R^2 = 0.63$; $p = 0.0012$; Pearson

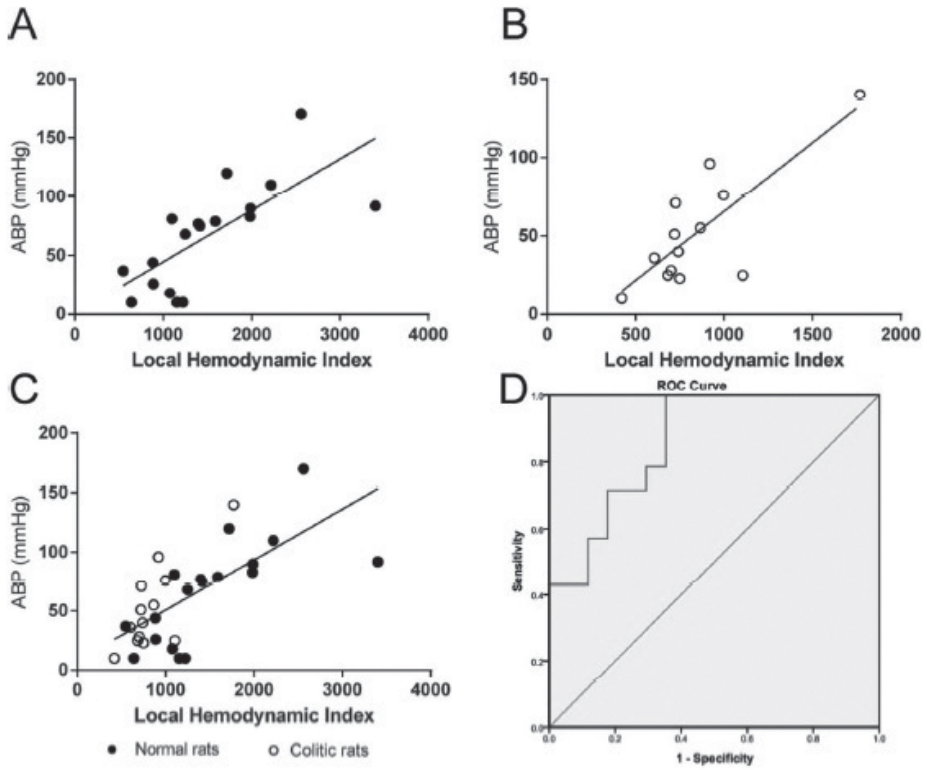

Figure 4. Correlation between the postoperative local hemodynamic index (LHI) and the anastomotic bursting pressure (ABP) on postoperative day (POD) 3. The X-axis is the anastomotic LHI on POD 3. The Y-axis is the ABP on POD 3. (A) In the normal rats, the linear regression analysis showed that Y = 0.044*X + 1.0, R2 = 0.52, $p < 0.001$, n = 18. (B) In the colitis rats, Y = 0.088*X - 22.22, R2 = 0.63, $p = 0.0012$, n = 13. (C) The correlation between the LHI and ABP remained significant when data of the normal and colitic rats were combined, Y = 0.043*X + 8.04, R2 = 0.49, $p<0.001$, n = 31. (D) ROC analysis showed LHI on POD3 had high accuracy for identifying ABP < 50 mmHg. Area under curve (AUC) = 0.86, S.E. = 0.065, 95%CI = 0.74 to 0.99, $p<0.001$, n = 31.

correlation test). Further linear regression analysis showed that $Y = 0.088 \times X - 22.22$; $P = 0.0012$ (Y stands for ABP; X stands for LHI; Figure 4).

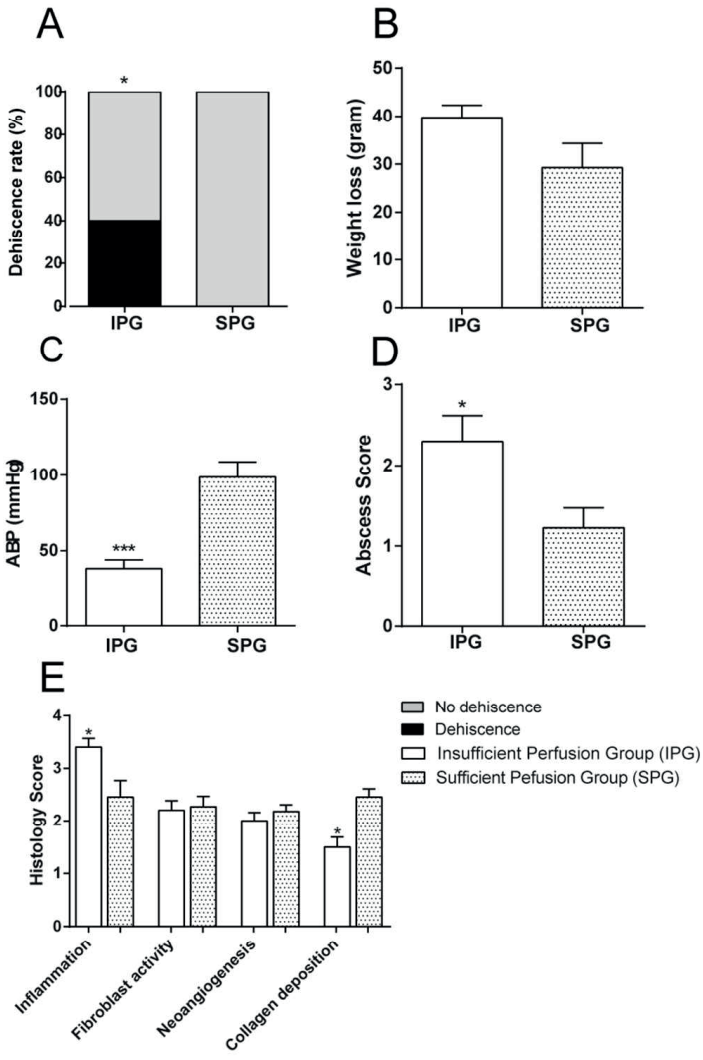

Figure 5. Comparison of clinical and histological outcomes between the insufficient perfusion group (IPG, n = 20) and the sufficient perfusion group (SPG, n = 11). (A) Anastomotic dehiscence rate was 40% (8/20) in the insufficient perfusion group and 0% (0/11) in the sufficient perfusion group, p = 0.028, Chi-square test. (B) Comparison of weight loss yielded a p = 0.048, t test. (C) Comparison of anastomotic bursting pressure (ABP) resulted in p < 0.001, Mann-Whitney test. (D) A significantly higher abscess score was found in the insufficient perfusion group than that in the sufficient perfusion group, p = 0.034, Mann-Whitney test. (E) In histological evaluations, the insufficient perfusion group showed a significantly worse status of inflammatory infiltration and collagen deposition, p < 0.05 respectively, Mann-Whitney test. * indicates p < 0.05, *** indicates p < 0.001. In (B-E) values are mean (± S.E.).

Diagnostic value of postoperative LHI measurement

Combining the data from the 2 experiments, we still observed a significant correlation between LHI and ABP (Figure 4). Moreover, we found that the postoperative LHI showed high accuracy in detecting failed anastomotic healing. Based on our previous rat experiments, ABP <50 mm Hg was set to determine if an anastomosis was mechanically insufficient [24]. As is shown in Figure 4, anastomotic LHI on POD3 had high accuracy for identifying ABP <50 mm Hg. Area under the curve was 0.86 (standard error = 0.065; 95% confidence interval [CI] = 0.74 to 0.99; $p < 0.001$). Further analysis showed that a cutoff point of 1236 yielded a sensitivity of 100% (95% CI = 77%-100%) and a specificity of 65% (95% CI = 28%-86%), a positive predictive value of 70% and a negative predictive value of 100%, and an accuracy of 81%.

To enable comparisons between rats with sufficient/ insufficient anastomotic LHI on POD3, the rats were further assigned to 2 groups based on perfusion. Rats with a higher anastomotic LHI than the cutoff point (ie, 1236) were assigned to a sufficient-perfusion group (n = 11), including 10 normal rats and 1 rat with colitis, and the other rats were divided into an insufficient-perfusion group (n = 20), including 5 normal rats with the 12-suture anastomosis, all 3 normal rats with the 5-suture anastomosis, and 12 rats with colitis.

In total, 8 animals had an anastomotic dehiscence, all from the insufficient-perfusion group. Significantly higher weight loss (39.8 ± 11.4 g vs 29.3 ± 16.9 g; $p = 0.048$; t test) and higher abscess score (2.3 ± 1.4 vs 1.2 ± 0.8; P = .034; Mann-Whitney test) were found in the insufficient-perfusion group as well when compared with the sufficient-perfusion group (Figure 5). An average bursting pressure of 38.2 ± 26.4 mm Hg was seen in the insufficient-perfusion group, which was significantly lower than that in the sufficient-perfusion group (98.7 ± 34.0 mm Hg; $p < 0.001$; Mann-Whitney test; Figure 5). On histological evaluation, the insufficient-perfusion group showed a significantly worse status of inflammatory infiltration and collagen deposition ($p < 0.05$; MannWhitney test).

DISCUSSION

Evaluation of anastomotic perfusion may be a reliable strategy to reveal the wound healing status and to detect AL after colorectal surgery. In this study, we demonstrated a correlation between LHI and anastomotic healing in a rat colectomy model with a novel mDLS device and further validated it in a surgical colitis model, exploring its diagnostic value. These data encourage further investigation of the device in detecting AL in colorectal surgery.

Though recognizing the importance of tissue perfusion in anastomotic healing, few strategies exist to assist surgeons objectively in determining anastomotic perfusion once the laparotomy/laparoscopy is closed. Several early attempts from previous studies suggest that an elevation of indirect parameters (eg, pH, lactate, and pyruvate) might indicate the presence of AL [2,14,25]. Direct correlation between postoperative anastomotic perfusion and its healing process has not yet been established. In this study, such a correlation was observed in both experiments regardless of the primary disease, which, in combination with the ROC analysis, provided direct evidence regarding the diagnostic value of the LHI measurements. In addition to the satisfactory diagnostic value for identifying mechanically weak anastomoses, poorer clinical manifestations, including anastomotic dehiscence and abscess formation, were found in the rats. Such differences were in line with the histological findings revealing worse inflammation and collagen deposition in the rats with insufficient anastomotic perfusion. Our data, in correspondence with the previous literature, confirm the crucial role of tissue perfusion in the process of wound healing.

Different from postoperative measurements, many clinical trials have suggested a clinical value of the intraoperative perfusion measurement in detecting anastomotic ischemia [9,12,13,26]. However, the limitation of such methods is clearly illustrated by our data. The LHI data of the 3 rats with insufficient sutures demonstrate that bowel ischemia may not exist during operation under circumstances when AL is caused by certain intraoperative factors, let alone postoperative ones. Nevertheless, our data do not oppose any further application of intraoperative evaluation because rather than a standardized rat model without preoperative risk factors, substantial risk factors resulting from patient-related comorbidities (eg, uncontrolled diabetes, smoking, atherosclerotic calcification) may cause bowel hypoxemia before the operation, which is probably detectable with the intraoperative perfusion measurement [27-29]. Moreover, our ongoing studies revealed that certain preoperative or intraoperative risk factors also influenced LHI changes during surgery (unpublished data). Further investigations with comprehensive analysis in this regard are being planned.

The applications of postoperative perfusion measurement are in 2 areas. One consists of objectively evaluating bowel perfusion during examination or reintervention in patients suspicious for AL. Such an application is greatly necessary because in many cases bowel ischemia is not detectable by the naked eyes during reoperation [25]. Moreover, our study also showed that mechanically insufficient anastomoses were not always accompanied by anastomotic abscess formation or dehiscence. Perfusion measurement may aid in the formulation of a better surgical plan based on a real-time bowel perfusion level and speculation of the mechanical strength of a suspicious anastomosis in circumstances when

clinical manifestations remain inconclusive. These measurements can be implemented in combination with a laparoscopic approach because of the very small size of the device. It can also be applied in combination with endoscopy for postoperative examination because mucosal and serosal blood flow measurements are linearly correlated [30].

Another avenue is postoperative real-time monitoring of anastomotic perfusion. The main strength of this mDLS device is its small size, which enables such monitoring, but it remains technically challenging. How to place it properly near the anastomosis and how to retract it are still major questions to be verified. Combining the perfusion sensor with an intra-abdominal drainage or with an intra-gastrointestinal tube might be a promising strategy, [31] and further investigation in this regard is still required with large animal models. Although difficult with the current technique, this remains a promising approach in the future. Doctors may implement real-time monitoring in patients with Bluetooth and data processing software, which will alert them when an early-stage AL occurs.

In addition to the technological development of the medical device, further investigation should also focus on LHI data mining. The DLS system has been developed for decades for various clinical indications [15,32]. The device in our study has been previously tested in human subjects at different clinical conditions for assessment of capillary skin flow, RBC-endothelial interaction, and pulse wave hemodynamic characteristics [32]. We analyzed the data with a frequency between 1 and 5 kHz in the current study, which is considered to best represent tissue perfusion [16,17]. Substantial data can be further extracted to investigate the microcirculation with other characteristics. Integrating the data from different frequencies may reveal not only the hemodynamic characteristics but also the tissue oxygenation level, providing a comprehensive picture of the tissue environment. Investigations addressing these issues are currently under way in our research team.

In this rat model, we chose POD3 as the follow-up period, when ABP best represents the anastomotic healing process. In a shorter follow-up period, the mechanical strength of an anastomosis is still greatly influenced by the suture strength, whereas in the long term, many well-healed anastomoses usually burst outside the anastomotic area [33]. Both conditions attenuate the representativeness of the ABP, which may eventually influence the correlation analysis (LHI vs ABP) in this experimental study. The acute inflammatory response at the constructed anastomosis usually reaches its peak within 3 days. When AL occurs, a large number of inflammatory cells infiltrate the anastomotic area, which results in abscess formation and collagen degradation, both weakening the anastomotic strength that can be detected by the ABP test [24]. Similar phenomena were also observed in our study.

Given an ideal follow-up time, to determine the correlation pattern between anastomotic strength and anastomotic perfusion remained difficult with our study design, because there was very limited knowledge from the previous literature exploring this topic. Some previous data have shown that colonic oxygen tension was correlated with anastomotic breaking strength, [34] and bowel perfusion was in correspondence with severity of bowel injury in Inflammatory bowel disease (IBD) patients, [27] both of which seemed to fit a linear correlation. Hsieh et al [35] also developed an equation with a linear regression when they implemented a laser Doppler device to predict diabetic ulcer. However, we recognize that simply using a linear regression and the corresponding equation to predict the anastomotic strength may include relatively high risks of bias. The R^2 values in the results (ie, 0.52 and 0.63) further confirmed this and suggested the involvement of other factors that influenced the anastomotic strength. Therefore, we performed the ROC analysis to determine the diagnostic value of LHI and the cutoff point of a mechanically insufficient anastomosis. We believe that such an analysis is more reliable for future research and application of the LHI device.

In conclusion, our data suggest that postoperative blood flow evaluation with an mDLS device at the anastomotic site provides useful information regarding anastomotic healing and may facilitate detection of AL in colorectal surgery.

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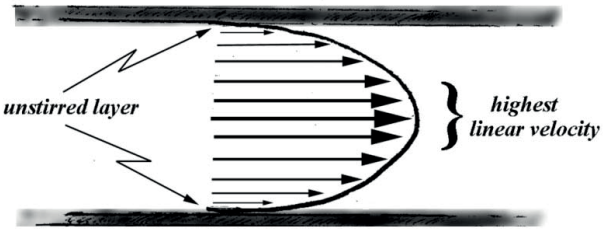
SUPPLEMENTARY DATA

How is the LHI calculated?

The m-DLS signal is originated from the relative movement of the scattering particles. In the case of the blood flow the relative movement is caused by the velocity profile of the flowing blood in small vessels(see the picture below).



The mDLS technology takes advantage of the RBC velocity differences and produces a signals that resemble other well knows physiological signals such as PPG, Laser Doppler Velocimetry (LDV), and Invasive Blood Pressure (IBP).

mDLS differs from the LDV in that LDV measures the local velocity of blood flow, whereas the mDLS measures the red blood cells (RBC) velocity gradient which is directly related to the shear rate. According to basic low of laminar flow the shear rate increases when the velocity goes up, so the mDLS signal is a function of flow velocity. The measured signal is formed by the difference in Doppler shifts of all correlated and uncorrelated particles in the scattering volume. The signal is derived from the fluctuations of the intensity signal *I(t)*. The measured parameter is the autocorrelation function ACF of *I(t)* which is defined by:

$$ACF(\tau) = \langle I(t) \cdot I(t+\tau) \rangle - \langle I \rangle^2$$

For the laminar blood flow the most important contribution to the correlation function measured by mDLS comes from all moving RBC pairs. These pairs are formed by the spatially related moving RBC's, which are located in close vicinity to each other. The more distant particles give negligible weight into the *g*. Therefore, the mDLS is sensitive to the velocity gradient in laminar or turbulent flow.

In the case of Poiseuille laminar blood flow the blood moves back and forth with oscillatory frequency ω in response to the oscillatory pressure gradient. The flow velocity *u(r,t)* is the function of radial location *r* in the vessel and time *t*. *u(r,t)* is described by:

$$u(r,t) = i\frac{k_s a^2}{\mu \cdot \Omega^2}\left(1 - \frac{J_0(\zeta)}{J_0(\Lambda)}\right) \cdot e^{iwt}$$

Where J_0 is Bessel function of order zero, $\Omega = \sqrt{\frac{\rho\omega}{\mu}}a$, *"a"* is the vessel radius and ρ is density. Additionally, $\Lambda = \left(\frac{i-1}{\sqrt{2}}\right)\cdot\Omega$, $\zeta(r) = \Lambda \cdot r/a$, k_s is amplitude of pressure gradient, and μ is the coefficient of viscosity. The signal of mDLS will be determined by the relative velocity of the paired RBC particles, or the value of $\frac{\partial(u(r,t))}{\partial r}$. It can be easily shown that $\frac{\partial(u(r,t))}{\partial r} \approx -\frac{k_s \cdot a}{\Lambda}\cdot\left(\frac{J_1(\Lambda)}{J_0(\Lambda)}\right)\cdot e^{iwt}$. In very simplified case if a vessel of radius *R*, axis symmetric velocity profiles *v(r,t)* can be described in cylindrical coordinates by the empirical relationship:

$$v(r,t) \approx v_{\max} * (1-(r/R)^{\xi}) * f(t)$$

where -1<(r/R)<1, *f(t)* is a periodic function of heart beat frequency, which is driven by systolic pressure wave and it is time phase-shifted with respect to the cardiac cycle, and *ξ* represents the degree of blunting. For example, in 30 micron arterioles, there is a range of *ξ* 2.4 – 4 at normal flow rates. If *ξ*=2, a parabolic velocity distribution is obtained. Blunting would occur even in larger arterioles at low flow rates. The standard deviation *d(v)* can be calculated by:

$$rms(dV) = v_{\max} * f(t)\sqrt{\frac{\int dv(r) * r^2 * dr}{\int dv(r) * dr}} = \frac{\xi * R^2}{2+\xi} * v_{\max} * f(t)$$

The rms(dV) is proportional to the blood flow velocity. In terms of ACF decay time of autocorrelation function can be estimated by $t_d \approx \frac{1}{dV(L)}$. Actually, the decay time of the process is very short and it means that high frequency component of the signal is closely is associated with the arterial blood flow signal.

For small arterials (around 20 microns), the fluctuation of velocity from systolic to diastolic phases ranges from 1.5mm/s to 2.5 mm/s. This results in a very significant fluctuation of standard deviation (Rms) during the systolic–diastolic cycle. Pulsatile signal, therefore, can be used for calculation of hemorheological parameters.

In (I. Fine et al, Journal of Biomedical Optics 17(8), 087002 (August 2012) it was shown the ACF , which is related to shear rate of blood, can be expressed in terms of power spectrum density so that as $LHI = \int_{\omega_1}^{\omega_2}\int_{-\infty}^{\infty} ACF(\tau, t_c)\cdot\exp(i\cdot\omega\cdot\tau)\cdot d\tau\cdot d\omega$

The value of I is driven by $\zeta(\omega)$ and by the range of ω values. Whenever the $\zeta(\omega)$ and range of $(\omega_1 - \omega_2)$ are adjusted toward the low frequency components then LHI value will represent a contribution of the slow moving RBC's. This kind of RBC's can be found mainly in very small vessels or in the vessels with very strong hemodynamic resistances. When the selected frequencies range from the LHI integral goes toward the higher range of the frequencies, it means that LHI index represents high levels of shear rate and velocities.

Chapter 4

Systemic levels of the inflammatory cytokines predict the infectious complications but not prolonged postoperative ileus after colorectal surgery

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Submitted

ABSTRACT

Aim

Postoperative ileus (POI), a transit cessation of bowel motility, is common after surgery. Previous knowledge from animal studies does indicate that the POI mechanism involves an inflammatory response, which is also activated during postoperative infectious complications. This study aimed to determine whether the selected inflammatory biomarkers might facilitate an early detection of prolonged POI (PPOI) or infectious complications.

Methods

Forty-seven adult patients who underwent oncological colorectal surgery were included. They filled out a perioperative diary to report their gastrointestinal symptoms. Blood samples were collected pre-operatively, and on day 1 and 3 after surgery. Levels of leucocytes, CRP, interleukin (IL)-6, TNF-α, and IL-1β were analyzed.

Results

Patients with PPOI had significantly longer stay in hospital than patients without (13.6±10.5 days versus 7.4±3.2 days, $p<0.001$); they also had higher levels of IL-6 ratios, leucocyte count, and CRP-levels after surgery, but not significant. Similar data were also found in the patients with infectious complications (incidence 48% in total, i.e. surgical site infection 26%, pneumonia 11%, anastomotic leakage 9%, urinary infection 9%), with higher levels of IL-6 ratios and CRP-levels after surgery ($p<0.05$ respectively). The ROC analysis found better diagnostic values of IL-6 ratio on both day 1 and 3 after surgery than that of CRP (POD 1; ROC 0.825, $p<0.001$).

Conclusion

Perioperative changes of the inflammatory cytokines cannot predict PPOI after colorectal surgery. Instead, systematic changes of IL-6 level on postoperative day 1 and 3 may predict the infectious complications with a better diagnostic value compared with leukocyte count and CRP changes.

INTRODUCTION

Surgical resection is still the cornerstone of colorectal cancer treatment. Nevertheless, colorectal surgery is associated with a high morbidity rate of 24-43% [1-3], which significantly compromise a fast recovery after surgery and quality of life after discharge. Infectious complications including surgical site infection and anastomotic leakage are the major causes of postoperative morbidity and mortality [4]. Moreover, many patients also develop postoperative ileus (POI) characterized by a transient impairment of bowel function and reduced motility. In some of them, prolonged POI (PPOI) is diagnosed when POI does not resolve after 5 postoperative days or recurs after an apparent resolution. Such delayed recovery of bowel function leads to other serious outcomes such as longer hospitalization, hospital-acquired infections, pulmonary compromise and a large increase of medical cost as well [5].

Many studies on animal models have revealed that the mechanism of POI includes an inflammatory response caused by the intestinal manipulation and surgical trauma [6-8]. Therefore, inflammatory markers such as interleukin (IL)-1β, IL-6, TNF-α, and CRP have been suggested to be valuable for the early detection of POI. Previous studies reported that the level of IL-1β, IL-6, and TNF-α in PPOI patients were significantly higher at postoperative day 5 in abdominal drain fluid than that in normal recoveries [9, 10]. However, due to the wide application of the ERAS (early recovery after surgery) program, peritoneal drainage is omitted in many colorectal patients anymore. In such cases, measuring systematic levels of the inflammatory cytokines seems to be a promising alternative since it can be easily integrated into postoperative blood tests.

This approach is supported by animal studies, which have revealed that elevation of the inflammatory cytokines is also detectable in the blood in POI models in addition to a localized change [6, 7]. Nevertheless, clinical data to support this are still not yet available. Moreover, it is important to note that the classic pro-inflammatory response is also activated in infectious complications, and increasing levels of the inflammatory cytokines were also reported in these complications [11-16]. Therefore, we collected the perioperative blood samples of the patients who underwent colorectal surgery, trying to determine whether measuring the systemic inflammatory markers may aid to the early detection of PPOI or infectious complications.

METHODS

Study population and design

Adult patients admitted to the Academic Colorectal Cancer Center Havenziekenhuis Rotterdam who underwent oncological colorectal surgery were included after informed consent. In total 50 patients were planned to be included in this prospective cohort during the period of November 2013 and November 2014. In accordance with the Dutch law on medical research in humans, this study was approved by the Medical Ethical Committee of the Erasmus University Medical Center, Rotterdam, the Netherlands (Permit number: MEC-2013-246, NL43053.078.13) and patients gave their written consent after receiving oral and written information.

All patients were asked to fill out a questionnaire before surgery and every day after surgery until postoperative day (POD) 7. The questions refer to their food and fluid intake, bowel movements and defecation, gastrointestinal symptoms and Visual Analogue Scale (VAS) pain score. Data collection included age, gender, body mass index (BMI), American Society of Anesthesiologists (ASA) score, medication use, smoking, operative procedure, postoperative complications including anastomotic leakage, fascia dehiscence, surgical site infection (SSI), urinary tract infection, pneumonia and postoperative course.

The primary goal of this study was to investigate whether the perioperative inflammatory cytokine levels can predict PPOI. Secondarily, we also tried to associate the cytokine levels with the infectious complications.

Selection of variables and definitions

To ensure the objectiveness of our primary endpoint, PPOI was not diagnosed by the participating surgeons but via the retrospective review of the patient diary and medical record. The participating doctors diagnosed the other complications according to the criteria listed in Table 1, which presents the variables and definitions of complications and outcome [17-19].

Blood sample analysis

Peripheral blood was drawn from each patient before surgery (baseline), and on the first and third postoperative day in the morning, together with the routine blood tests. Leucocytes and C-reactive protein (CRP) measurements were part of the standardized care and the outcomes were retrieved from the medical chart. Blood samples were centrifuged and plasma was stored at -80°C into two aliquots. Enzyme-linked immunosorbent assays (ELISAs) were performed according to manufacturer's instructions to quantify the concentrations of systematic inflammatory markers IL-6 and TNF-α (PeproTech Inc., Rocky Hill, USA), and IL-1β (R&D Systems, Minneapolis, MN, USA) in blood plasma.

ERAS (enhanced recovery after surgery) protocol

All patients were treated according to the ERAS protocol. Two hours before surgery patients pre-operatively received a carbon-hydrate loaded drink. In some cases of low anterior resection, an enema was given under prescription of the surgeon. In general, left sided colectomy and (low) anterior resections received bowel preparation with 2 liters of Macrogol 3350 (Klean prep 69 gr, Norgine Ltd, Harefield, United Kingdom). Immediately after surgery, nasogastric tubes were removed and patients are allowed to mobilize or drink fluid food. Normal diet was offered from the first postoperative day and on.

Table 1. The variables and definitions of complication and outcome

Complications/ outcome	Definition
PPOI*	Resolution of POI is defined as passage of feces with good toleration of solid food for at least 24 hours. PPOI is diagnosed if POI is not resolved after postoperative day 5; recurrent POI occurring after an apparent resolution of POI was also defined as PPOI [17, 19].
Anastomotic leakage	Defect of the bowel wall integrity at the anastomotic site. A pelvic abscess close to the anastomosis is also considered as anastomotic leakage. The diagnosed leakage were Grade B or C according to classification of Rahbari et al. [18].
Surgical site infection (SSI)	Erythema requiring initiation of antibiotic treatment or a wound requiring partial or complete opening for drainage of a purulent collection.
Pneumonia	Presentation of clinical symptoms including cough, fever, dyspnoea or consolidation on chest radiography requiring antibiotic treatment with or without a positive sputum culture.
Urinary tract infection	Presents of clinical symptoms e.g. fever, polyuria, and stranguria requiring antibiotic treatment.
Fascia defect	Dehiscence of the abdominal wall with or without the need for reoperation
Reoperation	During hospital stay, within 30 days postoperative or during readmission within 30 days after initial discharge
Length of hospital stay	Day of admission till the day a patient is ready for discharge, this means patient tolerate solid food, had passage of feces, and pain is adequately in control with oral analgesics
Readmission	Admission within 30 days after discharge for more than 24 hours
Mortality	Death occurring during hospital stay or within 30 days postoperative

* Prolonged postoperative ileus

Statistical analysis

The statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, USA, version 21.0 for Windows). Demographic data were presented in n (%), mean (SD) or median (95% confidence interval). Mann-Whitney *U* test, chi-square test, Pearson correlation test, and ROC analysis were employed according to a proper indication. A 2-tailed p value < 0.05 was considered to indicate statistical significance.

Table 2. Baseline and surgical characteristic comparison between the PPOI and no-PPOI patients.

	no-PPOI (%)	PPOI (%)
	n=34	**n=13**
Patient characteristics		
Age (yrs)	67.6 ± 10.4	71.2 ± 11.2
Gender		
Male	21 (62)	6 (46)
Female	13 (39)	7 (54)
BMI (kg/m2)	27.2 ± 4.7	24.7 ± 4.2
ASA score		
I	6 (18)	4 (31)
II	14 (41)	4 (31)
III	9 (26)	1 (8)
IV	0	0
Missing	5 (15)	4 (31)
Cardiac comorbidity	11 (32)	3 (23)
Diabetes Mellitus	6 (18)	1 (8)
Smoker	6 (18)	1 (8)
COPD	7 (21)	1 (8)
Use of statins	12 (36)	3 (23)
Use of antihypertensive	12 (36)	7 (54)
Neoadjuvant radiotherapy	2 (6)	0
Chemo-radiation	4 (12)	1 (8)
Abdominal surgery in history	12 (35)	3 (23)
Operation characteristics		
Type of operation		
Low anterior resection	10 (29)	2 (15)
Sigmoid resection	6 (18)	2 (15)
Hemicolectomy right	9 (26)	8 (62)
Hemicolectomy left	5 (15)	0
Colon transversum resection	1 (3)	1 (8)
APR	3 (9)	0
Approach		
Laparotomy	13 (38)	5 (38)
Laparoscopy	20 (59)	7 (54)
Conversion	1 (3)	1 (8)
Stapled vs. hand sutured#		
Sutured	19 (58)	9 (69)
Stapled	14 (42)	4 (31)

Table 2. Baseline and surgical characteristic comparison between the PPOI and no-PPOI patients. (continued)

	no-PPOI (%)	PPOI (%)
	n=34	**n=13**
Anastomotic configuration*		
Side-end	10 (29)	5 (42)
Side-side	14 (41)	7 (58)
End-end	6 (18)	0
Stoma	11 (32)	2 (13)
Profylactive drainage	4 (12)	1 (8)
Nasogastric tube**	10 (29)	6 (50)

Data are n (%), mean (SD). BMI=Body Mass Index, ASA= American Society of Anesthesiologists classification.
n=33 in no PPOI, n=13 in PPOI group
* n=30 in no PPOI group, n=12 in PPOI group
** n=12 in PPOI group

RESULTS

Between October 2013 and November 2014, 54 patients were included, three patients were excluded because of protocol violations of the inclusion criteria, and four patients retracted the informed consent. In total 47 patients were included for analysis.

PPOI versus no-PPOI

In total 72% (34/47) of the patients recovered from POI within five postoperative days (POD5) and were assigned to the no-PPOI group; 28% (13/47) patients recovered on or after 6 days postoperatively (8/13) or had recurrence of POI (5/13) and were therefore defined as PPOI. Univariate analysis showed a similar baseline and operative characteristics in the patients with or without PPOI (Table 2).

IL-6 levels were detectable in all samples in all three time-points in all patients. However, TNF-α and IL-1β were not detectable in the majority of samples. The detailed proportions of detectable samples are listed in Supplementary Table 1 (S1).

The absolute median values of cytokines of positive samples are presented in Supplementary Table 2 (S2). In the detected samples, we found several samples with substantially higher levels of cytokines compared with other samples, resulting in a large variation in results. We also found that cytokine levels of these patients remained high after surgery. Therefore a cytokine ratio was calculated with the following equation: ratio POD1 (or 3) = (cytokine level on POD1 (or 3))/(cytokine level before surgery) for further

analysis. Cytokine levels and ratios describe the ratio of cytokine levels at postoperative day 1 and 3 divided by the pre-operative cytokine level.

The PPOI group showed higher IL-6 ratios on POD3: 5.90 ± 9.11 than in the no-PPOI group: 2.44 ± 3.84 (Figure 1). Due to a low number of valid values, we found no differences in IL-1β ratio and TNF-α ratio between the two groups.

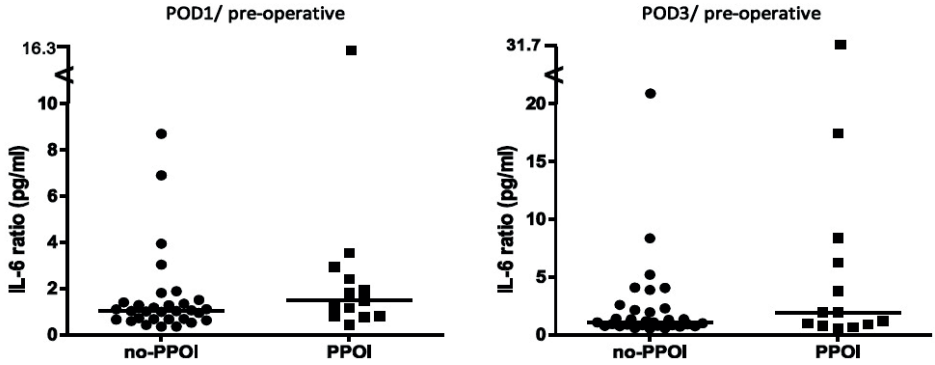

Figure 1A and 1B. IL-6 ratio in normal recoveries (no-PPOI) versus PPOI patients, every single dot represents a patient, the line indicates the median, and there are no significant differences.

Both leucocytes and CRP were higher in the PPOI group, but there were no significantly differences between no-PPOI and PPOI group at any time point (Figure 2). Also a higher postoperative VAS score was seen in the PPOI group, though no statistical difference was observed.

In total 13.0% (6/46) were diagnosed with colorectal anastomotic leakage. In the PPOI group a significantly higher percentage of anastomotic leakage was seen, 38.5% (5/13) versus 3.0% (1/33) in the no-PPOI group, $p = 0.005$. The hospital stay duration was significant longer in PPOI patients 13.6 ± 10.5 versus 7.4 ± 3.2 in the no-PPOI cases, $p < 0.001$ (Figure 2D).

Infectious versus no infectious complication

There were no significant differences between the baseline and surgical characteristic comparison of patients with and without infectious complications (Supplementary Table S3). Different from the PPOI patients, patients with the infectious complications had significantly higher IL-6 ratios and CRP levels on POD1 and POD3 ($p < 0.05$, respectively, Figure 3A), while the leucocytes count, though also higher, was not significantly different from patients without infectious complications, Figure 3C. Further detailed analysis

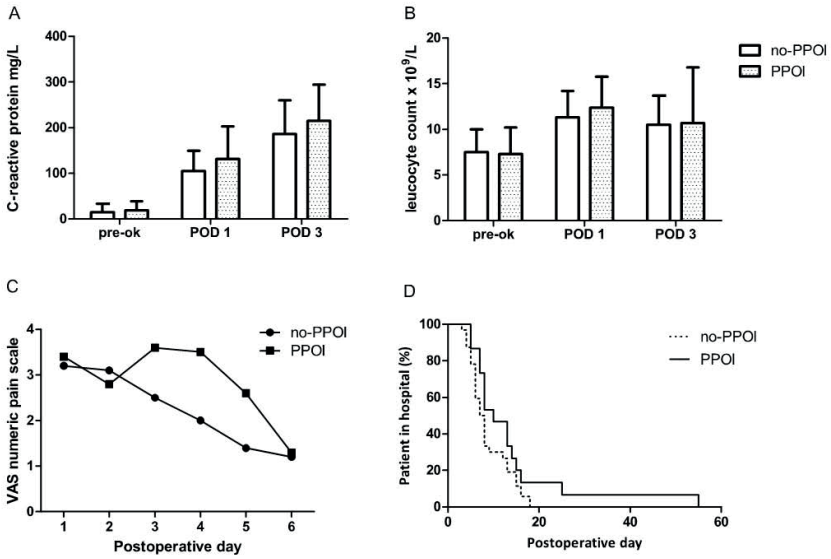

Figure 2. The Leucocyte count, CRP and VAS score in no-PPOI patients vs. PPOI. In 2A and 2B bars represent the mean and error bars the SD. There are no significant differences. Figure 2C presents the VAS (visual analogue scale for pain) score, from postoperative day 1 up to postoperative day 6. Figure 2D presents patients with or without PPOI and the time in days of being ready for discharge. Patients with PPOI had a significantly longer hospital stay $p < 0.001$.

showed that significantly higher levels of IL-6 ratios on POD1 and POD3 were found in SSI and colorectal anastomotic leakage (CAL) patients as is illustrated in Figure 3A, while the differences in CRP were not significant (Figure 3B). No differences were observed in IL-1β and TNF-α ratios between the groups.

We performed the ROC analysis to determine the diagnostic value of CRP and IL-6 ratio in detection of infectious complications. Both on POD1 and POD3, IL-6 ratio had a larger area under curve (AUC) than that of CRP (Figure 4). Further analysis showed that the diagnostic value was achieved on POD1 with a cutting-off point of 1.21 of IL-6 ratio, which yielded a sensitivity of 76% and a specificity of 86%. Although the sensitivity was relatively low (43%), a cutting-off point of 1.93 on POD1 reached a specificity of 100%, meaning all patients with an IL-6 ratio higher than 1.93 on POD1 were diagnosed with infectious complications later on.

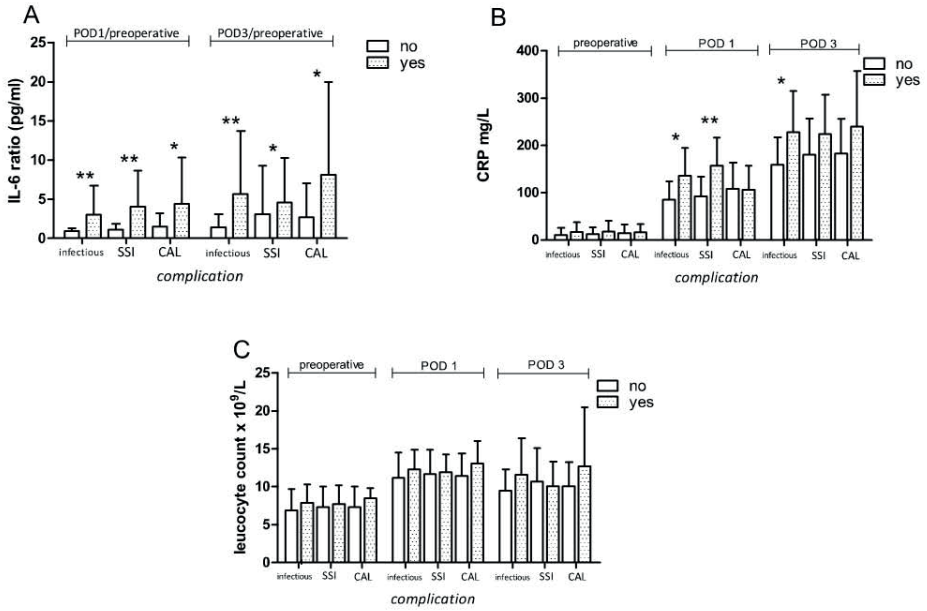

Figure 3. Comparison of IL-6, leucocyte count, and CRP between the patient group with infectious complication(s) (SSI, AL, pneumonia, UWI) and without infectious complication or with or without SSI (surgical site infection) or with or without CAL (colorectal anastomotic leakage). A) shows that all IL6-ratio are significant higher on both time points between all three groups; the infectious group POD1 $p< 0.001$ and POD3 $p=0.001$, SSI; POD1 $p=0.001$ and POD3 $p=0.017$, CAL; POD1 $p= 0.027$ and POD3 $p=0.050$. B) On POD1 and POD3 the CRP levels were significantly higher in the infectious complication groups (POD1 $p=0.009$, POD3 $p=0.008$). In the SSI groups CRP levels were significantly higher in patients with SSI compared to patients without SSI on POD1, $p < 0.001$. Also in the groups with CAL had higher numbers of CRP though not significant. C) Although the leucocyte count are higher in the infectious and CAL groups there were no significant differences. Bars represent the mean, error bars the SD, p-values are indicated with an asterisk; * p-value ≤ 0.05, ** p-value ≤ 0.001.

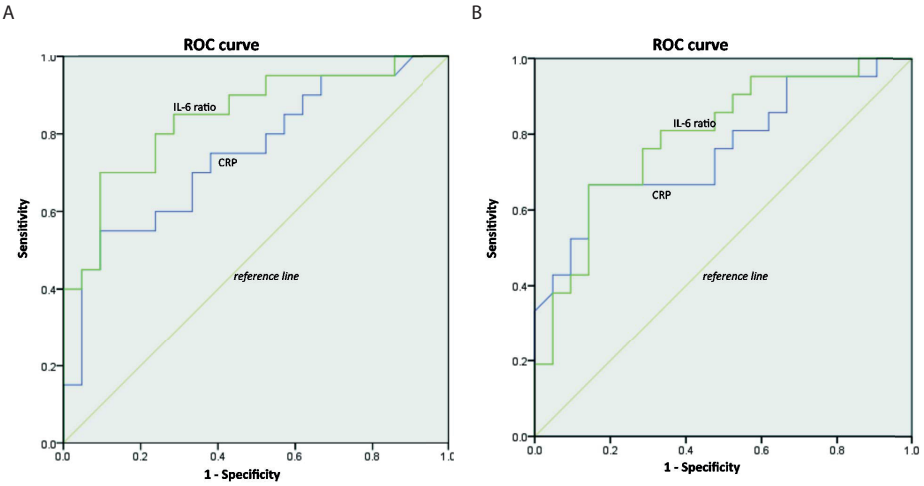

Figure 4. ROC analysis showed CRP and IL-6 ratio on POD1 (A), and POD3 (B), on both days the AUC was higher in for IL-6 ratio.

POD	Biomarker	AUC	SE	p	95% CI	
POD1	CRP	0.732	0.078	0.010	0.579	0.884
	IL-6	0.825	0.067	0.000	0.693	0.956
POD3	CRP	0.731	0.077	0.008	0.580	0.882
	IL-6	0.801	0.068	0.001	0.668	0.934

DISCUSSION

The importance of developing effective strategies to predict and eventually to treat the postoperative complications cannot be overemphasized. In this study, we investigated the association between the inflammatory cytokines and the postoperative complications. We found that systematic changes of IL-6 predicted the infectious complications but not prolonged postoperative ileus after colorectal surgery.

To develop the effective strategies, researchers depend on the translational knowledge from animal studies, which have been continuously contributing to the understanding of POI mechanism. Several experimental studies have reported the important role of IL-6 in the development of POI. Even little manipulation of the bowel induces activity of IL-6, which results in activation of nitric oxide and prostaglandins, and cause migration of leucocytes into the circular muscle of the bowel, and resulted in PPOI eventually [20-23]. However, with fruitful data obtained from animal studies, clinical attempts to predict POI by determining inflammatory mediator levels, the important mechanism in PPOI pathophysiology, remain limited. Zhu et al. found that peritoneal levels of IL-1, IL-6, and pro-calcitonin were higher in PPOI patients [24], indicating localized parameters are sensitive for PPOI prediction. Clinical data also found that IL-6 levels are higher in patients undergoing open surgery when compared with patients undergoing laparoscopic surgery [20], while open procedures had been demonstrated to delay recovery of POI [8].

In this study, we report a prospective cohort investigating the association between the inflammatory cytokines and the postoperative complications. We found that systematic changes of IL-6 predicted infectious complications but did not predict PPOI after colorectal surgery. In contrast to many previous animal studies, our results indicate that systematic cytokine levels yields poor predictive value in PPOI diagnosis. This can be partly explained by inevitable confounding factors (e.g. sex, age, type of surgery, preoperative risk factors etc.) in patient subjects which dilute the influence of POI on systematic inflammatory response [25], while those factors are usually controlled in animal studies.

Nevertheless, we believe that our included patients properly represent the common colorectal patient population. An ideal parameter should be able to identify the high-risk patients. In addition, many animal models used in POI research have very different inflammatory response compared with human [26, 27]. For instance, different from the animal data, our study found that systematic TNF-α and IL-1β levels were extremely low. This was also reported by Ellebæk et al. [28], and our previous meta-analysis, in which we found that in peritoneal samples, IL-6 is already significantly higher in CAL patients on POD1, while elevation of TNF-α and IL-1β, both at much lower concentration, were not observed in the first 3 postoperative days [29].

As is shown in our results, cytokine levels are individually dependent. This has not yet been previously investigated in surgical patients. Previous studies also reported great variation in systematic IL-6 levels [30], therefore we chose ratio instead of absolute levels of cytokines to rule out the individual baseline variations, which resulted in a higher diagnostic value of the infectious complications than CRP in the ROC analysis.

Based on our results, it seems that only in severe complications but not PPOI, the overwhelming inflammatory response can be detected in serum in clinical settings. For those complications, leukocyte count and CRP are commonly used to assist an early diagnosis [31, 32], thus we also included them into our analysis. In accordance to the previous studies, our data also support the value of CRP in the diagnosis of infectious complications. Nevertheless, the ROC analysis further demonstrates that IL-6 yielded better diagnostic value than CRP in predicting the infectious complications. It is important to note that the diagnostic value of IL-6 became evident very early on POD1. All patients had a ratio higher than 1.93 developed infectious complications, indicating the importance of IL-6 evaluation as a promising innovative biomarker for clinical practice.

Although many previous studies exclude patients with other complications from the PPOI group, we still included them to represent a common patient population. This is because it is possible to exclude those patients with complications (e.g. anastomotic leakage) from the POI or PPOI group in a retrospective database. But in a prospective cohort or clinical practice, a surgeon has to differentiate POI or PPOI from other severe complications that require more invasive interventions because many infectious complications first manifest abdominal symptoms before systematic manifestations. This may explain the significantly higher rate of the complications in the PPOI patients in our data.

CONCLUSION

Postoperative ileus remains the most common complication after gastrointestinal surgery, without a satisfactory parameter for its early detection or prediction. In this study, we report a prospective cohort study investigating the association between inflammatory cytokines and postoperative complications. We found that serum IL-6 changes can predict the infectious complications but not prolonged postoperative ileus after colorectal surgery. How to translate knowledge from rodent POI studies to clinical practice is evidently an urgent issue to be addressed. Further exploration of IL-6 seems promising and may assist an early detection of the infectious complications after surgery.

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SUPPLEMENTARY DATA

Table S1. Percentage of positive samples in the no-PPOI and PPOI group.

	no-PPOI			PPOI		
	pre-OK (n=31)	POD1 (n=34)	POD3 (n=34)	pre-OK (n=13)	POD1 (n=13)	POD3 (n=13)
Positive samples (%)						
IL-6	100	100	100	100	100	100
IL-1β	25.8	29.4	32.4	15.4	7.7	15.4
TNF-α	12.9	20.6	35.3	7.7	30.8	46.2

Table S2. Absolute level of cytokine in plasma of the positive samples

		no-PPOI			PPOI		
		pre-OK (n=31)	POD1 (n=34)	POD3 (n=34)	pre-OK (n=13)	POD1 (n=13)	POD3 (n=13)
IL-6	Median	377	418	498	100	126	219
IL-1β	Median	13	14	13	12	15	14
TNF-α	Median	163	40	30	67	78	103

no significantly differences

Table S3. Baseline and surgical characteristic comparison between patients with infectious complications and normal recovery.

	No infectious complication	Infectious complication
	n=24	**n=22**
Patient characteristics		
Age (years)	70.5 ± 10.3	68.8 ± 11.1
Gender		
male	15 (63)	12 (55)
female	9 (37)	10 (45)
BMI (kg/m2)	26.1 ± 5.0	26.9 ± 4.2
ASA score		
I	5 (21)	5 (23)
II	11 (46)	7 (32)
III	5 (21)	5 (23)
IV	0	0
missing	3 (13)	5 (23)
Cardiac comorbidity	10 (42)	4 (18)
Diabetes Mellitus	3 (13)	4 (18)
Smoker	3 (13)	4 (18)
COPD	3 (13)	5 (23)
Use of statins	7 (29)	8 (36)

Table S3. Baseline and surgical characteristic comparison between patients with infectious complications and normal recovery. (continued)

	No infectious complication	Infectious complication
	n=24	**n=22**
Use of antihypertensiva	9 (38)	10 (45)
Neoadjuvant radiotherapy	1 (4)	1 (5)
Chemoradiation	3 (13)	2 (9)
Abdominal surgery in history	9 (38)	6 (27)
Operation characteristics		
Type of operation		
low anterior resection	7 (29)	5 (23)
sigmoid resection	2 (8)	5 (23)
hemicolectomy right	9 (38)	8 (36)
hemicolectomy left	5 (21)	0
colon transversum resection	1 (4)	1 (5)
APR	0	3 (14)
Approach		
laparotomy	7 (29)	10 (45)
laparoscopy	16 (67)	11 (50)
conversion	1 (4)	1 (5)
Stapled vs. hand sutured*		
sutured	17 (71)	10 (48)
stapled	7 (29)	11 (52)
Anastomotic configuration**		
side-end	6 (27)	8 (38)
side-side	13 (59)	8 (38)
end-end	3 (14)	3 (14)
Stoma	6 (25)	7 (32)
Profylactive drainage	1 (4)	4 (18)
Nasogastric tube*	8 (33)	8 (36)

Data are n (%), mean (SD). BMI=Body Mass Index, ASA= American Society of Aneshtesiologists classification.

* n=21 in infectious group

** n=22 in no infectious group and n=21 in infectious group

Chapter 5

Clinical endpoint, early detection, and differential diagnosis of postoperative ileus: a systematic review of the literature

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ABSTRACT

Background

This systematic review summarizes evidence regarding clinical endpoints, early detection, and differential diagnosis of postoperative ileus (POI).

Methods

Using MEDLINE, EMBASE, Cochrane, and Web-of-Science, we identified 2,084 articles. Risk of bias and level of evidence (LOE) of the included articles were determined, and relevant results were summarized.

Results

Eleven articles were included, most of which with substantial risks of bias. Bowel motility studies revealed that defecation together with solid food tolerance is the most representative clinical endpoint of POI (LOE: 2b); other clinical signs (e.g. bowel sounds, passage of flatus) did not correlate with a full recovery of bowel motility. Inflammatory parameters including interleukin (IL)-6, IL-1, and TNF-α might assist in an early detection of prolonged POI (LOE: 4). Clinical manifestations (e.g. nausea, vomiting, abdominal distension, bowel sounds, flatus) and X-ray examinations provided limited aid to the differential diagnosis of POI, while CT with Gastrografin had the best specificity and sensitivity (both 100%; LOE: 1c).

Conclusions

Postoperative defecation together with tolerance of solid food intake seems to be the best clinical endpoint of POI. CT has the best differential diagnostic value between POI and other complications. Prospective studies with a high LOE are in great need.

INTRODUCTION

Postoperative ileus (POI) is a transient cessation of bowel motility after abdominal surgery. It is generally considered as a self-limiting process, which recovers within 3–5 days after operation [1–3]. However, postoperative recovery of bowel function often extends beyond the expected duration and may cause other serious adverse outcomes such as hospital acquired infections, pulmonary complications, as well as an increase in medical costs [4–6].

Although various clinical manifestations, including the absence of bowel sounds, flatus, and defecation, or intolerance of an oral diet, have been used to indicate postoperative bowel function, substantial variations exist in the definition and clinical endpoints of POI among different studies [7]. To surmount that, Delaney et al. [8] published a clinical consensus update on the definition of POI in 2006, in which they defined POI as 'the time from surgical intervention until passage of flatus or stool and until initiation of adequate oral intake that is tolerated and maintains hydration during 24 h'. However, the application of this consensus was not satisfactory. According to a recently published review, less than 20% (6/31) of new trials confirmed the definition provided by the consensus [7]. Choosing wrong clinical endpoints may substantially influence clinical practice and investigations. We noticed that most of the above-mentioned POI endpoints are lacking evidence support, and in many cases these endpoints failed to detect an earlier recovery of bowel function in laparoscopic surgery than in open surgery, while in contrast such difference has been constantly reported with bowel motility measurements [3, 9, 10]. It is thus questionable if these endpoints are able to represent a full resolution of POI.

Another important issue is that POI is usually diagnosed based on clinical symptoms and signs such as nausea, vomiting, abdominal distension, and lacking of bowel sounds, flatus, and defecation [11, 12]. However, these manifestations are also common in postoperative small bowel obstruction, and may be seen in major surgical complications such as internal herniation, adhesions, anastomotic leakage, or intraperitoneal bleeding [13–18]. Many of these complications require immediate intervention; otherwise they may cause direct morbidity and mortality [19]. Uncomplicated POI, on the other hand, needs supportive care in most cases. Therefore, an early and accurate diagnosis of POI differentiating it from other complications becomes a crucial first step before further treatments. Unfortunately, evidence-based suggestions regarding how to establish an early and accurate diagnosis of POI are limited.

To give an overview of the current evidence regarding the clinical endpoints, early detection, and differential diagnosis of POI, we conducted this systematic review. Relevant

studies were included and summarized, indicating their risk of bias and level of evidence (LOE), and evidence-based suggestions were also provided accordingly.

METHODS

Literature search strategy

The literature search for this systematic review was performed in August 2013 according to the PRISMA (Preferred Items for Reporting of Systematic Reviews and Meta-Analyses) guidelines in the databases including MEDLINE, EMBASE, Cochrane, and Web-of-Science libraries. There were no restrictions used during the search related to the publication year, publication language and type of study. The following search strategy was used in EMBASE and was modified accordingly for other databases:

('postoperative ileus'/de OR (ileus/de AND 'postoperative complication'/de) OR ((post-operative OR 'post operative' OR postsurg * OR 'post surgical' OR 'post surgery') NEAR/3 (ileus OR paraly * OR motilit * OR obstruct *)):ab,ti) AND (diagnosis/exp OR (diagnos * OR detect *):ab,ti) AND ('sensitivity and specificity'/exp OR 'prediction and forecasting'/exp OR monitoring/exp OR (sensitive * OR specific * OR predict * OR monitor * OR efficac * OR evaluat *):ab,ti) NOT ([animals]/lim NOT [humans]/lim).

Study Selection

The study selection criteria are shown in Figure 1. Two researchers (Z.W. and G.S.A.B.) screened the titles and abstracts of the identified articles independently for relevance to the subject. Only English articles targeting the clinical endpoints, early detection, and differential diagnosis of POI were included. For studies regarding risk factors of POI, a predictive analysis was required for inclusion. Reviews, Letters to the Editor, and Congress Abstracts were excluded. Afterwards, the references of these selected articles were screened for relevant articles.

Quality assessment and data extraction

The revised Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) tool [20] was used to assess the study quality by 2 independent authors. The tool assesses the risk of bias and applicability concerns by means of four key domains including patient selection, index test, reference standard, and flow and timing. After that, the LOE of each included article was also evaluated according to the Centre for Evidence-Based Medicine (2011).

All the included articles were divided into three different categories: clinical endpoints, early detection, and differential diagnosis. The following data were extracted from the included articles: first author, year of publication, study design, number of patients, surgery type, POI definition/diagnosis, research subjective/ tool, and results/outcomes.

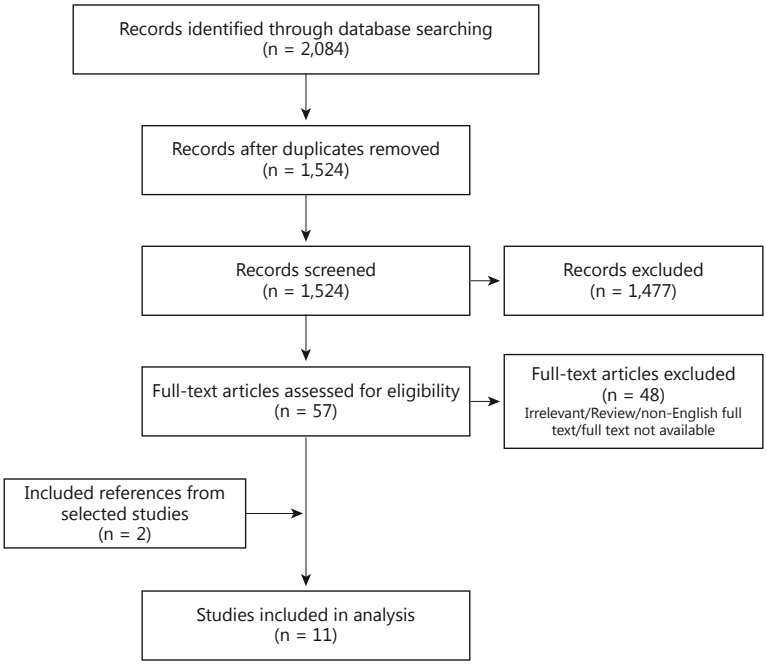

Figure 1. Study selection for relevant articles

RESULTS

Eleven articles were included in this systematic review. These articles were divided into the three categories clinical endpoints, early detection, differential diagnosis, and some of them were analyzed in more than one category.

The quality of each included study was evaluated according to the revised QUADAS-2 tool [20] and is shown in Table 1. Most included studies have substantial risks of bias, but the applicability concerns of the patient selection and index test are comparably limited. Due to a very small inclusion number in each category, the risk of bias analysis across the studies was not applicable.

Table 1. Risk of bias evaluation based on Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2)

First author [ref.]	Risk of Bias				Applicability concerns		
	patient selection	index test	reference standard	flow and timing	patient selection	index test	reference standard
Quan [17]	-	-	-	-	-	+	-
Coletti [13]	-	-	-	-	+	+	-
Zer [21]	-	-	-	-	-	+	-
Quatromoni [14]	-	-	-	-	-	+	-
Waldhausen [2]	+	+	-	?	+	+	-
Frager [25]	+	+	-	-	+	+	-
Heinberg [19]	+	-	-	-	+	+	-
Kronberg [24]	-	+	-	+	-	+	-
Van Bree [16]	+	?	+	-	+	+	+
Zhu [22, 23]	-	+	-	+	+	+	-

+ = Low risk; – = high risk.

Different terminologies of POI were found in the included studies (Table 2). In the early studies, the terminology of 'postoperative paralytic ileus' was often used [13, 14, 21]. In the 90s, the nomenclature 'postoperative ileus' (POI) started to be used in addition to 'paralytic ileus' [2], and both of them have been found in recent studies. In this review, we chose to use postoperative ileus (POI). More than half of the included studies provided a definition of POI. Many of them defined it as the inhibition of gastrointestinal motility after surgery [16, 22, 23]. Other studies used clinical signs to characterize POI [2, 19, 24].

The diagnosis of POI was made according to clinical symptoms and signs in all included articles. Substantial differences, however, were found in diagnostic details such as timing and clinical endpoints (Table 2). Radiological examinations were mainly used for differential diagnosis [13, 14, 17, 19, 21–23, 25], and bowel motility examinations were only used for investigational purpose in the included studies [2, 16].

Clinical endpoints

Only two articles were included in the group of clinical endpoints (Table 3) [2, 16]. Van Bree et al. [16] performed a prospective cohort study (LOE: 2b), in which they compared clinical endpoints of POI and analyzed their correlations with colon transit time. The best correlation was found in the time to tolerance of solid food and passing stool (area under curve = 0.9). When the authors retrospectively analyzed their previous database, this new endpoint was also able to detect an earlier recovery of bowel function in laparoscopic than in open surgery, which was not found by using traditional indicators (passage of flatus, passage of feces, or tolerance of food intake) [16].

Table 2. Terminology and clinical diagnosis of POI

First author [ref.]	Year	Patients: n	Terminology	Diagnostic tools	Clinical diagnostic criteria
Quan [17]	1961	60	Postoperative intestinal ileus	Clinical signs + X-ray	Nausea, vomiting, abdominal discomfort or severe pain and distention
Coletti [13]	1964	90	Paralytic ileus	Clinical signs + X-ray	Fever, vomiting, abdominal distention, peristaltic sounds, bowel movement
Zer [21]	1977	41	Paralytic ileus	Clinical signs + X-ray (with Gastrografin)	Not specified
Quatromoni [14]	1980	41	Paralytic ileus	Clinical signs + X-ray	Abdominal distention, intestinal colic, bowel sounds, or absence of flatus or stool
Waldhausen [2]	1990	44	POI	Clinical signs + motility test	Nausea, bloating, and cramps, all of which resolved by postoperative day 7 or earlier
Frager [25]	1995	36	POI, paralytic ileus	Clinical signs + CT scan	Abdominal pain and distension, nausea, vomiting, and diminished bowel peristalsis within 10 days of laparotomy
Heinberg [19]	2003	84	POI, paralytic ileus	Clinical signs + X-ray	Abdominal pain and distension, nausea, vomiting, absent flatus, absent bowel sounds, fever, and leukocytosis
Kronberg [24]	2011	413	POI	Clinical signs	Absence of bowel function for ≥5 days or need for nasogastric tube reinsertion after starting oral diet in the absence of mechanical obstruction
van Bree [16]	2013	84	POI, paralytic ileus	Clinical signs + motility test	Nausea, vomiting, bloating, delayed passage of flatus and stool, and inability to tolerate solid food
Zhu [22, 23]	2013	100	POI, prolonged POI	Clinical signs + radiography	Prolonged POI: ileus that persists >6 days following surgery with evident clinical symptoms and radiological imaging which must be diagnosed at an early stage for immediate treatment

Van Bree et al. [16] also reported that bowel sounds were recorded in most paralytic ileus patients (6/7) during the first 3 days after surgery. Similar results were also reported by Waldhausen et al. [2], who found that the recovery of normal colon discrete and continuous electric response activity took an average of 5.9 days after open surgery, which was much longer than the recovery of bowel sounds (2.4 days) but similar to passage of flatus and stool (5.1 days; LOE: 3b –). However, this similarity was lacking correlation analysis, which substantially weakened its reliability.

Table 3. Clinical endpoints of POI

First author [ref.]	Year	Patientsn	Study type	LOE	Surgery type	Diagnostic tests	Subject/tool	Signs correlated with motility measurement	Signs not correlated with motility measurement
van Bree [16]	2013	84	Prospective Cohort	2b	Laparoscopic or open colectomy	Scintigraphy	Scintigraphic recordings of gastrointestinal transit	Tolerance of solid food plus first defecation; positive predictive value of 93% on day 3	Flatus, bowel sounds
Waldhausen [2]	1990	44	Prospective cohort	3b	Open surgery	Myoelectric activity	28-gauge stainless steel bipolar recording electrodes	Pass flatus or stool	Bowel sounds

Table 4. Early detection of POI

First author [ref.]	Year	Patients n	Study type	LOE	Surgery type	Study tools	Useful tools	Results (useful tools)	Non-useful tools	Results (nonuseful tools)
Kronberg [24]	2011	413	RR	2b	Laparoscopic partial colectomy	Analysis of perioperative risk factors	POI score	Score (POI rate): 0 (2.7%); 1 (9.4%); 2 or 3 (18.3%).	-	-
Zhu [22, 23]	2013	100	PC	4	Open resection of sigmoid or rectum	Laboratory tests and data analysis	IL-1β, IL-6, TNF-α and procalcitonin levels on day 5	Significantly higher in patients with prolonged POI	-	-
van Bree [16]	2013	84	PC	2b*	Laparoscopic or open colectomy	Scintigraphy and data analysis	Reinsertion of nasogastric tube within 3 days	100% in paralytic ileus patients vs. 6.5% in patients without paralytic ileus	Bowel sounds	85.7% in paralytic patients within 3 days
Heinberg [19]	2003	84	RR	3b*	Intraabdominal gynecologic surgery	Radiography and clinical findings	Patient selection for plain films	Longer recovery to regular diet	Single clinical finding	Not correlated with either the decision to obtain films or final X-ray diagnosis
							ASA score	Predicted a radiographic diagnosis of ileus ($p = 0.002$)	-	-

* Not primary data.

RR = retrospective review; PC = prospective cohort

Early detection of POI

Five studies [16, 19, 22–24] were included in this category, and the synopsis of the results is listed in Table 4.

Preoperative prediction

Although many studies on POI risk factors were found in our analysis, most of them were excluded because the predictive value of the risk factors was not evaluated. Only one study by Kronberg et al. [24] analyzed the predictive value of 3 independent risk factors including age ≥ 60 years, preoperative narcotic use, and previous abdominal operation. They found that 18.3% of patients with 2 or 3 risk factors developed prolonged POI, which was significantly higher than the rate of 2.7% in patients without risk factors. This study had a LOE of 2b, but the researchers excluded patients with other severe complications in their data analysis, and thus had a high risk of bias in patient selection. Another study from Heinberg et al. [19] retrospectively reviewed 84 patients who underwent gynecological surgery. They reported that a higher American Society of Anaesthesia (ASA) score was related to a radiographic diagnosis of POI.

Clinical symptoms and signs

Two studies focusing on clinical endpoints and the radiological differential diagnosis of POI also reported predictive values of clinical symptoms and signs in the early detection of POI [16, 19]. Van Bree et al. [16] reported that nasogastric tube reinsertion due to distension was required in all paralytic ileus patients (7/7) during the first 3 days, while only few of normal gastrointestinal transit patients (5/77) required it on day 3. Heinberg et al. [19] found that patients selected for plain films had longer time to resumption of clear liquids and regular diet; however, no single clinical manifestation had a significant correlation with the decision to obtain X-ray films.

Laboratory tests

Two recent studies by Zhu et al. [22, 23] evaluated the levels of C-reactive protein, pro-calcitonin, interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha (TNF-α) for the prediction of prolonged POI. Due to a case-control design, both studies had a LOE of 4 and a high risk in patient selection. They found that IL-1β, IL-6, and pro-calcitonin levels in peritoneal exudate on postoperative day 5 were significantly higher in 8 patients (8/100) who developed prolonged POI. The levels of C-reactive protein and TNF-α were irregular from day 1 to 5 but not significantly different between the case and control groups [22, 23].

Differential diagnosis of POI

Six articles were included for a differential diagnosis of POI (Table 5) [13, 14, 17, 19, 21, 25]. Except for the studies by Frager et al. [25] and Heinberg et al. [19], other studies regarding differential diagnosis all contained substantial risks of bias. Most of them had a case-control or case-series design, and thus only had a LOE of 4 [13, 14, 17, 21].

Clinical Manifestations

Quan and Stearns [17] reported that 2 patients (2/27) who were diagnosed with uncomplicated POI actually died of peritonitis or anastomotic leakage, which was confirmed by postmortem examinations. They also found that elevations of temperature, pulse, respiration rate, or white blood count did not give much additional information for the differentiation between POI and other complications [17]. Postoperative gastrointestinal symptoms such as nausea, vomiting, and abdominal distension were reported to provide limited information for differential diagnosis [13]. Even obstructive bowel sounds, a typical sign of mechanical obstruction, had little diagnostic value, as it only occurred in 39% (16/41) of patients with postoperative mechanical obstruction [14]. Among all clinical manifestations, only cramp like abdominal pain seemed to be an adequate symptom for mechanical obstruction according to the study by Coletti et al. [13] , in which they found no patients with paralytic ileus complaining of cramp-like pain, while most patients (34/45) with mechanical obstruction felt such pain.

Radiology

A series of studies showed that X-ray examinations were often inconclusive. It provided a low rate of diagnosis (42.9% in the study by Heinberg et al. [19]), and the results were also not consistent with clinical impressions of POI, varying from 28.5% (4/14) [17] to 44.4% (20/45) [13]. Such variation also existed between different radiologists. In the study by Quatromoni et al. [14], the plain film of 26 patients who were initially diagnosed with mechanical obstruction were submitted to a senior radiologist, who was blinded to clinical signs or patient outcomes and was invited to retrospectively review the films again. Half of the patients were differently diagnosed with paralytic ileus, of which 2 patients had a definite mechanical obstruction, which was confirmed at reoperation. Different from the previous studies, Zer et al. [21] reported that the use of Gastrografin provided a reliable diagnosis in 68.3% of controversial cases (28/41) when clinical signs and X-ray were inconclusive. When distinguishing between mechanical small-bowel obstruction and POI, the highest accuracy was found with regard to CT. Frager et al. [25] compared the value of CT with that of X-ray and clinical findings to distinguish postoperatively between complete or partial small-bowel obstruction and paralytic ileus in 36 patients. The laparotomy findings (20 patients), clinical follow-up (13 patients), and contrast studies (3 patients) were used as the reference standard to evaluate the diagnostic value of

Table 5. Differential diagnosis of POI

First author [ref.]	Year	Patients n	Study type	LOE	Surgery type	Study tools	Useful tools	Results (useful tools)	Non-useful tools	Results (nonuseful tools)
Frager [25]	1995	36	PC	1c	Laparotomy	CT scan with oral contrast	CT scan with oral contrast	Sensitivity (100%) and specificity (100%)	X-ray	Sensitivity 13 – 19% small-bowel obstruction (but specificity 100%)
Heinberg [19]	2003	84	RR	3b	Intraabdominal gynecologic surgery	Radiography and clinical findings	-	-	X-ray	Only 42.9% (24/56) with diagnosis of paralytic ileus or small-bowel obstruction
Quan [17]	1961	60	RR	4	Open colorectal surgery	Radiography and clinical findings	Pain	None (0/27) of the ileus patients complained of pain	Temperature, pulse, respiration rate, and white blood count	Similar among patients with different complications
									X-ray	Only 28.5% (4/14) of patients clinically diagnosed with paralytic ileus had the same X-ray diagnosis
Coletti [13]	1964	90	RR	4	Open surgery	Radiography and clinical indings	Crampy pain	None (0/45) of paralytic ileus patientsvs. 75.6% (34/45) of mechanical obstruction patients		Similar between mechanical obstruction and paralytic ileus patients
									X-ray	Only 44.4% (20/45) of patients with paralytic ileus were supported with X-ray
Zer [21]	1977	41	RR	4	Open surgery	X-ray with Gastrografin	X-ray with Gastrografin			
Quatromoni [14]	1980	41	RR	4	Open surgery	Radiography and clinical findings	-	-	Obstructive bowel sounds	39% (16/41) of postoperative mechanical obstruction patients
									X-ray	50% (13/26) of cases had different diagnosis between radiologists

RR = retrospective review; PC = prospective cohort

CT and X-ray, showing that CT was very sensitive (100%) and specific (100%). Due to a 100% sensitivity and specificity, its LOE was determined as 1c.

DISCUSSION

POI is the most common complication after abdominal surgery. Substantial heterogeneity exists in the diagnosis of POI, and evidence-based recommendations regarding how to establish an early and accurate diagnosis of POI are still limited. This systematic review summarizes the limited evidence from previous studies, indicating their risk of bias and LOE, and it provides evidence-based recommendations regarding the early detection, clinical endpoints, and differential diagnosis of POI.

Various clinical endpoints of POI have been used not only in studies regarding POI diagnosis (our included studies) but also in studies targeting POI treatments [7]. The traditional endpoints of POI (e.g. occurrence of flatus or stool), though easy to determine, were often not able to present differences in bowel function detected by bowel motility measurements [3, 9, 10]. As is shown in this review, only van Bree et al. [16] performed a statistical analysis to evaluate the predictive value of those indicators and provided convincing evidence (LOE: 2b) in their study on bowel motility. Their methodology was the best one among our included studies. In their retrospective analysis, the proposed endpoint showed better sensitivity and predictive value than the traditional endpoints. These data support future applications of their proposed endpoint.

An inflammatory response caused by surgical procedures is considered as the main pathophysiology of POI [12, 26, 27]. Substantial experimental studies demonstrated that POI severity was correlated with levels of inflammatory cytokines such as TNF-α, IL-1, and IL-6, and the activation level of inflammatory cells (e.g. macrophages, mast cells) [27–34]. A pilot study from The et al. [35] revealed promising results in the prevention of POI with mast cell stabilization treatment. These data imply the possibility of predicting POI by evaluating these immune mediators, but only Zhu et al. [22, 23] tested it in clinical practice. With a low LOE, their studies suggested a clinical value of IL-1, IL-6, and pro-calcitonin tests in detecting prolonged POI. Nevertheless, it is necessary to notice that due to a case-control design, these studies contain a potential risk of overestimation of the test sensitivity. These inflammatory cytokines will also increase in other inflammatory or infectious complications such as anastomotic leakage and peritonitis [36], and an increased pro-calcitonin level was also found in patients with ischemic bowel obstruction [37]. An abnormal level of these cytokines may have more importance as a general alert for postoperative complications, warning surgeons to take

necessary interventions. To reach a higher reliability and LOE, further research searching a biomarker for POI or prolonged POI should include better statistical analysis methods (e.g. regression analysis) and avoid a case-control design. Using continuous variables as an endpoint (e.g. passage of feces and tolerance of a diet) may assist a better analysis.

Data from large-scale retrospective studies revealed many risk factors of POI including male sex, high ASA score, respiratory disease, and stoma construction [38, 39]. Unfortunately, it remains unclear from the literature whether those identified risk factors entail a satisfactory predictive value that allows a prospective application for predicting POI, and more importantly, whether patients with these risk factors require different interventions for the purpose of preventing POI. Based on our analysis, only Kronberg et al. [24] analyzed their data with regard to the former question and introduced a scoring system for the preoperative evaluation of POI. However, the applicability of a retrospective analysis might greatly vary in different centers, depending on patients' characteristics. For instance, none of the three independent risk factors in this scoring system reached significance in the analyses from other databases [38–40]. Different centers, therefore, need to consider or even reevaluate the applicability of the predictive scoring systems from other databases. Of course, the scoring system of Kronberg et al. [24] seems more useful in excluding patients without risk factors of POI rather than selecting high-risk patients based on their results. Such new perspectives may also assist surgeons to develop individualized approaches for different patients.

The importance of the correct diagnosis of POI prior to further interventions cannot be overemphasized, since surgical management absolutely depends on its cause. After abdominal operation, bowel dismotility occurs in all patients with or without other complications. Current understanding of POI has increased the tendency to attribute a failed recovery of bowel function to POI, or even uncomplicated POI, while many patients with POI manifestations may suffer from other severe complications [14–17]. Given that a delayed diagnosis of anastomotic leakage, small-bowel obstruction, and peritonitis may cause direct mortality and morbidity, we suggest that POI, especially prolonged POI, should be considered as a diagnosis by exclusion, and thus can only be made when necessary examinations and tests are done to exclude other complications. Our review shows that CT with Gastrografin provided the most reliable differential diagnosis between POI and mechanical obstruction (LOE: 1c) [25]. Our previous systematic review regarding the detection of anastomotic leakage also demonstrated it to be a reliable diagnostic tool [41], which may assist the differential diagnosis between POI and septic ileus, due to anastomotic leakage by radiological manifestations such as the existence of extraluminal contrast, perianastomotic air, and fluid collections in the CT scan as

adjuvants to the clinical manifestations and laboratory tests [42, 43]. These integrated results suggest that CT scan should be recommended when suspicion is aroused.

To find a good timing for postoperative CT, seeking clinical manifestations or biomarkers might be of assistance. Unfortunately, our review found that among the well-recognized bowel obstruction manifestations, only cramp-like pain remained to have differential diagnostic value with high specificity [13]; no biomarker has good specificity for a differential diagnosis of POI from other inflammatory complications [18]. Thus, the surgeons' awareness remains one of the most important values to identify patients who are suspected to have postoperative complications with the currently available evidence.

In conclusion, currently available evidence regarding the clinical endpoints, early detection, and differential diagnosis of POI is limited with substantial risks of bias and low LOE. Based on the currently available evidence, postoperative defecation together with tolerance of solid food intake seems to be the best clinical endpoint of POI. CT has the best differential diagnostic value between POI and other complications. More prospective studies with high LOE are in great need.

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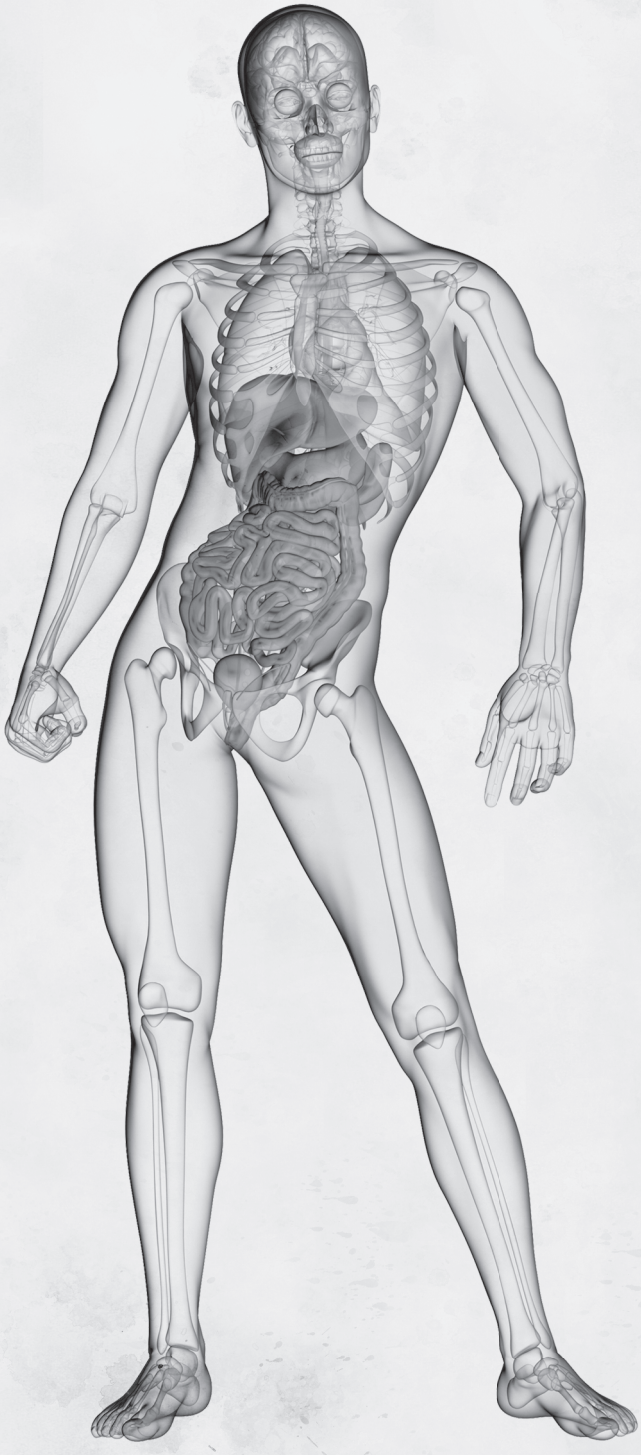

PART 2

Surgical complications: prevention

Chapter 6

Nicotine gum chewing: a novel strategy to shorten duration of postoperative ileus via vagus nerve activation

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ABSTRACT

Rationale

Postoperative ileus (POI) is a transit cessation of bowel motility after surgery. Substantial evidences suggest that gum chewing accelerate the recovery of bowel motility after surgery. Perioperative nicotine administration reduces postoperative opioid use and prevents postoperative nausea and vomiting. Nicotine gum chewing combines stimulation of the cephalic-vagal reflex by gum chewing, and activation of the cholinergic anti-inflammatory pathway by nicotine administration. We therefore hypothesized that nicotine gum chewing reduces POI and improves patient outcomes such as shortening the length of hospitalization as well as saving medical costs. As nicotine gum is commercially available, inexpensive, and has been in use for many years without any severe side effects, it may have a wide clinical application in POI prevention.

INTRODUCTION

Postoperative ileus (POI) is a common complication after abdominal surgery. It is a transit cessation of bowel mobility after surgery. Clinical manifestation of POI include nausea, vomiting, abdominal distension, and lack of flatus and defecation [1]. Delayed recovery of bowel function leads to other serious morbidity such as pulmonary complications, hospital-acquired infections, and longer hospitalization, and it also lead to higher medical cost [2]. The economic burden of POI in the USA health care system is estimated to surpass $1.5 billion per year [3].

Numerous risk factors such as previous surgery, general anesthesia and postoperative opioid consumption have been reported, contributing to prolonged bowel dismotility [4]. Targeting on them, multimodal fast-track perioperative care programs such as Enhanced Recovery After Surgery (ERAS) including adequate pain relief, minimal invasive surgery, and early enteral nutrition, are now being implemented with promising results. Previous research revealed that surgical procedures trigger two different phases of POI: an early neurogenic phase and a late inflammatory phase. The latter one is considered to be a more clinically relevant cause of gastrointestinal dismotility [4], but efficient strategies targeting it remains unavailable in clinical practice. In experimental studies anti-inflammation therapy with stimulation of the vagus nerve showed promising results in preventing POI. Activation the vagus nerve increases bowel motility by controlling inflammatory cell recruitment via the cholinergic anti-inflammatory pathway [1,4,5]. With the promising experimental results, different strategies of vagus nerve stimulation (e.g. physiological, pharmacological, and electrical stimulation) are now being attempted in human patients.

THE HYPOTHESIS

Nicotine chewing gum may have double effect on stimulating the vagus nerve via physiological pathways by chewing, and via pharmacological pathways by the nicotine administration. Although no direct evidence is available to date, accumulative data from clinical and experimental studies are in favor of the hypothesis: nicotine gum chewing reduces the time of POI and improve patient's outcomes via vagus nerve activation. We evaluated the current evidence supporting this hypothesis in the following parts.

EVALUATION OF THE HYPOTHESIS

Part I. Influence of gum chewing in preventing POI

Gum chewing is a form of sham feeding, which mimics the cephalic phase of digestion, stimulates the gastrointestinal motility via the vagal pathways [6]. In addition to the vagal pathway, ingesting contents of maxitols in sugar-free chewing gum may also accelerate the intestinal transit [7]. Since Asao et al. first demonstrated that gum chewing stimulated bowel motility and aided early recovery from POI in surgical patients [8], many randomized clinical trials have reported similar results [2,9–12]. In general, consensus has been reached that gum chewing reduces the duration of POI in abdominal surgery in the recent decade [13]. The postoperative gum chewing is supported by many systemic reviews and meta-analysis [6,14–17], which reported that chewing gum could shorten both POI duration and postoperative hospitalization for approximately one day [18]. Importantly, gum chewing is not only beneficial in POI prevention after gastrointestinal surgery. It has shown satisfactory results in the prevention of POI in other types of intra-abdominal surgery (e.g. gynecological operations) as well [19,20].

Part II. Influence of perioperative administration of nicotine

Nicotine, a selective cholinergic agonist, is an essential regulator of the cholinergic anti-inflammatory pathway [21]. In animal models, nicotine has been shown to improve survival rates of sepsis by stimulating a7 nicotinic acetylcholine receptor (nAchR) [22]. Experimental studies have also shown the effect of specific a7 receptor agonist that ameliorates POI in rats [5], while other nAchR also play important roles mediating the cholinergic anti-inflammatory pathway [5,23].

Despite the nicotine replacement therapy (NRT), nicotine administration has also been investigated for other medical indications, and many of them provide us indirect evidence of its possible effect on preventing POI. Perioperative administration of nicotine (e.g. nicotine patch) has been shown to reduce postoperative nausea and vomiting [24–26], which are common clinical manifestations of POI. Prevention of those symptoms may significantly accelerate the recovery of bowel function after surgery. Preoperative nicotine administration also reduces postoperative opioid consumption [27–30], while reducing opioid consumption is an important strategy of shortening POI [1,4,31]. This effect might be more evident to smoker patients because they may require more opioid compared to nonsmoker patients after surgery [32– 34]. Given that approximately 25–40% of colorectal patients are smokers [35,36], respective recommendations of nicotine gum chewing (i.e. dosage, frequency) should be considered between smoker and non-smoker patients due to their different requirements in postoperative analgesia and varied responses to nicotine administration.

It is important to notice that postoperative nausea and vomiting are strongly influenced by opioid administration in a dose dependent pattern [37]. Also, the a7 nicotinic receptor seems to mediate the nicotine-induced analgesia [38], so its activation by nicotine administration may benefit the control of both postoperative pain and POI.

One main concern of perioperative nicotine use is its potential systemic influence or intoxication [39], especially in non-smoker patients. However, no obvious adverse events (e.g. cardiovascular events) were reported in the studies included in Part II, many of which only included non-smoker patients. There was no significant difference between the groups in postoperative heart rate, arterial blood pressure, respiratory rate, or oxygen saturation after temporal nicotine administration [28,29]. The effective nicotine dosage in those studies were lower than that in NRT, let alone the dosage in cigarette smoking [40]. However, localized side effects such as dislike the taste, irritation of the tongue, mouth, and throat, and occasional nausea caused by nicotine gum chewing should be expected [41]. But the incidence of these side effects is now much lower than it was with the earlier forms of the gum.

CONSEQUENCES OF THE HYPOTHESIS AND CONCLUSION

Substantial evidence suggests gum chewing accelerates the recovery of bowel motility after surgery. Nicotine gum chewing combines the stimulation of the cephalic-vagal reflex by gum chewing, and the activation of the cholinergic anti-inflammatory pathway by nicotine administration, it might be beneficial to the prevention of POI. As nicotine gum is commercially available, inexpensive, and has been in use for many years without any severe side effects, it may have a wide clinical application in POI prevention. Nicotine gum chewing may reduce POI via activating the vagal pathway and result in better patient outcomes such as shortening the length of hospitalization as well as saving medical costs.

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Chapter 7

Hyperbaric oxygen therapy improves colorectal anastomotic healing

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ABSTRACT

Purpose

Hyperbaric oxygen treatment (HBOT) has been found to improve the healing of poorly oxygenated tissues. This study aimed to investigate the influence of HBOT on the healing in ischemic colorectal anastomosis.

Methods

Forty Wistar rats were randomly divided into a treatment group that received HBOT for 10 consecutive days (7 days before and 3 days after surgery), or in a control group, which did not receive the therapy. Colectomy with an ischemic anastomosis was performed in all rats. In each group, the rats were followed for 3 or 7 days after surgery to determine the influence of HBOT on anastomotic healing.

Results

Five rats from each group died during follow up. No anastomotic dehiscence was seen in the HBOT group, compared to 37.5% and 28.6% dehiscence in the control group on postoperative day (POD)-3 and 7 respectively. The HBOT group had a significantly higher bursting pressure (130.9 ± 17.0 mmHg) than the control group (88.4 ± 46.7 mmHg; $p = 0.03$) on POD3. On POD3 and POD7 the adhesion severity was significantly higher in the control groups than in the HBOT groups ($p < 0.005$). Kidney function (creatinine level) of the HBOT group was significantly better than of the control group on POD7 ($p = 0.001$). Interestingly, a significantly higher number of CD206+ cells (marker for type 2 macrophages) was observed in the HBOT group at the anastomotic area on POD3.

Conclusion

Hyperbaric oxygen enhanced the healing of ischemic anastomoses in rats and improved the postoperative kidney function.

INTRODUCTION

Colorectal anastomotic leakage (CAL) is the most serious complication following colorectal surgery, causing substantial morbidity and mortality as high as 33% [1]. With continuous improvements in surgical techniques and perioperative care, the incidence of this complication still varies between 10-13% [2, 3], hardly decreasing in recent decades despite developments in medical science and technology [4].

Regional ischemia has been considered as one of the main causes of anastomotic leakage [5-9]. Poor blood supply and perfusion of the rectal stump and ascending loop of bowel, or prolonged hypoxia are detrimental for wound healing and thus increase the risk of CAL [5, 10, 11]. Poor perfusion delays wound healing processes [12, 13]. The blood supply and perfusion of the preserved bowel, especially at the cutting edge, significantly affects the outcome of patients. Injury to the colon following an ischemic event is due to hypoxia and to reperfusion injury. Bowel ischemia results in hypoxia of the cells. Within one hour of ischemia, injury in the superficial part of the mucosa is already detectable. Prolonged severe ischemia causes necrosis of the mucosa layer, and lead to transmural infarction within 8 to 16 hours [14].

To prevent ischemia, one direct intervention is to provide oxygen. Oxygen is an essential component in tissue repair and wound healing. Oxygen stimulates collagen synthesis, matrix deposition, angiogenesis, epithelialization, and the eradication of bacteria [15-17]. Perioperative use of oxygen has been reported to reduce CAL and improve patient's outcome after colorectal surgery [18]. Clinical data have shown that perioperative hyperoxygenation reduced the occurrence of surgical site infections [19]. However, consensus in the interpretation of the data in this regard has not yet been reached and the mechanism of the oxygen therapy is still yet to be established.

The use of hyperbaric oxygen therapy (HBOT) is based on the same principle of hyperoxygenation and has been introduced in the treatment of surgical patients as well as in treatment of patients with chronic wounds [20, 21]. A positive influence of HBOT on anastomotic healing was first reported by Hamzaoglu et al. in 1998 [22]. Although this therapy is widely used in medical practice, its mechanism of action is still poorly understood. Previous study from Attard et al. has suggested that application of HBOT may reduce the production of inducible nitric oxide synthase protein (iNOS) expression [23], which is actively involved during the occurrence of CAL [24]. Many previous studies focused on localized changes such as collagen deposition and MMP (matrix metallopeptidase) activities [25].

The accumulated data supports the hypothesis that HBOT may improve anastomotic wound healing via suppression of pro-inflammatory agents and stimulation of anti-inflammatory agents [26]. This study was carried out to verify this hypothesis in an experimental rat model. HBOT was applied daily to all the rats 7 days prior to a partial colectomy with ischemic anastomosis, until 3 days after surgery. Macroscopic evaluation of anastomotic healing evaluation and immunohistochemistry were performed to investigate involvement of different inflammatory agents.

METHODS

Animals

Forty male Wistar-Albino conventional rats, 300-350 grams, were purchased from a licensed breeder (Harlan Laboratories, Boxmeer, The Netherlands). All rats were bred under specific pathogen-free conditions and kept under standard laboratory conditions in individually ventilated cages, and had ad libitum excess to water and regular rat chow. The experimental protocol was approved by the local Ethical Committee of Animal Experimentation of Erasmus University Rotterdam.

Experimental design

The animals were randomly divided into four different groups: control group, 3 days follow up; control group, 7 days follow up; HBOT group, 3 days follow up; HBOT group, 7 days follow-up.

The HBOT groups received HBOT for 10 consecutive days from seven days prior to surgery until 3 days after surgery. Each HBO (hyperbaric oxygen) session consisted of 100% oxygen under a pressure of 2.4 atm absolute for 90 minutes in the HBO Test Vessel P1460[27]. Animals were placed in a large transportation box (ten animals together) during the session. To exclude the possible bias due the HBOT procedure itself rather than the therapy itself, the control groups were also placed in a transportation box for the same time period in the same room, however, without undergoing HBOT.

On the day of operation, rats were anesthetized using 2% isoflurane/ O2; in addition, preoperatively, 0.05 mg/kg buprenorphine was administered as pain medication. After shaving and disinfecting the abdomen, the abdominal wall was opened through a 5-cm laparotomy. Subsequently, the ileocecolic arteries, the right colic artery, the middle colic artery, and the left colic artery were ligated (Silkam 4/0, B. Braun, Germany) to create an ischemic anastomosis (Figure 1). A partial colectomy was performed and proximal and distal ends of the colon were invertedly anastomosed with Dafilon 8/0 (B. Braun,

Germany). This model is earlier described, but in short, the colonic segment between 1.0 cm aborally to the cecum and 0.5 cm above the caudal mesenteric artery was resected [28]. The end-to-end anastomosis in all groups was made with 12 continuous sutures. Finally, the abdomen was closed in two layers and the rats were resuscitated with 5 mL of normal saline solution subcutaneously to prevent dehydration. No antibiotics were used. The HBO groups received HBOT immediately after surgery, which continued on postoperative day (POD)1 and POD2, and on POD3 just before the re-operation. During follow-up the rats were weighted and observed daily and had ad libitum access to water and food.

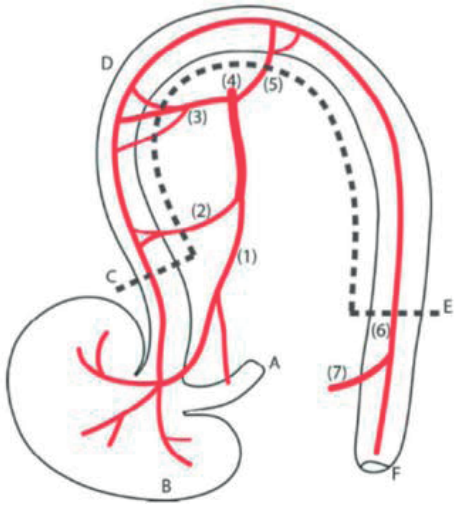

Figure 1. Schematic overview of artery distribution in rat colon, anterior view. (1) ileocecocolic artery, (2) colic branch of ileocecocolic artery, (3) right colic artery, (4) cranial mesenteric artery, (5) middle colic artery, (6) left colic artery, (7) caudal mesenteric artery, (A) terminal ileum, (B) cecum, (C) proximal cutting edge, (D) colon, (E) distal cutting edge, (F) anus. Adapted from Wu et al. [28]

ASSESSMENT OF THE ANASTOMOSES

Clinical observation and physical examination

On POD 3 or POD7, rats were anesthetized again. Anastomotic healing was assessed by observational, physical, and histological examination. The abdomen was checked for signs of anastomotic dehiscence. Abscess formation was scored according to the following scoring system: 0 = no abscess; 0.5 = one very small abscess; 1 = several small abscesses; 2 = medium abscess; 3 = large or several medium abscesses; 4 = one very large or several large abscesses [29, 30]. Adhesion strength and amount were recorded using the Zühlke score [31]. After clinical observation, the anastomotic bursting pressure test (ABP) was recorded in the same way as described previously [28]. In short, the

ABP was determined by insufflation of air in the closed segment of the colon, and the first leak of air was noted as the bursting pressure, the location was also noted.

Serum measurement

On POD3 or POD7 creatinine levels were measured in serum using the QuantiChrom assay kits (DIUR-500 and DICt-500, Gentaur Europe, Brussels, Belgium).

Histopathological evaluation

After the measurement of bursting pressure, a 1-cm-long colonic segment, 0.5 cm on each side of the anastomotic line, was resected and prepared for histopathological examination using standard procedures [28]. HE staining was performed and the anastomotic area of the slides was scored using the Ehrlich and Hunt numerical scale as modified by Phillips et al. [32]. The scoring system evaluated 4 parameters: inflammatory cell infiltration, fibroblast activity, development of new blood vessels, and collagen deposition. The parameters were graded from 0 to 4 as follows: 0 = no evidence, 1 = occasional evidence, 2 = light scattering, 3 = abundant evidence, 4 = confluent cells or fibers. The samples were scored by three investigators (G.B, Z.W. K.L.), who were blinded for the clinical findings, group allocation, and the treatment. Immunohistochemical staining for iNOS (marker for macrophage type 1; M1, 1:400, Abcam plc, Cambridge, UK) and CD206 (marker for macrophage type 2; M2, 1:1600, Abcam) were also performed on anastomotic samples with the same method described previously [24]. After overnight incubation at 4°C, the slides were incubated with Envision secondary antibody (DAKO, Glostrup, Denmark). After 30 minutes, Diaminobenzidine (DAKO, Glostrup, Denmark) was used for visualization of antigen-antibody reactivity. Slides were counterstained with hematoxylin.

To determine the positive target cell number on each slide, the same five fields were selected at the anastomotic site on each slide using a microscope with an imaging system (Olympus DP25, Tokyo, Japan), under 20 x 10 magnification (2560 x 1960 pixels). The cell numbers were counted with ImageJ (National Institutes of Health, Bethesda, MD). The average cell number of the selected fields was used for analysis. An M2/M1 index was calculated with the following equitation. The natural logarithm was used to adjust the data to normal distribution.

$$M2/M1 \text{ index} = \ln (\text{Number of } CD206^{+} \text{ cells})/(\text{Number of } iNOS^{+} \text{ cells})$$

Statistical analysis

Statistical analysis was performed with SPSS 21.0 (IBM Inc., Chicago, USA). Data are presented as mean ± standard deviation (S.D.) or as median or as percentage. The Mann-Whitney U test, t-test, and Pearson correlation test were used according to proper indications. We used the Levene's test to test equality of variances. The one-way analysis of variance

was performed with the Kruskall-Wallis test for non-parametric parameters. All reported *p* values were two-sided; a *p*-value < 0.05 was considered to indicate statistical significance.

RESULTS

Overall and general observation

In both the control groups and HBOT groups 5 animals died, all deaths are unrelated to anastomotic leakage: 5 colon ischemia, 4 colon ischemic necrosis, 1 overdosed anaesthesia. Postoperative weight loss occurred in all rats without significant difference between the groups.

HBOT improves clinical parameters

Anastomotic dehiscence was strictly limited to the control groups. The POD3 group had 37.5% (3/8) leakage and the POD7 group 28.6% (2/7) versus a rate of 0% in both HBOT groups (p = 0.021). The number of abscesses between groups was not significantly higher in the control groups (p = 0.08) (Table 1). HBOT resulted in significantly less anastomotic adhesions, which were significantly less severe as without oxygen therapy on both POD3 and POD7 evaluated with the Zühlke score(Figure 2).

Table 1. Comparison of anastomotic leakage and colon anastomoses abscess rate on POD 3 and POD 7 in the control and HBO groups.

	POD 3		POD 7		*p* value
	control	HBO	control	HBO	
Number of rats	10	10	10	10	
Mortality (%)	20 (2/10)	20 (2/10)	30 (3/10)	30 (3/10)	*NS*
Anastomotic dehiscence (%)	37.5	0	28.6	0	0.02
Colon anastomoses					
Abscess (mean number)	4	2	2	0	0.08

Dehiscence and abscess rate only include surviving animals

On POD3, the anastomotic bursting pressure (ABP) was significantly higher in the HBOT group than in the control group: 130.9 ± 17.0 mmHg vs. 88.4 ± 46.7 mmHg (p = 0.03) (Figure 3). The variance of ABP in POD3 in the HBOT group was also significantly lower (p = 0.004). ABP was not significantly different between the HBOT group and control group at POD7: 162.4 ± 39.7 mmHg vs. 141.1 ± 73.3 mmHg, (p = 0.51), but the variance of ABP was significantly lower in the HBO group (p = 0.009).

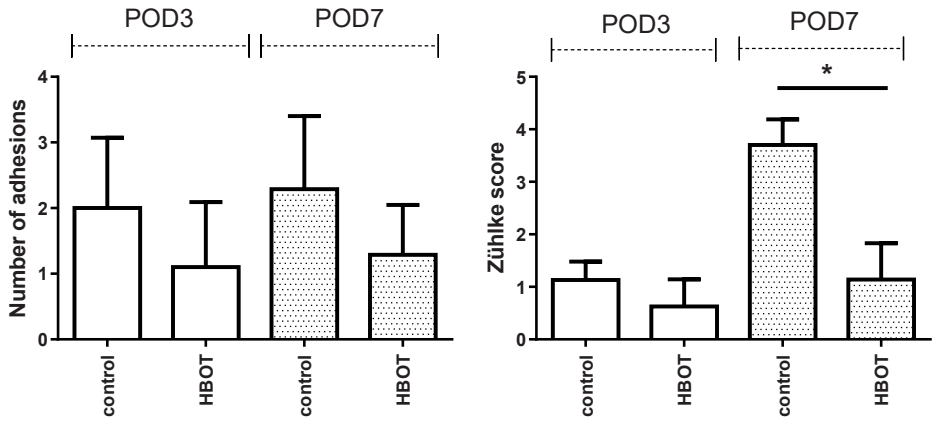

Figure 2. Adhesion score (number of adhesions) and Zühlke score (strength of adhesions) around the anastomosis [31]. The adhesions on POD7 in the HBOT were significantly less firm; control 3.7 ± 1.1 versus HBOT 1.29 ± 0.8. *indicate significance ($p = 0.001$)

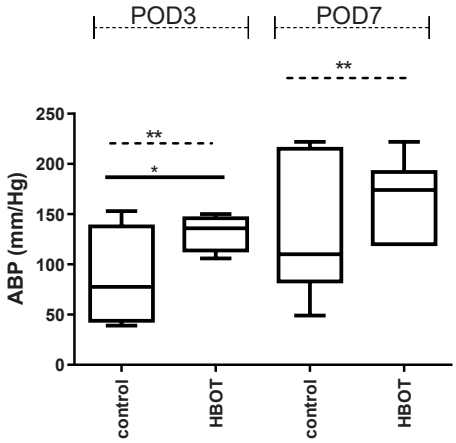

Figure 3. The anastomotic bursting pressure (ABP) in mmHg, was significantly higher on POD3 in the HBOT group 130.9 ± 17.0 mmHg vs. 88.4 ± 46.7 mmHg, p = 0.03. On POD7 we found a trend to an higher ABP in the HBOT group, but not significant (p =0.098), although the variance was significantly lower in the HBOT POD7 group. The variance of ABP between the groups was significantly different and is indicated with the p value on the dash line. *indicate $p < 0.05$, **indicate $p < 0.01$.

The mean creatinine level in the HBOT group on POD3 was lower, though not significantly, than in the control group; 13.4 ± 9.0 vs. 30.3 ± 28.6 mg/dl ($p = 0.07$). On POD7 the creatinine levels were significantly lower in the HBOT group than in the control group: 9.0 ± 12.1 vs. 52.0 ± 25.2 mg/dl (Figure 4).

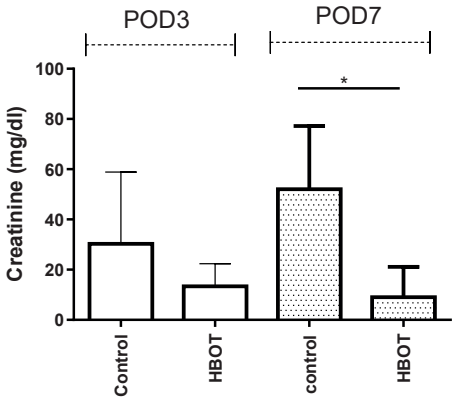

Figure 4. Creatinine levels measured as mg/dl were significantly higher in the control group than in the HBOT group on POD7; 9.0 ± 12.1 vs. 52.0 ± 25.2 mg/dl, $p = 0.003$ (*)

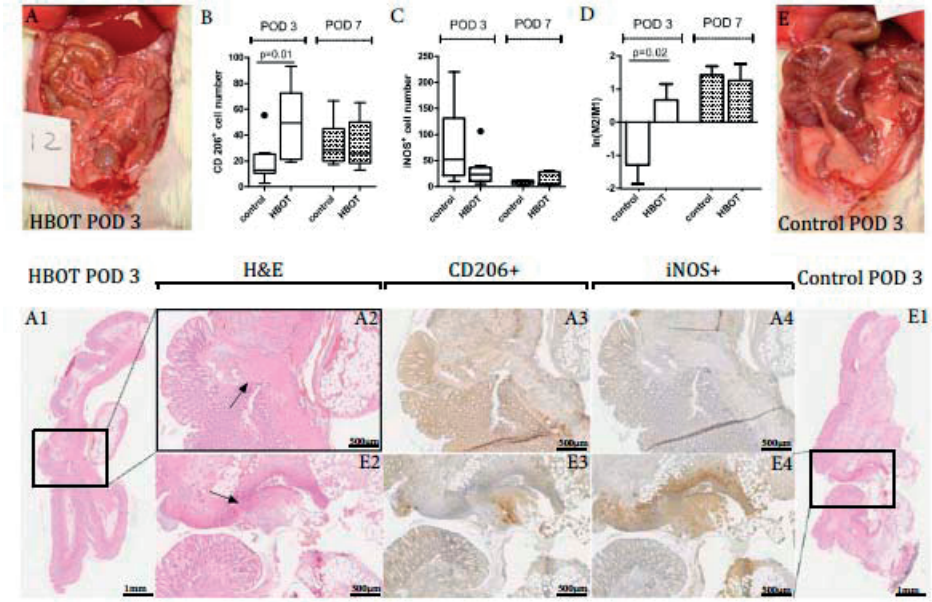

Figure 5. Comparison of macrophage numbers at the anastomotic site on POD 3 and POD 7 of the HBOT groups and control groups. A and E are representative pictures of an overview of the abdomen at POD 3. A A picture of a rat from the HBOT group which has no necrosis or ischemia. E shows ischemia of the cecum. B Illustrates the $CD206^+$ cell number (indication of M2 macrophages), the dot above the first bar indicate an outlier. C Illustrates the $iNOS^+$ cell number (indication of M1 macrophages), the dot above the second bar indicate an outlier. d Illustrates the M2/M1 index which is significantly in advantage of the HBOT POD3 group; p = 0.02. The left side of the image represents an anastomosis of the HBOT POD 3 group, the left side of this image is the intraluminal side of the colon. The selected area in A1 is represented in A2. Of the same area the immunohistochemistry staining of $CD206^+$ and $iNOS^+$ is represented in A3 and A4, positive cells are colored with diaminobenzidine. The same is true for the representative histology slides for the control POD3 groups as shown in figure E1–E4. The arrows in A2 and E2 indicate the anastomotic line (20 x 10 magnification)

Histology evaluation

The inflammatory cell infiltration, fibroblast activity, neoangiogenesis, and collagen deposition based on the HE staining were not evidently different between the groups (supplementary data 1). After 7 days more angiogenesis was observed in the HBOT group. Based on immunochemistry significantly more CD206+ cells (M2) were present in the HBOT group than in the control group on POD3; 50.0 ± 28.1 vs. 19.4 ± 16.2 cells, $p = 0.016$ (Figure 5). Also the M2/M1 index was significantly higher in the HBOT short-term group; $p = 0.02$. The number of iNOS+ cells as a marker for type 1 macrophages (M1, pro-inflammatory marker) was higher in the short term control group although not significantly different (Figure 5).

DISCUSSION

Colorectal anastomotic leakage is a dangerous short-term complication after colorectal surgery and may cause substantial immediate mortality if not treated as soon as possible. In this study, we evaluated the influence of HBOT on ischemic anastomotic wound healing and we found that HBOT improved the wound healing in ischemic colorectal anastomosis as shown with a higher ABP and less firm adhesions. We also observed improved postoperative recovery based on higher creatinine levels in the rats that received HBOT.

When comparing the data from this ischemic colorectal anastomosis model to the standard rat colectomy model [28] and to the other ischemic anastomosis models, the ligation of the arteries of the ascending stump resulted in catastrophic outcomes including evident ischemia at the cecum after surgery and a mortality rate as high as 25%. This is comparable to the clinical situation in ischemic bowel patients [33]. To our knowledge, this model best mimics clinical outcomes also because most ischemic anastomosis models only cause a lower bursting pressure and localized changes after surgery. Our previous study showed that functional failure of the ascending stump perfusion results in CAL in patients [34]. Impaired tissue perfusion may result from patient-related factors such as smoking inflammatory bowel disease or diabetes [35-37]. Meanwhile, different technique-related factors such as the level of artery ligation and anastomosis configuration may also influence anastomotic perfusion [34]. Our model inflicted a severe ischemic injury to the standard colorectal anastomosis, providing a satisfactory environment for evaluating the influence of HBOT.

Previous studies have reported the beneficial influence of HBOT on anastomotic healing such as increasing ABP [22, 38, 39] and reducing anastomotic adhesions. This was

also observed in our study. Moreover, the reduction of intra-abdominal abscess formation indicates an anti-infection effect of HBOT playing an important role in its overall beneficial effect. Because tissue necrosis and ischemia-reperfusion injury caused by the anastomotic ischemia also impair the systematic condition, we expected such beneficial effect of HBOT might also be observed in addition to the localized changes. Though failed to reduce the mortality rate, the HBOT group resulted in better kidney function as a marker for general health on POD7, indicating a beneficial systematic effect. More importantly, the preconditioning effect remained after cessation of HBOT.

Perioperative oxygen therapy providing 100% oxygen under higher pressure has been demonstrated to be effective to prevent CAL, but the mechanism is not fully understood. Moreover, it also influences the vascular response. The high concentration of oxygen causes vessel constriction, which has also been reported on HBO therapy cases [40, 41]. Though not fully understood, our preliminary observation found a different response pattern in the HBOT groups after artery ligation (unpublished data), which may eventually influence the blood supply and oxygenation of the tissues after anastomosis construction.

Substantial amount of data demonstrate that the effect of HBOT is not limited to the direct oxygenation [12, 13]. Previous studies showed that HBO therapy increased the ABP even when anastomoses were constructed under contaminated conditions [42, 43]. HBOT has been demonstrated to increase expression of the anti-inflammatory genes as well as influencing the local production of inflammatory cytokines [43], many of which are productions of macrophages. In accordance with that, our data suggest that the influence of HBOT on the inflammatory response via alternatively activating the macrophages. In addition to the increased ABP, the higher M2/M1 index also explains for a reduction of adhesion formation on anastomosis, probably because of earlier onset of regeneration as M2 macrophages enhance the regenerative responses as production of collagen [44, 45].

We investigated the mechanism of the HBOT on colorectal healing using perioperative treatment-format which according to the literature has demonstrated the most beneficial effect using perioperative HBOT [39]. We are aware that it is difficult to select the patients who would have a greater risk of ischemic anastomosis and start the treatment preoperatively. However in some patients such disposition can be presumed preoperatively. Clinical data remain in query to investigate whether application of HBOT in high-risk patients (e.g. smokers, patients with atherosclerosis, diabetic mellitus, cardiovascular disease, and low colon perfusion during operation due to low blood pressure, blood loss or multiple organ failure) may reduce CAL rate and improve clinical outcome. Whether

such application would benefit in a larger scale of patients (i.e. patients without clear risk of CAL) needs further investigation in the future.

CONCLUSION

Perioperative hyperbaric oxygen therapy prevents anastomotic leakage in ischemic colorectal anastomosis in the rat. The presence of anti-inflammatory macrophages is associated with the anastomotic healing. Application of HBOT as adjuvant therapy might be useful in the clinic when the patient is critically ill and giving the results of this study this needs testing in the near future.

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Chapter 8

Effects of adipose stem cell sheets on colon anastomotic leakage in an experimental model: Proof of principle

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ABSTRACT

The most dreaded complication of colorectal surgery is anastomotic leakage. Adipose tissue-derived stem cell sheets (ASC sheets) prepared from temperature-responsive culture surfaces can be easily transplanted onto tissues. These sheets are proposed to improve cell transplant efficiency and enhance wound healing. The aim of this study was to investigate whether application of ASC sheets could prevent leakage of sutured colorectal anastomoses. Insufficient suturing of colorectal anastomoses was performed in Wistar rats to create a colorectal anastomotic leakage model. Rats were randomized to ASC sheet application or control group. Leakage, abscess formation, adhesion formation, anastomotic bursting pressure (ABP), and histology were evaluated on postoperative day 3 or 7. ASC sheet application significantly reduced anastomotic leakage compared to controls, without increased adhesion formation. ASC sheet transplantation resulted in more CD3+ T-cells and CD163+ anti-inflammatory macrophages at the anastomotic site than the control group. ABP, vessel density and collagen deposition were not different between groups. Using cell sheet technology, we generated ASC sheets that prevented disruption of sutured colorectal anastomoses as shown by reduced leakage. Increased numbers of anti-inflammatory macrophages and T-cells might have contributed to this positive effect.

INTRODUCTION

Gastrointestinal (GI) disease is the third most common cause of death, the leading cause of cancer death, and the most common cause of hospital admission (Source: British Society of Gastroenterology, clinical services 2007). In 2009, the US National Institutes of Health reported 20 million ambulatory surgical GI procedures, to resect tumors, to relieve obstruction, after trauma, or as part of bariatric surgery [1]. An important and life-threatening complication of GI surgery is anastomotic leakage. Despite years of research, the incidence of impaired wound healing and consequent leakage remains high, ranging from 3% in small bowel surgery to 25-27% in colorectal surgery with mortality rates of up to 22% [2, 3]. Anastomotic leakage has a multifactorial etiology. Besides technical failures, restricted blood supply and uncontrolled inflammation are important reasons that contribute to impaired wound healing [4, 5].

Studies using human adipose tissue-derived mesenchymal stem cells (ASCs) have been successful in regenerating various tissues and promoting wound healing [6]. More importantly, several reports indicate that ASCs help to ameliorate tissue inflammation and can accelerate new blood vessel formation. These capacities seem among others to be attributable to the ability of ASCs to secrete a myriad of growth factors and cytokines that can promote repair of injured tissue or improve the quality of tissues that are regenerated [7, 8]. The beneficial characteristics of ASCs could be exploited to promote the healing process in the intestinal wall after surgery, thus helping to prevent postoperative complications. Recent studies using intraperitoneal ASC injections or ASC-coated bio-sutures showed no prevention from anastomotic leakage [9, 10]. Injection of ASCs as suspension or combined with biomaterials such as fibrin, collagen or gelatin is either associated with cell washout, insufficient cell retention or an inadequate distribution of transplanted cells.

Currently, cell sheet technology is a promising technique for cell transplantation improving cell retention. Cell sheets are typically prepared on special culture dishes that are coated with a temperature-responsive polymer. This polymer changes from being hydrophobic to hydrophilic when the temperature is lowered. In this way, intact cell sheets can be removed as one piece from the culture dishes without enzymatic treatment, preventing destruction of cellular and cell-extracellular matrix interactions within the sheet [11, 12].

Despite the application of various tissue sealants and other biomaterials, no significant reduction in the incidence of anastomotic leakage has been accomplished over the last 30 years [13]. Clearly, there is a need for new strategies therefore we propose a novel

approach in which ASCs are applied to the surgical wound area in cell sheets that are permeable to growth factors secreted by the ASCs and can be handled with ease by the surgeon. Therapeutic potential of ASC sheets was shown to improve cardiac tissue regeneration in the treatment of myocardial infarction [14], dilated cardiomyopathy [15], healing of hind limb ischemia [16] and chronic non-healing skin wounds [17]. Since cell sheet technology has been successfully used in improving healing of other soft tissues (i.e., heart, skin) as we mentioned above, we explored whether ASC sheets could serve as an ideal approach for the local delivery of cells to enhance healing and prevent leakage after intestinal anastomosis. To our knowledge this is the first attempt of ASC sheet application on intestinal surgical wounds.

The aim of this proof of principle study is to validate the efficacy of ASC sheets to prevent anastomotic disruption and leakage 3 and 7 days postoperatively in a colorectal leakage model in rats.

MATERIALS AND METHODS

ASC sheet preparation

ASCs were isolated from subcutaneous abdominal adipose tissue (all women, mean age 40 ± 9 years old). ASCs were seeded at 400,000 cells/cm^2 on 100% FBS pre-coated temperature responsive dishes (3.5 cm diameter, CellSeed, Tokyo, Japan). Every single sheet was made from one ASCs donor. ASCs were cultured in Dulbecco's Modified Eagle Medium 1 g/l glucose (LG-DMEM, Gibco, Life technologies, Paisley, UK) supplement with 10% fetal bovine serum (FBS, Lonza, Verviers, Belgium) with 50µg/ml gentamicin (Gibco) and 1.5µg/ml fungizone®(Gibco). After 48 hours, ASCs formed a coherent cell sheet. Thirty minutes before *in vivo* application, culture dishes were placed at room temperature to enable spontaneous detachment of the ASC sheets then culture medium was removed and refreshed with serum-free medium.

ASC sheet viability and histology in vitro

ASC sheet viability was evaluated by fluorescence microscopy on 0, 3, and 6 hours after detachment using a live/dead staining (Invitrogen, Life Technology, foster City, CA, USA). Briefly, ASC sheets were washed with Phosphate Buffered Saline (PBS, Gibco) after detachment and incubated for 30 minutes in calcein AM dye 1 µl/ml and ethidium bromide dye 1.5 µl/ml at 37°C in a humid atmosphere with 5% CO_2. The sheets were analyzed with fluorescence microscopy using Olympus IX71 inverted microscope, and images were captured with Cell F Imaging Software (Olympus: Hamburg, Germany, 2008). ASC sheets were fixed overnight in 4% buffered formaldehyde (Sigma, St Louis, Missouri and

Merck, Billerica, Massachusetts), set in in 2% agarose (Eurogentec, Liege, Belgium) and embedded in paraffin. Cross sections were stained with hematoxylin and eosin (H&E, Sigma).

SPIO-labeling of ASC sheet for pilot experiment

In a pilot study, ASCs were labeled with the so-called superparamagnetic iron oxide particles (SPIO) using ferumoxides (Endorem, Guerbet S.A., Paris, France) complexed to protamine sulphate (LEO Pharma N.V., Wilrijk, Belgium) as previously described [18]. For removal of extracellular iron, cells were washed with PBS containing heparin 10 U/ml (LEO Pharma B.V., Breda, the Netherlands). SPIO labeling mix was made at a constant concentration of 100 µg/ml ferumoxides with 5 µg/ml protamine to ensure identical particle formation. SPIO-labeled ASC were combined with unlabeled ASCs in a 30:70 ratio to form ASC sheets. Labeled ASC sheets were transplanted to 6 pilot rats after colorectal anastomosis to examine ASC sheet survival at POD3 (n=4) and 7 (n=2). Only for this pilot study, ASCs were labeled. In the following experiments, unlabeled ASCs were used to avoid any unwanted effects of labeling on ASC sheet function.

Animals

Male Wistar rats (weight 250–350g) were purchased from a licensed breeder (Harlan Laboratories, Boxmeer, The Netherlands). All rats were bred under specific pathogen-free conditions and randomly kept under standard laboratory conditions. Standard rat chow and water were supplied *ad libitum*. The research protocol was approved by the Ethical Committee of Animal Experimentation, Erasmus University Rotterdam (133-14-01). Sixty rats were randomly allocated to 4 experimental groups. The ASC sheet groups received local application of an ASC sheet around the colorectal anastomosis, anastomotic healing being evaluated on postoperative day 3 (n = 15) or 7 (n = 15). Rats in the control groups received no ASC sheet and anastomotic healing was evaluated at postoperative day 3 (n = 15) or 7 (n = 15).

Surgical technique and application of ASC sheets

To evaluate the ability of ASC sheet to prevent leakage the colorectal anastomotic leakage rat model previously described by Wu et al. was used [19]. Briefly, the rats were anesthetized with isoflurane/oxygen inhalation, and 0.05 mg/kg buprenorphine intramuscularly. After aseptic preparation, a midline abdominal incision was made and the right, middle, and left colic arteries were ligated (Silkam 4/0; B Braun, Melsungen, Germany). The colonic segment was resected 1 cm aborally to the cecum and 0.5 cm above the caudal mesenteric artery. An insufficiently sutured end-to-end anastomosis was created by one-layer inverting suturing with 5 interrupted sutures (Dafilon 8/0, B Braun) (Fig. 1A). Rats were randomly divided into control or ASC sheet groups. To make

sure the same side of the sheet was always applied and to avoid variation because of different orientation of the sheets, the dish-side of cell sheet was always applied to the serosal surface of the colon in every rat. In the ASC sheet group, the dish-side of an ASC sheet was wrapped around the anastomosis line (Fig. 1B). The ASC sheet adhered spontaneously to the serosal surface of the colon and required no further suturing or glue. The control group received no other intervention except for the insufficiently sutured end-to-end colorectal anastomosis. Immediately after ASC sheet application, the abdomen was closed in two layers of running sutures (Safil 5/0, B. Braun). Immediately postoperatively, rats were rehydrated and warmed. The rats returned to a normal diet after recovery from surgery.

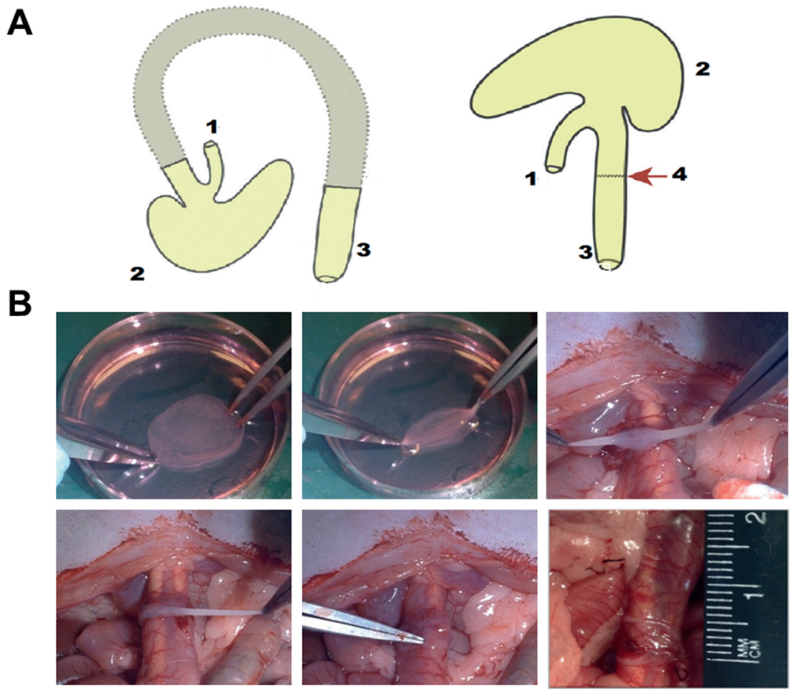

Figure 1. Rat partial colectomy and ASC sheet application:(A) Schematic overview of the rat colon before and after partial colectomy*. Anatomy: (1) terminal ileum, (2) cecum, (3) rectum and anus, (4) anastomosis. (B) ASC sheet transplantation (from upper left to lower right); ASC sheets were grasped with forceps at two rims and applied on top of the colorectal anastomosis. Following application, the sheet was unfolded and wrapped around the anastmosis thereby ensuring to cover it completely. *The schematic overview was adapted with permission from Wu Z, et al. Reducing colorectal anastomotic leakage with tissue adhesive in experimental inflammatory bowel disease. Inflamm Bowel Dis. 2015; 21(5):1038-46. Copyright© 2016 copyright Clearance center, Inc.

Follow up

Since both undifferentiated and differentiated ASCs have been used successfully for xenotransplantation in different other applications [20], no immunosuppressive drugs were used in this experiment. Wellness and weight of all rats were evaluated daily. On postoperative day (POD)3 or 7, rats were re-anesthetized and a re-laparotomy was performed. The abdomen was checked for signs of peritonitis, adhesions and abscesses (defined as "elsewhere"). At the anastomotic area, signs of stricture, disruption, adhesion, and abscesses were checked. Two observers (PS and GB) performed all macroscopic observations and evaluations for each rat.

Macroscopic observation

Abscess severity was scored according to the previously described abscess score, in short: 0 = no abscess, 0.5 = one small abscess (< 1 mm), 1 = several small abscesses, 2 = medium abscess (1-3 mm), 3 = one large (3-5 mm) or several medium abscesses, 4 = one very large (>5 mm) or several large abscesses [21]. Adhesions were recorded using the Zühlke score. In short: 0 = no adhesions, 1 = firm adhesions which can be separated with blunt dissection, 2 = strong adhesions which can only be separated with sharp dissection, 3 = very strong vascularized adhesions which can only be separated with sharp dissection and damage to surrounding tissue is hardly preventable. Anastomotic bursting pressure (ABP) was determined after macroscopic observation by a third blinded researcher (AC). In short, ABP was determined by insufflation of air in a closed segment of the colon and pressure at time of the first air leak was noted as bursting pressure. The location of the burst was also noted. After the ABP measurement, the colorectal anastomosis was resected and prepared for histological examination.

Histological evaluation after transplantation

Histological and immunohistochemically assessment were conducted by two independent blinded observers (PS and AC) in a fully randomized order to evaluate healing of the anastomosis. A segment of colon containing the colorectal anastomosis was harvested, washed with phosphate-buffered saline and opened longitudinally. After overnight fixation in 4% buffered formaldehyde, segments were embedded in paraffin. Paraffin-embedded sections (6 µm) were deparaffinized and rehydrated. For morphological evaluation, sections were stained with H&E. Perl's iron stain (Klinipath BVBA, Duiven, the Netherlands) was used for staining SPIO-labeled ASC sheets at POD3 and POD7 after transplantation in the pilot study. Picro Sirius red staining (Sigma, St Louis, Missouri) was used for collagen deposition. Immunohistochemistry was performed to assess vascular density (rat, CD34+), presence of T-cells (CD3+) and anti-inflammatory M2 macrophages (CD163+), ASC sheet survival (human mitochondria) and endothelial differentiation

(human CD31+) of ASCs at the colorectal anastomosis. Details of the methods used for staining are described in supporting information.

Statistical analysis

Data are presented as mean ± standard deviation (SD) and analyzed with the Mann-Whitney U test and Kruskal-Wallis one-way analysis of variance. To compare the incidence of anastomosis disruption between groups at each time point the two-tailed Chi-squared test was used. All reported *P* values are two-sided; a *P* value of < 0.05 indicated statistical significance. All statistical analyses were done using SPSS 21.0 (IBM Inc., Armonk, NY, USA).

RESULTS

ASC sheet characteristics in vitro

ASCs cultured at 400,000 cells/cm^2 in a temperature responsive culture dish for 48 hours formed cell sheets that could be spontaneously detached at room temperature (Fig. 2A). ASC sheets consisted of several cell layers (Fig. 2B). Viability of ASC sheets in medium at room temperature remained high until 6 hours after detachment (Fig. 2C).

In vivo overall observations

During the first postoperative days, all rats lost weight and had a lower wellness score than before surgery. From POD4 onwards, all animals started to gain weight. The weight loss and wellness scores in the control and ASC sheet groups were not significantly different (supplementary data; Fig. S1). Three out of 60 rats (5%) died within 24 hours after surgery in both control (POD3) and ASC sheet groups (POD3 and POD7). Autopsy demonstrated that these deaths were caused by acute (e.g., bleeding and anesthesia-related) complications; therefore, these animals were excluded from further analysis.

Postoperative intra-abdominal evaluation

At re-laparotomy, the ASC sheet structure was visible around the anastomosis in all rats of the ASC sheet group at POD3 and POD7 (Fig 3A-B). Ten out of 14 rats in the control group had anastomotic disruption on POD3 (71%) versus 2 out of 14 (14%) in the ASC sheet group ($P = 0.002$). No significant difference in the anastomotic disruption rate was observed at POD7 between the two groups. Significantly more rats in the control group had abscesses at POD7 around the anastomosis (10/15: 67%) compared to the group receiving an ASC sheet (4/14: 28%, $P = 0.04$). There were no differences in the occurrence of peritonitis, stricture formation, adhesions, and abscess formation elsewhere (Table 1). The average abscess number around the anastomosis was significantly higher in the

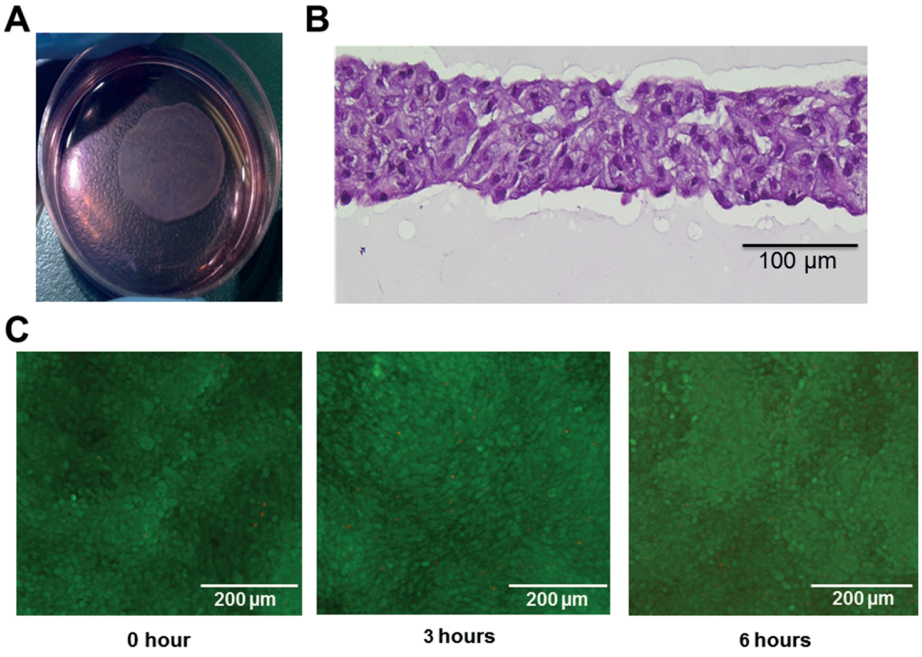

Figure 2. ASC sheet viability and morphology prior to implantation. (A) ASC sheet after detachment from the temperature responsive dish. (B) Cross section of ASC sheet stained with hematoxylin & eosin. (C) Live/dead staining of ASC sheets at 0, 3 and 6 hours after detachment. Viable cells stained with calcein (green), non-viable cells stained with ethidium bromide (red).

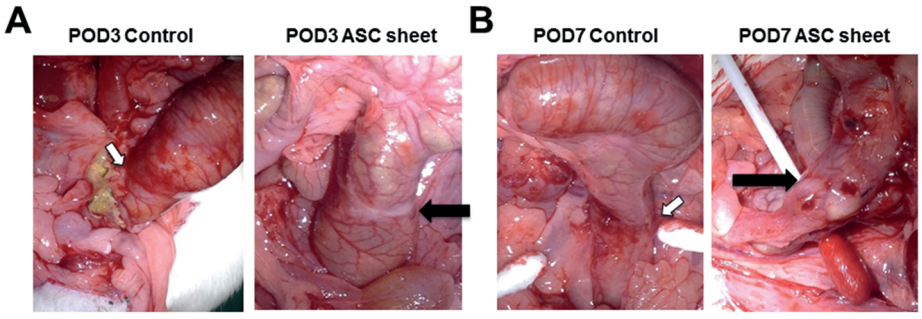

Figure 3. ASC sheet is visible around the anastomosis. (A) POD3 and 7 (B), white arrows point at anastomotic area and black arrows point at transplanted ASC sheets.

control group than in the ASC sheet group on both POD3 (1.6 ± 0.5 versus 1.0 ± 0.7, $P = 0.004$) and POD7 (1.2 ± 1.2 versus 0.4 ± 0.6, $P = 0.028$), (Fig. 4A). The abscess score between the ASC sheet and control groups did not differ on POD3 but the abscess score of the ASC sheet group was significantly lower than the control group on POD7 ($P = 0.048$, Fig. 4B). The number of intra-abdominal adhesions was significantly lower in the

Table 1. Post operative macroscopic findings

	POD3			POD7		
	Control (%)	**ASC sheet (%)**	***p*-value**	**Control (%)**	**ASC sheet (%)**	***p*-value**
Peritonitis	1/14 (7.1)	0/14 (0)	NS	0/15 (0)	0/14(0)	NS
Anastomotic disruption	10/14 (71.4)	2/14(14.3)	0.002	3/15(20)	2/14(14.3)	NS
Stricture	2/14 (14.3)	2/14(14.3)	NS	2/15 (13)	2/14(14.3)	NS
Abscess at anastomosis	14/14(100)	12/14(85.7)	NS	10/15(66.7)	4/14(28.6)	0.04
Adhesion at anastomosis	14/14(100)	14/14(100)	NS	15/15(100)	14/14(100)	NS
Abscess elsewhere	11/14(78.5)	8/14(57.1)	NS	6/15(40)	4/14(28.6)	NS
Adhesion elsewhere	8/14(57.1)	3/14(21.42)	NS	9/15(60)	7/14(50)	NS

Values are presented as the number of rats and relative percentage. POD3= postoperative day 3, POD7= postoperative day 7. ASC = adipose tissue derived stem cell. NS; not significant by Chi-square test.

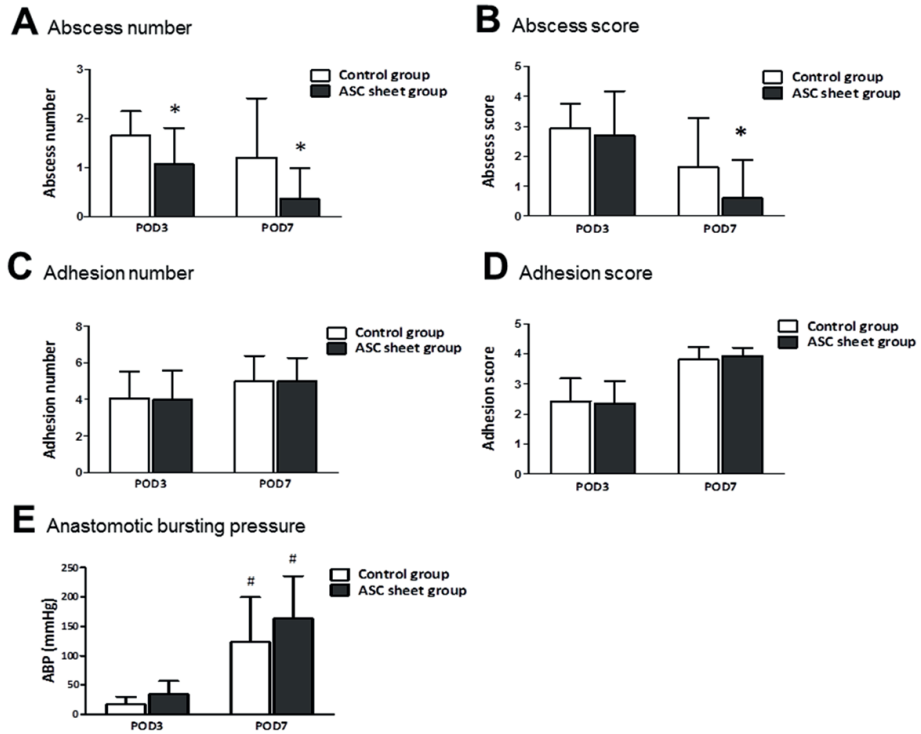

Figure 4. Intra-abdominal macroscopic findings at colorectal anastomosis. (A) abscess number, (B) abscess score (0-4), (C) adhesion number, (D) adhesion score (0-4) and (E) anastomotic bursting pressure (ABP). ASC = adipose tissue-derived stem cells. POD3 = postoperative day 3. POD7 = postoperative day 7. Each bar represents average number +/- standard deviation from control (n= 14 in the control and ASC sheet group at POD3; n= 15 in the control group and n= 14 in the ASC sheet group at POD7). * P <0.05 between groups. # P <0.05 between different time points within the same group.

ASC sheet group (P = 0.043) on POD3 (supplementary data, Fig. S2), but no significant differences in the number of adhesions (Fig. 4C) and adhesion scores (Fig. 4D) around the anastomosis were seen between the two groups on POD3 and POD7 .

On POD7 the average bursting pressure was higher compared to POD3 in both groups but was not significantly different between groups (Fig. 4E). On POD3 bursting of all anastomoses in both the control and ASC sheet groups occurred at the anastomosis line. On POD7 bursting occurred predominantly at the anastomosis line in the control group (10 rats (66%), whereas in the ASC sheet group bursting mostly (8 rats: 57%) occurred proximally or distally to the anastomosis line.

Histological evaluation

To evaluate whether ASC sheets would still be present 3 and 7 days after transplantation, Perl's iron stain was used for detection of SPIO-labeled ASC sheets. At both POD3 and POD7, SPIO-labeled ASCs were present at the colorectal anastomotic serosal side. (Fig. 5A, B). Further evaluation of ASC sheet state was performed by using a human mitochondrial staining. At POD3 ASC sheets showed abundant positive staining for human mitochondria, while at POD7 positive cells were severely diminished (Fig. 6A,B).

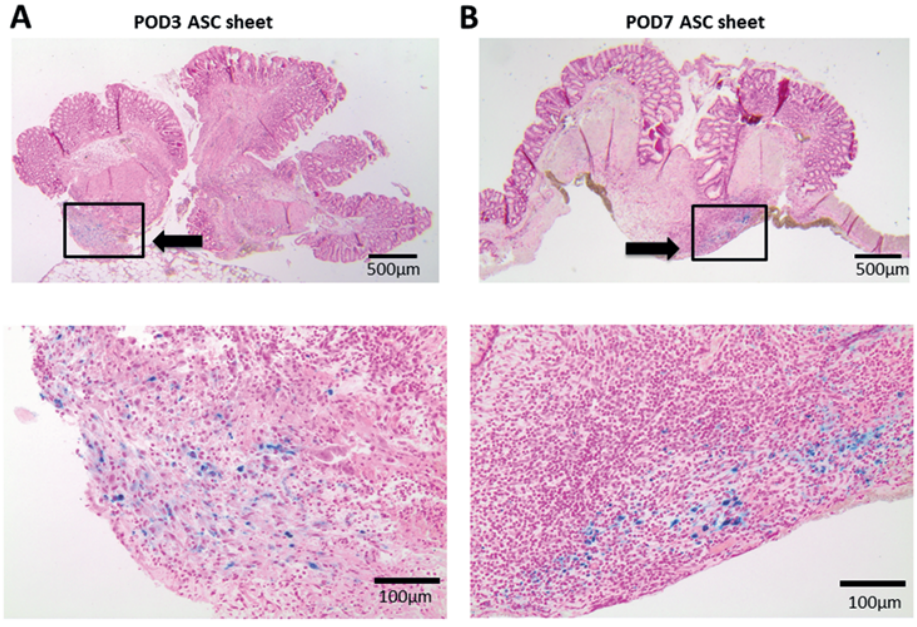

Figure 5. Tracking SPIO-labeled ASC sheet in a pilot experiment. SPIO-labeled ASC sheet stained with Perl's iron stain (blue). The selected area with solid line (see also black arrows) indicates the anastomotic area, which is enlarged and illustrated below for representation of positively stained cells A-B. Blue iron stained SPIO-labeled ASCs present in ASC sheet on serosal side of the colorectal anastomosis at (A) POD3 and (B) POD7.

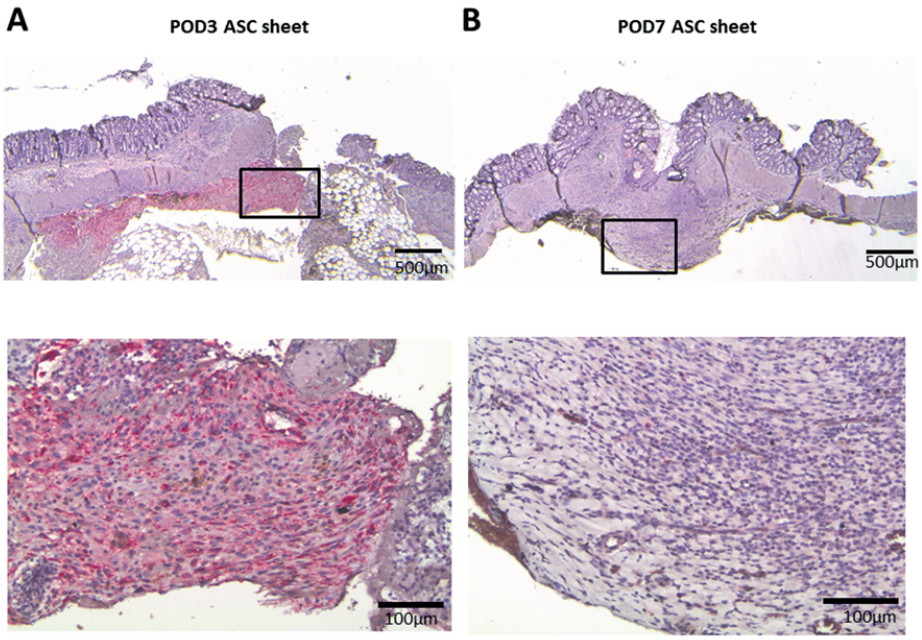

Figure 6. ASC sheet survival at anastomotic site. The selected area with solid line indicates the anastomotic area with ASC sheet, which is enlarged and illustrated below for representation of positive staining for human mitochondria A-B. (A) ASC sheet showed positive red staining of human mitochondria at POD3 and (B) severely diminished positive staining at POD7

To evaluate the effect of the ASC sheets on colorectal anastomosis vascularization, collagen deposition and infiltration of inflammatory cells, several specific immunohistochemically stainings were performed. Local application of ASC sheets did not affect CD34 positive capillaries or collagen deposition at the colorectal anastomosis site. Although the capillary density increased from POD3 to POD7 in both the control and ASC sheet groups ($P < 0.01$), there was no difference in the capillary density between groups at either time points (POD3, ASC sheet group 172.4 ± 44 vessels/cm^2 versus control group 150.5 ± 42 vessels/cm^2 and POD7, ASC sheet group 277.2 ± 95 vessels/cm^2 versus control group 276.5 ± 83 vessels/cm^2, Fig. 7A,E). No human ASC-derived CD31 positive endothelial cells were detectable within the ASC sheets before or after transplantation (data not shown).

Similar findings regarding collagen deposition at the anastomotic site were found. Despite an increase of collagen deposition from POD3 to POD7 in both groups, groups were not significantly different from each other (POD3, ASC sheet group 2.2 ± 2.4% versus control group 0.7 ± 0.6%, and POD7, ASC sheet group 19.5 ± 0.6% versus control group 22.7 ± 10.5%, Fig. 7B,F).

The number of T-cells and M2 macrophages was used to evaluate the inflammatory response at the anastomosis site. At POD3 a nonsignificant different number of CD3 positive T-cells were found in the ASC sheet group (81.8 ± 36 cells/cm^2) as compared to the control group (103.6 ± 45 cells/cm^2). The number of CD3 positive cells increased in both groups from POD3 to POD7. At POD7 a significantly higher number of CD3 positive T-cells was detected in the ASC sheet group (508.6 ± 243 cells/cm^2) than in the control group (204.6 ± 107 cells/cm^2, $P = 0.001$, Fig. 7C, G).

In the control group, the number of CD163 positive cells indicating M2 macrophages decreased significantly between POD3 (140.3 ± 67 cells/mm^2) and POD7 (55.9 ± 37 cells/mm^2, $P < 0.001$) whereas in the ASC sheet group, the number of CD163 positive cells did not differ between POD3 (159.6 ± 89 cells/mm^2) and POD7 (122.8 ± 128 cells/mm^2, Fig. 7D, H).

DISCUSSION

Cell sheets are a recent development in regenerative medicine. They allow for the delivery of cultured cells and their deposited extracellular matrix and prevent cell loss associated with administering cells via injection or scaffold [22]. Previous studies demonstrated that ASC sheets show great promise in promoting the healing of several organ injuries. In this study, application of an ASC sheet to an insufficiently sutured rat colorectal anastomosis resulted in reduced anastomotic disruption and abscess formation. ASC sheets facilitated an increase in the number of anti-inflammatory macrophages and T-cells at the anastomosis site, which might have contributed to this positive effect.

Application of ASC sheets to colorectal anastomosis is feasible since ASC sheets are flexible and spontaneously adhere to the serosal surface rapidly. This might be due to adhesive proteins present at the dish-side of ASC sheet that remain after harvesting from a temperature responsive dish [23]. ASC sheet survival after in vivo transplantation has been shown in other in vivo models and varied form 3 days [24] up to 8 weeks [25]. In our in vivo model ASCs were still present in the sheets at POD3 and 7 and mitochondrial staining was present until day 3. These findings indicate that ASC sheet were present and contained living cells for at least 3 days after transplantation allowing them to exert their potential paracrine effects for several days.

The recording of bursting pressure in colorectal anastomoses showed that on POD7, the average bursting pressure was not different between the groups. However, the bursting site in the ASC sheet group was mainly remote from the anastomotic line compared

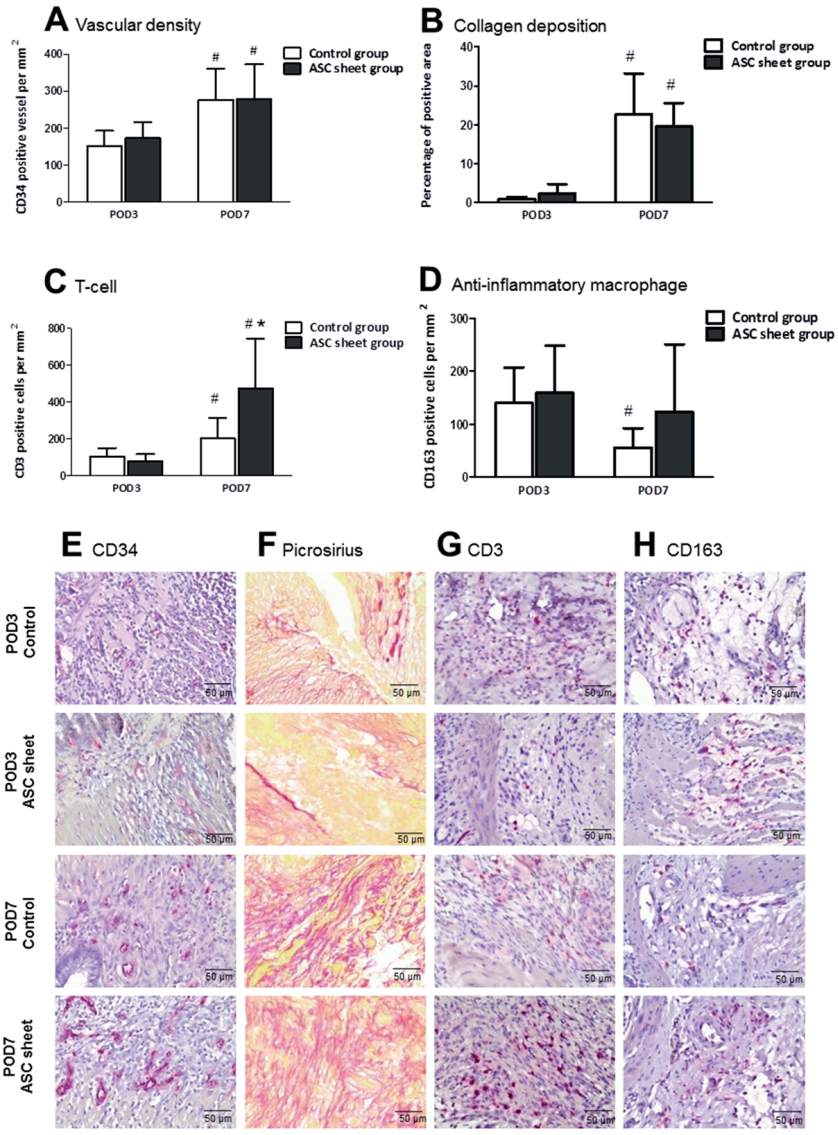

Figure 7. Comparison of vascular density, collagen deposition, T-cell and anti-inflammatory macrophage amount at the anastomotic site on POD3 and POD7 between ASC sheet and control group. The comparisons were listed in A-D and illustrated in E-H. (A) Vascular density as assessed by average number of CD34+ vessels/mm^2. (B) Collagen deposition determined by area percentage of Picrosirius red staining. (C) T-cell response determined by number of CD3+ cells/mm^2. (D) Anti-inflammatory macrophage response assessed by average CD163+ cells/mm^2. POD3 = postoperative day 3. POD7 = postoperative day 7. ASC = adipose tissue-derived stem cells. Each bar represents average value +/- standard deviation from control (n=14 in the control and ASC sheet group at POD3; n=15 in the control group and n=14 in the ASC sheet group at POD7. * P <0.05 between groups, # P <0.05 between different time points within the same group. Representative examples of immunohistochemically staining of colorectal anastomosis for (E) CD34, (F) Picrosirius red, (G) CD3, and (H) CD163 of each group (200x).

with the control group in which the burst occurred mainly at the anastomosis itself. These results suggest that when an intraluminal pressure is exerted to the colonic wall, anastomosis that is reinforced by ASC sheet is less susceptible to perforation. Alternative techniques that have been used previously in an attempt to prevent leakage of gastrointestinal anastomosis have been based on the sealing of the anastomosis with tissue adhesives or synthetic meshes [26, 27]. Although these techniques for leakage prevention have various levels of clinical efficacy, they are imperfect solutions that can give burdens for patients. The therapeutic potential of ASC sheets is generally thought to be attributable to ASCs paracrine ability, not the mechanical/physical strength of the ASC sheet to seal the wound [28-30]. Although ASC sheets are composed of only a thin layer of cells, it cannot be excluded that the reduction in dehiscence, and abscess formation in this study might be possible due to the mechanical ability of ASC sheets to seal the anastomosis and prevent leakage. However, merely external coating of colonic anastomoses has yet failed to show convincing results [31, 32]. Future experimental studies are warranted to determine and compare the mechanical role of external coating materials and ASC sheets for prevention of colon anastomotic leakage.

To investigate whether the ASC sheets contributed to the healing of the anastomosis, capillary density and collagen deposition were evaluated, both being of great importance for anastomotic healing. Although mesenchymal stem cells are believed to promote vascularization and stimulate collagen deposition, capillary density was not significantly different between the control and the ASC sheet groups in this model. Collagen deposition was also similar in the control and experimental groups on POD7. The lack of increased capillary density in the ASC sheet treated group conflicts with previously published data [16, 17] indicating that ASCs promoted angiogenesis in animal models characterized by low blood perfusion and ischemia such as hind limb ischemia [16], myocardial infarction, and skin wounds in diabetic rats [17]. However, no such effects were seen in a dilated cardiomyopathy model [15] or in gastric ulcer healing [33]. Possibly, the tissue to which the sheet was applied and whether or not this was ischemic might explain these differences. Transplanted human ASCs have been suggested to be able to differentiate into endothelial cells and thereby contributing to angiogenesis [34], however, this was not observed in the study presented here. The absence of increase in collagen deposition may indicate that although ASCs are able to produce collagen themselves [35] they do not promote collagen synthesis at the anastomosis site up to POD7. However, as suggested by Rabau et al [36] total collagen amount might be less important for anastomosis' tensile strength than structure and arrangement of collagen matrix.

Inflammatory cells play a key role in the healing of intestinal anastomoses [37]. Macrophages and activated lymphocytes are the main inflammatory cells in wound healing and in the development of fibrosis. Macrophages fulfil multiple roles in inflammation: besides phagocytosis and encouraging inflammation, macrophages also have important anti-inflammatory properties. Macrophages that stimulate inflammation are called M1 macrophages, whereas those that decrease inflammation and stimulate tissue repair are called M2 macrophages. Since the M2 subpopulation plays an important role in wound healing and ASCs have been shown to induce macrophage polarization towards the M2 subtype [38], we focused on the presence of M2 macrophages at the anastomosis area. As shown before in fetal-membrane mesenchymal stem cell sheets [39], the number of M2 macrophages did not decline between POD3 and POD7 in the ASC sheet group when compared to the control group and might have stimulated the healing process at the anastomosis.

T-lymphocytes and more specific T-regulatory cells (T-regs), have the ability to release cytokines and growth factors that regulate other immune cells and positively affect wound healing [40]. On the other hand, in case of rejection of transplanted tissue, high numbers of cytotoxic T-cells are seen around the implanted tissue, together with abscesses and inflammation. In this study, the ASC sheet group had significantly more CD3+ T-cells within the anastomotic area than the control group at POD7, whereas the number of abscesses decreased instead of increased. Since the higher number of CD3+ T-cells at the anastomosis was accompanied with lower abscess number and anastomotic disruption, it is very unlikely that the increase of CD3+ T-cells is the results of rejection of the implanted ASC sheet. A more likely scenario is that the ASC sheets resulted in increased numbers of T-regs around the anastomosis thereby contributing to better healing of the wound. However, to our knowledge, a good staining for FoxP3, the classical marker for T-regs, is not available for rat at the moment.

Several studies suggest that human ASCs are immune-privileged and can survive in immunocompetent animals [41]. Both undifferentiated and differentiated ASCs have been used successfully for xenotransplantation [20]. Based on rat wellness score, and the positive effect of ASC sheets on colorectal anastomosis leakage we consider the presence of an (hyper)acute rejection in our study unlikely. However, future better understanding of the functionality of allo- or xenogeneic-derived ASCs for therapy is warranted.

Unfortunately, it was not possible to fully blind the observers from the treatment group since the ASC sheet structure was obvious in macroscopic and microscopic evaluations. Moreover, we evaluated the effect of ASC sheets on colorectal anastomosis after POD3 and POD7 since dehiscence is most likely to occur in this critical phase of colonic anas-

tomotic healing[13]. We are aware of the fact that at this time point the wound healing process is not complete yet and further maturation and collagen remodeling will occur at later stages of anastomotic healing [42].

The present study suggests that application of human ASC sheets to experimental colorectal anastomosis is a promising technique to prevent short-term post-operative complications such as anastomotic disruption and abscess formation without increasing adhesion formation. To translate the potential of ASC sheets for future clinical application, our results must be complemented with further short and long-term studies and defining the role of ASC sheets in the technique of colorectal anastomoses with regards to complications such as abdominal contamination and severe peritonitis.

CONCLUSIONS

This study explored the proof of principle of a therapeutic potential of ASC sheet for preventing disruption of sutured colorectal anastomoses. ASC sheet application significantly enhanced healing of colorectal anastomoses with decreased incidence of disruption and abscess formation. The increased numbers of anti-inflammatory macrophages and T-cells might have contributed to promotion of the healing process. This preclinical study indicates that ASC sheet application may have a therapeutic role in promoting colorectal healing and prevention of anastomosis-related complications.

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SUPPORTING METHODS

ASCs isolation and culture

Human ASCs were isolated from subcutaneous abdominal adipose tissue from eight healthy female donors (age 25-50 years) as leftover adipose tissue from breast reconstruction surgery, with approval of the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam (MEC-2014-092). ASCs were isolated as described previously.[8] Briefly, after digestion of the tissue, ASCs were plated in 40,000 cells/cm^2 and grown in Dulbecco's Modified Eagle Medium 1 g/l glucose (LG-DMEM, Gibco, Life technologies, Paisley, UK), containing 20% fetal bovine serum (FBS, Lonza, Verviers, Belgium), 50 µg/ml gentamicin (Gibco), 1.5 µg/mL fungizone® (Gibco). Ascorbic acid-2-phosphate (25 µg/ml, Sigma-Aldrich, St. Louis, MO, USA) and 1 ng/ml human recombinant fibroblast growth factor 2 (FGF2, AbDSerotec, Kidlington, UK)] were freshly added. After 24 hours, cultures were washed with Dulbecco's phosphate-buffered saline (PBS, Gibco) with 2% FBS to remove non-adherent cells and erythrocytes and refreshed with expansion medium (LG-DMEM with 10% FBS, 50 µg/ml gentamicin, 1.5 µg/mL fungizone® and freshly added ascorbic acid-2-phosphate and FGF2). Isolated ASCs were cultured at 37°C under humidified conditions in an atmosphere with 5% CO_2. When cultures reached 90% confluence, ASCs were sub-cultured with 0.25% trypsin EDTA (Gibco) and reseeded at a density of 8,000 cells/cm^2 for two passages and thereafter. ASCs at passages 2–5 were used for following experiments.

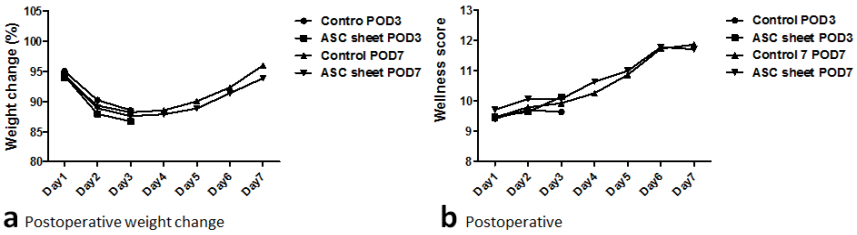
Immunohistochemistry

All sections were treated with xylene and ethanol series to deparaffinize. Heat induced antigen retrieval was used by boiling sections at 95 °C for 20 minutes in citrate buffer or Tris-EDTA buffer solution. Vascular density was evaluated using antibodies against CD34 (AF4117, dilution 1:200; R&D systems, Minneapolis, Minnesota). T-cells were identified by CD3 staining (Ab16669, dilution 1:100; ab16669; Abcam Cambridge, UK). M2 macrophages were identified by CD163 staining (MCA3426A, dilution 1:100; AbDSerotec, Raleigh, NC, USA). Anti-human mitochondria antibody (Abcam AB92824, dilution 1:500, Cambridge, UK) was used to identify viable ASC sheet. Human endothelial cells were identified with a mouse anti human antibody against CD31 (M0823, dilution 1:100, Dako, Glostrup, Denmark). After blocking with normal goat serum each primary antibody was diluted in 1% bovine serum albumin in PBS and sections were incubated for 60 minutes at room temperature. The slides were then incubated with biotinylated goat-anti-rabbit link (BioGenex Laboratories, San Ramon, CA, USA) for 30 minutes followed by incubation with streptavidin-AP (Biogenex) for 30 minutes. Neu Fuchsin (Chroma, Kongen, Germany), napthtol AS-MX phosphate (Sigma), sodium nitrate (Sigma), levamisole (Sigma)

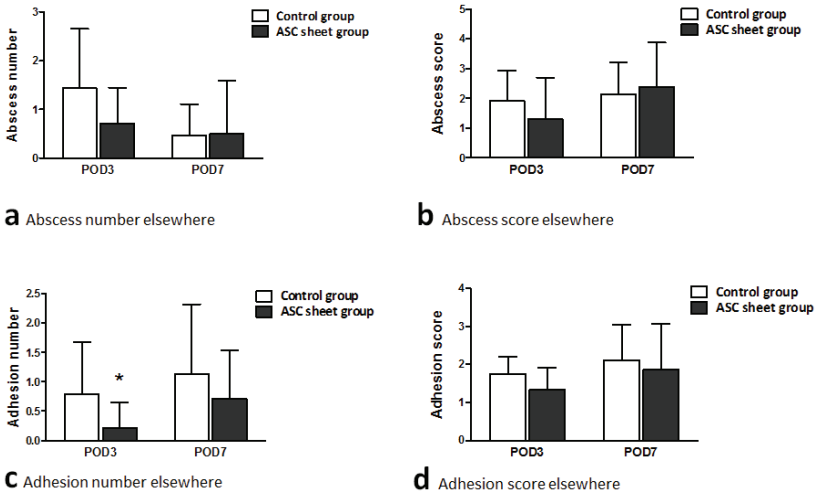
and dimethyformamid (Sigma) were used in the substrate. Hematoxylin was used as a counter stain. Isotype-matched antibodies were used as negative controls.

The histological and immunohistochemical assessments were performed by two independent blinded observers (PS and AC). Seven images of each slide were taken for analysis including the anastomotic area and adjacent area in every layer. The number of positive cells for each photograph from high power field (200x magnification, Olympus BX50, Olympus, Hamburg, Germany) was counted with ImageJ (National Institutes of Health, Bethesda, MD). For CD3 and CD163, the values were expressed as average number of positive cells per mm^2. For capillary density, values were expressed as average number of CD34 positive vessels per mm^2. Quantification of collagen deposition within the anastomotic healing area was calculated as percentage of Picrosirius red positive staining area. Two sections were randomly selected from each slide and 3 images of the anastomotic area were evaluated using Image J software.

SUPPLEMENTARY RESULTS



Supplementary Figure 1. (A) Postoperative weight changes and (B) wellness score (0-12). ASC= adipose tissue-derived stem cells. POD3 = 3 days post operation, POD7 = 7 days post operation.



Supplementary Figure 2. Intra-abdominal macroscopic findings distant from colorectal anastomosis: (A) Abscess number, (B) abscess score (0-4), (C) adhesion number and (D) adhesion score (0-4). ASC = adipose tissue-derived stem cells. POD3 = postoperative day 3. POD7 = postoperative day 7. Each bar represents average number +/- standard deviation from control (n= 14 in the control and ASC sheet group at POD3; n= 15 in the control group and n = 14 in the ASC sheet group at POD7), * P <0.05 between groups.

Chapter 9

Critical analysis of cyanoacrylate in intestinal and colorectal anastomosis

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ABSTRACT

Background

Although cyanoacrylate glue (CA) has been widely used in various kinds of medical applications, its application in gastrointestinal anastomosis remains limited, and outcomes of experimental studies have not been satisfactory. This systematic review summarizes research regarding CA application in intestinal and colorectal anastomosis, and correlates methodological aspects to experimental outcomes.

Methods

A systematic literature search was performed using Medline, Embase, Cochrane and Web-of-Science libraries. Articles were selected when CA was applied to intestinal or colorectal anastomoses. Included articles were categorized according to CA molecular structure; the method details in each study were extracted and analyzed.

Results

Twenty-two articles were included. More than half of the inclusions reported positive outcomes (7 articles) or neutral outcomes (8 articles). Analysis of the methods revealed that methodological details such as CA dosage, time of polymerization are not consistently reported. Porcine studies, inverted anastomosis, and n-butyl-cyanoacrylate studies showed more positive outcomes; everted anastomosis, and oversized sutures may negatively influence the outcomes.

Conclusions

Due to the positive outcome from the porcine studies, application of CA in GI anastomosis still seems promising. To achieve a better consistency, more methodological details need to be provided in future studies. Optimizing the dosage of CA, choice of animal model, inverted anastomosis construction, and other method details may improve intestinal and colorectal anastomoses with CA application in future studies.

INTRODUCTION

Cyanoacrylate (CA) was invented more than 60 years ago for industrial applications [1, 2]. Famous for its strong adhesiveness, various commercial names such as "crazy glue" or "instant glue" are well known in daily life. Moreover, the strong adhesiveness of CA also made it an ideal candidate for replacement of conventional sutures in medical use, such as wound closure. In addition to a strong bond, a fully and evenly sealed anastomosis can be created with CA, avoiding excessive tissue approximation that can induce disturbances in the microcirculation [3, 4]. In 1998, the Food and Drug Administration (FDA) approved Dermabond (2-octyl-cyanoacrylate) for topical skin wound closure [5], which was the first FDA approved CA for medical use. Ever since then, more and more medical-use CA have appeared on the market for different indications [6, 7].

Except for skin wounds, the gastrointestinal anastomosis is another important type of wound closure. However, the use of CA in this field is still limited, and no clearly documented clinical attempts have been made so far. Though substantial experimental efforts have been made, the results of animal studies have not yet been encouraging. Some experimental studies reported anastomoses could be well constructed with CA [8, 9]; while others reported a mortality rate as high as 30-40% [10, 11]. Besides large variations in results, inconsistencies with regard to the methodology were also noticed in those experiments. As it has been demonstrated that the anastomotic technique used in clinical gastrointestinal surgery influences the outcomes, we hypothesized that the inconsistent results of experimental studies are partly due to differences in their methods. Thus, the purpose of this systematic review is to summarize the experimental studies regarding CA application in intestinal and colorectal anastomosis, correlating the methodological details to the experimental outcomes.

METHODS

Search methods

This systematic review was performed according to the PRISMA (Preferred Items for Reporting of Systematic Reviews and Meta-Analyses) guidelines [12]. The systematic literature search was performed on the 5th of November 2012. The systematic search of literature was performed using the databases of Medline, Embase, Cochrane and Web-of-Science libraries. The same search strategy was used in all the databases. The search strategy encompasses the following:

(cyanoacrylate/de OR 'cyanoacrylate derivative'/de OR 'cyanoacrylic acid octyl ester'/de OR enbucrilate/de OR bucrilate/de OR 'poly (ethyl 2 cyanoacrylate)'/de OR (cyanoacryl* OR 'cyano acrylate' OR 'cyanoacrylic acid' OR 'octylcyano acrylate' OR enbucrilate* OR bucrilate* OR enbucrylate* OR bucrylate* OR butylcyanoacryl* OR fimomed OR histacryl OR histoacryl OR sicomet OR isobutylcyanoacrylat* OR ocrilate OR ocrylate OR octylcyanoacrylat* OR dermabond OR omnex OR glubran OR surgiseal OR floraseal OR 'derma flex qs' OR gluseal OR octyseal OR wormglu OR periacryl OR indermil OR liquiand OR xion):ab,ti) AND ('gastrointestinal surgery'/exp OR (('gastrointestinal tract'/exp OR 'digestive system'/exp) AND (surgery/exp OR (surg* OR operat* OR preoperat* OR postoperat* OR perioperat* OR intraoperat*):ab,ti)) OR (((gastr* OR digestiv* OR intestin* OR anal OR anus OR anorect* OR rect* OR bariatr* OR pancrea* OR stomach* OR antireflux* OR colon* OR colorect* OR bowel* OR duoden* OR esophag* OR oesophag*) NEAR/3 (surg* OR operat* OR postoperat* OR preoperat* OR perioperat* OR intraoperat* OR anastom* OR suture* OR adhesi* OR glue* OR sealant* OR hemosta* OR coat* OR lesion* OR wound* OR dehisc* OR disattach* OR attach*)) OR vagotom* OR colectom* OR gastrostom* OR stoma* OR appendectom*):ab,ti)

Study selection

Two independent researchers (Z.W. and G.B.) screened all the articles (the title and the abstract) in a standardized manner. Articles were included only if the CA glue was applied in an intestinal or colorectal anastomosis. The search was restricted to publications in English. Presentations, reviews and letters to editor were not included. All references from the selected articles were screened for further possible inclusions.

Data extraction

For all selected studies, a standard data extraction form was filled in, and the following data were extracted: year of publication, first author, subject (animal species), number of animals, glue (chemical name), glue (commercial name), usage (CA sutureless anastomosis / sealant), dosage, curing time, anastomotic material (additional material to create the anastomosis other than CA), suture material (chemical component), suture size, suturing technique, GI level, and outcome (positive / negative, judged according to conclusions of the articles).

RESULTS

A total number of 962 articles were found, from which 22 studies were included for final data analysis (Figure 1). Among these, seven articles had positive outcomes; eight had

neutral outcomes; the others had negative outcomes. As is listed in Table 1, CA with different molecular structures produced by different manufacturers were used and tested. The included articles were divided according to the chemical structure of the CA used, and their chemical names (commercial names if applicable) were listed. Further subdivisions were made according to the use of CA with regard to anastomosis (sutureless anastomosis or sealant).

Table 1. Cyanoacrylate adhesives used in the included studies

Chemical structure	Abbreviation	Trade name	Manufacturer
Metho-cyanoacrylate	MCA	910 Easterman	Ethicon (Somerville, New Jersey, USA)
Etho-cyanoacrylate	ECA	Pattex	Henkel (Dusseldorf, Germany)
N-butyl-cyanoacrylate	NBCA	Histoacryl (blue)	B. Braun (Melsungen, Germany)
	NBCA	Glubran 2	GEM Italia (Via reggio, Italy)
2-octyl-cyanoacrylate	OCA	Dermabond	Ethicon (Norderstedt, USA)
	OCA	Gluseal	GluStitch, Inc (Delta, BC, Canada)

Metho-cyanoacrylate (MCA)

Four studies were included that report the use of MCA [2, 10, 11, 13]. A sutureless anastomosis was created in all of them, and none of these studies had positive outcomes (Table 2).

In 1962, O'Neill et al. used MCA (Eastman 910) to create a sutureless anastomosis in canines' small intestine or colon. In this model, a clamp was used to construct an everted anastomosis [11]. They found that most of the intestinal anastomoses (11 / 12) were satisfactory and no death occurred, but 28.6% (4 / 14) of canines died when CA anastomoses were created in the colon [11]. A similar clamp was also used by Weilbaecher et al., who performed the intestinal anastomosis with a greater number of canines. Mortality rate as high as 34% (34/101), and no advantage of MCA were found when compared with conventional suture methods [10]. A high mortality rate of 22% (8 / 35) was also found when Gennaro et al. used an intraluminal gelatin stent to create a colonic MCA anastomosis in a rat model [2]. Different from those experiments, Linn et al. reported a canine study [13], in which no anastomosis-related mortality occurred. Anastomoses with MCA had less inflammation than the conventional group, but stricture occurred in 40% of the anastomoses when a new invagination technique to construct the MCA anastomoses was used [13].

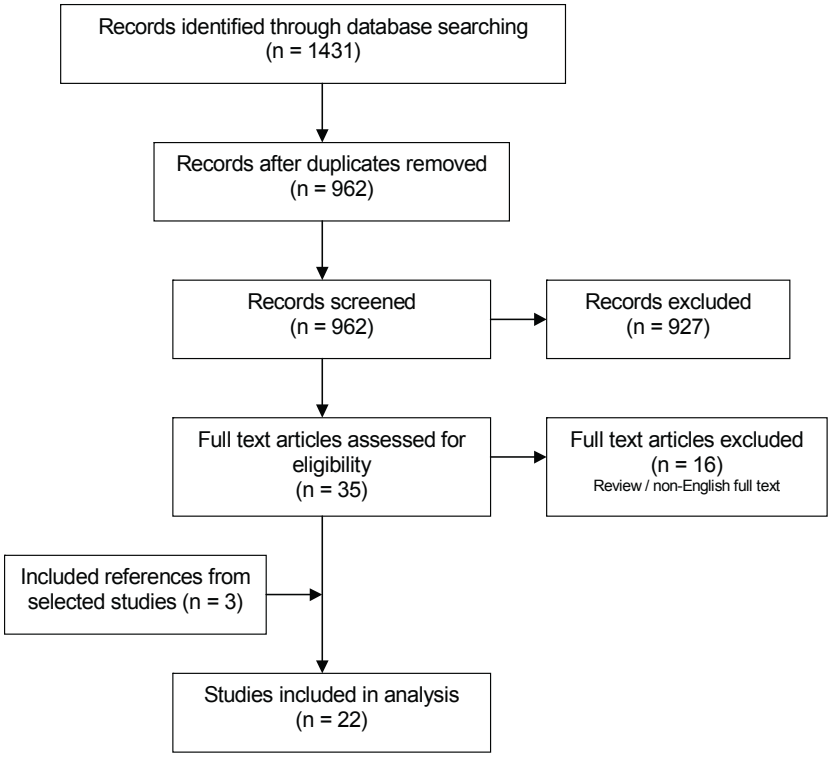

Figure 1. Study selection for relevant articles

Etho-cyanoacrylate (ECA)

Only one study used ECA to create the CA sutureless anastomosis [3], and no study used MCA as an anastomotic sealant (Table 2).

In 2009, Elemen et al. used ECA (Pattex) to construct end-to-end, side-to-side, or side-to-end intestinal anastomoses in a rat model. No deaths occurred during follow up, and no differences in bursting pressure were found between the CA anastomosis and sutured anastomosis, while higher hydroxyproline levels (a parameter of anastomotic wound healing) and shorter operating time were found in the CA groups [3].

N-butyl-cyanoacrylate (NBCA)

Nine studies regarding NBCA were included [8, 9, 14-20] (Table 2). Among these, three studies focused on the sutureless anastomosis [8, 16, 17], three studies looked into NBCA sealant [9, 18, 20], and the other three tested both applications [14, 15, 19]. In the NBCA studies, all four large animal studies had positive results [8, 9, 14, 15], while of the other five rat studies, only one had positive outcomes [20].

Matsumoto et al. reported a comparison between CA in different molecular structures (N-butyl-, Amyl-, Heptyl-cyanoacrylate) in a canine model of intestinal anastomosis. Only NBCA showed good wound healing without stenosis after four or twelve weeks [21]. Another comparison between NBCA (Glubran 2) and OCA (Dermabond) was performed in a porcine model [8]. The CA sutureless anastomoses were constructed in the colorectum with a modified stapling device, in which all the staples were taken out in advance. All the NBCA anastomoses were satisfactory, while two leakages occurred in the OCA group; NBCA was also superior to OCA regarding to the adhesion and stenosis severity [8]. Tebala et al. also tested NBCA with different suturing techniques in a porcine model [15]. They performed 11 different types of anastomosis. Good wound healing was observed in macroscopic, histological and angiographic examinations; foreign body reaction was even less in the sutureless anastomosis group than the sealant group [15]. Tebala et al. also created an insufficient anastomosis in a pig model by removing 1/5 of the sutures or staples from a normal anastomosis [9]. NBCA was then used to seal the defect. Anastomotic healing was sufficient, and no ileus occurred during the follow up [9].

Positive results of CA use were reported in a rat study by Ensari et al. [20]. In this study, the authors constructed an ischemic-reperfused intestinal anastomosis and used NBCA (Glubran 2) to reinforce it. Higher bursting pressures were found after the CA reinforcement with or without the initial ischemic intervention, while more adhesions were found in the CA groups [20]. Weiss et al. tested another NBCA (Histoacryl) and created gastrojejunal anastomoses in a rat model, comparing it with resorbable sutures. In this study, anastomotic healing regarding leakage rate, stricture, peritonitis, and mortality were similar between both groups. The only significant difference was a shorter operating time in the NBCA group [16]. Bae et al. tested the same glue in a rat model, in which they created NBCA (Histoacryl) reinforced anastomoses and the NBCA sutureless anastomoses in the rat colon. No leakage occurred in any of these groups, but more strictures, lower bursting pressure and more severe inflammation was found in the CA reinforced group and the CA sutureless group [19]. Similarly, a lower bursting pressure was also reported by Ozmen et al. in a CA sutureless colonic anastomosis with two holding sutures [17]. NBCA has also been tested in high-risk animal models. Kayaoglu et al. used 0.2 mL NBCA (Glubran 2) as sealant to reinforce the anastomosis in a fecally contaminated environment. Similar macroscopic wound healing and bursting pressure were found on day 3 and day 7 in both the CA group and the suture group; however, more inflammation and necrosis were found in the CA group [18].

Iso-butyl-cyanoacrylate (isoBCA)

Four studies regarding isoBCA were included [22-25], of which no study had positive results. Dating back to 1980, Kirkegaard et al. used isoBCA to create the sutureless anastomosis with a gelatin stent [25]. They found more stenosis and inflammation in the CA group, however these complications were significantly reduced when the CA anastomosis was covered with an omental tag [25].

High mortality was reported by all the other isoBCA studies. Stirling et al. used isoBCA to create the sutureless everted anastomosis, which resulted in a mortality rate of 27.0% (10/37) of canines [22]. In 1968, Hale et al. first used a rat model to compare the influence of isoBCA as sutureless anastomosis or as suture reinforcement. Twelve of 16 canines (75%) died in the sutureless anastomosis group, while conventional anastomoses or CA reinforced anastomoses were mostly satisfactory [23]. In 1971, Uroskie et al. used a canine model and performed two intestinal anastomoses in each animal, in which the distal anastomosis was sealed with isoBCA. Sixty percent (9/15) of the animals died during the follow-up due to anastomosis-related complications, mostly due to AL in the CA reinforced anastomoses [24].

2-octyl-cyanoacrylate (OCA)

Three studies on OCA were included [26-28]. None reported additional advantages in anastomotic healing when OCA was applied.

Kanellos et al. resected a segment of 1.0 cm in the rat transverse colon, and randomly chose OCA (Dermabond) or sutures to create the sutureless anastomosis. Similar leakage rates, bursting pressures and histological results were found between the CA and suture groups [26]. In 2009 Irkorucu et al. also used OCA (Gluseal) to seal or construct rat colonic anastomoses after inducing wound ischemia. Similar bursting pressure and hydroxyproline concentrations were found between groups, while more adhesions were found in the CA reinforced and the sutureless groups than the conventionally sutured groups [28]. However, in an ischemic anastomosis model by Nursal et al., the mechanical strength of the OCA (Dermabond) anastomosis was significantly lower on day 7 than the conventionally sutured groups; furthermore, a higher inflammatory response and necrosis were found in the OCA group [27].

Other

Galvao et al. used CA to assist a cuff apparatus to create an invaginated anastomosis on rat intestine. The chemical structure of the used CA was not described in this study, but satisfactory anastomoses were still found in both macroscopic and histological evalua-

tions, the CA anastomosis also cost much less time. However, after one and three days, tissue lesions due to CA toxicity were observed [29].

Method details

As is shown in Table 2, methodological details of each included study were listed. These details mainly focused on the material and technique used for the anastomosis construction.

CA dosage and curing time

Of all 22 included studies, only four studies specified the amount of CA used in each anastomosis. One study used 1.0 mL CA to create the sutureless anastomosis in a pig model [8], obtaining positive outcomes. 0.5 mL and 0.2 mL CA were also used in three rat models for creating sutureless anastomoses or as an anastomotic sealant [18, 20, 27]. In these rat studies, only one reported positive conclusions [20]. Only eight studies listed the curing time after CA application, which varied from 10 seconds to 4 minutes [2, 10, 11, 16, 22, 24, 29].

Animal species

Three different animal species were used in the included studies. Most studies used animal was the rat (14 studies), and four of them had positive outcomes [3, 20, 25, 29]. Six canine studies were included. All of them were performed in the 1960's and 1970's, while only one had positive conclusions [14]. Only three porcine studies were included, all showing positive conclusions [8, 9, 15].

Anastomotic construction

Fourteen studies described or had figures demonstrating the anastomotic pattern such as inverted (serosa to serosa), everted (mucosa to mucosa) or invaginated (mucosa to serosa) anastomosis. Six studies employed an inverted anastomosis [8, 9, 15, 16, 18, 24], among which three had positive outcomes [8, 9, 15]. Five studies used an everted anastomosis [2, 10, 11, 19, 22]; none of these had positive results. Three studies constructed an invaginated anastomosis [13, 14, 29], and two of them showed positive outcomes [14, 29].

Sutureless anastomosis constructed with CA was tested in 18 studies, of which five reported positive outcomes [3, 8, 14, 15, 29]. Different materials such as clamps, stents, modified staplers or holding sutures were used to approximate the two cutting edges, as is shown in Table 2. Within those materials, none of the studies that used an anastomotic clamp (3 studies [10, 11, 22]) showed positive outcomes. In the other studies which used holding sutures or a modified stapler to create CA anastomosis, mostly the canine and porcine studies (3/4) had positive results [8, 14, 15]. In the contrast, only one rat study (1/8) with holding sutures had positive results [3].

Nine studies tested CA as a sealant after construction of a primary anastomosis; among these, four reported positive results (two porcine studies [9, 15], one rat study [20]). Most of these studies used different suture materials (silk, polypropylene or glycolic acid) and varying suture techniques for the construction of the primary anastomosis. Except for materials, different suture sizes were tested as well. Two porcine studies used 3/0 sutures, both of these having positive outcomes [9, 15]. Five studies used 5/0 or 6/0 sutures, mostly in rat models [18, 19, 23, 24, 28], and none of them conclude positively. One rat study used 7/0 sutures, and it had positive outcomes [20].

DISCUSSION

Substantial efforts have been made to test the feasibility, effect and safety of the use of CA in intestinal and colorectal anastomosis. Using CA as suture-replacement, early experiments in the 1960s and the 1970s failed to create a successful sutureless anastomosis [10, 30], some recent results, though promising, still vary from one to another. Previous opinions mainly put the blames on the chemical characteristics of CA [2, 7]. Indeed, intra-abdominal (actually intra-peritoneal) application of CA is distinct from its topical use such as skin wound closure, because intra-abdominally applied CA can only be absorbed, metabolized, and degraded by the body instead of falling off by itself. However, this still does not explain everything, as most current available CA contain longer molecular chain, which are less toxic than short length CA [7]. Creating anastomoses with artificial materials not only requires a good mechanical strength, but should also induce a good physiological wound healing which eventually supports the bowel continuity and biomechanical strength by itself. All these influences indicate the importance to investigate methodological details in CA application, such as selection of CA molecular structure, dosage, animal model, and anastomotic technique. With this aim, this review summarizes the studies regarding application of CA in intestinal and colorectal anastomosis, linking the method details to the outcomes. We found that these studies contained great inconsistencies in the methods. Furthermore, some important factors and details in the methods might influence outcomes, which are discussed respectively below.

CA molecular structure

CA was tested as a potential suture replacement because of its strong adhesiveness, which makes it possible to seal a technically flawed anastomosis, and even to create a sutureless anastomosis. Our previous ex-vivo study showed that adhesiveness is similar among different types of CA, but is much stronger than that adhesive strength in other categories of tissue adhesives (unpublished data). When choosing CA for specific surgical applications, it is therefore more important to take other factors into account, such as tissue toxicity [31, 32].

Table 2. Synopsis of results: cyanoacrylate application in intestinal and colorectal anastomosis

TABLE II. Synopsis of Results: Cyanoacrylate Application in Intestinal and Colorectal Anastomosis

Year	Author	Subject	*n*	Glue (Chemical Name)	Glue (Trade Name)	Usage	Dosage	Curing Time	Anastomotic Material	Suture Material	Suture Size	Anastomotic Pattern	GI Level	Outcome
1962	O'Neill et al.[11]	Canine	26	MCA	910 Easterman	Anastomosis	NS	60s	Clamp	–	–	Evert	Intestine Colon	+/–
1964	Weilbaecher et al.[10]	Canine	101	MCA	910 Easterman	Anastomosis	NS	3 min	Clamp	–	–	Evert	Intestine	–
1966	Linn et al.[13]	Canine	30	MCA	910 Easterman	Anastomosis	NS	NS	Invaginate	–	–	Invaginate	Intestine	+/–
1976	Gennaro et al.[2]	Rat	35	MCA	910 Easterman	Anastomosis	NS	10–20s	Gelatine stent	–	–	Evert[a]	Colon	–
2009	Elemen et al.[3]	Rat	96	ECA	Pattex	Anastomosis	NS	NS	Holding suture	Polyglactin 910	5/0	NS	Intestine	+
2011	Paral et al.[8]	Porcine	12	NBCA vs. OCA	Glubran 2 Dermabond	Anastomosis	1.0ml		Modified stapler	–	–	Invert	Colon	+ BCA +/– OCA
2001	Weiss and Haj[16]	Rat	64	NBCA	Histoacryl	Anastomosis	NS	3–4min	Holding suture	Vicryl sutures	6/0	Invert	Stomach-jejunal	+/–
2004	Ozmen et al.[17]	Rat	40	NBCA	Histoacryl	Anastomosis	NS	NS	Holding suture	Polypropylene	5/0	NS	Colon	–
1995	Tebala et al.[9]	Porcine	10	NBCA	NS	Sealant	NS	NS	Suture Stapler	Silk suture	3/0	Invert	Intestine Colon	+
2009	Kayaoglu[18]	Rat	80	NBCA	Glubran 2	Sealant	0.2 ml	NS	Suture	Glycolic acid	5/0	Invert	Colon	–
2010	Ensari[20]	Rat	40	NBCA	Glubran 2	Sealant	0.2 ml		Suture	Polypropylene	7/0	NS	Intestine	+
1967	Matsumoto[14]	Canine	70	NBCA vs. other CA	NS	Anastomosis Sealant	NS	NS	Invaginate	NS	NS	Invaginate	Intestine	+ BCA – others
2010	Bae[19]	Rat	60	NBCA	Histoacryl	Anastomosis Sealant	NS	NS	Suture	Polypropylene	5/0	Evert[a]	Colon	–
1994	Tebala et al.[15]	Porcine/ Rat	55/30	NBCA	NS	Anastomosis Sealant	NS	NS	11 kinds of anastomosis	Silk suture	3/0	Invert	Intestine Colon	+
1965	Stirling and Cohn[22]	Canine	37	isoBCA	NS	Anastomosis	NS	2–3min	Clamp	–	–	Evert	Intestine	+/–
1980	Kirkegaard et al.[25]	Rat	60	isoBCA	NS	Anastomosis	NS	NS	Stent	–	–	NS	Colon	+/–
1968	Hale and Ellis[23]	Rat	66	isoBCA	NS	Anastomosis Sealant	NS	60s	Holding suture	Silk suture	5/0	NS	Intestine Colon	+/–
1971	Uroskie et al.[24]	Canine	15	isoBCA	NS	Sealant	NS	3–4 min	Suture	Silk suture	5/0	Invert	Intestine	–
2002	Kanellos et al.[26]	Rat	40	OCA	Dermabond	Anastomosis	NS	NS	Holding suture	Polypropylene	6/0	NS	Colon	+/–
2004	Nursal et al.[27]	Rat	90	OCA	Dermabond	Anastomosis	0.5 ml	NS	Holding suture	Polypropylene	7/0	NS	Colon	–
2009	Irkorucu et al.[28]	Rat	40	OCA	Gluseal	Anastomosis Sealant	NS	NS	Holding suture/Suture	Polypropylene	6/0	NS	Colon	–
2007	Galvao et al.[29]	Rat	18	NS	NS	Anastomosis	NS	2–4 min	Cuff	–	–	Invaginate	Intestine	+

[a] Shown on the picture of the inclusion.
Abbreviation of different cyanoacrylate is specified on Table I. NS = not specified.

In general, shorter chain CA monomers (i.e. methyl-cyanoacrylate) create significant amounts of heat during polymerization, and are known to degrade into toxic end-products, resulting in severe tissue reaction and inflammation, while longer chain-length CA is associated with more hydrophobic and bacteriostatic properties and less tissue toxicity [2, 7]. However, in intestinal and colorectal anastomoses, data from the studies that compared different CA seem to prefer in NBCA to other shorter or longer monomers [21, 27, 33]. Our results in this review also agree with this, as most CA studies with MCA, isoBCA or OCA had negative outcomes, and more than half of the NBCA studies reported positive ones [8, 9, 14, 15, 20]. Nevertheless, one must note that, with the current limited data, it is still too early to conclude which CA is the best for intestinal and colorectal anastomoses. The biological properties of CA are influenced not only by its molecule structure, but also by the additional components added into the adhesives. Developments in biochemistry may bring further improvements in CA molecule structure for specific use as intestinal and colorectal anastomotic.

CA dosage

As well as the molecular structure, an important role in the tissue reaction of CA is also played by CA dosage. Unfortunately most studies did not provide details on this. One can imagine that an overdose of CA, comparable to a very high number of sutures or staples around the anastomosis, may lead to more side effects rather than a further increase in anastomotic strength. As CA is known to react exothermically during polymerization, CA overdose may cause direct tissue damage during polymerization, and increase adhesion formation, lengthening the long-term degradation time.

The currently available information is not enough to allow an analysis of the optimal amount of CA for intestinal and colorectal anastomosis in different animal models. According to the study of Paral et al., 1.0 mL of CA should be enough to construct a sutureless anastomosis in the porcine model [8]. Compared with the dosage for porcine anastomosis, 0.5 mL and 0.2 mL CA might be too much for rat anastomosis, as the rat colon is more than ten times smaller. Some clues on optimal CA dosage can be found from data in vascular surgery, where only 0.4 µl CA was enough to create vessel anastomosis in rats [34]. While the manufacturers' original applicator can be directly used in porcine or other big animal models, a small syringe with a blunt needle is recommended in rodent models to ensure accurate CA application.

Animal model

Not only due to the poor outcomes from the previous literatures, but also because of ethical concerns, canine models might not be suitable for future CA studies. This review shows that all previous studies using porcine models had positive results, implying that

this might be the best large animal model for future CA studies regarding to intestinal and colorectal anastomosis. This is also supported by the previous systematic review, which also found the porcine model to be superior to those with other animal species, as the pig's GI tract is much more similar to a human's than a rodents' [35]; this enables human-size surgical tools and human-dose CA to be used directly on porcine. However, the high costs of large animal models result in most animal studies on CA being performed on rat models. As stated earlier, most of the previous rat studies in this field were not a success. This is most probably due to the small size of the rat. Almost all techniques, and also the material size and dosage will thus need to be specifically adjusted for rats.

Anastomotic technique

Construction of a successful anastomosis is not simply connecting two endings together and reaching a mechanical strength as high as possible. A good and safe physiological wound healing without complications (i.e. anastomotic leakage, adhesion, stenosis) is more important from a clinical perspective [36]. For anastomosis of the digestive tract, the inverted-suture technique has been demonstrated to lead to a sufficient biomechanical strength as well as a better wound healing than the everted pattern; invaginated anastomosis is hardly used in clinic due to higher risks to develop stenosis and other complications [36-39]. Outcomes from CA research also confirm this, as all the studies using everted anastomosis had negative results, while more than half of those using inverted anastomosis had positive outcomes [8, 9, 15]. Comparing data from the included studies, we recommend that an inverted-suturing technique should also be used in future CA studies.

Overall, the use of CA in intestinal and colorectal anastomosis has two functions: to construct a sutureless anastomosis, or to reinforce a primary anastomosis as an anastomotic sealant. For sutureless anastomosis, various materials have been used to approximate the two bowel endings before CA application. Among these materials, the modified circular stapler (in which the staples are removed prior to use) in large animal models might be a good option because the CA can easily be applied on the inverted anastomosis [33]. As a small stapler for rodents is lacking, the use of holding sutures was described in most of the rat studies. However, it does not yet seem to be satisfactory according to our results. One possible reason is that the holding sutures are not able to guarantee the inverted connection, thus creating an everted anastomosis that may complicate wound healing if CA is polymerized between the two wound edges. Also, instructions for topical usage of CA in skin wound closure indicate that the application of CA between the wound edges should be prohibited [7]. To ensure an inverted anastomosis, a special stent might be a good replacement for holding sutures, but more work on this is still required.

For the use of CA as a sealant, the suture material and its size are also important factors for a good anastomosis. Our data shows that 3/0 sutures, often used in human intestinal and colorectal anastomosis, are suitable for large animal models; 5/0 sutures may be inappropriate for the rat intestinal and colorectal anastomosis, as no study reported positive outcomes with these. This may due to the large size of the 5/0 sutures (diameter of absorbable 5/0 suture: 0.15-0.199 mm [40]) relative to that of the rat colon (thickness of adult male rats: around 0.6 mm [41]). The 3/0 sutures (0.30-0.349 mm [40]) are much smaller and lighter compared to the human colon (thickness: 2.6 mm [42]) or porcine colon. For rat intestinal and colorectal anastomosis, smaller size sutures such as 7/0 (0.07-0.099 mm [40]) or 8/0 (0.05-0.069 mm [40]) seemed to be proper while more evidence is still required.

CONCLUSION

In view of the positive outcomes of the large animal experiments, the application of CA in intestinal and colorectal anastomosis seems promising. However, the great inconsistency and lack of detailed information in the previous literature made comparison of methodology difficult. To achieve a better consistency, studies should provide more details in the methods. If the dosage of CA, the choice of animal model, inverted anastomosis construction, and other method details also are improved, future studies will achieve better intestinal and colorectal anastomoses with CA.

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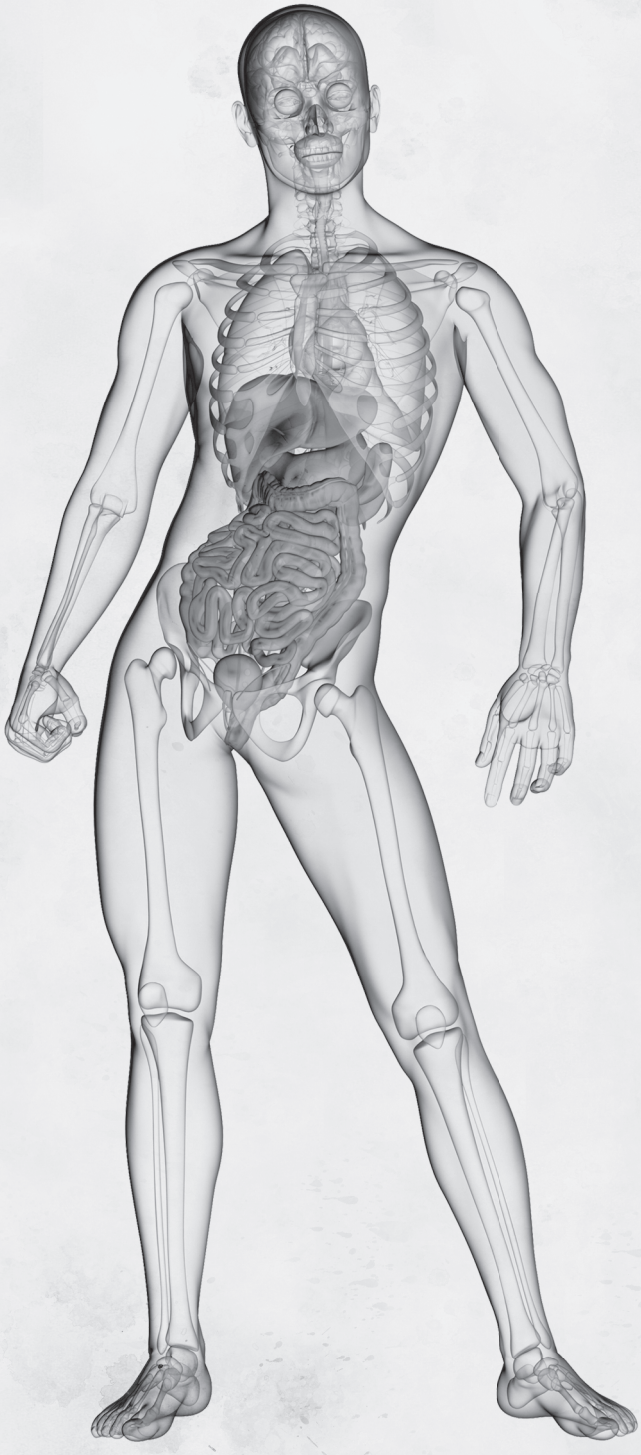

PART 3

Surgical complications: prevention in patients at risk

Chapter 10

The prevention of colorectal anastomotic leakage with tissue adhesives in a contaminated environment is associated with the presence of anti-inflammatory macrophages

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ABSTRACT

Background

Colorectal anastomoses created in a contaminated environment result in a high leakage rate. This study investigated whether using anastomotic sealants (TissuCol®, Histoacryl® Flex, and Duraseal®) prevents leakage in a rat peritonitis model.

Methods

Sixty-seven Wistar rats were divided into control and experimental groups (TissuCol-, Histoacryl-, and Duraseal-group). Peritonitis was induced one day before surgery with the caecal ligation puncture model. On day 0, colonic anastomosis was constructed with sutures and then sealed with no adhesive (control group) or one select adhesive (experimental groups). Bursting pressure, abscess formation and adhesion severity were evaluated on day 3 or day 14. Hematoxylin and eosin staining and immunohistochemical staining for CD4, CD8, CD206 and iNOS were performed.

Results

On day 3 bursting pressures of the TissuCol-group (120.1±25.3 mmHg), Histoacryl-group (117.3±20.2 mmHg), and Duraseal-group (123.6±35.4 mmHg) were significantly higher than the control-group (24.4±31.7 mmHg, $p<0.001$). Abscesses around the anastomosis were found in the control-group (6/7) and Duraseal-group (2/9), but not in the TissuCol-group or Histoacryl-group. A higher number of CD206+ cells (M2-macrophages), a lower number of iNOS+ cells (M1-macrophages), a higher M2/M1 index, and a higher CD4+/CD8+ index were seen at the anastomotic site in all experimental groups compared with the control group on day 3. On day 14 abscesses were only found in the control group. Adhesion severity in the Duraseal-group was significantly lower than that in the control group ($p=0.001$).

Conclusions

Anastomotic sealing using TissuCol®, Histoacryl® Flex, or Duraseal® seems to be an effective and safe option to prevent leakage in contaminated colorectal surgery. The presence of large numbers of anti-inflammatory macrophages seems to be involved in preventing the leakage.

INTRODUCTION

Under certain conditions such as abdominal trauma or perforation in diverticulitis and colorectal carcinoma, emergency surgery is initiated in order to repair bowel defects in a contaminated environment. Instead of a Hartmann's procedure for perforated diverticulitis, primary anastomosis has become a well-accepted intervention in selected patients [1-4], resulting in similar or even better clinical outcomes regarding postoperative mortality and complication rates than Hartmann's procedure [1, 5, 6]. However, performing primary anastomosis in a contaminated environment is challenging and still causes substantial leakage [7-9], especially in urgent situations. Patients with perforated diverticulitis who underwent primary anastomosis alone suffered a leakage rate of 19.3% [10], which is much higher than the leakage rate of approximately 9% following a low anterior resection for rectal cancer [11].

Intra-abdominal sepsis induces nitric oxide production at the anastomotic site, which activates substantial inflammatory responses and subsequently impairs the collagen synthesis thereby delaying anastomotic healing [12, 13]. Macrophages are one of the main factors in the inflammatory response, and based on their behavior this response is either pro-inflammatory (M1) impairing wound healing or anti-inflammatory (M2) promoting wound healing; other immune cells such as T lymphocytes, though not fully understood, were also reported to be involved in the response [14]. Interestingly, the deleterious influence of inflammation is localized on the anastomosis and does not affect new collagen synthesis in the uninjured colon, where the biological barrier is intact [12]. This suggests that using a tissue adhesive or sealant as an artificial barrier to obstruct contact between intra-abdominal pathogens and anastomosis may reduce the deleterious effects of inflammation, thus preventing anastomotic leakage. Among different tissue adhesive compounds, fibrin glue and cyanoacrylate glue have been substantially investigated in both experimental and clinical studies with promising results [15-17]. Other sealants, such as polyethylene glycol glue, also had satisfactory results as an adhesion barrier system [18]. These tissue adhesives have been tested in different environments [17, 19, 20], however knowledge regarding their influence on anastomotic healing in a contaminated environment is still limited. We therefore conducted an experiment under conditions of peritonitis induced by the rat caecal ligation puncture (CLP) model that has been used in previous studies [21, 22]. Colonic anastomoses were constructed and sealed with select tissue adhesives. This study aimed to investigate the influence of the select tissue adhesives on anastomotic healing, and to determine whether they are safe and effective solutions to prevent anastomotic leakage under contaminated conditions.

METHODS

Animals

Male Wistar rats, weighing 250-350 grams, were purchased from a licensed breeder (Harlan Laboratories, Boxmeer, the Netherlands). All rats were bred under specific pathogen-free conditions, and kept under standard laboratory conditions. Standard rat chow and water were supplied ad libitum. The research protocols of all experiments were approved by the Ethical Committee on Animal Experimentation of Erasmus University Rotterdam.

Peritonitis model

To induce peritonitis, the rat caecal ligation puncture (CLP) model was used [21, 22]. In brief, rats were anaesthetized with isoflurane/oxygen inhalation, and the abdomen was opened through a midline incision. Then, the caecum was ligated distally to the ileocecal valve with a non-absorbable nylon suture (Ethilon 4-0, Ethicon, Somerville, USA), maintaining the continuity of the bowel. The distal caecum was punctured once with an 18-gauge needle, and was gently compressed until feces were extruded. The abdomen was closed with two layers of running sutures (Safil 5-0, B Braun, Melsungen, Germany). Wellness of all rats was evaluated during the follow-up, and animal with severely compromised wellness (i.e. ceased food intake, circulatory or respiratory difficulty, severe weight loss, severely abnormal locomotion) would be euthanized and examined prematurely for humane endpoint.

Surgical technique and follow up

Twenty-four hours later, the rat was anesthetized again. The abdomen was reopened, and a culture swab was taken to confirm peritonitis. After that, 6 mg/kg of gentamicin (Centrafarm, Etten-Leur, The Netherlands) was injected intramuscularly. The ligated caecum was resected, the abdominal cavity was rinsed with at least 20mL phosphate buffered saline (PBS, 37°C), and colorectal anastomosis was performed afterwards. A colon segment of 1 cm in length was resected approximately 3 cm proximally to the peritoneal reflection. An end-to-end one-layer continuous anastomosis was constructed in an inverted fashion with Dafilon 8-0 (B. Braun, Melsungen, Germany). One researcher (ZW) performed all anastomoses under microscopic vision enhancement. Following that, one tissue adhesive was selected and applied at two parts: at the descending colon around the anastomosis as a sealant (distal segment), and 1 cm in length at the beginning of the ascending colon (proximal segment). The tissue adhesives were prepared according to the instruction manuals. Because all tissue adhesive were designed for human patient with much larger amount of adhesive than the amount we applied on rats, one tissue adhesive was randomly chosen on the operation day and reused within the manual-instructed time period until reaching the planned group size. If necessary,

a blunt needle was used to guide an accurate adhesive application around the anastomosis. The average amount of applied tissue adhesive is listed in Table 1. According to the applied tissue adhesive, rats were divided into the control group, TissuCol-group, Histoacryl-group, and Duraseal-group. To ensure full polymerization of tissue adhesives after application, we allowed the adhesives to set for five minutes before closing the abdomen with a running suture (Safil 5-0, B Braun, Melsungen, Germany).

On postoperative day (POD) 3 or POD14, rats were anesthetized again and re-laparotomy with a U-shape incision was performed. The abdomen was examined for manifestations of abscess formation, anastomotic dehiscence, and adhesions. Adhesion severity was recorded using the Zühlke score [23]. Bursting pressure was determined afterwards, and the bursting location was noted. The samples from the distal and proximal segments were harvested for histological examination, then the rat was euthanized.

Table 1. Chemical components and postoperative mortality

	Chemical components	Manufacturer	Amount per anastomosis (mL)	Number of animals	Postoperative deaths
Control	-	-	-	17	3
TissuCol®	Fibrin glue, with aprotinin	Baxter (Deerfield, USA)	0.1	16	0
Histoacryl® Flex	n-butyl-2-cyanoacrylate	B. Braun, (Melsungen, Germany)	0.02	17	3
Duraseal®	Polyethylene glycol	Covidien, (Mansfield, USA)	0.1	17	1
Total	-		-	67	7

Note: Overall mortality 7 / 67 = 10.4%.

Histology and immunohistochemistry

All the harvested segments were fixed overnight in 4% buffered formaldehyde and embedded in paraffin. The distal samples were cut longitudinally and the proximal samples were cut transversely, both in a depth of 5 micrometers. Hematoxylin and eosin (HE) staining was performed in all samples.

Three parameters including inflammatory cell infiltration, fibroblast activity, and collagen deposition at the anastomotic site were evaluated for all the HE stained distal samples. For each parameter a ranking was made for all the slides with the following strategy: first, one researcher (ZW) and one pathologist (KL) performed a blind evaluation of each slide under a microscope using the Ehrlich and Hunt numerical scale as modified by Phillips et al. [24]. After scoring, a ranking was made within the slides having the same score by

cross-comparing the slides. Then the slides on the margin of each score were compared and adjusted again (e.g. compare highest ranked ones with score 1 with lowest ranked ones with score 2). Finally all the ranked slides were consecutively rearranged (sorted from the lowest to highest rank) and minor modifications were made if necessary.

Immunohistochemical staining for CD4 (T helper cells), CD8 (T suppressor cells), CD20 (B lymphocytes), iNOS (M1-macrophages), and CD206 (M2-macrophages) was performed on distal samples. The slides were first deparaffinized and boiled in Tris/EDTA pH 9.0 for 15 min. Endogenous peroxidase activity was blocked with 1.5% H2O2 in PBS for 10 min. Slides were blocked for 30 min with a 5% non-fat dry milk in PBS solution. The primary antibody of CD4 (1:100, Emelca Bioscience, Breda, Netherlands), CD8 (1:200, AbD Serotec, Kidlington, UK), CD20 (1:100, Emelca Bioscience, Breda, Netherlands), CD206 (1:400, Abcam plc, Cambridge, UK), or iNOS (1:1600, Abcam plc, Cambridge, UK) was applied respectively, and the slides were incubated overnight at 4°C. On the second day the slides were washed with PBS, and subsequently incubated with Envision secondary rabbit-anti-mouse or rabbit-anti-rabbit antibody (DAKO, Glostrup, Denmark) for 30 min. Diaminobenzidine (DAKO, Glostrup, Denmark) was used for visualization of antigen-antibody reactivity. Finally all slides were counterstained with hematoxylin, dehydrated and mounted.

A blinded investigator using a microscope under 40x10 magnification counted the positive cell amount of each staining. For the anastomotic site, three fields were selected: one on each cutting edge, the other one on the interface. For the serosa-glue site, three fields were also chosen at the interface of the tissue adhesive and adjacent tissue; in the control group, three fields were chosen at the interface of adhesion and adjacent tissue. The number of positive cells for each staining was counted, and the average of the three fields was used for analysis. An M2/M1 index was calculated with the equitation blew. A natural logarithm was used to adjust the data from exponential distribution into linear distribution. A similar equitation was also used for the CD4/CD8 index.

$$M2/M1\ index = \ln (Number\ of\ CD206^{+}\ cells)/(Number\ of\ iNOS^{+}\ cells)$$

Statistical analysis

Statistical analysis was performed with SPSS 21.0 (IBM software, USA). Data were presented as mean (S.D.) or percentage. The one-way analysis of variance was performed with the Kruskal Wallis Test or Chi-square Test, and a p-value < 0.05 was considered to indicate statistical significance. In multiple comparisons, α was corrected with the number of comparisons with the following formula: $\alpha' = \alpha / N$ ($\alpha = 0.05$, N = number of comparisons). When a significant difference was reached in the Kruskal Wallis Tests,

multiple comparisons were made between the control group and each corresponding tissue adhesive the Mann-Whitney U Tests or Chi-square Tests in this study, so N = 3 was chosen for correction. Only a p'-value < 0.017 (0.05/3) was considered to indicate statistical significance in the multiple comparisons. All reported p-values were two-sided.

RESULTS

A total number of 67 rats were used in the experiment and seven rats (10.4%) died during follow-up. Six deaths occurred on the day of the operation due to septicemia, but no anastomotic related complications were observed during autopsy. One rat had bloody stools after its operation and died on POD3, with its autopsy showing abscess formation on the anastomosis. There was no statistical difference in mortality rate between groups (Table 1).

After induction of fecal peritonitis with CLP model, all rats manifested septic symptoms such as compromised activities, nasal/ocular exudates, fluffy hair, diarrhea and weight loss, but no rat was prematurely euthanized for humane endpoint. On the operation day, weight loss were seen in all rats with an average of 10 to 12 grams in each group; no significant difference was found between groups. Abdominal fecal peritonitis were observed in all rats, manifesting as existence of ascites with fecal content. Abdominal culture tests of the ascites further confirmed bacterial contamination with Gram-positive (e.g. Enterococcus, Staphylococcus) and Gram-negative (e.g. Escherichia coli, Proteus) flora.

Intra-abdominal observations

On POD3, anastomotic dehiscence occurred in 28.6% (2/7) of rats in the control group, but not in other groups. Abscesses on the anastomosis was found in 85.7% (6/7) of rats in the control group, 22.2% (2/9) of rats in the Duraseal-group, but not in the TissuCol-group or Histoacryl-group, and it was significantly different between groups ($p < 0.0001$). On POD14, abscess formation on the anastomosis were found in one rat from the control group whereas none was found in the other groups.

The bursting pressure in the control group was 24.4 ± 31.7 mmHg. This was significantly lower than that in the TissuCol-group (120.1 ± 25.3 mmHg, p = 0.001), Histoacryl-group (117.3 ± 20.2 mmHg, p = 0.001), and Duraseal-group (123.6 ± 35.4 mmHg, p = 0.001, Figure 1). In the control group, 85.7% of the segments burst at the anastomotic line during the test, while the rates in the other groups differed between 28.6% (Histoacryl-group) and 50% (TissuCol-group). On POD14, most anastomotic segments did not burst at the site of the anastomosis in the ABP tests.

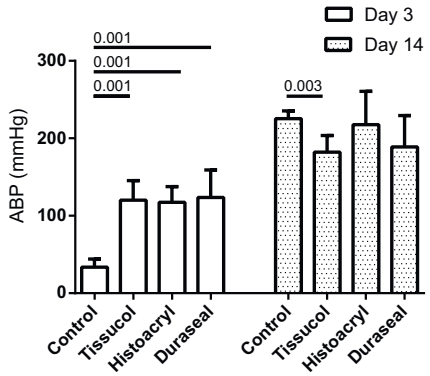

Figure 1. Comparison of anastomotic bursting pressures (ABP) on postoperative day (POD) 3 and 14. Values are mean (S.E.M.). The On POD3, overall comparison, p = 0.001, Kruskal-Wallis Test. On POD14, overall comparison, p = 0.039, Kruskal-Wallis Test. The p values of Mann-Whitney pairwise comparisons are illustrated in the figure when relevant.

Anastomotic adhesion formation was found in all rats. On POD3, the number of adhesions was significantly different between groups (p = 0.012). An average adhesion number of 3.7 was found in the control group, which was significantly higher than the TissuCol-group (average: 1.8; p = 0.004) and Histoacryl-group (average: 1.9; p = 0.015), but not significantly higher than the Duraseal-group (average: 2.8). Most rats were scored 2 in the Zühlke score (blunt dissection possible but partly sharp dissection possible; beginning of vascularization), and no difference was found between groups. In contrast, on POD14, the number of adhesions was similar between groups (average varied between 1.4 and 1.7), while their severity significantly differed (p = 0.004). The lowest adhesion score was found in the Duraseal-group (average of 1.3; firm adhesion, easy to separate by blunt dissection; no vascularization). It was significantly lower than the control group (p = 0.001), which had an average severity of 3.1 (lysis possible but sharp dissection only, clear vascularization).

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Anastomotic site

All the distal-segment slides showed an acute inflammatory response at the anastomotic site on POD3. The inflammatory cell infiltration was highest in the control group, which was significantly higher than that in the Histoacryl-group (p = 0.009). Lower fibroblast activity and collagen deposition was seen in the control group compared with the experimental groups, but their differences were not statistically significant (Figure 2). A lower number of CD206+ cells (M2) and a higher number of iNOS+ cells (M1) were seen

in the control group, and the M2/M1 index for the control group was also statistically significantly lower than all the tissue adhesive groups respectively (p = 0.002; Figure 3). A significant correlation was found between the ABP value and the M2/M1 index (R = 0.682; $p < 0.0001$). Similar changes were also seen in the CD4+ cells (T helper cells), CD8+ cells (T suppressor cells), and CD4+/CD8+ index (Figure 4), although only the difference between the control group and the TissuCol-group regarding CD8+ cells was statistically significant.

On POD14 most slides demonstrated sufficient wound healing on the anastomosis, showing as re-continuity of mucosal and muscle layers, less inflammatory cell infiltration and higher collagen deposition. There was no significant difference in fibroblast activity and collagen deposition between the control group and the adhesive groups. A higher number of iNOS+ cells (M1) and a lower M2/M1 index was seen in the control group compared with the tissue adhesive groups (Figure 3).

Serosa-glue interface

On POD3, similar numbers of iNOS+ cells and CD206+ cells were seen at the serosa-glue interface. The M2/M1 index, though higher in the tissue adhesive groups, was not significantly different between groups. On POD14, a significant higher M2/M1 index was observed in the Histoacryl-group compared with the control group at the serosa-glue interface (Figure 5).

Proximal samples

In proximal samples, except for a minimal number of inflammatory cells, evidence of an inflammatory response was not seen in the control group. Similar to the distal samples, a moderate reaction was seen at the serosa-glue interface in the TissuCol-group, Histoacryl-group and Duraseal-group on POD3, which manifested as macrophage infiltration around the tissue adhesive, without interrupting the continuity of the mucosal, sub-mucosal and muscle layers of the colon. On POD14, the foreign body reaction in the TissuCol-group was observed to have significantly reduced, and the glue was not observed (neither macroscopically nor microscopically) in 75% (6/8) of the rats. The reaction in the Histoacryl-group and Duraseal-group was still moderate.

DISCUSSION

Performing anastomosis in a contaminated environment results in a high leakage rate of colorectal anastomosis and poor clinical outcomes, threatening patient's safety. To investigate whether using tissue-adhesive sealants prevents leakage in a contaminated

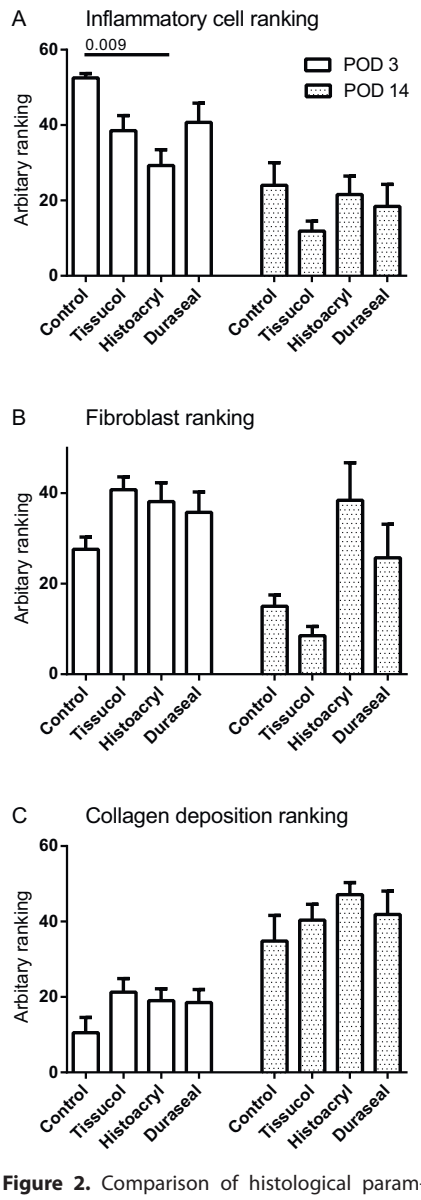

Figure 2. Comparison of histological parameters on postoperative day (POD) 3 and 14. Values are mean ranking (S.E.M.). On POD3, overall comparison of inflammatory cell infiltration ranking (2.A) yielded a p = 0.003 with the Kruskal-Wallis Test. Overall comparisons resulted a p > 0.05 in the other parameters, Kruskal-Wallis Test. The p values of Mann-Whitney pairwise comparisons are illustrated in the figure when relevant.

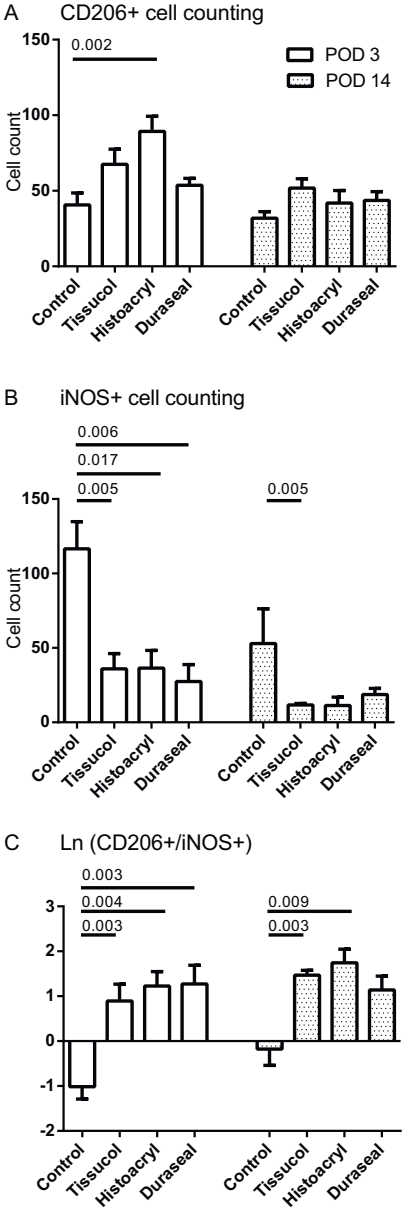

Figure 3. Comparison of macrophage subtype (i.e. M2 and M1) amount at anastomotic site on postoperative day (POD) 3 and 14. Values are mean of cell count (S.E.M.). On POD3, overall comparison of M2 (3.A), p = 0.003; M1 (3.B), p = 0.011; M2/M1, (3.C) p = 0.002, Kruskal-Wallis Test. On POD14, overall comparison of M2 (3.A), p > 0.05; M1 (3.B), p = 0.016; M2/M1, (3.C) p = 0.010, Kruskal-Wallis Test. The p values of Mann-Whitney pairwise comparisons are illustrated in the figure when relevant.

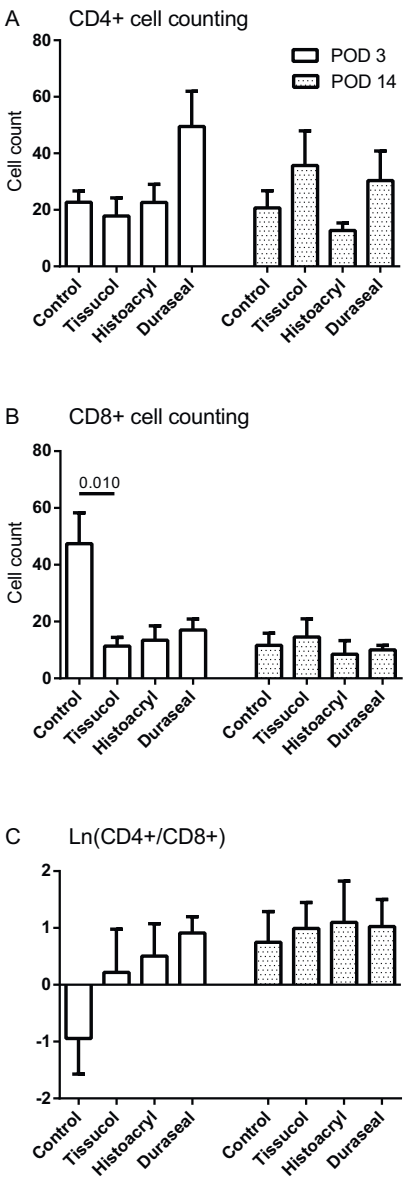

Figure 4. Comparison of T lymphocyte amount at anastomotic site on postoperative day (POD) 3 and 14. Values are mean of cell count (S.E.M.). On POD3, overall comparison of CD4+ (4.A), p = 0.042; CD8+ (4.B), p = 0.027; CD4+/CD8+, p > 0.05 (4.C), Kruskal-Wallis Test. Overall comparisons on POD14 all resulted a p > 0.05, Kruskal-Wallis Test. The p values of Mann-Whitney pairwise comparisons are illustrated in the figure.

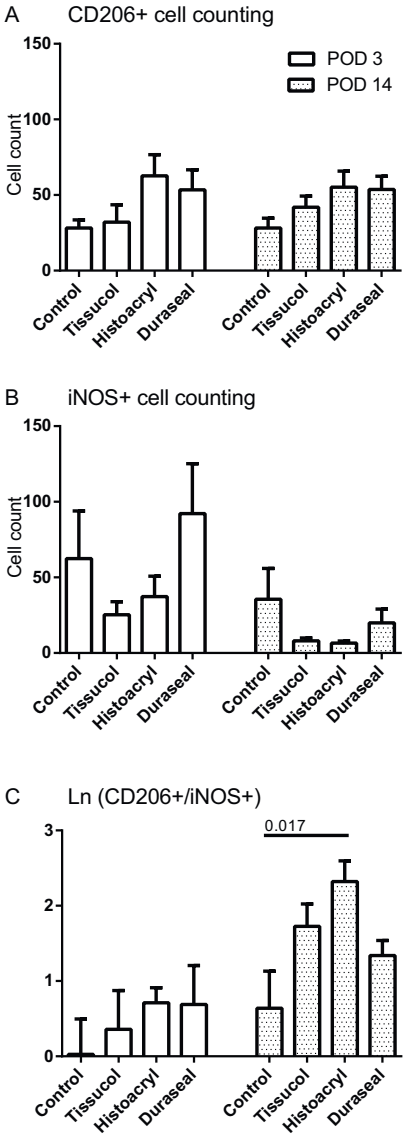

Figure 5. Comparison of macrophage subtype (i.e. M2 and M1) amount at serosa-adhesive site on post-operative day (POD) 3 and 14. Values are mean of cell count (S.E.M.). Despite overall comparison of M2/M1 on POD14 (5.C) yielded a p = 0.036, all the other overall comparisons on POD3 or 14 resulted a p > 0.05, Kruskal-Wallis Test. The p values of Mann-Whitney pairwise comparisons are illustrated in the figure.

environment, we constructed colorectal anastomoses under fecal contamination using the rat CLP model. All three chosen tissue adhesives increased the biomechanical strength of anastomosis in the short term, without increasing the risk on adhesion formation in the long term. The increased presence of anti-inflammatory macrophages (M2) and the decrease of the presence of pro-inflammatory (M1) macrophages is associated with increased biomechanical strength and most likely contributed to the positive effect of the tissue adhesives. These results support the further application of these tissue adhesives in a contaminated environment.

In this study, bacterial peritonitis induced with the CLP model caused high mortality, intra-abdominal abscess formation, anastomotic dehiscence, and low bursting pressures in the control group rats on POD3. These results are in line with previous studies [22, 25, 26]. In comparison, we found that under sterile conditions colonic anastomotic bursting pressure approximated an average of 80-90 mmHg (unpublished data). Lipopolysaccharide (LPS) from intra-abdominal bacteria triggers the classical activation of macrophages (M1), secreting nitric oxide, proinflammatory cytokines (i.e. tumor necrosis factor (TNF)-α, interleukin (IL)-12), subsequently enhancing cell-mediated immunity [27]. Although the exact mechanisms of nitric oxide in anastomotic healing is not yet determined, previous studies found that sepsis-induced nitric oxide production subsequently impaired collagen synthesis and thus delayed colonic anastomotic healing in the early phases [12, 13]. Accumulation of nitric oxide also causes apoptosis of neutrophils [28], manifesting as abscess formation. These histological changes were also observed in the control group, and more importantly correlation between the M2/M1 index and the bursting pressure was also found on POD3. These data demonstrate that bacterial peritonitis impaired anastomotic healing in the short term in our CLP model and that macrophages play a role in this process.

We also observed that tissue adhesives reduced abscess formation and increased the bursting pressure in the short term. These positive influences on wound healing are unlikely to be completely determined by the sealant adhesiveness. We previously evaluated the adhesiveness of twelve commercially available tissue adhesives in an ex-vivo rat colon model [29]. According to that study, only cyanoacrylate (including Histoacryl® Flex) had a strong adhesiveness that may instantly increase anastomotic strength. In contrast, all tested fibrin and PEG glues (including TissuCol® and Duraseal®) had very limited adhesiveness, which might hardly give additional strength to the anastomosis [29, 30]. However, as is shown in our results, both TissuCol® and Duraseal® significantly increased the bursting pressure on POD3, indicating involvement of other mechanisms.

Activation of macrophages is critical in the acute phase of wound healing. In the early phase, the wound strength mainly comes from the sutures and type III collagen produced by fibroblasts [31]. The fibrogenesis by fibroblasts is enhanced by alternatively activated macrophages (M2), while the classical activation of macrophages (M1) has negative influence on collagen deposition [27, 32]. In addition to the actual cell count, the M2/M1 index is also a representative parameter for macrophage function in tissue reaction [33, 34]. Our data showed a lower number of M1, a higher number of M2, and a higher M2/M1 index in the tissue adhesive groups in the short term. The data suggest an alteration in the macrophage activation. We hypothesized that the tissue adhesive might isolate the contact between the anastomosis and intra-abdominal bacteria, and thus prevented the endotoxin-induced proinflammatory responses. This was further supported by the results of the Duraseal-group. In that group, a similar amount of iNOS+ cells as in the control group was found at the serosa-glue site (macroscopically presenting as abscess formation), but the anastomotic site was protected from this deleterious environment by the adhesive sealant, and far fewer iNOS+ cells were seen around the anastomosis, probably contributing to the high bursting pressure on POD3.

T lymphocytes also play an important role in the wound healing process. Previous studies reported that accumulation of CD8+ cells had a negative influence on collagen deposition and thus impairing early phase wound healing [35], which was also seen in our data accordingly. After exposure to LPS, type I interferon (IFN-α, β) produced by antigen-presenting cells activates CD8+ cells (T suppressor lymphocytes) [36], which trigger the apoptosis process of the infected somatic cells via the caspase cascade. The involvement of CD4+ cells (T helper cells) in wound healing is complicated. T helper 1 cells are involved in cellular immunity, and enhance iNOS production in macrophages [37]. T helper 2 lymphocytes and mast cells, however, produce IL-4 and other cytokines, which stimulates fibroblasts to produce extracellular matrix proteins, fibronectin, and collagen [38]. Although we did not further differentiate between the subpopulations of T helper cells, in a previous study on thermal injury, a decrease in CD4+/CD8+ ratio could only be found in infected wounds but not in the wounds without infection [14, 39]. A similar phenomenon was only seen in our control group which had a contaminated anastomotic wound. These data are in line with the observation in macrophage activation, and they further elucidate that applying the tissue adhesives prevents localized infection on the anastomosis and thus activates an alternative inflammatory response.

Previous studies found that the protective effect of tissue adhesive on the anastomosis was temporary, mainly in the short term [20, 40, 41]. De Hingh et al. reported that biomechanical strength of anastomoses is most vulnerable on POD3, and then gradually recovers in the CLP model [26]. Other studies showed that the strength at

the anastomosis was higher than the intact colon in the long term, when the bursting location was not at the anastomotic site [25, 42]. Similar changes in bursting pressure and inflammatory cells were also seen in our data. Such phenomena are consistent with clinical observations, as most clinical anastomotic leakages occur within the first seven postoperative days, especially after contaminated procedures [25, 43]. In this regard tissue adhesives are required to provide effective protection during the first critical days after surgery prior to a long-lasting protection. Our results showed application of select tissue adhesives assists wound healing during the crucial period.

Increasing the anastomotic strength in the short term, it is also important that the applied tissue adhesives do not cause other adverse events in the long term. Among those events, adhesion formation and foreign body reaction are the most concerning ones for intra-abdominal application of biomaterials [19, 20]. Our data showed that the tissue adhesives used did not increase adhesion formation. The foreign body reaction after adhesive application were moderate in all tissue adhesive groups, which were also shown in the M2/M1 index in the long term.

Among the select tissue adhesives, fibrin glue has been known as inert [41, 44, 45], and polyethylene glycol glue has been used as an adhesion barrier [18]. A moderate foreign body reaction and adhesion formation after application of the cyanoacrylate glue, however, was not expected because it was reported to increase inflammatory reactions, necrosis and adhesion formation in normal or high-risk conditions [46-48]. The inconsistency between previous cyanoacrylate studies and our data can be explained by several reasons. First, our amount of cyanoacrylate, 0.02 mL, was much smaller than that in the previous studies [20, 46], and thus fewer adverse events could be expected. In addition, most cyanoacrylate studies with positive results used n-butyl-cyanoacrylate, which causes a less inflammatory response and tissue toxicity than other cyanoacrylate molecules [20]. Therefore Histoacryl® Flex was also used in this study which is made of n-butyl-cyanoacrylate. These differences, though small, might significantly influence outcomes.

In conclusion, the application of the select tissue adhesives (i.e. TissuCol®, Histoacryl® Flex, and Duraseal®) increased anastomotic strength in the short term without increasing the long-term risk for adhesion formation. The alternative activation of macrophages and T cells most likely mediated these positive effects. Our results support the further application of anastomotic sealants in contaminated colorectal surgery.

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Chapter 11

Reducing colorectal anastomotic leakage with tissue adhesive in experimental inflammatory bowel disease

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ABSTRACT

Background

Anastomotic leakage after gastrointestinal surgery remains a challenging clinical problem. This study aimed to investigate the effectiveness of TissuCol (fibrin glue), Histoacryl Flex (n-butyl-2-cyanoacrylate), and Duraseal (polyethylene glycol) on colorectal anastomotic healing during experimental colitis.

Methods

We first performed colectomy 7 days after 10 mg trinitrobenzene sulfonic acid (TNBS)-induced colitis to validate a rat TNBS-colitis- colectomy model. Subsequently, this TNBS-colitis-colectomy model was used in 73Wistar rats that were stratified into a colitis group (CG, no adhesive), a TissuCol group (TG), a Histoacryl group (HG), and a Duraseal group (DG). Anastomotic sealant was applied with one adhesive after constructing an end-to-end hand-sewn anastomosis. Clinical manifestations, anastomotic bursting pressure, and immunohistochemistry of macrophage type-one (M1) and type-two (M2) was performed on postoperative day (POD)3 or POD7.

Results

TNBS-caused mucosal and submucosal colon damage and compromised anastomotic healing (i.e. abscess formation and low anastomotic bursting pressure). On POD3, higher severity of abscesses was seen in CG. Average anastomotic bursting pressure was 53.2 ± 35.5 mmHg in CG, which was significantly lower than HG (134.4 ± 27.5 mmHg) and DG (95.1 ± 54.3 mmHg) but not TG (83.4 ± 46.7 mmHg). Furthermore, a significantly higher M2/M1 index was found in HG. On POD7, abscesses were only seen in CG (6/9) but not in other groups; HG had the lowest severity of adhesion.

Conclusions

We describe the first surgical IBD model by performing colectomy in rats with TNBS-induced colitis, which causes intra-abdominal abscess formation and compromises anastomotic healing. Anastomotic sealing with Histoacryl Flex prevents these complications in this model. Alternative activation of macrophages seems to be involved in its influence on anastomotic healing.

INTRODUCTION

Inflammatory Bowel Disease (IBD) is a chronic relapsing disorder, and significantly impairs patient's quality of life. Although oral and parenteral therapies are the mainstays in the management of IBD, surgical treatment is still required in case of unsuccessful medical management or occurrence of severe complications. Approximately 20-30% of patients with chronic ulcerative colitis (UC) ultimately require surgical treatment, and 30% of them need a total colectomy within five years after diagnosis [1-3].

Despite a residual chance for relapse, these patients still suffer substantial risk of short- and long-term surgical complications after operation. After surgical intervention such as ileal-pouch-anal anastomosis, an overall complication rate approximating 30% to 40% in IBD patients has been reported. The patients have even higher rates of complications, especially the infectious ones (68%) when receiving high doses steroids [3, 4]. A major course of the infectious complications (e.g. pelvic sepsis) is leakage at the sutured or stapled anastomotic site [3, 5]. Therefore, preventing anastomotic leakage is a major objective in the request for preventing postoperative complications. Unfortunately, however, effective strategies are still lacking to date.

Using tissue adhesives as an artificial barrier at the anastomotic site seems to be a promising avenue for combating anastomotic leakage rates [6]. Previously, we evaluated the mechanical strength and biological reaction of tissue adhesive in rat models and showed that several tissue adhesives (i.e. TissuCol®, Histoacryl® Flex, and Duraseal®) effectively prevented leakage in contaminated conditions [7, 8]. However, the potential applicability of these results in the setting of inflammatory bowel disease is obscure at best and further investigation in this regard is urgently needed before human testing can commence.

In the microbiologically contaminated environment of the inflamed intestine, prevention of colorectal anastomotic leakage critically depends on skewing the ratio between pro-inflammatory macrophages (M1) and regulatory macrophages (M2) [7]. In addition, macrophages also play an important role in IBD pathology per se, but their exact role remains controversial. Though not fully understood, it has been reported that nitric oxide (NO) production through inducible nitric oxide synthase (iNOS) by M1 macrophages, perpetuates chronic inflammation [9]. Conversely, accumulation of M2 macrophages has been reported to reduce the severity of IBD [10]. In view of their principal and antagonistic roles in anastomotic healing, further investigation as to strategies to increase the M2 component and to decrease the M1 component of the macrophage compartment

is of evident importance to devise rational clinical strategies for furthering anastomotic healing in IBD patients.

Although many IBD models have been established and contributed greatly to mechanistic insight in IBD as well as to potential treatment [11-15], a model focused on the surgical treatment of this disease is conspicuously lacking. To this end in this study we developed a novel surgical IBD model by combining the rat colectomy model with the trinitrobenzene sulfonic acid (TNBS) induced colitis model to study these questions [16, 17]. Colonic anastomoses were constructed in rats with colitis and sealed with one selected tissue adhesive. Clinical manifestations, anastomotic healing, and the ratio of M2- and M1-macrophages using immunohistochemistry were evaluated after surgery to determine the effect of the adhesives. The results show that tissue adhesives and especially Histoacryl® Flex improves outcome, which open the way for human trials testing this adhesive in the surgical treatment of IBD patients

METHODS

Animals

Male Wistar rats, weighing 300-350 grams, were purchased from a licensed breeder (Harlan Laboratories, Boxmeer, the Netherlands). The rats were bred under specific pathogen-free conditions and kept in individually ventilated cages. The study was performed according to a research protocol approved by the Ethical Committee on Animal Experimentation of Erasmus University Rotterdam.

Establishing a novel rat TNBS-colitis-colectomy (TCC) model

We developed the TCC model by combining the TNBS colitis model with our previously validated rat colectomy model [16, 18].

The rats were fasted for one day before TNBS-colitis induction to ensure a generally empty colon. On the day of colitis induction, the rats were anaesthetized with isoflurane/oxygen inhalation, and a plastic cannula (7.5 cm in length) was inserted trans-anally into the rat colon. TNBS solution (10 mg diluted in 25% ethanol, 0.25 ml; TNBS group, 5 rats) or 25% ethanol (0.25 ml; control group, 10 rats) was injected respectively. The rats were put in a head-down position for 5 minutes and then returned to the cages with free access to food and water.

Seven days later, a partial colectomy was performed in all rats according to our previously published method and conditions (Figure 1) [18], resecting the major part of the

colon distal to the caecum. Subsequently, an end-to-end one-layer continuous anastomosis was constructed in an inverted fashion with Dafilon 8-0 (B. Braun, Melsungen, Germany). One trained researcher (ZW) performed all anastomoses under microscope. After closing the abdominal wall, 5 mL saline was subcutaneously injected to prevent dehydration. TNBS-caused colon damage was evaluated during surgery; functional, macroscopic and histological changes regarding anastomotic healing were evaluated on postoperative day (POD)-3.

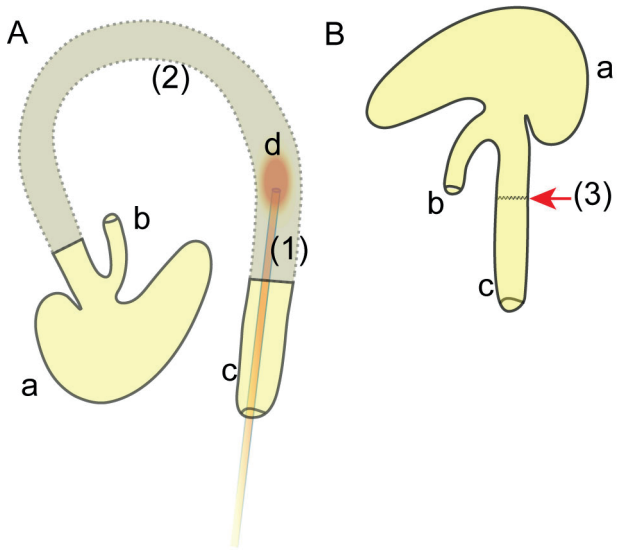


Figure 1. Schematic overview of the rat colon and the methodology employed for the trinitrobenzene sulfonic acid (TNBS)-colitis-colectomy model, anterior view. (A) Rat colon before colectomy. (B) Rat colon after colectomy. Anatomy: a. caecum, b. terminal ileum, c. rectum and anus, d. colitis lesion (intra-luminal). Procedure: (1) Seven days before the colectomy, a TNBS solution (10 mg diluted in 25% ethanol, 0.25 ml) is injected trans-anally with a plastic cannula and this causes colon damage (d); (2) During colectomy, a major part of the colon (i.e. selected with dashed line and indicated with grey colour) is resected ; (3) After colon resection, an end-to-end one-layer continuous anastomosis (red arrow) was constructed in an inverted fashion.

Evaluating tissue adhesives in the rat TCC model

Evaluation of tissue adhesives in the rat TCC model was performed according to procedures essentially similar as those described above for development and validation of this model. Seventy-three rats with TNBS-colitis were used in this experiment.

Following anastomosis construction, one selected tissue adhesive was applied around the anastomosis as a sealant. The tissue adhesive was prepared according to the instruction manual. According to the applied tissue adhesive, rats were divided into the colitis

group (no adhesive applied), TissuCol group, Histoacryl group, and Duraseal group. A blunt needle was used to guide adhesive application when necessary. A standard amount tissue adhesive was applied as in our previously published study and listed in Table 1[7]. After application, a five-minute curing time was allowed to ensure full polymerization of the adhesive. The colitis group, which serves as the non-adhesive negative control, was alternatingly operated between the tissue adhesive groups in order to rule out any systematic bias. TNBS-induced colon damage was also evaluated during surgery; functional, macroscopic and histological changes regarding anastomotic healing were evaluated on POD3 and POD7.

Table 1. Chemical components and dosage of the selected tissue adhesives employed in the study.

Commercial name	Chemical components	Manufacturer	Adhesive dosage per anastomosis (mL)
TissuCol®	Fibrin glue, with aprotinin	Baxter, (Deerfield, USA)	0.1
Histoacryl® Flex	n-butyl-2-cyanoacrylate	B. Braun, (Melsungen, Germany)	0.02
Duraseal®	Polyethylene glycol	Covidien, (Mansfield, USA)	0.1

Evaluation of colon damage

During the colectomy, the rat colon was carefully examined for signs of adhesions to adjacent organs and other pathological changes. Immediately after resection the resected colon was opened longitudinally and flushed with PBS solution. One blinded researcher (SV) weighted and measured the length of the resected colon, and a colon weight ratio was calculated (i.e. colon weight/length ratio, g/cm). Subsequently, the colon sample was scored according to the system described by Monozzi et al. (see Supplemental Digital Content, Table S1.a) [16]. H&E staining of the colon damage specimens was performed. The slides were scored according to the histological scoring system which was used by Monozzi et al. (see Supplemental Digital Content, Table S1.b) [16].

Evaluation of intra-abdominal manifestations and histology

On POD3 or POD7, rats were anesthetized and the abdomen was examined for manifestations including intra-abdominal abscess formation, anastomotic dehiscence and adhesion. Abscess severity was scored according to the previously described abscess score, which was further adapted for its use in this rat model [19, 20] (see Supplemental Digital Content, Table S2). Adhesion severity was recorded according to the Zühlke score [21]. Anastomotic bursting pressure (ABP) was determined after macroscopic observation with the previously described method [7, 18].

H&E stained slides of the anastomotic area of the slides were scored using the Ehrlich and Hunt numerical scale as modified by Phillips et al. [21]. One pathologist (KL) and

one researcher evaluated all the slides in a fully randomized order. Immunohistochemical staining for CD206 as a marker for M2 macrophages and iNOS as a marker for M1 macrophages was performed on anastomotic samples employing the same method described in our previous study [7]. The primary antibody against CD206 (1:400, Abcam plc, Cambridge, UK) or iNOS (1:1600, Abcam plc, Cambridge, UK) was applied. To determine the positive cell count on each slide, eight fields were selected on each slide using a microscope with an imaging system (Olympus DP25, Japan), under 20x10 magnification (2560x1960 pixels). Five fields were selected at the anastomotic site and three fields were selected at the serosa-adhesive site, the interface of the tissue adhesive and adjacent tissue (see Supplemental Digital Content, Figure S1.). In the groups without tissue adhesive application, the latter three fields were chosen at interface of serosa and adjacent tissue (anastomotic adhesion). Following the randomization of the slide number with a computer program, the number of positive cells for each field was counted with ImageJ (National Institutes of Health, USA). The average number of the five fields (i.e. macrophage at anastomotic site) and the three fields (i.e. macrophage at serosa-adhesive site) was used for analysis respectively. An M2/M1 index, using the natural logarithm to adjust the data to normal distribution, was calculated according to the following equitation.

$$M2/M1\ index = \ln(\text{Number of } CD206^{+} \text{ cells})/(\text{Number of } iNOS^{+} \text{ cells})$$

Statistical analysis

Data of the validation experiment were first analysed separately to validate the TCC model, but were thereafter pooled with the other data for final analysis to improve statistical power. Statistical analysis was performed employing SPSS 21.0 (IBM software, USA). Data were presented as mean ± standard deviation (S.D.) unless stated otherwise. To detect differences in the variance between groups, Levene's test was used. The one-way analysis of variance was performed with the Kruskal-Wallis Test (for non-parametric parameters) or one-way ANOVA (for normal distributed parameters), and a p-value < 0.05 was considered to indicate statistical significance. For post-hoc comparisons, the Fisher's least significant difference (LSD) test was used for normal distributed parameter. In pairwise comparisons, the Mann-Whitney U test was used. Comparisons were made between the colitis control group and each of the tissue adhesive groups, not between the tissue adhesive groups. All reported p values are two-sided.

RESULTS

Validation of the TNBS-colitis colectomy (TCC) model

We set out to establish a model that would allow us to investigate the effects of different tissue adhesives in an IBD-relevant context. To this end we decided to combine TNBS colitis with colectomy in experimental rodents. For validating this approach a total number of 15 rats were employed, 10 rats allotted to a control group, whereas 5 other ones were subjected to TNBS treatment. Seven days after TNBS administration, colon hyperemia and ulceration were clearly evident in the TNBS group, resulting in an average gross colon damage score of 2.6 ± 1.1, which was significantly higher than the score of 0.4 ± 0.5 in the control group ($p = 0.002$). The resected colon weight ratio was 0.13 ± 0.04 g/cm in the TNBS group, which was higher but not significantly different from the control group (0.11 ± 0.01 g/cm).

On POD3, one rat from the TNBS group had anastomotic dehiscence. An average bursting pressure of 47.0 ± 34.3 mmHg was observed in the TNBS group, which was lower than that in the control group (84.0 ± 34.9 mmHg, $p = 0.129$). We concluded that our novel TCC model would allow us to test the influence of tissue adhesives in an IBD-relevant setting.

TNBS-Colitis damage evaluation

A total number of 73 rats were used for evaluating the influence of tissue adhesives on anastomotic healing. Of these experimental animals, 9.6% (7/73) died within 24 hours after surgery, with no difference being evident between groups in this respect. Autopsy showed that all deaths were due to acute complications (e.g. bleeding, anaesthesia) other than anastomotic leakage and thus these animals were excluded from further analysis.

Comparisons of the colitis rats from the validation experiment with the ones from the tissue adhesive experiment resulted in satisfactory similarity with regard to the colon damage and anastomotic healing (see Supplemental Digital Content, Figure S2), allowing the data to be pooled together (named colitis group) for further analysis.

Seven days after TNBS-colitis induction, colon damage such as hyperaemia, ulceration and adhesion formation was seen in all colitis rats (Figure 2). The average gross colon damage score of each group varied between 2.0 to 3.0, with no significant difference between groups; the resected colon weight/length ratio was not different between the groups as well (see Supplemental Digital Content, Figure S2), suggesting that application of tissue adhesives is not associated with any systematic experimental bias per se.

Compared to the normal rats (i.e. the control group), the resected colon weight/length ratio, and macroscopic damage score of the colitis rats was significantly higher (p < 0.001 respectively; Figure 2.). Inflammatory cell infiltration, mucosal and sub-mucosal damage, and poor regeneration were seen in colitis rats during histological evaluation, resulting in an average histological damage score of 5.9 ± 2.6, which was significantly higher than an average of 3.0 ± 1.9 in the normal rats (p = 0.002; Figure 2).

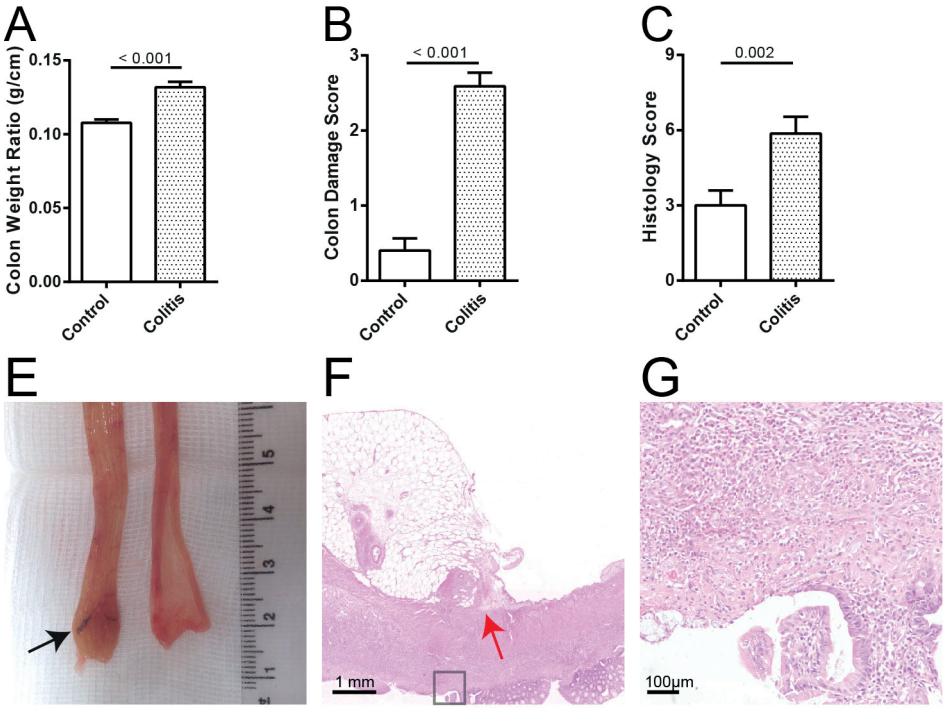


Figure 2. Comparison of the colonic damage between control rats and rats with trinitrobenzene sulfonic acid (TNBS)-induced colitis. In the control rats, 0.25 ml of 25% ethanol was trans-anally injected, n = 10; in the colitis rats, 0.25 ml of 10 mg TNBS diluted in 25% ethanol, n = 78 in A and B, n = 15 in C. (A) Resected colon weight ratio (i.e. wet weight over segment length). (B) Gross colonic damage score. (C) Histology score of colon damage. Values are mean (± S.E.M.). The p values of Mann-Whitney analysis of statistical significance between the groups are provided as well. (D) Compared to the normal rat colon (on the right), a colitic colon (on the left) manifests wall thickening, ulceration, and necrosis (black arrow) as is illustrated with the intra-luminal view of the rat colon sample. (E) Histological manifestation of TNBS colitis. The top part of the figure represents the extra-colon side, and the bottom side image represents the intra-colon side. The selected area is enlarged and illustrated in F. Normal structure of colon mucosa and sub-mucosa is destructed, and substantial inflammatory infiltration and adhesion (red arrow) is seen instead.

Postoperative intra-abdominal evaluation

On POD3, 73.7% of colitis rats (28/38) displayed intra-abdominal abscess formation but no significant difference between the groups was noted. Most abscesses were formed

near the anastomotic site, and higher severity of abscess formation was seen in the colitis group, but this did not reach statistical significance in the overall comparison between the groups. Significantly different adhesion severity was observed ($p = 0.021$; Figure 3). Though not significant, higher severity of adhesion formation was seen in the colitis group and the TissuCol group.

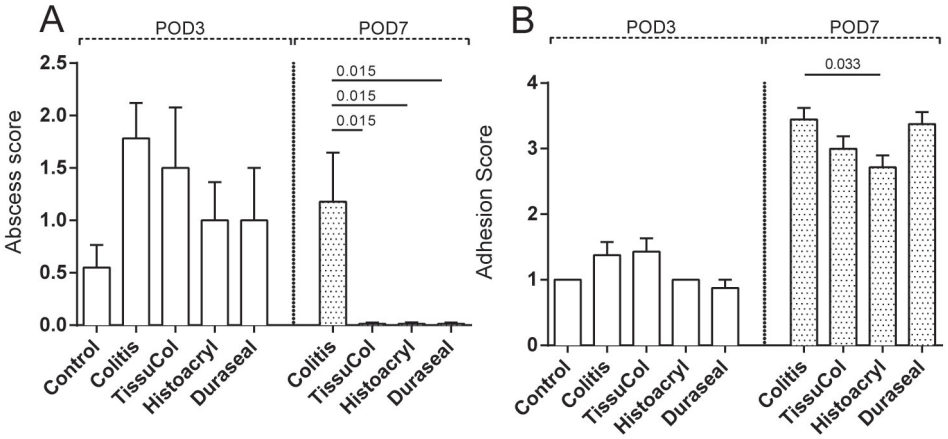


Figure 3. Comparison of abscess and adhesion severity on postoperative day (POD)-3 and POD7 between the various experimental groups employed in this study. (A) Overall comparison of abscess severity yielded a $p > 0.05$ on POD3 but a $p < 0.001$ on POD7 as determined using the Kruskal-Wallis test. On POD7, abscess formation was only observed in the colitis group, which yielded significantly higher abscess severity compared to the other groups on POD7. (B) Overall comparison of adhesion formation resulted in $p = 0.021$ on POD3 and $p = 0.040$ on POD7 when analyzed with the Kruskal-Wallis test. The Histoacryl group had significantly lower anastomotic adhesion severity than the colitis group. The figure also provides the p values of Mann-Whitney pairwise comparisons between the colitis group and other groups when relevant. Values are mean (± S.E.M.), n = 16 in the colitis group on POD3, n = 7-10 in the other groups.

An average bursting pressure of 53.2 ± 35.5 mmHg was seen in the colitis group on POD3, which was significantly lower than the control group (84.0 ± 34.9 mmHg, $p = 0.027$) and the Histoacryl group (134.4 ± 27.5 mmHg, $p < 0.001$), and lower than the Duraseal group with marginal significance (95.1 ± 54.3 mmHg, $p = 0.049$) but not the TissuCol group (83.4 ± 46.7 mmHg, $p = 0.12$; Figure 4).

On POD7, intra-abdominal abscess formation was found in 66.7% rats of the colitis group (6/9) but not in any other group ($p = 0.002$; Figure 3). Most rats had anastomotic adhesions that were strong or firm adhesions based on the Zühlke score. The highest adhesion score was found in the colitis group (3.4 ± 0.5), which was significantly higher than that observed in the Histoacryl group (2.7 ± 0.5; $p = 0.033$) but not different from the other two groups (Figure 3).

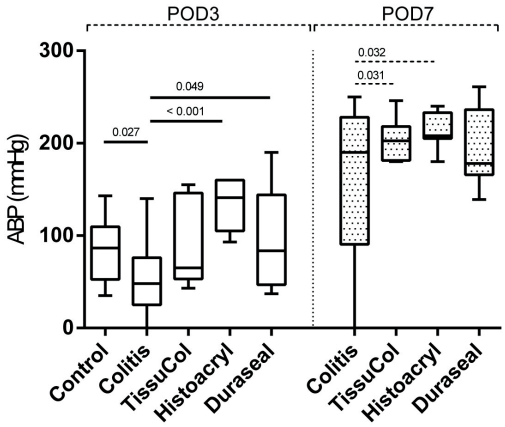
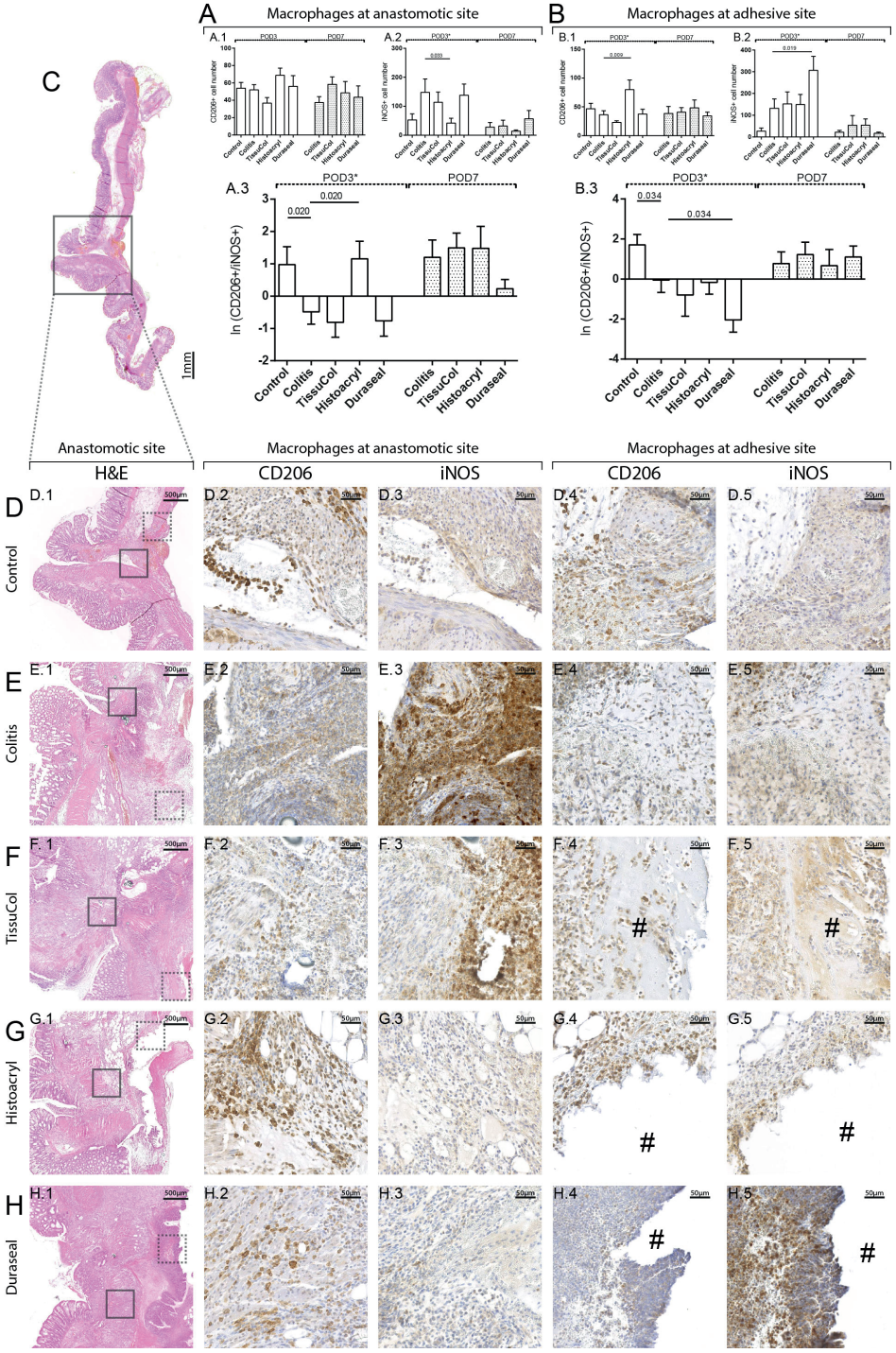


Figure 4. Comparison of anastomotic bursting pressure (ABP) on postoperative day (POD)-3 and POD7 between the various experimental groups employed in this study. Values are bars and whiskers (min to max, middle line at median); n = 15 in the colitis group on POD3; n = 7-10 in the other groups. On POD3, overall comparison, p = 0.002, Kruskal-Wallis Test. The control group and the Histoacryl group yielded significantly higher ABP compared to the colitis group. The p values of Mann-Whitney pairwise analysis are provided as well. On POD7, an overall comparison yielded a p > 0.05 as determined with the Kruskal-Wallis test. The TissuCol group and Histoacryl group showed significantly less variation in ABP when compared to the colitis group. The p values, as determined with Levene's test, are provided and were indicated with a dashed line.

On POD7, the highest bursting pressure was observed in Histoacryl group (213.3 ± 20.1 mmHg), but it was not significantly higher than the control, Duraseal, or TissuCol group (160.6 ± 90.2 mmHg, 195.0 ± 43.4 mmHg, 203.8 ± 23.1 mmHg; p > 0.05). The intra-experimental group variation of the ABP in the colitis group was significant larger from the TissuCol group (p = 0.031) and Histoacryl group (p = 0.032).

Histological evaluation

H&E scores were similar between groups with no significant difference, and acute inflammation was seen on H&E stained slides on POD3 in all groups (see Supplemental Digital Content, Figure S3). The number of CD206+ cells (indicating M2-macrophages) at the anastomotic site was similar between groups on POD3, while a higher number of iNOS+ cells (indicating M1 macrophages) were seen in the colitis group. The M2/M1 index was significantly different between groups on POD3 (p = 0.016), and a higher M2/M1 index was found in the control group and Histoacryl group (Figure 5). At serosa-adhesive interface, a significantly higher number CD206+ cells was found in the Histoacryl group; similar numbers of iNOS+ cells were seen in all the colitis rats on POD3, which were all higher than the control group. The M2/M1 index was similar between the colitis rats, which were all lower in average than the control group.


A
Macrophages at anastomotic site
B
Macrophages at adhesive site
C
A.1
A.2
A.3
B.1
B.2
B.3
POD3
POD7
POD3*
CD206+ cell number
iNOS+ cell number
ln (CD206+/iNOS+)
0.020
0.033
0.009
0.019
0.034
Control
Colitis
TissuCol
Histoacryl
Duraseal
1mm
Anastomotic site
H&E
Macrophages at anastomotic site
CD206
iNOS
Macrophages at adhesive site
CD206
iNOS
D
Control
E
Colitis
F
TissuCol
G
Histoacryl
H
Duraseal
D.1
D.2
D.3
D.4
D.5
E.1
E.2
E.3
E.4
E.5
F. 1
F. 2
F. 3
F. 4
F. 5
G.1
G.2
G.3
G.4
G.5
H.1
H.2
H.3
H.4
H.5
500µm
50µm
#

← **Figure 5.** Comparison of macrophage amount at the anastomotic site and the serosal-adhesive site on postoperative day (POD)-3 and POD7 between the various experimental groups employed in this study. The comparisons were listed in A and B, and illustrated in C-H. Values are the means (S.E.M) of cell counts, n = 16 in the colitis group on POD3, n = 7-10 in the other groups. (A) Comparison of macrophage amount at the anastomotic site. (B) Comparison of macrophage amount at the adhesive site. A.1 and B.1 illustrates comparison of regulatory M2-macrophage (indicated as CD206+) amount; A.2 and B.2 illustrates comparison of inflammatory M1-macrophages (indicated as inducible nitric oxide synthase positive, iNOS+) amount; A.3 and B.3 illustrates comparison of M2/M1 index, i.e. ln(CD206+/iNOS+). * indicates $p < 0.05$ in the overall comparison using the Kruskal-Wallis Test. The *p* values of Mann-Whitney pairwise comparisons between the colitis group and other groups were also provided when relevant. (C) Histological observation of an H&E stained anastomosis from the control group. The right side of the image represents the extra-luminal side, and the left side represents the intra-luminal side of the colon. The selected area indicates the anastomotic area which were also chosen in one rat from each group respectively on POD3. The chosen anastomoses are listed in the Column 1 in D-H. (D-H) Immunohistochemistry staining of CD206 and iNOS at the anastomotic site and the adhesive site. In each row, column 1 illustrates the H&E stained anastomotic area of the selected sample. The selected area with solid line indicates the anastomotic area which is enlarged and illustrated in column 2 and 3 for representation of the CD206+ and iNOS+ cells respectively. The selected area with dashed line indicates the adhesive area which is enlarged and illustrated in column 4 and 5 for representation of the CD206+ and iNOS+ cells respectively. # indicates area where the adhesive is present (i.e. in the TissuCol group) or existed but dissolved during staining (i.e. in the Histoacryl group and Duraseal group).

Histology with H&E staining revealed less inflammatory cell infiltration, more fibroblasts and more collagen deposition at the anastomotic site on POD7 than those on POD3 (see Supplemental Digital Content, Figure S3). The number of iNOS+ cells was significantly reduced compared to that on POD3, without difference between groups. A higher M2/M1 index as compared to the earlier time points was also found in each experimental group, but no difference between the groups was detected. Pooling the M2/M1 index data of POD7 with those of POD3, a significant correlation was found between the ABP value and the M2/M1 index (R square = 0.105; p = 0.004). At serosa-adhesive interface, similar numbers of CD206+ cells and iNOS+ cells, and similar M2/M1 indexes were observed between the groups (Figure 5).

DISCUSSION

Surgical treatment is required in many IBD patients, and with limited preventive strategies, substantial severe short-term postoperative complications still occur. In this study, we developed a novel experimental surgical IBD model (i.e. the TNBS-colitis-colectomy model: TCC model) and investigated the influence of three tissue adhesives on anastomotic healing in this model. Among the selected adhesives, especially Histoacryl® Flex significantly increased the biomechanical strength of anastomosis, and reduced accumulation of pro-inflammatory macrophages at the anastomotic site.

The rodent TNBS-colitis model has been widely used for IBD research and has appropriate visual, histological and also gene-expressional changes resembling those observed in human ulcerative colitis [16, 22-24]. Our gross observations and histological results are in line with the previous data with consistent colon damage in the colitis rats. Although we did not perform subtotal colectomy or ileal pouch-anal anastomosis in the rat model, performing partial colectomy on basis of the colitis model also allows us to resect most part of the diseased segment [17], which is also the clinical purpose of surgical intervention in IBD patients. Our data demonstrate that the presence of colitis compromises surgical outcomes with main features including lower ABP, higher intra-abdominal abscess and adhesion severity, which are in agreement with clinical findings in IBD patients. Accumulation of M1 macrophages at the anastomotic site is likely to play a role in the impaired anastomotic healing process in the colitis group. We base this statement on two observations: first, compromised ABP and higher number of M1-macrophage was observed in the colitis group as compared with the control group; second, the significant increase of ABP in the Histoacryl group was accompanied with a lower number of M1 macrophages and a higher M2/M1 index at anastomotic site. It is well known that IBD has a chronic and recurrent course with mucosal damage and over-recruitment of inflammatory cells. The M1 macrophages at anastomotic site in our TCC model may have been accumulated due to both colitis and anastomotic healing. Unfortunately, our results do not allow us to determine the origin of M1-macrophage at the anastomotic site in the TCC model and further investigation in this respect is needed.

The mechanism as to how tissue adhesive may act in preventing anastomotic leakage is not yet fully understood. We observed lower severity of abscess formation on POD3 and full clearance of intra-abdominal abscesses in the tissue adhesive groups on POD7. These data indicate tissue adhesives may act in this model through prevention of intra-abdominal abscess formation and correspond to earlier data from our laboratory, albeit that this previous study was not performed in an IBD-relevant context [7]. The most obvious cause of the prevention of such abscesses is a sealing effect blocking intra-luminal content from leaking into the abdominal cavity. Among our selected tissue adhesives, compositions of Duraseal, a synthetic hydrogel (i.e. polyethelene glycol and trilysine), provided us the possibility to evaluate the effect of a pure watertight anastomotic sealant. It has no component influencing the wound healing process [6]. These data further suggest that the sealing effect observed in our study may not entirely depend on the mechanical strength of tissue adhesives.

The pathogenic differences of IBD from other disease are important factors influencing the effectiveness of tissue adhesives. It has been reported that fibrin glue used as a non-surgical treatment of anal fistula failed to reach satisfactory outcomes in IBD patients

[25]. In line with this, our data also suggest that in the context of colitis these mechanically weak sealants with or without wound-healing components are less effective when compared with the cyanoacrylate. This is mainly based on two observations in our study. First, although the average was higher, ABP of 40 mmHg or lower on POD3 was still observed in both the TissuCol and Duraseal groups, while ABP in the Histoacryl group were all higher than 90 mmHg. Moreover, the M2/M1 index of the TissuCol and Duraseal groups on POD3 still demonstrated an inflammatory rather than an anti-inflammatory status as observed in the control and Histoacryl group. The M2/M1 index at the serosa-adhesive site also indicates an acute foreign body reaction, especially in the Duraseal group. These data may also partly explain the unsatisfactory clinical results of fibrin glue application.

In addition to a watertight sealing effect, the strength of Histoacryl® Flex is much higher than the other selected adhesives, providing sufficient additional mechanical strength to a primary anastomosis regardless of its original configuration (i.e. sufficient, insufficient or even sutureless anastomosis) [17, 26, 27]. Such reinforcement may further provide a firm contact between the cutting edges, creating an ideal environment for anastomotic healing. Moreover, more M2 macrophages were observed at serosa-adhesive interface in the Histoacryl group, which are known to foster regenerative responses through connective tissue inducing characteristics including the stimulation of collagen production [28, 29]. It is not an easy task to further verify whether strong adhesiveness of Histoacryl® Flex is the main mechanism of preventing leakage, because modification of the adhesiveness of cyanoacrylate may also change its biological properties. However, from a practical point of view, our data, together with the evidence from other animal studies, have revealed the effectiveness and safety of n-butyl-cyanoacrylate as an anastomotic sealant [30]. It seems to be a promising adjuvant in a surgeon's toolbox.

We recognize that one main limitation of the TCC model is that it only partially mimics human IBD pathology. In addition, most of the diseased colon segment was resected in during colectomy, which may limit the severity of the inflammatory response in long-term after surgery. We therefore chose POD7 (14 days after colitis induction) but not any longer as our second follow-up time. This limitation probably explains the high ABP values in all the groups on POD7. Nevertheless, significant variations were still observed in the colitis group. Different from increasing the bursting pressure on POD3, the selected tissue adhesive seemed to prevent the failure of wound healing on POD7. It is known that application of cyanoacrylate provides an immediate reinforcement with much reduced ABP variation [17]. The increase of ABP was accompanied with a decrease in M1 macrophages in all groups. Combining the short- and long-term data revealed a significant correlation between the M2/M1 index and ABP, which is in line that

timely switching of the macrophages from the M1 to the M2 phenotype is important to enhance anastomotic healing.

The use of anastomotic sealant is only one strategy to prevent postoperative complications and results may further improve when combined with other interventions. Especially pharmacological treatments are of great importance in current IBD medical regime, also in a post-operative setting. We think that the model introduced in the present study might aid efforts to understand the influence of pharmacological therapy (e.g. anti-TNF-α, anti-IL-6) on postoperative complications in IBD patients and help understanding the factors governing impaired wound healing process. Investigations addressing this possibility are currently underway in our laboratory.

CONCLUSION

In conclusion, we describe here a novel surgical IBD model by performing colectomy in rats with TNBS-induced colitis, which causes substantial intra-abdominal abscess formation, compromises anastomotic healing. We also demonstrate that application of the selected tissue adhesives, especially Histoacryl® Flex, has a favourable effect to the colorectal anastomotic healing and prevents postoperative complications in the this model. Timely switching of the macrophages from M1 to M2 phenotype seems to be associated with less complications during the early phases of anastomotic healing.

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Chapter 12

Reinforcement of the colonic anastomosis with cyanoacrylate glue prevents anastomotic leakage in a high risk porcine model

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ABSTRACT

Background

Previous experimental studies on the use of cyanoacrylate glue (CA) for prevention of anastomotic leakage (AL) have shown promising results. The aim of this study is to investigate the effect of CA in prevention of AL in a high risk porcine model.

Methods

Twenty-four pigs were divided into four groups with ischemic anastomosis and; 1) sufficient complete suture technique (ISCH), 2) sufficient suture, and CA reinforcement (CA-ISCH), 3) insufficient suture technique (ISCH-AI), 4) insufficient suture, and CA (CA-ISCH-AI). In CA-groups, N-butyl-2-cyanoacrylate was applied between the colon ends. Anastomotic bursting pressure (ABP), abscess, and adhesion formation were evaluated on postoperative day 7. Tissue samples were obtained for histology.

Results

The AL-rate was 4/6 in the ISCH-AI group compared to none in the other three groups. The mean ABP was 167±54mmHg in the ISCH-group versus 213±43mmHg in the CA-ISCH-group (p=NS) and 145±102mmHg in the ISCH-AI-group versus 187±19mmHg in the CA-ISCH-AI-group (p=NS). The adhesion score was greater in the ISCH-group than in the CA-ISCH-group (4.2±1.3 vs 1.7±0.82; p=0.019). Stricture of the anastomosis occurred only in non-CA-groups (3/12, 25%).

Conclusion

Anastomotic reinforcement with cyanoacrylate is effective and safe to prevent leakage in a high risk, colo-colonic anastomosis in a porcine model.

INTRODUCTION

Anastomotic leakage (AL), with an incidence between 3-19%, is a severe complication after colorectal surgery and is associated with high morbidity and mortality rates [1, 2]. Despite proper patient selection and operative technique, AL cannot be completely avoided.

Staple line or anastomotic reinforcement techniques have been developed in attempts to decrease leakage in the first week postoperatively. A recent review published by Betzold et al. described three main categories of products that reinforce the anastomosis including permanent, semi-absorbable, and bio-absorbable materials [3]. Of the bio-absorbable materials, only fibrin glue and the polyglycolic acid/ trimethylene carbonate reinforcement of staple lines (Gore-Tex Bio absorbable Seamguard, W.L Gore and Associates, Flagstaff, AZ) have been investigated in clinical trials of colorectal surgery [4-7]. The fibrin glue study included 223 patients undergoing laparoscopic rectal surgery but did not show a significantly decrease in AL [7]. Also, in the largest, randomized, controlled trial including 258 patients with application of Gore®Seamguard® did not show a decrease in the rate of AL [6].

The use of a synthetic, bio-absorbable reinforcement material for the anastomosis has considerable advantages over non- or semi-absorbable staple line reinforcement and may help to decrease anastomotic leakage while avoiding the risk of strictures [1, 4]. N-butyl-2-cyanoacrylate (CA) is a biodegradable tissue adhesive. In our previous ex vivo porcine study and in vivo rat studies, CA had the greatest mechanical strength in either normal anastomoses, incomplete 'insufficient' anastomoses, and anastomoses in a bacterially contaminated field, compared to other tissue adhesives [8]. Besides the good adhesiveness of CA to the colonic surface, CA also enhanced the anastomotic healing by influencing the amount of anti-inflammatory macrophages, and no adverse foreign body reaction could be observed [9, 10].

The promising results from these previous experimental studies suggest that CA may be used safely to reinforce colorectal anastomoses. The objective of the present study was to investigate if the application of CA could prevent anastomotic leakage defined as either fecal peritonitis or macroscopic dehiscence of the anastomosis in a validated porcine model of a partially ischemic colo-colonic anastomosis.

METHODS

Study design

This study was a prospective, randomized, controlled animal experiment evaluating the effectiveness of N-butyl-2-cyanoacrylate, to prevent colo-colonic AL in pigs with a partially ischemic colo-colonic anastomosis using AL as the primary outcome parameter. All experiments were performed with approval of the Erasmus University Medical Center Animal Care Committee in accordance with national law regarding the protection of animals, approval number DEC-3167;105-13-07. The manuscript was written according to the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines.

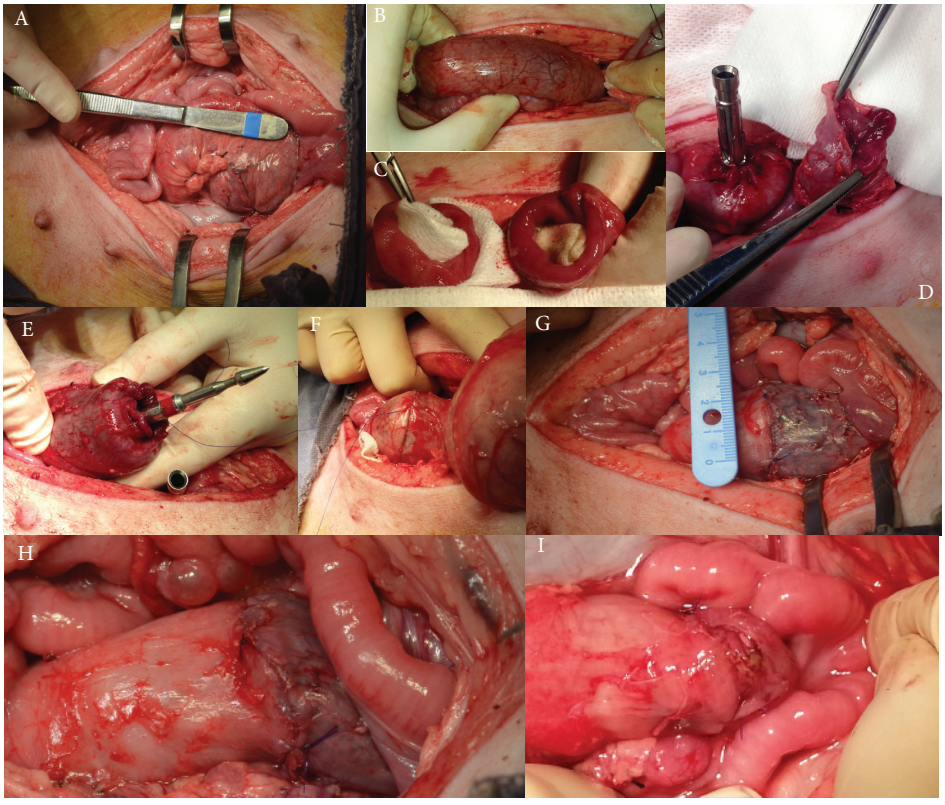

Figure 1. A. sigmoid after ligation of mesentery arteries (10cm), B. sigmoid after dissection of serosa, C. sigmoid after transection, D. proximal cutting edge secured around the anvil, E. lower cutting edge secured around the fully extended trocar, F. click anvil on distal part of the stapler, application of glue or not and anastomosis was made by hand suture, G. gap of 2.1cm in anastomotic leakage model, H. anastomosis after removal of stapler device, with glue, I. anastomosis after removal of stapler device, without glue.

Experimental animals

Twenty-four female domestic pigs (Yorkshire x Landrace), with an average weight of 40.7 ± 3.0 kg, were purchased from a licensed breeder in The Netherlands. The sex of the pigs was chosen based on previous literature [11, 12]. The pigs were randomly divided into four groups of six animals per group; the randomization occurred based on the assigned ear number of the pigs by the breeder. The pigs were operated in pairs, and the animal with the lowest ear number was assigned to a glue group. In the next session the opposite order was used with the animal with the lowest ear number assigned to a non-glue group. All animals were housed in pairs and had an acclimatization period of one week prior to operation. Pigs were fed 2 kg of pig feed daily and had free access to water.

Anesthesia and operative procedure

The animals were fasted 1 day prior to surgery but with free access to fluid. Preoperatively, the animals were sedated with tiletamine/zolazepam (5 mg/kg), xylazine (2.25 mg/kg), and atropine (0.03 mg/kg) intramuscularly (IM). Colon preparation was achieved by administration of sodium phosphate enema (Colex Klysma, Tramedico BV, the Netherlands) directly after induction of anesthesia to allow easier transrectal introduction of the empty circular stapler. An ear vein was used for intravenous fluid administration of 500ml of 5% glucose throughout the procedure. General anesthesia was maintained by intubation and ventilation with O2/N2 (1/3 v/v) enriched with 1-3% isoflurane. Animals received a single IM dose of procaïnebenzylpenicilline/ dihydrostreptomycine (per ml. 200.000 I.E./200mg; 0.05 ml/kg) as antibiotic prophylaxis, and buprenorphine (0.015 mg/kg) for analgesia. Depth of anesthesia was monitored by regular checking for absence of pain-reflexes and with cardiac monitoring.

A midline laparotomy was conducted after standard aseptic preparation of the surgical field, and the distal colon was exposed. Subsequently, the mesenteric branches were ligated over a length of 10 cm and the mesentery was cleared from the serosa of the colon in the same area to achieve an ischemic 10 cm segment. Before and after ligation of the mesenteric vessels, the Local Hemodynamic Index (LHI) was measured with a miniaturized, Dynamic Light Scattering (mDLS) device (Elfor, Elfi-Tech Ltd.) to evaluate the degree of ischemia [13].

In all groups, the distal colon was transected at approximately 40 cm from the anal verge. The colo-colonic anastomosis was constructed in a hand sewn, end-to-end fashion after applying the CA. In attempt to both standardize and facilitate application of the CA we used a circular 28 mm stapler, with the staples removed (Chex™ CS surgical staplers, Arseus Medical, distributor of Frankenman International Limited, HongKong)[12]. To approximate the edges and to apply the CA glue between both on the serosal surface, the

stapler assured inversion of the mucosa and application of the CA directly onto the serosal surface. Before performing a hand sewn anastomosis as follows, the proximal colon was secured around the anvil with a purse-string suture (4.0 PDS, Johnson & Johnson, Medical BV, the Netherlands). The cartridge of the stapler was inserted transanally, and the distal end of the colon was secured with a purse-string suture. The circular stapler was connected and partially closed with the proximal and distal ends 5 mm apart. In the CA-groups (II and IV), 0.3 mL of CA (Histoacryl® Flex, N-butyl-2-cyanoacrylate, B. Braun, Melsungen, Germany) was applied between the two ends of the colon parts, and the stapler was then closed completely. In the control groups (I and III), the stapler was closed without application of CA. In all groups, the anastomosis was then constructed with a single-layer, continuous, hand suture (4.0 PDS) before removal of the stapler. In group III and group IV, the anastomosis was constructed insufficient: before removal of the stapler, a gap of 21 mm length was left on the anti-mesenterial side according to the validated porcine AL model of Nordentoft et al [11].

The abdominal cavity was lavaged with 500 ml of 0.9%NaCl. The abdominal wall was closed in one layer, similar to a 'mass closure', with an absorbable monofilament (Monosyn 2.0, B. Braun, Melsungen, Germany). The abdominal skin was closed with a continuous dermal suture (Vicryl 2.0, Ethicon, Somerville, NJ). After the operation, the animal was extubated and placed into a warm recovery booth. A fentanyl patch (25mcg/h) was applied for postoperative analgesia for three days. Animals were allowed to drink immediately and were given access to solid food the next morning.

Follow up

During the 7 day postoperative period the researchers were blinded for the allocated treatment. The animals were weighed daily, and their health was scored based on a short version of the pig health scoring system proposed by Husa et al. (supplementary data; Table 1)[14]. On postoperative day (POD) 7, all pigs were anesthetized and relaparotomy was performed to evaluate the integrity and condition of the anastomosis. AL was defined as either fecal peritonitis or dehiscence of the anastomosis. In order to assess the severity of AL including abscess formation around the anastomosis, the grading system according to Wu et al. was used: 0 = no leakage, 1 = abscess formation only at the side of the anastomosis, 2 = presence of fecal peritonitis with or without abscess formation, and 3 = leakage-related death [15]. The abdomen was examined for presence of other distant abscesses, contaminated fluid, or other forms of inflammation in the area of anastomosis, other pathologicprocesses in the abdominal cavity and the incidence and the extent of adhesions. Abscess formation was scored according to the following scoring system: 0 = no abscess; 0.5 = one very small abscess; 1 = several small abscesses; 2 = medium abscess; 3 = large or several medium abscesses; 4 = one very

large or several large abscesses [16, 17]. Adhesion severity was recorded using the Zühlke score [18]. Abscesses and adhesions where scored separate for both the anastomotic site and other intra-abdominal locations. Anastomotic bursting pressure (ABP) was determined by insufflation of air in the closed clamped segment of the colon in a saline filled abdomen, and the ABP and location was noted. The anastomotic segment was harvested for histologic examination, and the animal was euthanized using a sodium pentobarbital solution (Euthasol® 200mg/ml; 100mg/kg IV).

Histology

After measuring the ABP, we collected the entire anastomosis and opened it at the antimesenteric side. A 1x1cm sample of the anastomosis was collected for histology. After fixation in 10% neutrally buffered formalin, dehydration and embedding in paraffin the specimen was cut in 5µm sections and mounted on glass slides. The paraffin sections were stained with hematoxylin and eosin (H&E), and scored using the Ehrlich and Hunt numerical scale [19]. Inflammatory cell infiltration, angiogenesis, fibroblast activity, and collagen deposition at the anastomotic site were evaluated. Each parameter was graded from 0 to 4 as follows: 0 = no evidence, 1 = occasional evidence, 2 = light scattering, 3 = abundant evidence, 4 = confluent cells or fibers. Two independent 'blinded' investigators (G.B. and K.L.), scored all samples.

Statistical analysis

Statistical analysis was performed with SPSS 21.0 (IBM software, USA). Data are presented as mean (S.D.) or number and percentage. The Mann-Whitney U test or the one-way analysis of variance was performed with the Kruskal-Wallis test for non-parametric parameters. The Levene's test was used to test equality of variances. All reported p values were two-sided; a p-value < 0.05 was considered to indicate statistical significance.

RESULTS

Perfusion data

To evaluate standardization of the ischemic anastomosis, we measured the LHI at the anastomotic site. A similar anastomotic perfusion was found among different groups, indicating a standardized ischemic condition (supplementary data; Table 1).

General observations

All operations were performed with an empty stapler device and all 'donuts' were complete. No mortality occurred in any of the groups. Postoperative weight loss occurred up to POD 3 or 4 in all groups, and on POD 7 the weight loss was fully compensated with

a higher weight compared to the day of surgery. There were no statistically significant differences in weight between any of the four groups. The health scores in the CA groups were more favorable than those of the control groups during the first 4 postoperative days (supplementary data; Table 2).

Intra-abdominal observations

The ISCH and ISCH-AI groups had significantly higher AL scores; 0.8 ± 0.4 and 1.5 ± 0.8 vs. 0.2 ± 0.4 (CA-ISCH) and 0 in the CA-ISCH-AI group respectively, (ISCH vs CA-ISCH: p = 0.027 and ISCH-AI vs CA-ISCH-AI: p = 0.006). Anastomotic dehiscence was recorded in four of six animals in the ISCH-AI group (67%) compared to none in the other three groups. These four animals had a low but measurable ABP as the dehiscence was covered by an abscess. Anastomotic abscesses were observed in one animal in the CA-ISCH-AI group , none of the CA-ISCH group and 83% (5/6) in the ISCH group (p = 0.005) and 67% (4/6) in the ISCH-AI group (p = 0.030) (Figure 2).

Adhesions around the anastomosis were present in all animals. The average adhesion score was greater in the ISCH groups when compared with the CA-ISCH groups (4.2 ± 1.3 vs 1.7 ± 0.8; p = 0.019). Complete covering of the anastomosis by strong adhesions were only seen in both none-CA groups (Figure 2). The anastomoses reinforced with CA did not show any stricture of the anastomosis. In contrast, 25% (3/12) of the control pigs developed a stricture of the anastomosis.

Distant intra-abdominal abscesses occurred in 67% (8/12) of pigs in the control groups (ISCH and ISCH-AI). In the CA-ISCH group 50% (3/6) of the animals had a distant abscess, one intra-abdominal and two located in the abdominal wall. In the CA-ISCH-AI group 17% (1/6) had an intra-abdominal abscess (Figure 2).

Anastomotic Bursting Pressure (ABP)

The mean ABP was 167 ± 53.9 mmHg in the ISCH group versus 213 ± 43 mmHg in the CA-ISCH group (p= NS). The majority (67%) of bursts occurred in native tissue proximal or distal to the anastomosis. In the insufficient anastomosis groups, the mean ABP for the ISCH-AI group was 145 ± 102 mmHg versus 187 ± 19 mmHg in the CA-ISCH-AI group. Although this difference did not reach levels of statistical significance, the variance of the ABP in the CA-ISCH-AI group was significantly lower than in the ISCH-AI (p = 0.005). The anastomotic bursting pressures and bursting locations are shown in Figure 3.

Histology

On HE-stained paraffin sections, the inflammatory cells, angiogenesis, fibroblasts, and collagen cells were scored. The CA-ISCH-AI group scored the least inflammation and

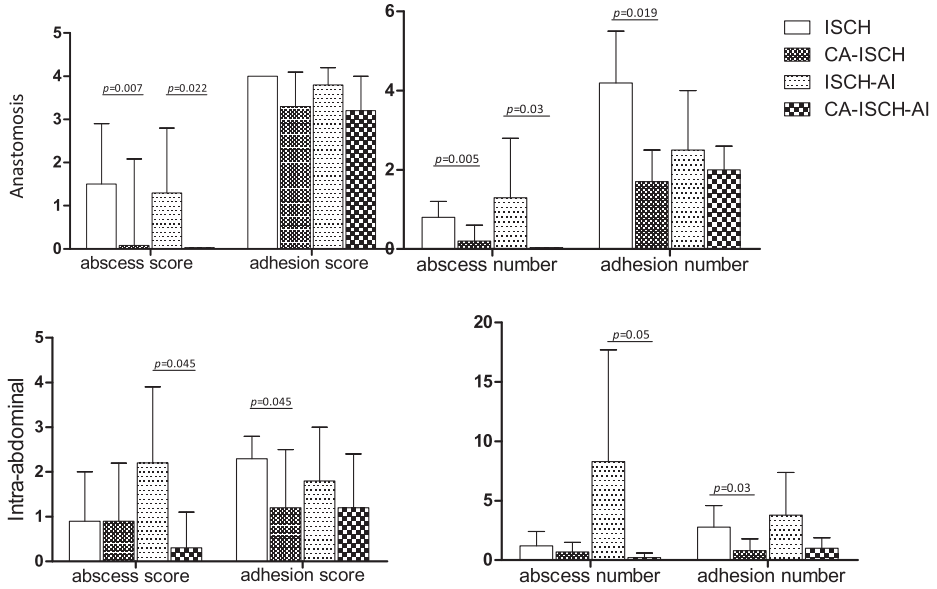

Figure 2. Comparison of the number and severity of abscesses on the anastomotic site and intra-abdominally, between the different groups. Both glue groups (CA-ISCH and CA-ISCH-AI) show a significantly lower abscess number and score, compared to respective control groups. Also the abscesses elsewhere were significantly lower in the glue anastomotic leakage group (CA-ISCH-AI) than in the control groups.

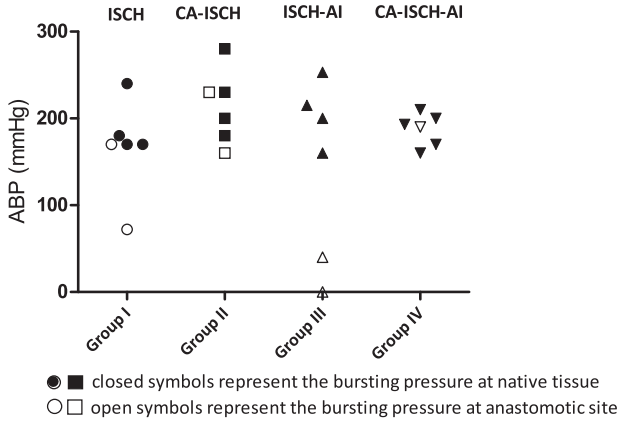

Figure 3. The anastomotic bursting pressures (ABP) are shown in this figure; every data point represents an individual animal. Open symbols represent the bursting pressure at the anastomotic site. Closed symbols represent the bursting pressure at native tissue, either proximal or distal from the anastomosis. The mean ABP was higher in the in the CA-ISCH group compared to ISCH, although not significant. The animal from group ISCH-AI with an ABP of 0 mmHg had fecal peritonitis.

most collagen formation compared to the other groups. The CA-ISCH group showed the greatest angiogenesis score, but also a greater collagen score compared to the ISCH group. None of these scores were statistically significant (supplementary data; Table 3). There were no multinucleated giant cells seen in any of the groups as a sign of foreign body reaction.

DISCUSSION

This experimental study on colorectal anastomotic leakage in an ischemic and insufficient porcine model showed that cyanoacrylate tissue adhesive can reduce anastomotic leakage scores and anastomotic abscess formation, while adhesion formation was reduced in comparison to the control (no cyanoacrylate) groups.

During the last decades colorectal surgery has improved by perioperative standardization. However, many risk factors for AL, such as age, gender and comorbidity, are patient dependent and can hardly be avoided. Several changes in techniques and perioperative protocols such as standardization of the anastomosis, by for example a stapler device, could not reduce the anastomotic leakage rate. Several intraluminal devices have been developed to reduce colorectal AL [20]. Despite positive results from some clinical studies the use of these protective devices has not been widely implemented [21-23].

Staple line reinforcement is a relatively new method for colorectal surgery, although it is well recognized in the field of bariatric and pulmonary surgery [24-29]. The available investigational and clinically used staple line reinforcement materials in gastrointestinal surgery consists of a large range of different biologic and non-biologic materials [4, 30-34]. Most of the experimental studies showed reduced leakage rates, but one study showed an increased leakage rate in the reinforcement group [35]. Reliable clinical data is lacking as only a few clinical pilot studies on Bio-absorbable Seamguard (BSG) were published. These studies showed a lower reduced incidence of AL between 0%-3.4%, although the groups are very small. BSG is more biocompatible than compared to the other reinforcement materials [4, 5, 36]. Previous data on the use of cyanoacrylate for colorectal anastomotic reinforcement is only reported in experimental animal studies.

The majority of these animal studies have been performed in rats [8, 37]. Our own previous rat studies showed that the occurrence of AL could be significantly reduced by application of tissue adhesives. Cyanoacrylate (Histoacryl flex®) showed very promising results with an increase of anastomotic strength and reduction of abscess formation [8-10]. The CA used in this study is strong, inert, and, moreover, it stays flexible after

polarization, representing advantageous properties that facilitate its application on a difficult surface like the colon. An important limitation of Histoacryl flexible® (N-butyl-2-cyanoacrylate monomer with myristic acid and triacetin) is the lack of CE approval for intra-abdominal use, at this moment it is only CE approved for topical use. However, another cyanoacrylate variant Glubran 2® (N-butyl cyanoacrylate with metacryloxisulfolane) is CE certified for internal and external use.

A limitation of rat studies is that these species have the ability to withstand severe abdominal infections, while mice and pigs seem to have a more comparable response to humans [11, 38]. Although, only four pig studies have been carried out since 1994 regarding this subject [12, 39-41]. Paral et al. compared Glubran 2® with Dermabond®, two types of CA. After 14 days follow up all anastomoses constructed with Glubran 2® healed with no sign of pathology, but the Dermabond group showed stenosis of the anastomosis [12]. In this study the anastomosis was constructed under optimal conditions in young healthy animals [16]. The most recent study published in 2014, showed that the glued colon anastomoses without sutures or staples were resistant to lower intraluminal pressures than stapled anastomoses, but the glued anastomoses did resist significantly higher pressures than the physiological intraluminal pressures in the colon [41].

Strengths of this study include the use of a high-risk colorectal anastomosis leakage porcine model in combination with CA. All animals were allocated in the different groups by randomization and operated under the same standardized and sterile circumstances. The use of a large-size animal is more comparable to human surgical procedures. The use of a stapler aids application of the glue and standardization of the hand-sewn anastomosis. Therefore these results may be applied to both stapled and hand-sewn anastomosis. However, the method of application is also the biggest challenge. It is difficult to reach the dorsal part of a low colorectal anastomosis or to apply the adhesive on an intra-corporal anastomosis during laparoscopic surgery. For practical use it is preferable that, for example, CA will be incorporated within the circular stapler.

Absorbable staple line reinforcement is preferred over non-absorbable materials because of the lower foreign body reaction. Cyanoacrylate is a biodegradable tissue adhesive, and is therefore expected to elicit only a low inflammatory response. Our histological data showed no multinucleated giant cells as a sign of foreign body reaction. Histological investigation also showed a trend for more collagen formation in the CA-groups. Type 1 macrophages (pro-inflammatory macrophages) impair collagen deposition by nitro oxide production. There is always a balance between type 1 macrophages and type 2 macrophages (anti-inflammatory). Previous studies showed that cyanoacrylate

induces more type 2 macrophages and it is plausible that this occurred in this study too [9]. The application of only a small dosage of N-butyl-2-cyanoacrylate could also have resulted in less inflammation, using 0.3 ml of glue, representing half of the dosage used in another porcine study [12].

Our study demonstrates that reinforcement of the colonic anastomosis with N-butyl-2-cyanoacrylate glue and prevents leakage in a high-risk colorectal anastomosis. No anastomotic dehiscence and only one small abscess occurred in the CA groups. Furthermore, no undesirable effects, like stricture, were observed. This porcine study strongly supports the introduction of future clinical studies on the application of cyanoacrylate glue for the prevention of colorectal AL.

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SUPPLEMENTARY DATA

Supplementary Table 1. Median values of Local Hemodynamic Index (LHI).The ischemic condition was similar in all groups; *p*=NS.

	Local Hemodynamic Index (LHI) mean ± S.E.M.			
	ISCH	CA-ISCH	ISCH-AI	CA-ISCH-AI
Time points during surgery				
ascending; before ligation	530 ± 76	643 ± 44	727 ± 67	676 ± 48
decending; before ligation	534 ± 59	636 ± 37	862 ± 151	696 ± 92
ascending; after ligation	503 ± 50	923 ± 168	993 ± 234	700 ± 67
decending; after ligation	549 ± 69	541 ± 38	767 ± 158	712 ± 194
Anastomosis	516 ± 115	597 ± 91	657 ± 73	628 ± 79

Supplementary Table 2. Clinical observations record sheet adapted with modifications from Husa et al. A record sheet was completed each observation day.

Pig ID	Stools	Behavior	Appetite	Ambulation
1	1 2 3 4	1 2 3 4	1 2 3	1 2 3
2	1 2 3 4	1 2 3 4	1 2 3	1 2 3
3	1 2 3 4	1 2 3 4	1 2 3	1 2 3
-	1 2 3 4	1 2 3 4	1 2 3	1 2 3
-	1 2 3 4	1 2 3 4	1 2 3	1 2 3
	normal daily score = 4; max. score = 14			extracted from Husa et al.

Explanation
Stool consistency; 1 = normal, 2 = semi-formed, 3 = diarrhea, 4 = diarrhea with blood or tissue
Behavior; 1 = normal, 2 = lethargic, 3 = huddled, 4 = moribund
Appetite; 1 = normal, 2 = diminished, 3 = anorexic
Ambulation; 1 = normal, 2 = lame, 3 = down

Supplementary Table 3. Histological evaluation of anastomotic healing on postoperative day 7, values are mean. The *p* values of overall comparisons were all > 0.05.

	mean histological score			
	ISCH	CA-ISCH	ISCH-AI	CA-ISCH-AI
inflammation	2.3	2.7	2.5	1.8
angiogenesis	1.5	2.5	2.2	1.7
fibroblast activity	2.3	2	2.5	2.3
collagen	1.5	2.2	2.3	2.3
p = NS between the groups				

Histologic scoring
0 = no evidence; 1 = occasional evidence; 2 = light scattering; 3 = abundant evidence; 4 = confluent cells or fibers

Chapter 13

The effect of biomaterials used for tissue regeneration purposes on polarization of macrophages

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ABSTRACT

Activation of macrophages is critical in the acute phase of wound healing after implantation of surgical biomaterials. To understand the response of macrophages, they are often cultured *in vitro* on biomaterials. Since a wide range of biomaterials is currently used in the clinics, we undertook a systematic review of the macrophage polarization in response to these different surgical biomaterials *in vitro*. Beside the chemistry, material characteristics such as dimension, pore size, and surface topography are of great influence on the response of macrophages. The macrophage response also seems to depend on the differences in sterilization techniques that induce lasting biochemical changes or residues of chemicals and their byproducts used for sterilization. Regarding tissue-based biomaterials, macrophages on human or porcine dermis, strongly cross-linked by chemicals elicit in general a pro-inflammatory response with higher amounts of pro-inflammatory cytokines. Synthetic biomaterials such as polyethylene, PET + PAAm, PET + PAANa, PFPE with large posts, PEG-g-PA, and PDO always seem to elicit an anti-inflammatory response in macrophages, irrespective of origin of the macrophages, e.g. buffy coats or full blood. In conclusion, in general *in vitro* models contribute to evaluate the foreign body reaction on surgical biomaterials. Although it is difficult to simulate complexity of host response elicited by biomaterials, after their surgical implantation, an *in vitro* model gives indications of the initial foreign body response and allows the comparison of this response between biomaterials.

INTRODUCTION

A wide range of biomaterials are used as implantable medical devices, notably for soft tissue repair. These materials have their own characteristics with regards to composition, mechanical strength, topography, porosity, and chemistry. Implantation of biomaterials is always associated with tissue damage, more or less important, according to the invasiveness of the surgical procedure, i.e. surgical treatment of the disease and biomaterial delivery. Initially, the body response most often starts with blood coagulation followed by wound healing. This process is characterized by protein adsorption to the biomaterial, followed by recruitment of cells including macrophages already 60 minutes after implantation of the material. In response to the cytokines and chemokines produced by the macrophages, cells involved in wound healing are attracted [1]. The inflammatory response is very important following surgical tissue damage and material implantation, also called foreign body reaction.

Activation of macrophages is critical in the acute phase of wound healing [2,3]. Macrophages can be roughly divided into pro-inflammatory macrophages, also called M1 macrophages, and anti-inflammatory macrophages, also called M2 macrophages [4,5]. The balance between M1 and M2 plays a critical role in the phagocytosis of pathogens, the clearance of apoptotic cells and the healing and remodeling of injured tissues [6].

Almost immediately after implantation, macrophages are recruited to biomaterials. Depending on the biomaterial specific characteristics, these macrophages will determine the type and intensity of the host-response [6,7]. The eventual success of an implantable medical device strongly depends on this response.

The host-response after implantation is inter alia guided by soluble factors such as cytokines and growth factors, as communication agents between cells, active in the wound healing process. Several studies point out the cytokine classification according to their role in the foreign body response [8-10]. These soluble factors are, among other cell types, produced by macrophages and play pivotal roles in wound healing and serve as useful markers of M1/M2 activation [7,10-12].

The pivotal role of macrophages in the wound healing process, including tissue repair or regeneration supported by biomaterials, is a strong incentive to interrogate the macrophage response, elicited by biomaterials, in well-defined *in vitro* conditions, with reasonable prediction of the complex foreign body reaction by using simplified single cell approaches. For this purpose, human monocyte-derived macrophages, human monocyte cell lines, mouse bone marrow-derived macrophages and murine macrophage cell lines

are used as culture models. In these models, it is examined whether biomaterials elicit a pro-inflammatory, anti-inflammatory, pro-wound healing or an anti-wound healing response by macrophages. These models support the first step to analyze materials before use in the clinic. As nicely reviewed by Sridharan et al. [1] many different properties of the material influence the polarization of the macrophage, among others the mechanical properties, topography, and surface chemistry. Since many types of biomaterials are used in many different culture models with a large variety of read-out parameters, the purpose of this review was to provide an overview of which biomaterial leads to which response, in particular regarding the differentiation and activation of the macrophages and the associated production of soluble factors.

MATERIAL AND METHODS

Search methods

This systematic review was conducted in accordance to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.

Search strategy and study selection

On the 29th of June 2015 a systematic literature search was performed using Medline, EMBASE, Cochrane, PUBMED, Google Scholar, and Web-of-Science libraries, [appendix A]. There were no restrictions used during the search based on the publication year, publication language and type of study. Two researchers (G.B. and N.G.) screened all titles and abstracts of the identified articles independently for their relevance. From all articles that possibly met the inclusion criteria, the full-text version was retrieved and assessed for inclusion. Disagreement was resolved by discussion or requesting advice from a third author (Y.B.J).

An article was eligible for inclusion when it reported on macrophages and their response to biomaterials in an *in vitro* model. Presentations, reviews and letters to the editor were not included. All references from the selected articles were screened for further possible inclusions.

Data extraction and analysis

The extracted data are presented in separate tables. The following information was retrieved from each study: first author, year of publication, culture model, biomaterial, and cytokine expression. A meta-analysis could not be performed due to the lack of sufficient comparative studies and the important variability of the *in vitro* macrophage models (e.g. cell origin and isolation procedure, culture conditions, markers).

Appendix A We used the following search strategy in the Embase, and the searching strategy was modified in other databases accordingly.

('tissue adhesive'/exp OR 'adhesive agent'/de OR 'surgical mesh'/de OR 'surgical equipment'/de OR 'tissue scaffold'/de OR biomaterial/de OR (adhesive* OR glue* OR bioglue* OR tachocomb* OR bucrilate* OR enbucrilate* OR cyanoacryl* OR mesh* OR 4DDOME OR AIGISRx OR AlloDerm OR AlloMax OR 'Bard Composix EX patch' OR 'BIO-A Tissue Reinforcement prosthesis' OR CollaMend OR DermaMatrix OR DualMesh OR 'Evolution P3EM' OR FasLata OR FlexHD OR FortaGen OR 'IntePro Lite' OR InteXen OR NEOVEIL OR 'Optilene Mesh LP' OR 'Parietex composite' OR Pelvicol OR Pelvisoft OR Pelvitex OR PerFix OR 'Peri-Strips Dry' OR PeriGuard OR Permacol OR Physiomesh OR Strattice OR Surgisis OR TIGR OR Timesh OR 'TiMESH light' OR Tutomesh OR Tutopatch OR Ultrapro OR Ventralex OR Veritas OR Vivosorb OR Vypro OR X-Repair OR XenMatrix OR scaffold* OR biomaterial* OR biocompatib* OR Hemocompatib* OR Haemocompatib* OR resorbable OR (implant* NEAR/3 integrat*)):ab,ti) AND ('macrophage culture'/de OR 'monocyte culture'/de OR ((macrophage/exp OR 'macrophage activation'/de OR monocyte/de OR (macrophag* OR monocyte*):ab,ti) AND ('in vitro study'/exp OR monoculture/de OR ('in vitro' OR culture* OR monocultur*):ab,ti))) NOT ([Conference Abstract]/lim OR [Conference Paper]/lim OR [Review]/lim OR [Conference Review]/lim OR [Letter]/lim OR [Note]/lim OR [Editorial]/lim) AND [english]/lim

RESULTS

Search

After the exclusion of 2904 duplicates we identified 4275 references. After screening the titles and abstracts, we excluded another 4169 articles. The other 106 articles were regarded relevant and evaluated as full text. After careful reviewing the full text, another 90 were excluded. In addition seven articles were included via references, resulting in 23 included articles (Figure 1).

Culture models/ experimental conditions

All included studies cultured monocytes or macrophages on biomaterials. However, substantial differences were found in cell culture conditions between the studies. Monocytes isolated from a human buffy coat or human peripheral blood were used in 19/23 of the studies [7,8,11,13-28]. In the other four studies, one used monocytes derived from mouse bone marrow [4], two used the RAW 264.7 cell line (mouse leukaemia monocyte macrophage cell line)[9,29], and the other two used the THP-1 human monocyte cell

line [10,30] (Table 1). In most of the studies, no additional factors were added to the culture medium. However, some also added soluble factors to the media. Medium with LPS (lipopolysaccharides) was the most common, but media also contained LPS/ IFN-γ (interferon gamma, IL-4 (interleukin), IL-4/IL-13 or MCP (monocyte chemotactic protein) -1/IL-6/IFN-γ. The culture time varied from 2 hours to 14 days, but the majority cultured for 1, 3, 7 and/ or 10 days.

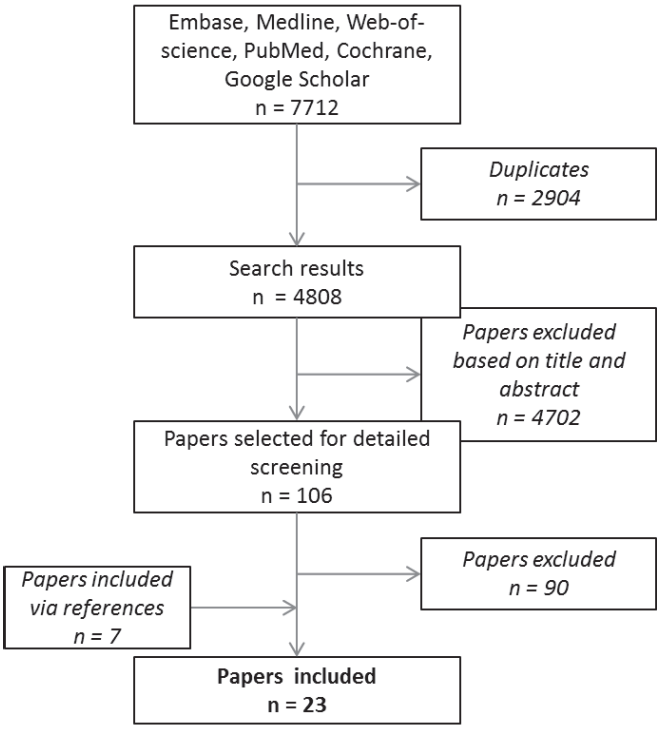

Figure 1. Study selection for relevant articles

Biomaterials

Biomaterials can be divided into three groups namely the non-biodegradable polymers (synthetic), biodegradable polymers (synthetic), and biologic materials [31]. In total 35 different materials were used in the included articles (Table 2).

Non-biodegradable synthetic polymers

Expanded polytetrafluoroethylene (ePTFE) and polytetrafluoroethylene (PTFE) are commonly applied hernia mesh and vascular grafts materials. PTFE, also known as Teflon®, is naturally hydrophobic and non-porous. ePTFE is stretched and nano-porous and was introduced under the trademark GORE-TEX®, in 1969. Monocytes (precursors

of macrophages) on PTFE produced low amounts of IL-1β and high amounts of TNF (tumor necrosis factor)-α and IL-6 in the first days of culture. IL-10 levels increased during culture time, it was mainly produced between culture day 2 and 6[11,16]. After a culture time for 8-10 days the production of TNF-α and IL-10 decreased, while IL-8 increased after 8 days of culture [16]. GM-CSF (granulocyte-macrophage colony-stimulating factor) was secreted during the whole culture time (1-10 days) [11,16,18]. Macrophages on PTFE also produced Platelet-Derived Growth Factor-BB (PDGF-BB), and Matrix Metalloproteinase (MMP) 9 but VEGF (vascular endothelial growth factor) was undetectable [26]. Macrophages on ePTFE produced more pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, TNF-α) and chemokines (MCP-1, MIP1-β, MCP-3) in association with an increase of the pore size of the material [18]. In contrast, immortalized human monocyte cell line (THP-1) cultured on ePTFE induced an anti-inflammatory and pro-wound healing profile characterized by a high IL-10 production in another study [10].

Table 1. Included studies cultured monocytes or macrophages on biomaterials.

Author	year	cells
Almeida et al.	2013	human buffy coat
Ballotta et al.	2014	human buffy coat
Bartneck et al.	2010	human peripheral blood
Bartneck et al.	2012	human peripheral blood
Bhardwaj et al.	2001	human buffy coat
Bhattacharjee et al.	2013	human peripheral blood
Bota et al.	2010	human peripheral blood
Brodbeck et al.	2002	human peripheral blood
DeFife et al.	1995	human peripheral blood
Fearing et al.	2014	THP-1 cell line*
Garg et al.	2013	mouse BMD (bone marrow-derived) Mφ
Gretzer et al.	2003	human buffy coat
Grotenhuis et al.	2013	human buffy coat
Jones et al.	2007	human peripheral blood
Oliveira et al.	2012	human buffy coat
Orenstein et al.	2009	human peripheral blood
Orenstein et al.	2009	human peripheral blood
Orenstein et al.	2010	human peripheral blood
Schachtrupp et al.	2002	human buffy coat
Schutte et al.	2007	THP-1 cell line*
Spiller et al.	2014	human buffy coat
Van den Beucken et al.	2007	RAW 264.7 & J744A.1#
Wagner et al.	2003	human peripheral blood

** THP human leukemic monocyte #RAW / J744 murine macrophage cell line*

Current surgical applications of polyethylene terephthalate (PET) are i.e. surgical meshes, vascular grafts, heart valves and sutures. Macrophages on PET produce predominantly pro-inflammatory cytokines, MCP-3, TNF-α, IL-6, IL-1β, MIP-1α [7,8,32] and pro-inflammatory chemokine IL-8 [32].

PET is also used in combination with different 'coatings'. These coatings affect biomaterial adherent monocyte/macrophage cytokine expression through modification of surface chemistry. Different coatings are used: PET + poly(styrene-co-benzyl N,N-diethyldithiocarbamate) *(BDEDTC; hydrophobic)*, PET + BDEDTC + polyacrylamide *(PAAm; hydrophilic and neutral),* PET + BDEDTC + sodium salt of poly(acrylic acid) *(PAANa; hydrophilic and anionic),* PET + BDEDTC + methyl iodide of poly [3-(dimethylamino)propyl]acrylamide *(DMAPAAmMeI; hydrophilic and cationic)*, and PET + absorbable, continuous and hydrophilic collagen film *(Parietex™ Composite)*. Macrophages on PAAm and PAANa surfaces reacted anti-inflammatory with a higher IL-10 production and lower IL-8 production than when cultured in PET without coating during the culture time from day 3 till day 10[8,32]. Monocytes adherent to PAAm produced the most IL-6, IL-1β, IL-10, IL-8, and MIP-1β at all-time points, compared to the other coatings in combination with PET [32]. Macrophages cultured for 3 to 7 days, produced the highest concentrations of IL-1β on PAAm and least on BDEDTC. MIP-1β concentrations were greatest with PAANa at day 3. DMAPAAmMeI promoted a decrease of IL-10 and IL-1RA in macrophages, but it did not influence on the expression levels of IL-1β, TNF-α and IL-6[7,30]. BDEDTC, PAAm, PAANa, and DMAPAAmMeI let the IL-1β, TNF-α and IL-6 expression levels relatively unchanged at the end of culture time [8,32]. Parietex™ Composite (Covidien) induced high levels of pro-inflammatory and anti-inflammatory proteins [7].

Macrophages cultured on polyethylene (PE), with versatile use such as catheters and joint prosthesis, produced low amounts of cytokines in general but the balance was more towards anti-inflammatory and pro-wound healing cytokines [10,19]. Both THP-1 cell line monocytes/ macrophages and macrophages isolated from human buffy coats cultured on polyurethane (PU), often used in blood contact applications, produced high levels of anti-inflammatory and pro-wound healing cytokines [10,16]. Perfluoropolyether (PFPE) is a non-degradable homopolymer that shows chemical inertness, lipophobicity and has very low surface energy [14]. This material was tested with different micro topographies and the effect on the response of macrophages. Different surface topographies resulted in different cytokine production by macrophages. An M1 surface marker, 27E10, had an enhanced expression in response to closely packed small posts, comparable to when macrophages were stimulated with LPS. In contrast, macrophages cultured on PFPE with large posts expressed the M2 surface marker CD163 the most. Large posts also resulted in significantly the highest M2-M1 index based on macrophages surface markers [14].

Poly(propylene) (PP) is also commonly used mesh and suture materials in surgery. Both an anti-inflammatory reaction characterized by high levels of CCL-18 and IL1-RA amongst others and a pro-inflammatory reaction characterized by production of IL-8, IL-6, and IL-1β by macrophages seeded both for 24h or 3 days on PP were observed [7,27]. When combined with polyglactin 910 materials (Vypro II®, Ethicon), monocyte/macrophages also released high amounts of TNF-α, IL-6, and low amounts of IL-10 after 5 days of culture, which indicates a pro-inflammatory response [11].

Poly(ethylene glycol):poly(acrylate) PEG-g-PA is also modified with cell adhesion promoting peptides (YRGDS and YEILDV, peptides recognized by integrins) to modulate the host cell response [27]. Culturing macrophages on PEG alone resulted in low production of TNF-α, IL-1β, IL-6, and IL-8. Macrophages on peptide modified PEG-*g*-PA produced even lower levels of TNF-α and IL-6 [27].

Poly-D-lysine (PDL) and poly(allylamine hydrochloride) (PAH), both synthetic polymers, were coated with DNA and seeded with two different cell line macrophages. All experiments showed decreased levels of TNF-α compared to the cultured polymers with LPS stimulated murine macrophages (density of 1 x 10^5 cells/cm^2)[9]. The cytokine secretion of IL-1β, IL-10, and TGF-β1 was not different between macrophages cultured on PDL and PAH with or without LPS stimulation [9]. Monocytes on silicone cultured for 10 days produced high GM-CSF and IL-8[16]. TNF-α and IL-10 were produced at high levels the first 2-6 days, where after the production decreased [16].

Biodegradable synthetic polymers

Synthetic biodegradable polymers were first used as biodegradable sutures in the 1960s. Synthetic biodegradable implants are mostly used in the clinic as soft / hard tissue reinforcement materials or temporary barriers / wound supports. Their purpose is to avoid a chronic foreign body reaction [33]. These polymeric biomaterials are based on lactic acid and glycolic acid, as well as other monomers, including dioxanone, trimethylene carbonate e-caprolactone as homopolymers and copolymers.

Polylactic acid (PLA) induces production of IL-6, IL-12/23 and IL-10, these cytokines are both pro-inflammatory and anti-inflammatory, it seemed like that human monocytes cultured on PLA exhibited a heterogeneous profile [13].

Poly(D,L-lactide-co-glycolide) (PLGA) represents a major class of materials widely used in surgical applications and tissue engineering [15]. Bartneck et al. generated 3D nanofibrous meshes in different porosities PLGA/ sP(EO-stat-PO) and a 2D NCO-sP(EO-stat-PO)

hydrogel. NCO-sP(EO-stat-PO) and sP(EO-stat-PO) are ethylene oxide derived polymers, used for preventing unspecific protein adsorption and cell adhesion.

Macrophages on the 2D materials formed clusters with an elevated release of IL-1β and TNF-α. Macrophages produced more IL-8 and CCL-4 (pro-angiogenic chemokines) on the more covered 3D nanofibers PLGA/ sP(EO-stat-PO)[15].

Macrophages seeded on a copolymer of glycolic acid and trimethylene carbonate, also known as GORE® BIO-A® Tissue Reinforcement (WL Gore Assoc), produced very low pro-inflammatory cytokine levels [24]. Polydioxanone (PDO) polymer is developed for biodegradable wound closure sutures. Bone marrow-derived macrophages were cultured on different PDO diameter fibers and pore sizes. An increase of the fiber/pore size resulted in an increased expression of anti-inflammatory and angiogenic markers as VEGF, TGF-β and FGF2[4].

The impact of mechanical cues on adherent monocytes on poly-e-caprolactone bisurea (PCL-U4U) was investigated. It has been demonstrated that strain affects macrophage response in terms of signaling and differentiation. Moderate strain (7%) elicits polarization towards a reparative M2 profile and enhance the expression of genes participating in the immune response [28]. Poly(urethane urea) (PUUR) elicited very small amounts of TNF-α and IL-10 [20].

Biologic materials

Biologic materials are either decellularized tissues such as human or porcine skin or porcine small intestine submucosa (SIS), or fabricated scaffolds or meshes made of natural molecules such as collagen, chitosan, silk, or keratin. The decellularized tissues can have additional chemical cross-links to alter the degradation speed [34].

After 7 days of culture CollaMend™ FM Implant (Bard/ Davol), a moderately chemically cross-linked porcine dermis, mostly elicited a pro-inflammatory response in macrophages with high IL-1β, IL-6, IL-8 and VEGF production [25]. Macrophages on Permacol™ (Covidien), a slightly chemically cross-linked porcine dermal matrix, produced high IL-1β, IL-6, IL-8 and VEGF levels after 7 days of culture [25]. But in other settings, low levels of both pro-inflammatory and anti-inflammatory proteins after 3 days of culture, were released by macrophages, in presence of Permacol™[7]. There were no differences in culture method between the two studies. AlloMax™ Surgical Graft (Bard / Davol) and FlexHD® (Ethicon), non-chemically crosslinked decellularized dermis but of human instead of porcine origin, also induced mainly pro-inflammatory reactions with high IL-1β, IL-6, IL-8, and VEGF cytokine production [23,24]. AlloDerm® Regenerative Tissue Matrix

(LifeCell) (non-chemically crosslinked decellularized human dermis) induced a lower pro-inflammatory response than the other decellularized human dermis, characterized by lower expression of IL-1β, IL-6, IL-8, and VEGF [23,24]. Macrophages seeded on the non-cross-linked porcine dermis, Strattice™ (LifeCell), or on the non-cross-linked porcine small intestine submucosa, Cook® Biodesign® Surgisis® (Cook), produced low levels of IL-1β, IL-6, IL-8, and VEGF [25].

Macrophages cultured on collagen coatings expressed mostly M1 surface markers (CD86+) and express both M1 and M2 markers [30,35]. These macrophages produced also high levels of pro-inflammatory cytokines. Another collagen-based biomaterial is Avitene™ UltraFoam™ Collagen Sponge (Bard / Davol; bovine source collagen sponge). Macrophages cultured on this gel did not produce IL-1β, and IL-6 production was only seen at day 1 and was lower produced at day 3, indicating that the response of the macrophages was not pro-inflammatory [17].

Other noncommercial biopolymers have been investigated. Bhattacharjee et al. studied the macrophage responses against silk-fibroin and silk-sericin based 2D films, and 3D silk-fibroin scaffolds [17]. These scaffolds are used for tissue engineering and drug delivery. The 3D fibroin scaffold induced gene expression of pro-inflammatory genes and accordingly the production of IL-1β and IL-6. Silk-sericin films also induced IL1-β gene expression [17].

Two other biologic biomaterials are keratin and chitosan. Keratin has been described for applications such as tissue regeneration, hemostasis, and wound healing. A low foreign body reaction against keratin was described characterized with predominantly M2 (CD206+) macrophages, high levels of IL-10 and low levels of IL-1β and IL-6[30]. Chitosan (a natural polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine) induced an M2 phenotype in one study based on low TNF-α that decreased with time and high IL-10 and TGF-β1 levels cytokines [22]. In another study chitosan induces a predominant M1 response based on high production of TNF-α and IL-12/IL-23 and low expression of IL-6, especially in the 3D geometry [13]. Oliveira et al. cultured on chitosan films instead of 3D geometry [13].

DISCUSSION

Macrophages are key components of tissue repair and remodeling in wound healing. Their polarization appears to depend on the type of biomaterial and their characteristics. The release of a variety of cytokines and chemokines is decisive for the differentiation

Table 2. This table present the reviewed biomaterials and their predominant reaction. This table shows results coming from different macrophage models, not necessarily equivalents. The results are adapted generally from 1 study.

BIOMATERIAL	Predominant reaction of macrophages in contact with biomaterial	Low/ high cytokine production	Ref.
Polytetrafluoroethylene (PTFE)	Mainly pro-inflammatory	High	10, 15
ePTFE	Pro-inflammatory and anti-inflammatory	High/high	9, 17, 25
Polyethylene terephthalate (PET)	Mainly pro-inflammatory	High	6, 7, 20
PET + BDEDTC	Mainly pro-inflammatory	High	7, 20
PET + BDEDTC + PAAm	Mainly anti-inflammatory	High	7, 20
PET + BDEDTC + PAANa	Mainly anti-inflammatory	High	7, 20
PET + BDEDTC + DMAPAAmMeI	Mainly pro-inflammatory	High	7, 20
Parietex™ Composite	Pro-inflammatory and anti-inflammatory	High/high	6
Polyethylene	Mainly anti-inflammatory	Low	9, 18
Polyurethane	Pro-inflammatory and anti-inflammatory	High/high	9, 15, 18
Perfluoropolyether (small posts)	Mainly pro-inflammatory	High	13
Perfluoropolyether (large posts)	Mainly anti-inflammatory	High	13
Polypropylene (PP)	Pro-inflammatory and anti-inflammatory	Low/low	6, 26
PP + polyglactin	Mainly pro-inflammatory	High	10
Poly(ethylene glycol):poly(acrylate)	Mainly anti-inflammatory	Low	27
Poly-D-lysine-PAH	Mainly pro-inflammatory	Low	8
Silicone	Pro-inflammatory and anti-inflammatory	High/high	15
Polylactic acid (PLA)	Pro-inflammatory and anti-inflammatory	High/high	12
Poly(ethylene oxide) (PEO)	Mainly pro-inflammatory	High	14
Bio-A	Mainly pro-inflammatory	Low	23
Polydioxanone (PDO)	Mainly anti-inflammatory	High	3
Poly-e-caprolactone bisurea	Mainly anti-inflammatory	High	27
Poly(urethane urea) (PUUR)	Pro-inflammatory and anti-inflammatory	Low/low	19
Collamend™	Mainly pro-inflammatory	High	24
Permacol™	Mainly pro-inflammatory/ Pro-inflammatory and anti-inflammatory	High/low	6, 24
Allomax	Mainly pro-inflammatory	High	22, 23
FlexHD	Mainly pro-inflammatory	High	22, 23
Alloderm	Mainly pro-inflammatory	Low	22, 23
Strattice™	Mainly pro-inflammatory	Low	24
Surgisis®	Mainly pro-inflammatory	Low	24
Collagen coating	Mainly pro-inflammatory	High	28
Ultrafoam	Mainly pro-inflammatory	Low	16, 25
Silk	Mainly pro-inflammatory	High	16
Keratin	Pro-inflammatory and anti-inflammatory	Low/high	28
Chitosan	Pro-inflammatory and anti-inflammatory	Low/high	12, 21

and activity of monocytes [36]. Here, we reviewed the macrophage response on different materials *in vitro* used in tissue repair and regeneration and provided an overview of commonly seen macrophage responses to these biomaterials.

Based on the literature review, we have shown that the dimensions of the cultured material is of great influence on the response of macrophages. This was (mostly) investigated in PFPE, ePTFE, chitosan and polydioxanone. The association was however different between increasing fiber/ pore size and the polarization or release profile of macrophages. Two synthetic biomaterials showed the opposite effect of pore size. Bartneck et al. showed a higher pro-inflammatory effect when the pore size was smaller in PFPE [14]. Bota et al. saw a higher pro-inflammatory effect of macrophages cultured on ePTFE when the pores are larger [18]. Almeida et al. saw the same effect, on scaffolds based on chitosan, a biologic material [13]. In contrast, Garg et al. cultured macrophages on polydioxanone, a synthetic biodegradable material, and they showed that large pores induced M2 phenotype and a decreased M1-marker expression. However in this study, mouse bone marrow-derived macrophages were used instead of human macrophages. In an in vivo study with biodegradable pHEMA (2-hydroxyethyl methacrylate) hydrogel scaffolds it was also shown that pore size affect macrophage response. Pore size of 34 µm was shown to reduce fibrous encapsulation, however more M1 cells were found than at those scaffolds with a larger pore size of 160 µm, this indicate that the initial M1 response is necessary [37].

As expected, macrophages on moderately chemically cross-linked human or porcine dermis responded in general pro-inflammatory with higher amounts of pro-inflammatory cytokines than the macrophages cultured on non-chemically cross-linked or slightly chemically cross-linked materials. This was also seen in *in vivo* studies were Collamend™ FM Implant (Bard/ Davol) induced a chronic foreign body response and downstream encapsulation [38,39]. This mainly pro-inflammatory response lead to chronic fibrosis [40]. Unfortunately, in all *in vitro* studies on these biologic materials, were only investigated IL-1β, IL-6, IL-8 and VEGF, known for their mainly pro-inflammatory response, no anti-inflammatory cytokines were measured. A recent review presented that moderately to strongly cross-linked collagen materials can alter normal wound healing. In particular, residues of chemical crosslinks in the material were associated with a M1 macrophage response, and inhibition of M2 macrophage polarization [34].

Chitosan, another biopolymer, showed a predominant M1 response with a very low IL-6 production [13]. The same effect was seen on the collagen gel; mainly pro-inflammatory cytokines were produced, but no production of IL-1β [17]. This can be considered a pleiotropic function of IL-6 and IL-1β. It is known that IL-6 can act either pro-inflammatory or

anti-inflammatory, depending on the environment [41]. IL1-β is a key cytokine that is important for wound healing, activating and recruiting fibroblasts, resulting in expression of extracellular matrix components like collagen, elastin and glycosaminoglycans [42-44].

Some materials induced different responses in different experiments such as acellular human dermis from different companies. This could be due to the differences in sterilization techniques that induce lasting biochemical changes or residues of the chemical used for sterilization; gamma radiation is used for AlloMax™ Surgical Graft (Bard Davol), FlexHD® (Ethicon)is sterilized by detergents, disinfectants and ethanol, and the sterilization process of AlloDerm® Regenerative Tissue Matrix is proprietary. AlloDerm® induced the least of the pro-inflammatory cytokines. Also the methods of decellularization and processing of the materials were different which can be an additional explanation for the different foreign body responses, notably explained by chemical residues, used for decellularization and fat removal.

Comparing all the responses of the different materials, it seems that polyethylene, PET + PAAm, PET + PAANa, PFPE (large posts), PEG-g-PA, and PDO always elicited an anti-inflammatory response in macrophages, irrespective of origin of the macrophages.

In vitro testing of macrophage response to biomaterial can be an initial means of assaying biocompatibility. Macrophages are certainly great drivers of the acute inflammation reaction, but not only. Also neutrophils (polymorphonuclear leukocytes, PMNs) characterize the acute inflammatory response. Mast cell degranulation with histamine release and fibrinogen adsorption is also known to mediate acute inflammatory responses to implanted biomaterials [45,46]. For a complete *in vitro* model, these factors should also take into account. For example, Surgisis® is known to strongly activate PMNs, particularly neutrophils [47]. Bryan et al. show a strong release of Reactive Oxygen Species by Surgisis® versus Alloderm® and Permacol™, in animal models [48].

In general, *in vitro* models are useful in the first step to evaluate the foreign body reaction on surgical biomaterials. Although it is difficult to simulate the environment during a surgical procedure, an *in vitro* model gives an indication of the initial foreign body response even in an environment that simulates an infection by for instance addition of LPS. Grotenhuis et al. proved this by simulating a bacterial infection in their *in vitro* model, but the macrophage response remained biomaterial dependent [49]. In this perspective it will be useful to test for example other surgical biomaterials like tissue adhesives that are used in the clinic.

Because of the complexity of host response to foreign body material it is difficult to predict the *in vivo* outcome from *in vitro* assays. Wolf et al. developed an *in silico* analysis by using an *in vitro* assay that characterized the dynamic inflammatory response of human monocyte derived macrophages to biomaterials in combination with quasi-mechanistic analysis [50]. This approach can be used to better predict the *in vivo* response. More sophisticated systems, like multi cellular approaches combining macrophages with fibroblasts, endothelial cells, stem cells, aiming at recreating a better mimicking system, should certainly be useful for the in-depth investigation of the behavior of materials *in vivo* [51].

Simple models as single cell approaches should be used for screening approaches, enabling the direct comparison of materials. Macrophage models can gain even higher interest by including monocytes from specific patient groups, like obesity, which may react differently to materials.

CONCLUSION

With this review, we provided an overview of *in vitro* responses of macrophages to many different biomaterials. Some materials performed comparable in different studies and it seems clear which response these biomaterials elicit in macrophages. Other materials behaved differently in different culture set-ups. Therefore all physical properties (e.g. stiffness, pore size, strain, topography, surface chemistry) of the biomaterial must be announced, because these features can induce different macrophage behavior [1,40]. Each step in cell culture is critical, the macrophage isolation, activation of the macrophage before culture or not, time duration of cell culture since it conditions the phenotype/ differentiation of cells, and the type of culture medium, minimal changes in culture methods can cause the different outcome [2,36,52,53]. *In vitro* culture models using macrophages on biomaterials are a valuable addition to the development of new biomaterials. Based on this review there is however to be a need for standardized culture models and a systematic comparison to the *in vivo* response.

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Chapter 14

Monocyte subsets in blood correlate with obesity related response of macrophages to biomaterials *in vitro*

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ABSTRACT

Macrophages play a key role in the foreign body response. In this study it was investigated whether obesity affects the acute response of macrophages to biomaterials *in vitro* and whether this response is associated with biomarkers in blood. CD14+ monocytes were isolated from blood from obese and age and gender matched lean persons. Monocyte subsets were determined based on CD14 and CD16 on their surface. C-reactive protein (CRP) was measured in peripheral blood. The response of monocyte-derived macrophages to polypropylene (PP), polylactic acid (PLA), polyethylene terephthalate (PET) monofilament, and PET-multifilament (mPET) in culture was based on cytokine production. More IL-6 (for PET), less CCL18 (all materials) and IL-1ra (for PLA) was produced by macrophages from obese patients than lean subjects. Body mass index, serum CRP and to a lesser extend percentages of monocyte subtypes correlated with IL-6, TNFα, CCL18, and IL-1ra production. Taken together, monocyte-derived macrophages of obese patients respond more pro-inflammatory and less anti-inflammatory to biomaterials than macrophages from lean subjects, depending on the material. These results are a step towards personalized medicine for the development of a model or even a blood test to decide which biomaterial might be suitable for each patient.

INTRODUCTION

Biomaterials are often used in several surgical disciplines such as urology, gynaecology and general surgery [1]. The foreign body response to implanted biomaterials is crucial for adapting the material in the human body. Macrophages play a key role in the foreign body reaction to biomaterials [2]. For regenerative biomaterials, an initial pro-inflammatory (M1) response is necessary for recruiting inflammatory cells to encourage the foreign body response, which are necessary events for wound healing including ingrowth. However, a prolonged M1 response results in fibrous capsule formation and extended inflammation. Therefore, a subsequent transition to the anti-inflammatory macrophages (M2), which promotes tissue repair and remodeling, is generally presumed to be the preferred modification [3]. Achieving the desired outcome is individual and biomaterial dependent.

In general, obesity seems to be an important factor for adverse outcomes after surgery. Observed complications are surgical site infections, impairment of cutaneous wound healing, wound failure, anastomotic leakage, and fascia dehiscence [4-6]. These complications are major risk factors to develop incisional hernia or a recurrent incisional hernia after repair [7, 8]. Potential factors that increase wound complications by obesity include intrinsic tenuous anatomic properties, poor vascularization, and cellular and molecular alterations. Inflammatory mediators such as tumor necrosis factor alpha (TNFα), interleukin 6 (IL-6), leptin, and angiotensin increase simultaneously with increasing mass of adipose tissue and adipocyte size [4]. These factors negatively affect wound healing and are most likely produced by many types of cells than macrophages alone. Obesity is also positively correlated with oxidative stress which can lead to decreased oxygen tension and impaired fibroblast proliferation and collagen synthesis [4].

Due to obesity, macrophages undergo a phenotypic switch from M2 to M1, which leads to a chronic low-grade systemic inflammation [9-13]. Monocytes, the precursors of macrophages, can be divided into subsets, according to their expression of the cell surface antigens CD16 (Fcg receptor III) and CD14 (a receptor for bacterial lipopolysaccharide (LPS))[14]. The classical monocyte has high CD14 ($CD14^{++}$) cell surface expression and is CD16 negative ($CD16^{-}$). The non-classical monocyte also expresses CD14 at its surface but at an approximately ten times lower level than the classical monocyte ($CD14^{+}$), and is positive for CD16 ($CD16^{++}$). The intermediate monocyte expresses CD14 at a high level ($CD14^{++}$), and CD16 at an approximately ten times lower level than the non-classical monocyte ($CD16^{+}$). In general, monocytes expressing CD16 have a high phagocytic capacity and produce more pro-inflammatory cytokines such as TNFα and IL-6, and are therefore considered pro-inflammatory [15]. The classical $CD14^{++}/CD16^{-}$ subset is the

predominant population and accounts for approximately 90% in healthy persons. It has been suggested that obesity leads to a shift from classical towards intermediate and non-classical monocytes [16, 17].

Previous *in vitro* models have shown that culturing macrophages isolated from healthy donors on different biomaterials leads to a biomaterial-specific reaction [18] and that even in a contaminated *in vitro* model, surgical biomaterials still elicit differential reactions in macrophages [19]. These *in vitro* models did not take into account patient specific characteristics, such as age, smoking, diabetes or obesity. Obesity is a growing healthcare issue in the clinics and a subgroup of these patients does receive a biomaterial for several reasons like abdominal wall hernia with an increased risk of unwanted reactions to the biomaterial or delayed wound healing [4, 5]. Therefore, the aims of this study were to investigate how obesity affects the acute host response of macrophages to biomaterials *in vitro* and to examine whether this *in vitro* response can be predicted beforehand by determining monocyte subsets in the blood or by measuring the systemic inflammation marker CRP that is a common used clinical parameter for inflammation.

METHODS

Study population

In total we included 20 obese patients and 20 age and gender matched healthy, lean (BMI 18-25 kg/m^2) volunteers. Obese patients with a BMI >30 kg/m^2 were included at the department of bariatric surgery at the Maasstad Hospital, Rotterdam. Exclusion criteria for both groups were smoking, diabetes mellitus, use of immunosuppressive drugs, autoimmune disease or chronic inflammatory disease, and medical history such as previous surgery or having a prosthesis (e.g. vascular implants, mesh, osteosynthesis material). This study was approved by the Medical Ethical Committee of the Erasmus University Medical Center, Rotterdam, Netherlands, in accordance with the Dutch law on medical research in humans. Permit number MEC-2014-221, NL47780.078.14. Written informed consent was obtained from all patients.

Biomaterials

Four types of biomaterials were selected for use in all experiments: polypropylene (PP; 0.9 g/cm^3), polyethylene terephthalate multifilament (mPET; 1.34 g/cm^3), polyethylene terephthalate monofilament (PET; 1.34 g/cm^3) and polylactic acid (PLA; 1.25 g/cm^3) (Figure 1). All materials were provided as yarns braided in the same conformation. All tested materials were braided according to a similar pattern and with same volumic density, corrected by the g/cm3 values by each material. The braided materials are created of a

mix of micro- and macro-porosity that favors cell attachment, particularly for monofilaments, and even more particularly, for polypropylene monofilaments.

Monocyte isolation and seeding on biomaterials

Peripheral blood mononuclear cells (PBMC) were isolated from 30 mL blood of obese patients and healthy volunteers by gradient density separation using Ficoll-Paque PLUS (GE Healthcare Life Sciences, Little Chalfont, UK). The blood from the obese patients was obtained preoperatively to bariatric surgery. Monocytes were isolated by $CD14^+$ selection. Briefly, the blood was diluted 1:1 with PBS (Gibco; Carlsbad, USA) supplemented with 0.1% BSA (Sigma-Aldrich, St. Louis, USA), applied on top of a Ficoll layer, and centrifuged at 900 x *g* for 30 minutes to acquire separation of layers. The interphases were collected and washed twice with PBS/0.1% BSA before a 20 minute incubation at 4^0C with anti-human CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Positive selection was performed by Magnetic-acitvated cell sorting (MACS). The isolated $CD14^+$ monocytes were kept in suspension in X-VIVO™ 15 medium (Lonza, Verviers, Belgium) containing 20% heat inactivated fetal calf serum (FCS; Lonza), 50 µg/mL gentamicin (Gibco) and 1.5 µg/mL amphotericin B (Fungizone; Gibco), from now on referred to as 'culture medium', until seeding. Prior to seeding, the biomaterials were pre-conditioned in non-heat inactivated FCS for 2 hours at 37^0C with agitation. After pre-conditioning, the monocytes were seeded by rotation onto the biomaterials for 2 hours at 37^0C at 20 rpm (VWR tube rotator, Radnor, Pennsylvania, USA). The materials were exposed to 850,000 monocytes per yarn at a concentration of 500,000 monocytes/mL. After seeding, the materials were carefully transferred to 96-well plates (Corning Costar, NY, USA) and cultured in 125 µL culture medium per well. Per patient, four different materials in triplicate were cultured. The culture system is shown in Figure 1. After 2 days of culture, the materials were transferred to new wells and medium was refreshed to only take into account the biomaterial adherent cells. Twenty-four hours after refresh, the medium was collected while keeping the three samples separate, centrifuged for 10 minutes at 300 x *g* and stored at -80^0C for later cytokine quantification. The macrophages adhering to the biomaterials were lysed in 125 µL PBS/0.1% Triton-X (Sigma-Aldrich) and stored at -20^0C before DNA quantification. The remaining $CD14^+$ monocytes that were not used for seeding were stored in 20% dimethyl sulfoxide (DMSO)/FCS in liquid nitrogen for flow cytometric analysis.

Protein adsorption by the biomaterials

To evaluate potential adsorption of the proteins by the materials, the materials were pre-conditoned for 2 hours in non-heat inactivated FCS with agitation, followed by 2 hours incubation in X-VIVO/20% FCS in a tube rotator at 37°C. Next, the materials were transferred to well plates and incubated in X-VIVO/20% FCS for 2 days. After this period, the materials were transferred to new well plates and incubated in medium containing

either 1 ng/mL IL-6 (Peprotech), 250 pg/mL CCL18 (R&D Systems), 1.25 ng/mL IL 1RA (R&D Systems), 500 pg/mL TNFα or no cytokine. After an additional incubation day, the media were harvested, centrifuged at 300 x g and stored at -80°C until cytokine quantification. The use dosages were based on the detection ranges of the enzyme-linked immunosorbent assays (ELISAs) that were used to determine cytokine concentrations.

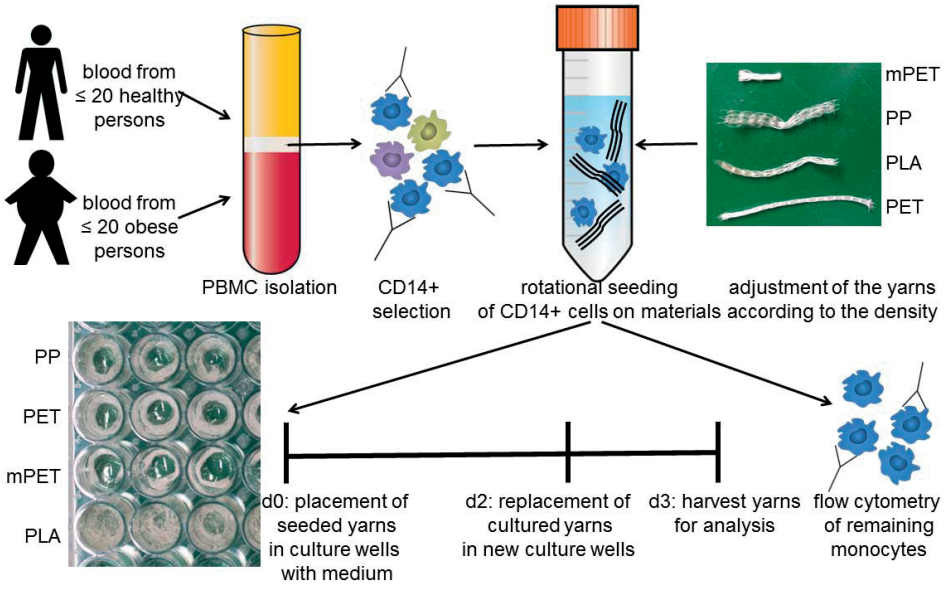

Figure 1. Experimental flow of our study, including pictures of the yarns and how the yarns were placed in the culture wells. CD14 = cluster of differentiation 14, PP = polypropylene, PLA = polylactic acid, PET = monofilament polyethylene terephthalate, and mPET = multifilament polyethylene terephthalate.

Cytokine quantification

Enzyme-linked immunosorbent assays (ELISAs) were performed according to manufacturer's instructions to quantify the concentrations of CCL18, IL-1ra, IL-6, and TNFα (R&D Systems, Minneapolis, MN, USA) released in the cell culture supernatants. These selected cytokines were chosen based on our previous research in which CCL18, IL-1ra, IL-6, and TNFα were the most discriminative for the different macrophages phenotypes [18, 20]. All measurements fitted within the standard curve, for every material and cytokine different dilutions had to be made of the culture medium, ranging from a 3 to 100 times dilution. C-Reactive Protein (CRP) levels in the plasma were determined using the standard technique at the hospital's laboratory (Dimension Vista® System, Flex reagent cartridge, Siemens Healthcare Diagnostics Products, Germany) and expressed in mg/L. CRP is a very common used parameter in all hospitals to detect early systemic inflammation, also prior to surgery, especially in obese patients.

DNA quantification

Since cell attachment was different between materials we normalized the protein content in the culture media to the amount of DNA on the biomaterial as an indication for the cell number. By normalizing for DNA, we adjust for variation in cell number allowing to determine the production of cytokines per cell, not influenced by the number of cells that adhered to the material. DNA was quantified with a modified CYQUANT® cell proliferation assay (Invitrogen, Carlsbad, California, USA), in order to normalize the cytokine production for the number of cells. In short, the samples were sonicated for 30 minutes at 48 kHz to completely disintegrate the cells. Next, a solution containing 250 IU heparin (LEO Pharma, Ballerup, Denmark) and 125 µg RNAse (Sigma-Aldrich) were added to the suspensions and incubated for 30 minutes at 37°C. Finally, 0.375 µL CyQUANT GR dye was added to each sample and fluorescence was immediately measured on a SpectraMax Gemini micro plate reader (Molecular Devices, Sunnyvale, USA) at 480 nm excitation and 520 nm emission.

Flow cytometric analysis

Monocytes were thawed from -80°C and re-suspended at 500,000 cells/mL in FACSFlow solution (BD Biosciences) and stained for 30 minutes at 4°C with antibodies against human CD14 conjugated with APC-H7 and CD16 conjugated with PE (both BD Biosciences), according to the manufacturer's guidelines. Unstained cells were used as negative control. Flow cytometric analysis was performed using the FACSJazz™ (BD Biosciences), and data were analyzed using FlowJo (FlowJo v7.6.4/v10; Ashland, OR, USA). As can be seen in supplementary Figure 1, cells changed in shape and granularity (A and C), and most likely because of cell death, less cells were stained with either of the two antibodies after freezing and thawing. Percentages of monocytes subsets remained however comparable (B and D).

Statistical analysis

Statistical analysis was performed with SPSS 21.0 (IBM Inc., Chicago, USA). Basic characteristics are presented as mean ± standard deviation (SD) and data related to cytokines are presented as mean and standard of mean (S.E.M.). An independent T-test was used for the age and BMI due to normal distribution of these parameters. Mann Whitney U analysis was used for statin use. To compare cytokine levels between macrophages obtained from lean and obese subjects and compare cytokine levels between the different materials within the obese and control group, a Kruskal-Wallis analysis followed by a post-hoc Mann Whitney U analysis was performed. An M1/M2-index per material was calculated based on the cytokine production of pro-inflammatory (M1) cytokines IL-6 and TNFα and anti-inflammatory (M2) cytokines CCL18 and IL-1ra. The mean of the relative M1 cytokine levels per patient to the overall M1 cytokine levels of all patients,

was divided over the mean of the relative M2 cytokine levels per patient to the overall M2 cytokine production of all patients, as shown in the following formula.

$$M1/M2 - index = \frac{\frac{(\mathrm{IL{-}6_{per\ patient}} + \mathrm{TNF\alpha_{per\ patient}})}{(\overline{\mathrm{IL{-}6_{all\ patients}}} + \overline{\mathrm{TNF\alpha_{all\ patients}}})}}{\frac{(\mathrm{CCL18_{per\ patient}} + \mathrm{IL1ra_{per\ patient}})}{(\overline{\mathrm{CCL18_{all\ patients}}} + \overline{\mathrm{IL1ra_{all\ patients}}})}}$$

To determine correlations, a non-parametric Spearman test was performed. All reported *p*-values were two-sided; a *p*-value < 0.05 was considered to indicate statistical significance. Since the analyses were exploratory and the groups sizes small, no adjustment for multiple testing was performed.

RESULTS

As a result of our inclusion criteria, BMI was significantly different between the included lean and obese subjects. Age, gender and the use of statins were not significantly different between the two groups (Table 1).

Table 1. Patient characteristics lean group vs obese patients.

	lean (*n*=20)	obese (*n*=20)	*p*-value
BMI (kg/m^2)	22.9 ± 2.6	43.8 ± 6.5	<0.001
gender (male/ female)	2/18	2/18	1.0
age (years)	41.8 ± 13.1	41.3 ± 13.5	0.916
use of statins	0/20	2/20	0.154

Values are means (SD), p-value was estimated by using independent sample T-test

Obesity influenced cytokine production by macrophages on biomaterials

The production of IL-6 and TNFα as indicators for a pro-inflammatory response and CCL18 and IL-1ra as indicators for an anti-inflammatory response were measured. Macrophages from obese patients produced significantly more IL-6 than macrophages from lean subjects when cultured on PET (144.0 pg IL-6/ng DNA vs 102.0 pg IL-6/ng DNA, $p = 0.022$). No significant differences were seen for the other materials regarding IL-6 production (Table 2 and Supplementary Figure 2A). TNFα production was not significantly different between the groups for any of the tested materials (Table 2 and Supplementary Figure 2B). CCL18 production was significantly higher for all materials in the lean group than in the obese group (Table 2 and Supplementary Figure 2C). IL-1ra production was higher in the lean group than in the obese group when cultured on PLA (34.6 pg IL-1ra/ng

DNA vs 15.5 pg IL-1ra/ng DNA, $p = 0.026$) but not on the other materials (Table 2 and Supplementary Figure 2D).

Table 2. The average production of IL-6, TNFα, CCL18, and IL-1ra corrected for DNA by macrophages on the different materials.

		cytokine production by macrophages (pg protein/ng DNA)		
cytokine	material	lean (mean ± SD)	obese (mean ± SD)	*p* value
IL-6	PP	116.6 ± 97.2	172.4 ± 114.1	0.106
	PLA	109.2 ± 67.1	157.4 ± 146.8	0.247
	PET	102.0 ± 73.9	144.0 ± 58.4	**0.022**
	mPET	39.2 ± 23.9	68.7 ± 64.0	0.140
TNFα	PP	7.9 ± 8.3	5.9 ± 4.9	0.300
	PLA	5.0 ± 4.4	3.3 ± 3.1	0.119
	PET	3.6 ± 3.8	3.3 ± 1.5	0.417
	mPET	1.0 ± 0.9	0.7 ± 0.5	0.421
CCL18	PP	0.8 ± 0.7	0.2 ± 0.3	**< 0.001**
	PLA	1.4 ± 0.9	0.4 ± 0.4	**0.002**
	PET	1.6 ± 1.3	0.5 ± 0.6	**0.002**
	mPET	0.7 ± 0.6	0.3 ± 0.3	**0.007**
IL-1ra	PP	49.4 ± 52.2	24.4 ± 16.8	0.128
	PLA	34.6 ± 28.5	15.5 ± 9.0	**0.026**
	PET	32.3 ± 22.6	20.2 ± 13.6	0.071
	mPET	18.0 ± 18.4	10.0 ± 12.3	0.057

Bold values denote statistical significance

No IL-6, TNFα, CCL18, and IL-1ra were detectable in medium with serum alone and thus also no difference was seen after incubation of the material in medium with serum but without adherent cells. When the proteins of interest were spiked in the culture medium, adsorption of these proteins was seen to the materials, with the most adsorption of all four proteins to PP, and PLA in the case of IL-6 (Figure 2).

The DNA concentration as an indication for the number of attached macrophages to the biomaterials, was not significantly different between the lean and obese patients in all biomaterials (Supplementary data Figure 3A). Absolute protein production per individual is shown in supplementary Figure 2B-D. When comparing the effect of the materials on macrophages within the obese and lean group and per material, PP induced higher levels of IL-6, TNFα, and IL-1ra than the other materials, especially when compared to mPET. Less clear differences between materials were seen for CCL18 production (Figure 3).

To compare overall response of the different materials in lean and obese subjects, an M1/M2 index was calculated for each condition. The M1/M2 index was significantly higher of the obese group than for the lean subjects for PP ($p < 0.001$), PET ($p = 0.001$), and

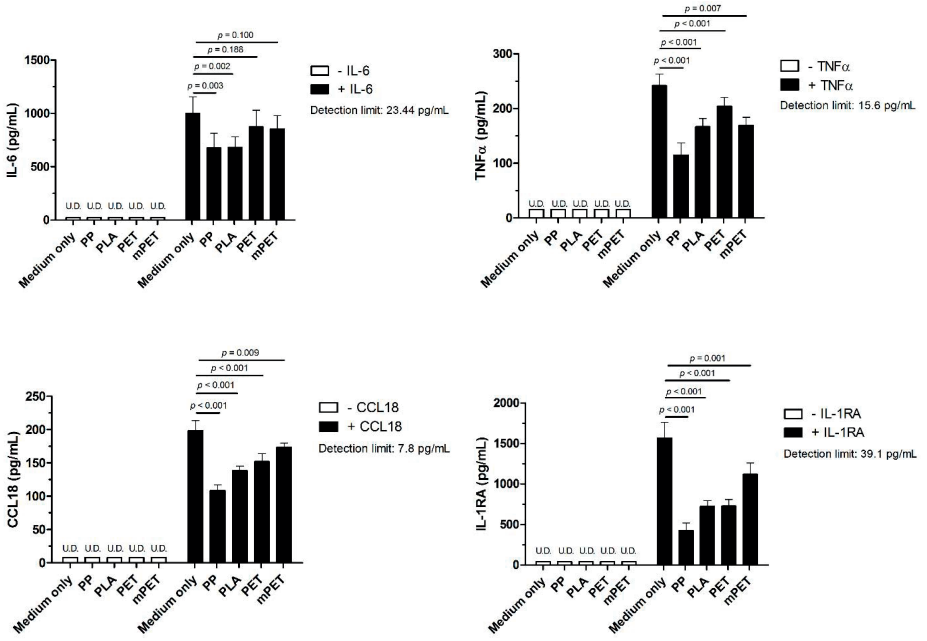

Figure 2. Measurements of IL-6 (A), TNFa (B), CCL18 (C), and IL-1ra (D) in the culture medium with and without the incubation of the biomaterials and with and without spiking of the protein of interest. White bar indicates measurements in medium with or without incubation of the materials. Black bars indicate measurements in medium alone or after incubation with the material in the presence of the spiked proteins. Bars represent n= 6 + sd for every bar.

mPET (p = 0.003) but not for PLA. No differences regarding the M1/M2 index were seen between materials for the lean subjects. In obese patients, PLA resulted in the lowest M1/M2 index, and mPET the highest (Figure 4).

Serum CRP and BMI correlate with cytokine production by macrophages

The average C-reactive protein level in lean subjects was 1.3 ± 1.8 mg/L versus 15.6 ± 17.1 mg/L in obese patients, p = 0.004 (Supplementary Figure 4). CRP concentration positively correlated with BMI (Table 3). A positive correlation was also seen between CRP and IL-6 production in response to the material for all materials, but only significant for PP and mPET. A significant negative correlation was seen between serum CRP concentration and CCL18 production by macrophages in response to PP, PLA, and mPET and between BMI and CCL18 production by macrophages in response to PP, PLA, and mPET. CRP also negatively correlated with IL-1ra production in response to PP, PLA, and mPET. CRP or BMI did not correlate with TNFα production (Table 3).

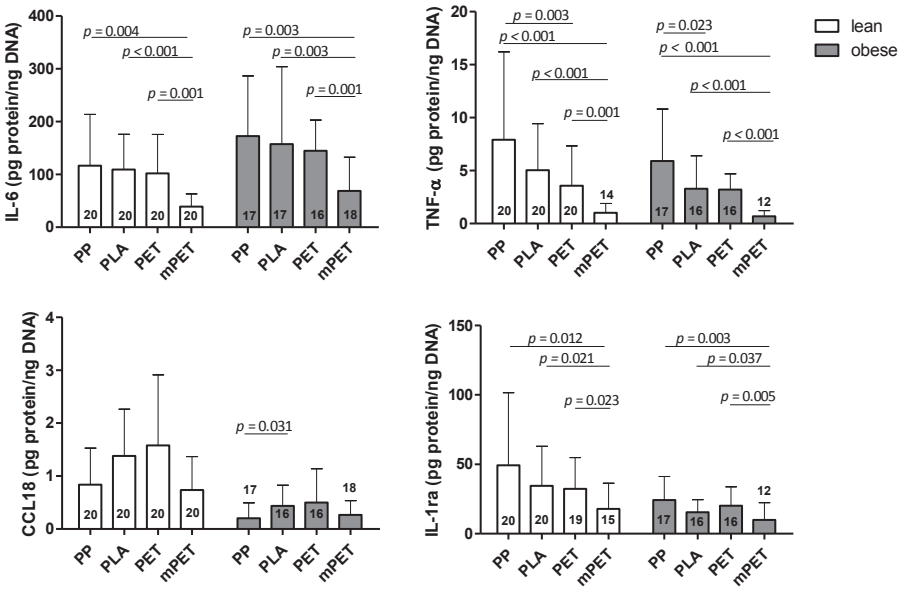

Figure 4. Cytokine production corrected for DNA compared per material, in lean subjects or in obese subjects. Number of patients per cytokine and per material are indicated in the bars or just above the error bar.

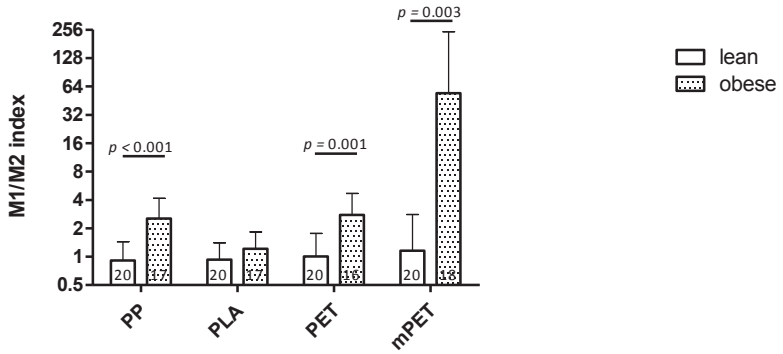

Figure 5. M1/M2 index between materials. Bars represent the mean, error bars the SD, *p*-values indicate significant differences. A base 2-log scale is used for the Y-axis. PP = polypropylene, PLA = polylactic acid, PET = monofilament polyethylene terephthalate, and mPET = multifilament polyethylene terephthalate. The number of patients included per material, per group are indicated in the bars.

Differences in monocyte subsets between lean and obese patients

The percentages of classical monocytes ($CD14^{++}CD16^{-}$), intermediate monocytes ($CD14^{++}CD16^{+}$), or non-classical monocytes ($CD14^{+}CD16^{++}$) in peripheral blood were not statistically significantly different between lean and obese subjects (Table 4). However, the percentages of intermediate monocytes correlated positively with IL-6 for PLA and

Table 3. Correlations between CRP concentration in peripheral blood, BMI of all subjects, and cytokine production by the macrophages.

		CRP		BMI	
	material	r	*p*- value	r	*p*-value
CRP	-	-	-	0.64	**< 0.001**
IL-6	PP	0.37	**0.046**	0.27	0.111
	PLA	0.20	0.310	0.22	0.198
	PET	0.40	0.035	0.27	0.146
	mPET	0.53	**0.003**	0.22	0.186
CCL18	PP	-0.45	**0.012**	-0.44	**0.006**
	PLA	-0.56	**0.002**	-0.37	**0.028**
	PET	-0.36	0.063	-0.39	**0.017**
	mPET	-0.54	**0.002**	-0.30	0.068
IL-1ra	PP	-0.36	**0.05**	-0.15	0.391
	PLA	-0.45	**0.015**	-0.20	0.245
	PET	-0.35	0.075	-0.22	0.211
	mPET	-0.54	**0.013**	-0.22	0.267
TNFα	PP	-0.17	0.382	-0.18	0.295
	PLA	-0.15	0.438	-0.24	0.16
	PET	0.14	0.492	0.02	0.903
	mPET	-0.17	0.476	-0.30	0.143

Bold values denote statistically significant *p*-values

negatively with the CCL18 protein production for PET and mPET, and with IL-1ra for mPET. The percentages of non-classical monocytes correlated negatively with CCL18 production when macrophages were cultured on mPET. No statistically significant correlations were seen between percentages of monocyte subsets and TNFα production by the macrophages cultured on any of the biomaterials (Table 5A). For PP and mPET the M1/M2 index significantly correlated with the percentage of classical monocytes. Intermediate monocytes significantly correlated negative with the M1/M2 index for mPET. Supplementary Figure 1 shows that the percentages of monocyte subsets are unaffected before and after thawing.

Table 4. Percentages of peripheral blood monocytes subsets in lean (n = 9) and obese (n = 8) subjects. Values are mean +/- sd.

% of monocyte	lean	obese	*p*-value
classical ($CD14^{++}CD16^{-}$)	90.9 ± 5.3	77.4 ± 22.0	0.290
intermediate ($CD14^{++}CD16^{+}$)	2.2 ± 3.4	7.9 ± 13.4	0.336
non-classical ($CD14^{+}CD16^{++}$)	4.0 ± 3.8	12.9 ± 13.7	0.211

Table 5. Spearman correlation between percentages of $CD14^{++}CD16^{-}$ (classical), $CD14^{++}CD16^{+}$ (intermediate), or $CD14^{+}CD16^{++}$ (non-classical) monocyte subsets and production of cytokines by cultured macrophages on the four different materials. The Spearman correlation coefficients (r) define the relationship between monocyte subsets from peripheral blood and the production of IL-6, CCL18, IL-1ra, and TNFα) by macrophages cultured on PP, PLA, PET, and mPET. Table 5B shows the correlation between the percentages of monocyte subsets with the M1/M2 index for the four different materials. PP = polypropylene, PLA = polylactic acid, PET = monofilament polyethylene terephthalate, and mPET = multifilament polyethylene terephthalate.

A

		$CD14^{++}CD16^{-}$		$CD14^{++}CD16^{+}$		$CD14^{+}CD16^{++}$	
	material	r	p- value	r	p-value	r	p-value
CRP	-	-0.42	0.120	0.35	0.198	0.35	0.203
BMI		-0.16	0.535	0.12	0.636	0.31	0.231
IL-6	PP	-0.17	0.541	0.27	0.334	0.26	0.355
	PLA	-0.43	0.086	0.53	**0.028**	0.41	0.103
	PET	-0.54	**0.038**	0.51	0.052	0.47	0.079
	mPET	-0.36	0.158	0.38	0.133	0.26	0.323
CCL18	PP	0.28	0.321	-0.13	0.639	-0.23	0.405
	PLA	0.16	0.549	-0.32	0.107	-0.18	0.370
	PET	0.21	0.451	-0.40	**0.045**	-0.36	0.073
	mPET	0.24	0.353	-0.50	**0.007**	-0.39	**0.039**
IL-1ra	PP	0.12	0.676	-0.43	0.108	0.18	0.516
	PLA	0.16	0.529	-0.29	0.252	0.04	0.889
	PET	0.28	0.334	-0.65	**0.011**	-0.03	0.911
	mPET	0.13	0.658	-0.53	0.052	0.16	0.594
TNFα	PP	-0.37	0.173	0.17	0.550	0.42	0.121
	PLA	-0.35	0.171	0.37	0.141	0.21	0.428
	PET	-0.46	0.084	0.30	0.283	0.30	0.296
	mPET	-0.41	0.167	0.01	0.986	0.42	0.152

B Spearman correlation between percentages of monocyte subsets and M1/M2 index

		$CD14^{++}CD16^{-}$		$CD14^{++}CD16^{+}$		$CD14^{+}CD16^{++}$	
	material	r	p-value	R	p-value	r	p-value
M1/M2	PP	-0.59	**0.020**	0.36	0.182	0.48	0.069
	PLA	-0.40	0.112	0.37	0.144	0.35	0.174
	PET	-0.45	0.092	0.26	0.341	0.27	0.328
	mPET	-0.51	**0.038**	0.58	**0.016**	0.41	0.098

Bold values denote statistically significant *p*-values

DISCUSSION

The use of biomaterials has become common in regenerative medicine. The reaction of primary human macrophages to biomaterials has been shown *in vitro* to be biomaterial specific, even when an inflammatory situation is simulated [18, 19]. However, the person-dependent foreign body response has not been taken into account in these models. In the current explorative study, we investigated the effect of obesity, a growing problem in the Western world, on the response of macrophages to biomaterials and found that on average macrophages from obese patients respond more pro-inflammatory to biomaterials as indicated by higher IL-6 and lower CCL18 and IL-1ra production than in macrophages from lean persons that were cultured on the same materials. In addition, we found that BMI, serum CRP and percentages of monocyte subsets in the peripheral blood correlate with the response of the macrophages to the biomaterials *in vitro*, and that these correlations were biomaterial specific. In addition, we showed that macrophages derived from monocytes from obese patients still respond pro-inflammatory, even when they are not in an obese environment anymore. To our knowledge, this is the first study that investigated the differences in macrophage response to biomaterials between lean and obese patients.

Obese patients included in this trial had no insulin resistance and therefore, according to the WHO criteria, no metabolic syndrome [21]. Because of the strict inclusion and exclusion criteria, such as no smoking, no implants, and the absence of diabetes mellitus we assume that the different responses to the biomaterials between lean and obese patients is the result of obesity only and not because of a difference in the presence of diabetes or previous operations in which biomaterials were used. However, certain risk factors that are unknown at the moment might have influenced our measurements and have resulted in the large variation that is sometimes observed in the cytokine measurements. Although these patients do not have a metabolic syndrome, 50% of them had a CRP level >10 mg/L, indicating systemic low-grade inflammation. CRP levels in the serum correlated positively with IL-6 production by the macrophages on PP and mPET and negatively with CCL18 and IL-1ra levels on PP, PLA and mPET *in vitro*, showing that CRP has a pro-inflammatory effect on macrophages. This was supported by an *in vitro* study, where it was shown that CRP polarizes human macrophages to an M1 phenotype [22]. A shift from classic monocytes in the peripheral blood to intermediate or non-classic monocytes has been seen before as a result of obesity [11, 23, 24], of which the latter two subsets are regarded as the pro-inflammatory subsets with increasing CD16 positivity [11, 23, 24]. We did not observe a statistically significant shift when comparing the presence of these subsets between lean persons and obese patients. This can be due to the fact that the inclusion criteria were strict and only obese patients without

a metabolic syndrome were included. In addition, the numbers of patients from who we were able to obtain a sufficient number of monocytes to perform additional flow cytometric analysis next to culture with biomaterials were low and thus resulting in a low power. Interestingly however, when comparing percentages of monocyte subsets in the peripheral blood with the cytokine production of monocyte-derived macrophages on biomaterials *in vitro*, CCL18 and IL-1ra production by macrophages on mPET and PET *in vitro* were correlated with the percentages of the different monocyte subsets in the peripheral blood. The percentages of classical monocytes correlated positively with CCL18 and IL-1ra levels produced by macrophages in culture, the percentages of the more pro-inflammatory intermediate and non-classical subsets correlated negatively with CCL18 production in culture. CCL18 is a chemokine that is predominantly made by anti-inflammatory macrophages [18, 25], indicating that the initial presence of classical monocytes is associated with the differentiation towards anti-inflammatory macrophages. As could be seen from the individual levels of IL-6, CCL18 and IL-1ra, not all obese patients had macrophages producing high levels of IL-6 and low levels of CCL18 or IL-1ra. No corrections for baseline production of the cytokines of interest were made however, because in our opinion, this best represents the in vivo situation. Even though no corrections were made, differences were still seen between the effects of different biomaterials on cells of the same patient. This underlines potential patient specific responses even when obesity already changed the metabolic status of the patient and these responses can be explained by serum CRP levels and percentages of monocyte subsets in the blood. The production of TNFα in our culture system was not influenced by obesity, this might be explained by the short time detection range of TNFα [26]. Based on our data, it seems that PLA followed by PP and PET, are more preferable for obese patients and that all tested materials can be more or less equivalently be used for lean for lean patients, assuming that a pre-dominant anti-inflammatory reaction is preferred. Although the choice of material may be better guided by the inflammatory reaction at the individual patient level rather than at the comorbidity category such as obesity. As shown in previous clinical studies, no enormous undesirable behavior of multifilament PET mesh (e.g. Parietex™ Composite mesh) for hernia repair in obese patients has been reported till now, therefore the clinical impact might be moderate [27, 28]. Nevertheless the patients outcome can always be improved with careful and personalized selection of meshes.

The polymers used in this study are commonly used for materials for soft tissue repair. The host-response to materials is not only material dependent but also the porosity, topography, and surface of the material influence the biocompatibility [3, 29-31]. The many different properties of the material influence the polarization of the macrophages [3]. In the current study, the materials were braided in the same way, but because of

different diameters of the individual fibers between the materials, the topography was not exactly the same. Therefore the length of the knitted yarn was adjusted to the diameter to achieve the best possible comparable material appearance. Interestingly, PET and mPET resulted in different M1/M2 indexes, especially when macrophages of obese patients were cultured on the materials. This demonstrates that indeed not only the polymer but also the architecture of the material is important for elicited responses. In this study, PP did not elicit an anti-inflammatory effect based on the cytokines measured. This underlines again that not only the polymer itself is important for the reaction the material elicits, but also the architecture of the material since in our earlier studies we have used meshes instead of yarns [18, 19]. Braided yarns were chosen in the current study to make the macrophage-biomaterial contact more optimal necessary for the low numbers of patient cells available for this study. After spiking of IL-6, TNFα, CCL18, and IL-1ra in the culture medium, adsorption was seen, and varying between the materials. Since PP had the most adsorption of our proteins of interest, the values for PP (and for PLA in the case of IL6) are most likely an underestimation. However, most of the associations and comparisons are made within a biomaterial and a cytokine, not comparing two different cytokines or materials with each other. These comparisons and associations are therefore unaffected in our opinion by the adsorption of the protein of interest. However, the difference in adsorption to each material, and especially the high adsorption to PP, might overshadow the differences in reactions elicited by the materials.

After implantation, the biomaterial eventually will be in contact with macrophages, but it will also be surrounded by non-adherent macrophages and extracellular matrix. We however specifically chose not to included non-adherent macrophages in our experimental set-up. The biomaterials were cultured plates made of tissue culture polystyrene (TCPS), also a polymer. By transferring the materials with their adherent cells to new wells, the medium contained mainly the cytokines from the macrophages adhering to the yarns. TCPS most likely will have a totally different effect than the extracellular matrix that normally surrounds an implanted biomaterial. In fact, we have seen that collagen indeed exerts different effect on macrophages than polymers [18, 19]. Therefore, we believed that including cytokine production from macrophages adhering to the TCPS would make the system even more artificial.

The proteins IL-6, TNFα, CCL18, and IL-1ra were selected as indicators of pro-inflammatory and anti-inflammatory responses. We are aware that these cytokines do not represent the full spectrum of mediators produced during the foreign body response. However previously, we have seen that these mediators are most discriminative between pro-inflammatory and anti-inflammatory macrophages [18, 25]. Studies to determine the

actual *in vivo* response to the biomaterial and correlating this with the parameters in the peripheral blood are necessary to draw more firm conclusions about the predictive value of monocyte subset percentages and serum CRP levels for the reaction biomaterials elicit in a certain patient.

CONCLUSION

Monocyte-derived macrophages of obese patients respond more pro-inflammatory and less anti-inflammatory to biomaterials than macrophages from lean subjects and this response depends on the type of biomaterial. This variation in cytokine production by the macrophages was associated by the percentages of monocyte subsets in the peripheral blood, serum CRP levels, or BMI of the patient. The results of this *in vitro* study offer possibilities and could stimulate future research towards personalized medicine, eventually leading to a model that can be used to test biomaterials for tissue repair and tissue engineering using patient's own cells prior to implantation of a biomaterial. In addition, our results offer the prospect that monocyte subsets in the blood or serum CRP might be measured prior to surgery to predict which biomaterial might be suitable for each patient. Studies indeed examining the clinical outcome after implantation of a biomaterial in relation to serum CRP, BMI, and monocyte subsets are however needed to confirm this.

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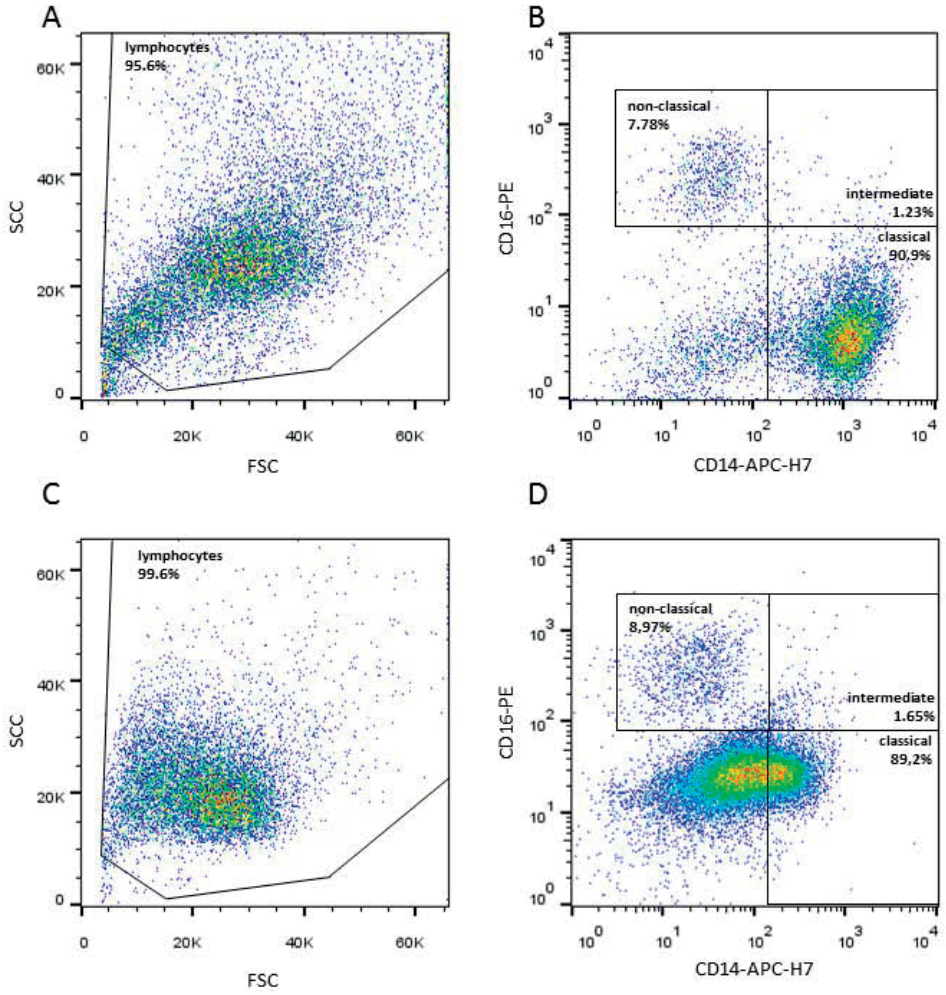
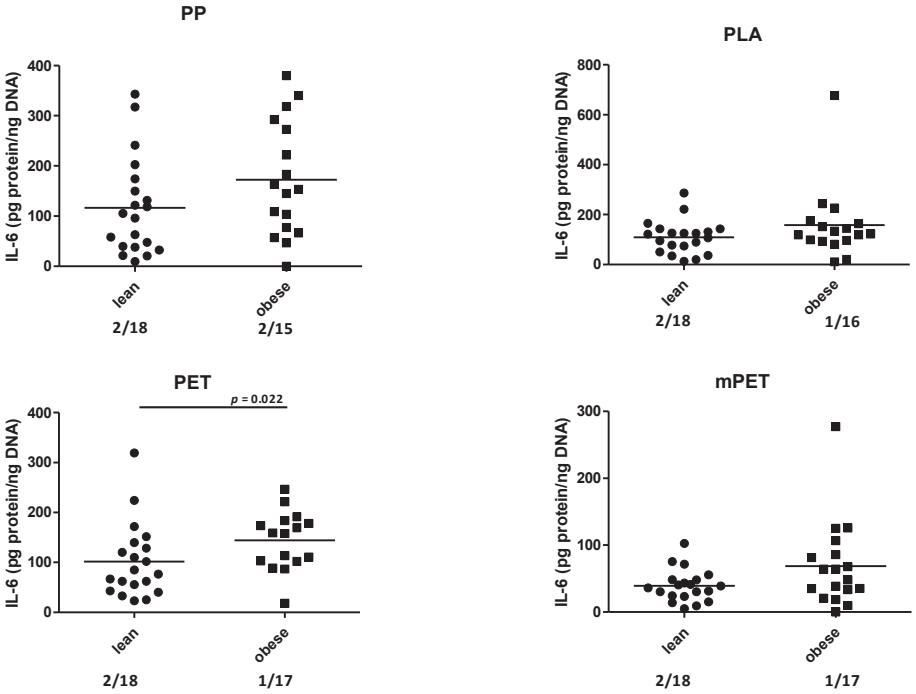
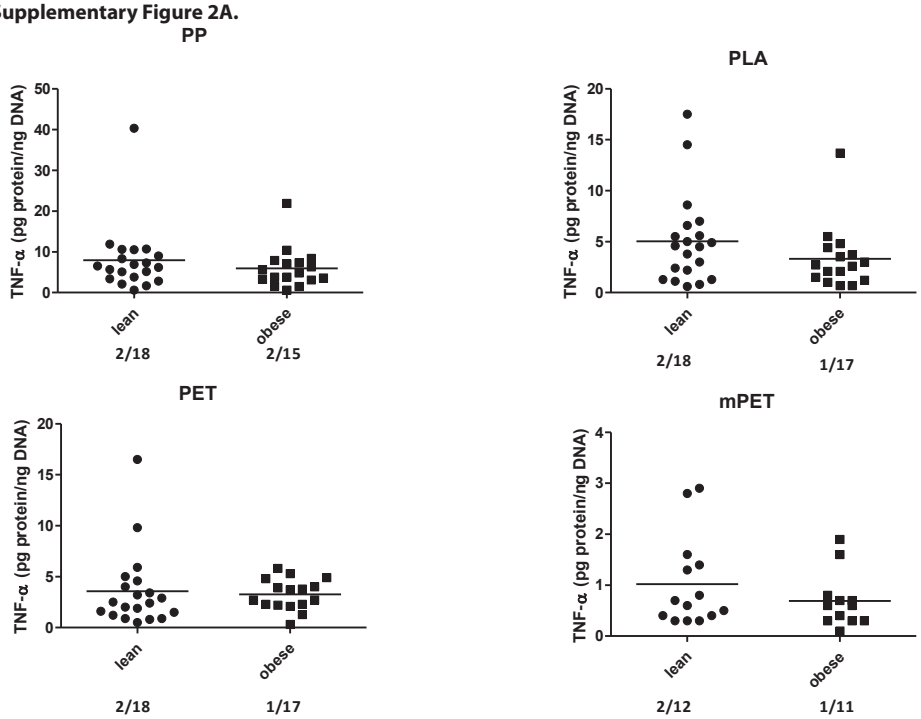
SUPPLEMENTARY DATA



Supplementary Figure 1. flow cytometric analysis of fresh (A, B), and frozen monocytes (C, D). Forward scatter (FSC) and sideward scatter (SSC) show size and granularity of the cells (A, C) and monocyte subsets were determined based on the presence of cluster of differentiation 14 (CD14) and CD16 (B, D).



Supplementary Figure 2A.



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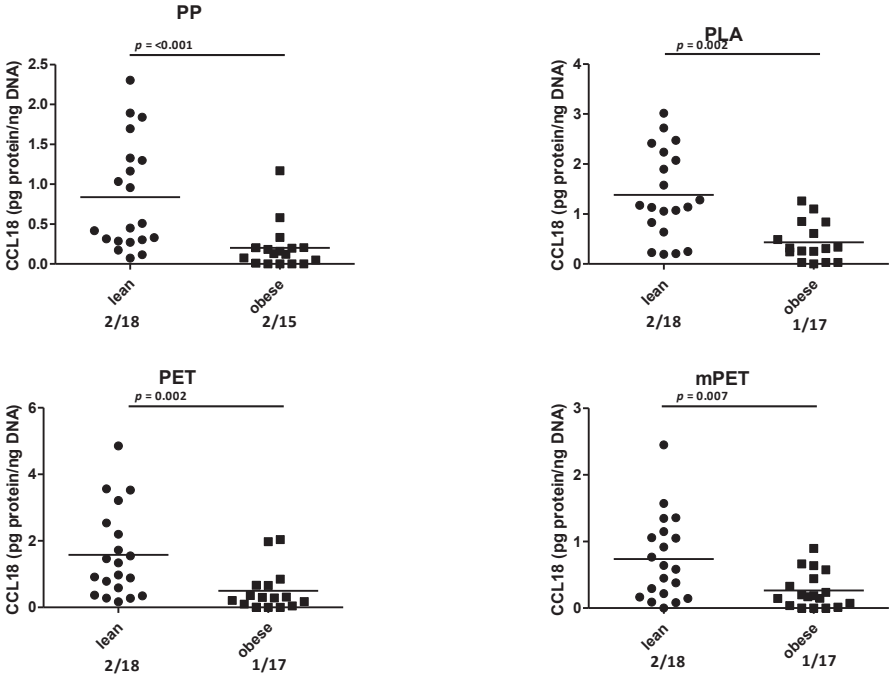

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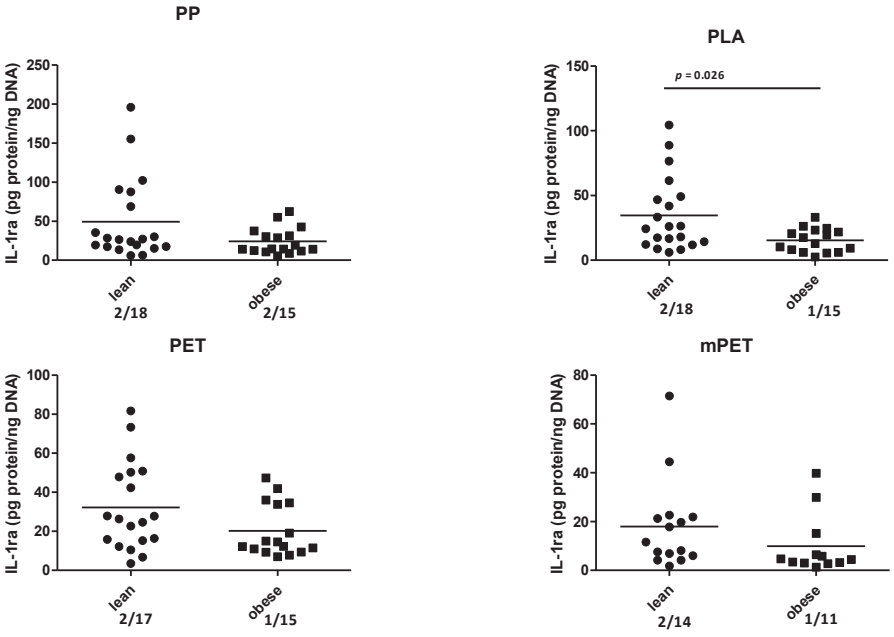
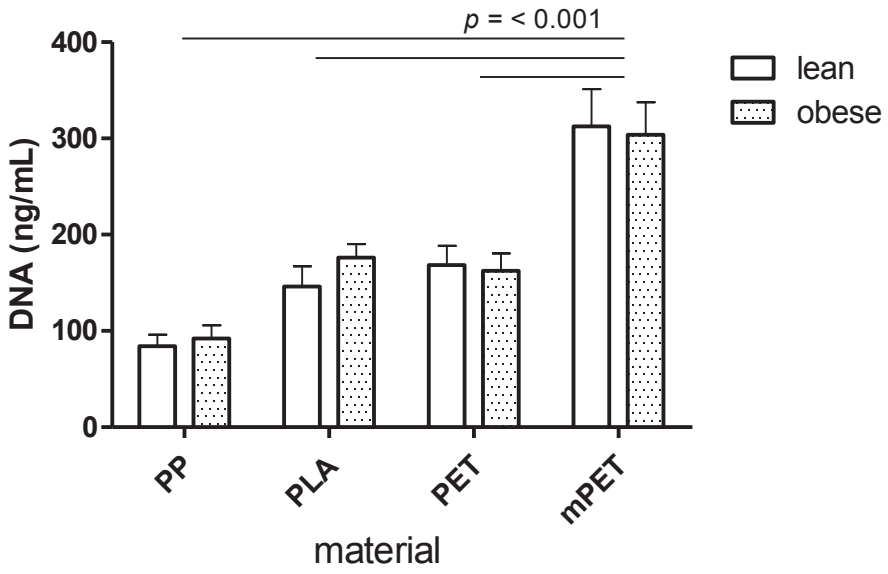
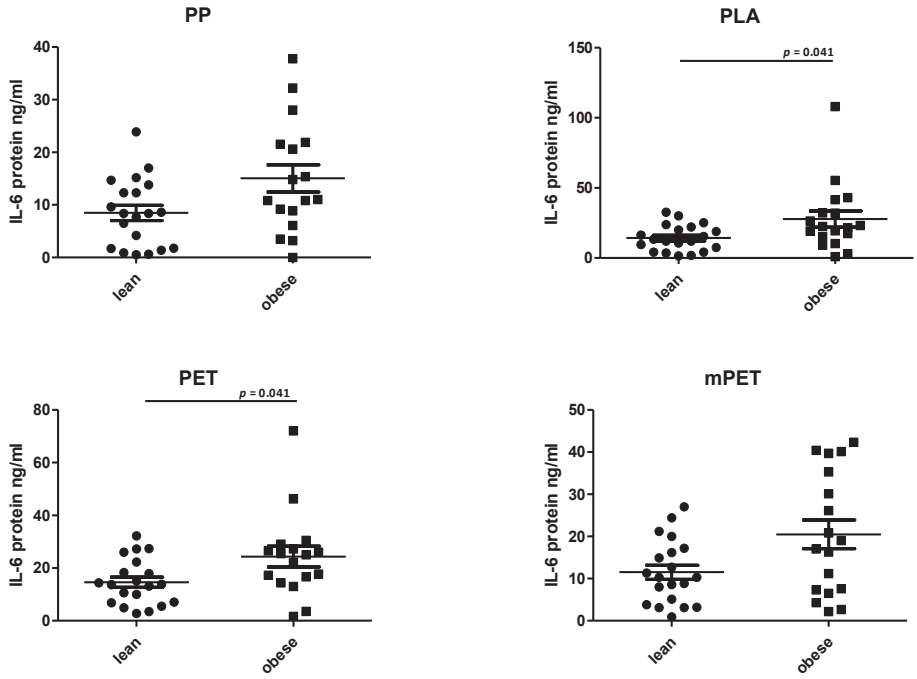


Supplementary Figure 2D.

Supplementary Figure 2. A: IL-6, B: TNFα, C CCL18, D IL-1ra production by macrophages seeded on different materials corrected for DNA, lean vs. obese groups shown per material. Every dot represents a single donor. The line indicates the mean, *p*-values indicate a statistically significant difference. Bars represent the mean, whiskers the SD. Ratios underneath the graphs indicate the male/female ratio per measurement and per material. PP = polypropylene, PLA = polylactic acid, PET = monofilament polyethylene terephthalate, and mPET = multifilament polyethylene terephthalate.



Supplementary Figure 3A.



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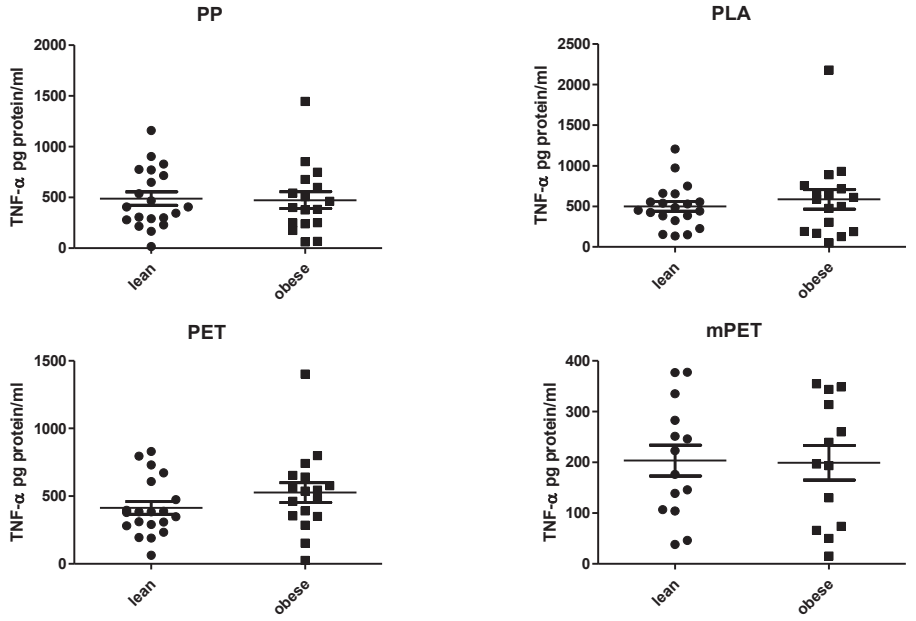

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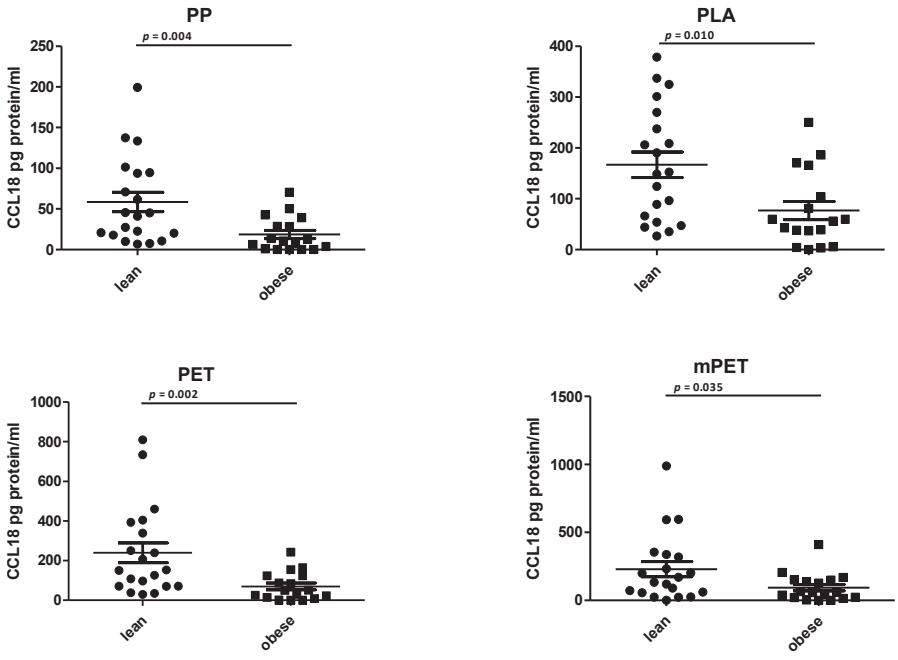

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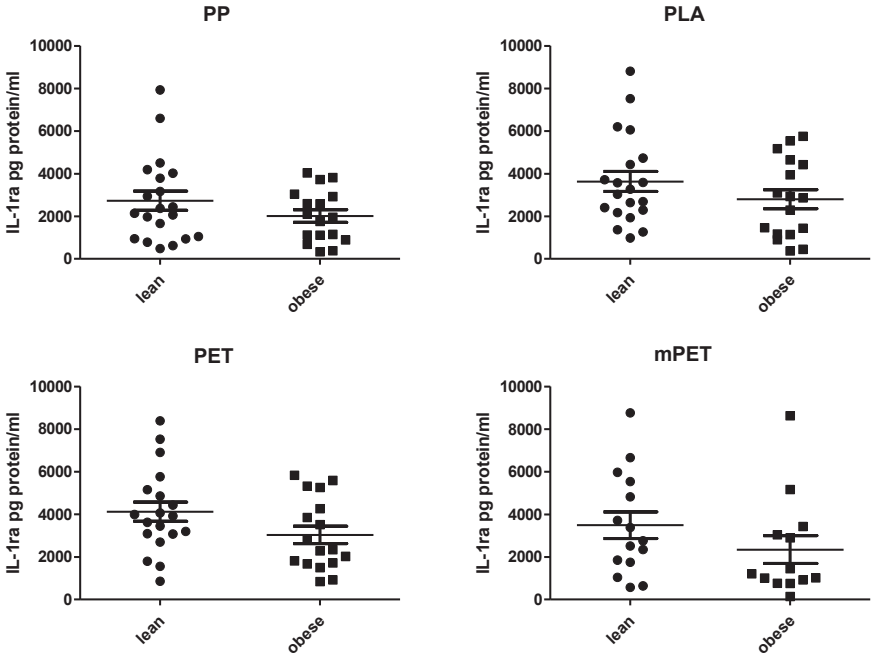
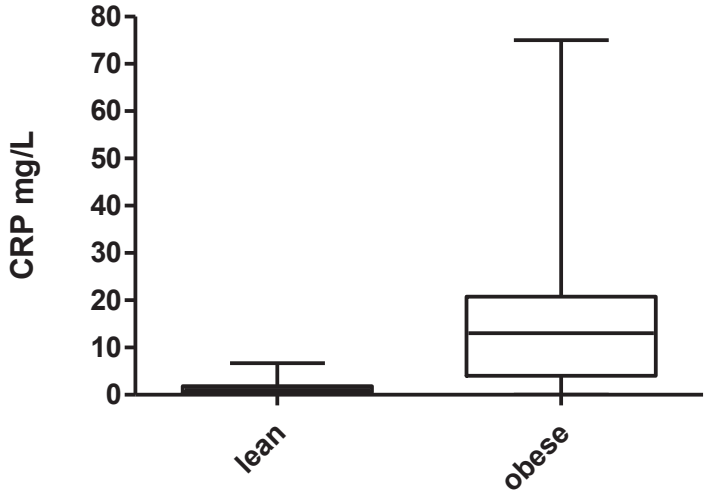
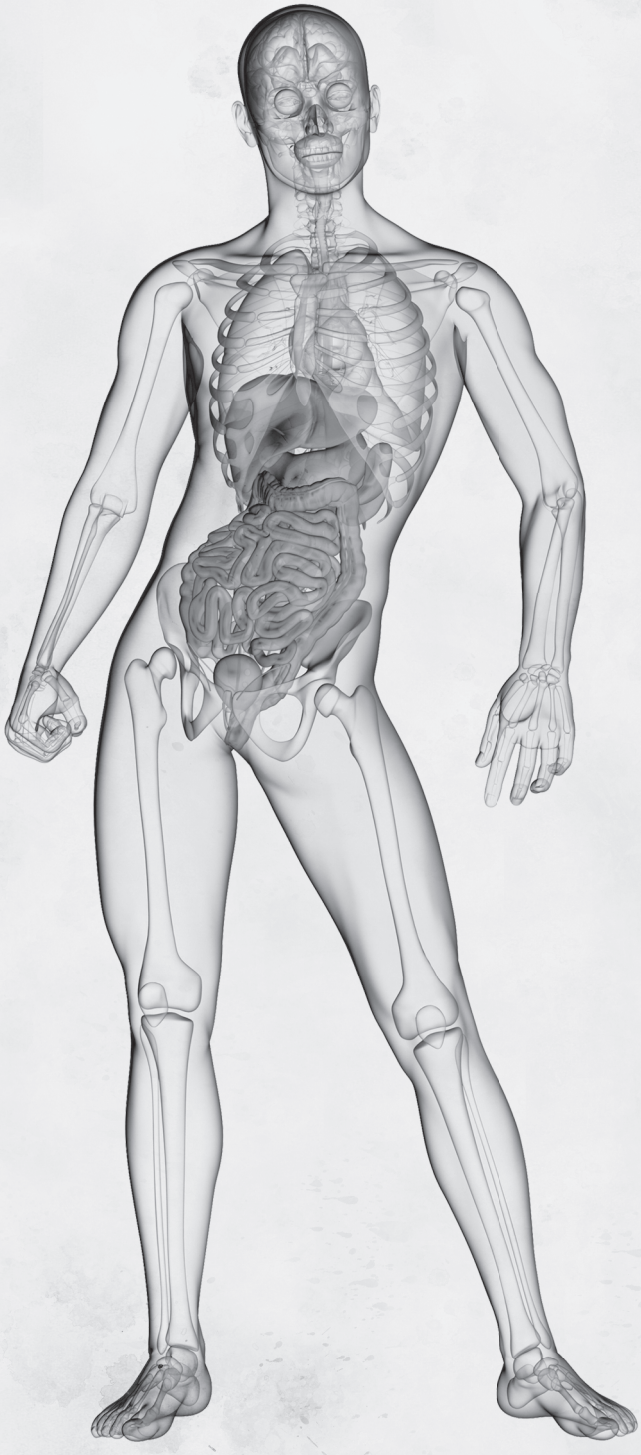


Supplementary Figure 3E.

Supplementary Figure 3. A. The amount of DNA as indication of the number of attached macrophages to the biomaterials. DNA is shown as ng/mL for polypropylene (PP), polylactic acid (PLA), monofilament polyethylene terephthalate (PET), and multifilament polyethylene terephthalate (mPET) for the lean (open bars) and obese (dotted bars) donors. Bars represent mean ± S.E.M. n = 20 donors/ group, three samples/ per material/ per donor, p-value indicates a significant difference. Figure 3B, C, D, E Comparing macrophages from lean and obese donors cultured on different materials regarding B) IL-6 production and C) TNFα production and D) CCL18 production and E) IL-1ra production in ng/ml after 3 days of culture. Every dot represents a single donor. Line and whiskers indicate mean ± S.E.M., p-values indicate a statistically significant difference. PP = polypropylene, PLA = polylactic acid, PET = monofilament polyethylene terephthalate, and mPET = multifilament polyethylene terephthalate.



Supplementary Figure 4. C-reactive protein (CRP) levels in mg/L in plasma of lean subjects vs. obese patients. The middle line in box represent the median and whiskers the minimum and maximum; lean (0-7 mg/L) and obese (0-75 mg/L).



PART 4

General discussion, future perspectives, summary, and appendices

Chapter 15

General discussion and future perspectives

In the best case scenario no patient would have a complication after surgery. Unfortunately, to date postoperative complications still happen very often after abdominal surgery, in a recent study a non-lethal complication rate of 33.5% in patients was found, while 15% of the patients had two or more complications [1]. Some complications can be general for surgery, others are specific to particular operations, like for example anastomotic leakage after colorectal surgery. Postoperative complications cause high morbidity, mortality, longer hospital stays, and increase costs. Therefore it would be ideal to avoid complications and when a complication happens, detect this as soon as possible to prevent from worse.

PREDICTION AND EARLY DETECTION OF COLORECTAL ANASTOMOTIC LEAKAGE AND POSTOPERATIVE ILEUS

Of course prevention is better than detection and cure, but when a complication happens, obviously early detection is crucial. Therefore it still is important that also preoperative risk factors are considered before choosing the best personalized surgical procedure. Sufficient blood supply to the wound is considered essential for anastomotic healing. Perioperative identification and monitoring of the vascularization of the colon is essential. These days most surgeons still mainly judge the perfusion of the anastomosis by eyesight. And unfortunately in most cases the preoperative vascular status of the patients is not available.

In **Chapter 2** the focus was on the amount of calcium/ atherosclerosis in the arteries that provide the blood supply of the anastomosis after *left-sided* colorectal surgery, because CAL rates are higher in this type of surgery. It was hypothesized that a high amount of calcium was associated with a higher leakage rate, because of its correlation with tissue ischemia and poor micro-circulation. Komen et al. investigated this hypothesis for colon anastomosis in as well right/ left sided colectomy, low anterior resections, and rectum resections [2]. The study of Komen et al. showed that the atherosclerotic calcifications in the left and right common iliac arteries are an independent risk factor for CAL. Our study however indicates that calcified atherosclerosis in the large abdominal arteries does not influence the perfusion of the anastomotic edges and is not related with the incidence of anastomotic leakage. The microperfusion at the cutting edges of the anastomosis, which is extremely important for tissue healing, is not only determined by the large arteries but also by the presence of a sufficient collateral network, which was not investigated in our study. Further research should also focus on the local tissue perfusion of the anastomosis itself.

To date, CAL is usually detected between day 5 and day 8 postoperatively based on clinical presentation, more than half of the detected leakages requiring reoperation. Many leakage cases are not detected until too late with this current strategy [3-5].

In **Chapter 3**, a novel miniaturized dynamic light scattering (mDLS) device to carry out postoperative evaluation of anastomotic microcirculation was used. In this study we evaluated whether the perfusion data correlates with anastomotic healing to determine whether blood flow measurement with the mDLS device may aid in the diagnosis of failed healing of the anastomosis prior to the clinical symptoms. Our data suggested that postoperative blood flow evaluation with an mDLS device at the anastomotic site indeed provides useful information regarding anastomotic healing and may facilitate detection of anastomotic leakage in colorectal surgery. Till now there are no devices that provide real-time monitoring information about the perfusion of the anastomosis postoperatively. There are only studies that investigated the perfusion during the construction of the primary anastomosis for example with Indocyanine green fluorescence angiography (ICG-FA)[6].

In addition to CAL, postoperative ileus (POI) is another important complication following colorectal surgery. Both complications are caused by local and systemic inflammatory response.

In **Chapter 4**, a prospective cohort study was carried out to investigate whether systemic inflammatory markers may aid to the early detection of anstomotic leakage, infectious complications or prolonged POI. Perioperative blood samples of patients who underwent colorectal surgery were collected. Based on our results, it seems that only in severe complications such as anastomotic leakage and surgical site infections but not PPOI, the overwhelming inflammatory response can be detected in a systematic manner in *clinical* settings. The diagnostic value of systemic levels of IL-6 yields better diagnostic value than CRP in predicting postoperative infections. In literature there are only two studies that describe the value of systemic cytokine levels and the link between CAL, showing no significant difference in the cytokine levels between patients with or without CAL [7, 8]. On the contrary, peritoneal levels of proinflammatory cytokines increase in the first postoperative days in CAL patients and also in POI patients [9-11]. Therefore, it seems like that postoperative peritoneal cytokine levels are more sensitive to predict PPOI than systemic levels.

PREVENTION OF POSTOPERATIVE ILEUS

Previous research revealed that surgical procedures trigger two different phases of POI: an early neurogenic phase and a late inflammatory phase. Prevention of POI and early detection are important because it affects the patients' recovery. Many interventions, such as early mobilization and nutrition, fluid restriction, prokinetic agents, epidural anesthesia, and analgesia are used for the management of POI [12].

Recently there is an increasing number of studies that investigated the effect of gum-chewing on the prevention and reduction of POI. A recent Cochrane review showed some benefits of gum chewing postoperatively [13]. Gum-chewing mimics the cephalic phase of digestion and stimulates the gastrointestinal motility via the vagal pathways. Asao et al. first demonstrated that gum chewing stimulated bowel motility and aided to early recovery from POI in surgical patients [14].

In **Chapter 6** it was hypothesized that a combination of chewing gum with nicotine stimulates the vagal nerve in two ways in a non-invasive way. Activation of the vagal nerve increases bowel motility by controlling inflammatory cell recruitment via the cholinergic anti-inflammatory pathway [15]. In the near future the first results of our study investigating this hypothesis will be presented.

COLORECTAL ANASTOMOTIC LEAKAGE: PREVENTION AND DETECTION

Colorectal surgery is the cornerstone for colorectal cancer however, despite new techniques colorectal anastomotic leakage (CAL) is still a common and most feared complication after colorectal surgery, with an occurrence in the colon of 3-7% and in the rectum of 13-18% [16-19], and a mortality rate of 0.8-27% [20, 21].

CAL is considered to be of multifactorial origin, the etiology of CAL being based on three main components: 1) communication between the intraluminal and extraluminal parts of the colon, 2) infection at the anastomotic site, and 3) healing disturbances that cause delay in healing of the anastomosis [22, 23]. Numerous risk factors are described for CAL, the risk factors being in line with the etiology of CAL. For example in a recent systematic review and meta-analysis 23 studies were described involving in total 110.272 patients in which the perioperative risk factors were evaluated [19]. In general these factors cannot be influenced and are patient related. The review showed that the anatomical level of anastomosis, gender, and preoperative radiotherapy are the main risk factors for CAL. Also very high and very low Body Mass Index (BMI) is associated with worse surgical

outcome [24-26]. Steroid treatment is also associated with impaired wound healing and therefore a higher risk of CAL [27-29].

Almost all patient-related risk factors cannot be influenced prior to surgery. Though, it is possible to change or adjust the technique that is used to perform the anastomosis. Results in the literature show that an inverting, continuous single-layer anastomosis with slowly absorbable monofilament material or using a stapler seems preferable [30]. There is no evidence that hand-sewn anastomosis is better than stapling the anastomosis is. Actually there is little known about the distance between the bites, how much tension should be given on the suture, and how much the distance should be to the anastomotic edge [30]. Even by standardizing the anastomotic technique by using a stapler device, becoming more and more common over the last four decades, the percentage of CAL did not decrease.

This thesis focuses on improving the anastomotic strength and healing of the anastomosis by applying hyperbaric oxygen therapy on the anastomosis **(Chapter 7)**, applying stem cell sheets around the anastomosis **(Chapter 8),** or using tissue adhesives at the anastomotic site **(Chapters 9-11)**.

In **Chapter 7** the role of hyperbaric oxygen therapy (HBOT) to improve anastomotic healing and lower the infection rate at the anastomotic site were investigated. Oxygenation is important for tissue repair. The mechanism of HBOT is still not fully understood, but it is known that oxygen stimulates collagen synthesis, matrix deposition, angiogenesis, epithelialization, and the eradication of bacteria [31-33]. The blood supply and perfusion of the preserved parts of the bowel proximally and distally to the anastomosis and especially around the cutting edge, significantly affects the outcome of the operation [34]. The increased oxygen pressure (100% oxygen under pressure) results in a three times more diffusion of oxygen from the plasma into the tissues increasing the diffusion distance. Perioperative application of HBOT in rats with an ischemic anastomosis, prevents anastomotic dehiscence, increases the anastomotic strength, and reduces anastomotic adhesions. The mechanism behind these observations is probably partly due to the inhibition of pro-inflammatory cytokines like TNF-α and IL-1β, produced by M1 macrophages [35, 36]. This hypothesis was supported by the fact that more M2 macrophages were observed at the anastomotic site at the histological level in rats that underwent HBOT, and significantly less M1 macrophages.

Other approaches to prevent anastomotic leakage or enhance anastomotic healing are using foreign body materials like tissue adhesives or using own cells or biological materials.

In **Chapter 8**, a study was described in which adipose tissue derived stem cells (ACS) were used, assembled to form a sheet, to apply on the colorectal anastomosis promoting wound healing. This is not the first study applying stem cells on the colonic anastomosis [37-39], although ours is the first study that applied stem cells as a sheet of one cell layer thick. The other studies showed improved intestinal regeneration and increased angiogenesis after intraperitoneal injection of ASC, however there were no differences found in CAL [38, 39]. In our study, the ASC sheets prevented anastomotic leakage as shown by a lower leakage score, less abscess formation, and slightly higher ABP. As with the cyanoacrylate, in this study with ASC sheets more M2 macrophages were found in the ASC group compared to the rats without ASC application.

Reinforcement of the anastomotic site is another strategy to prevent CAL. This is already used in pulmonary, vascular, and bariatric surgery [40-43], although not yet in colorectal anastomosis. The application of semi-absorbable or bio-absorbable materials reinforces the anastomosis by sealing the gaps between the staples, reducing the bleeding, and improves the resistance to tensile forces. Over the years different materials have been tested for linear staple reinforcement. Only two studies described circular reinforcement, one with semi-absorbable bovine pericardium strips and the other with polyglycolic acid:trimethylene carbonate [44, 45]. Also the C-seal: a biodegradable sheath that is attached to the inner surface of the bowel, just proximally the colorectal anastomosis in order to prevent intestinal contents leak intra-abdominally seemed not to lead to the optimal solution [46]. Several studies with protective intraluminal stents did not solve CAL either, the main disadvantage of stents being migration [47].

Therefore in **Chapters 10-12** focus was aimed at the prevention of communication between the intraluminal and extraluminal parts of the colon by application of tissue adhesives. The use of tissue adhesives to prevent CAL in experimental studies is not new: already in the 1960s' the first experimental studies started. Most of these were animal studies performed on rats or pigs, describing the use of fibrin glue or cyanoacrylate glue [48, 49]. The use in clinical studies is only recent: only one study in 2010 has been described with the use of fibrin glue, although no advantage of the glue has been reported [50]. In addition there is a wide variation between the used animal models and the amount and type of glue in the described studies. By comparing the mechanical strength and rheology of 12 different existing tissue adhesives was chosen we thought best for prevention of CAL [51]. After this study three glues were selected that had the most potential for preventing CAL: TissuCol (fibrin glue), Histoacryl Flex (cyanoacrylate), and Duraseal (polyethylene glycol adhesive). Eventually, based on another study from our group, cyanoacrylate glue was selected as the one to focus on. Cyanoacrylate is

one of the strongest adhesives with mild clinical and immune-histopathological effects, making it promising as a colonic sealant [52].

Research regarding cyanoacrylate application in intestinal and colorectal anastomosis was reviewed in **Chapter 9**. The conclusion of this review was indeed that application of cyanoacrylate seemed promising, although there was a great inconsistency and lack of detailed information on how the different animal studies were performed. Partly caused by this conclusion, our study group tried to set a gold standard for animal research in CAL and developed a new colorectal anastomotic leakage rat model. This rat model was of great value to perform research in this field [53].

In addition to strengthen the anastomosis with tissue adhesive it can also prevent infection of the anastomosis when the anastomosis is conducted in an contaminated environment.

Therefore, in **Chapter 10** TissuCol (fibrin glue), Histoacryl Flex (cyanoacrylate), and Duraseal (polyethylene glycol adhesive) and their influence on the anastomotic healing in a *contaminated environment* were evaluated. Cyanoacrylate did not only prevented CAL, but histologically there was also an association with anti-inflammatory M2 macrophages. M2 macrophages play an important role in the foreign body response to biomaterials by the production of anti-inflammatory cytokines. On the anastomotic site pro-inflammatory M1 macrophages were less common. M1 macrophages produce pro-inflammatory cytokines like TNF-α and are associated with impaired collagen deposition. Anti-inflammatory cytokines induce regenerative processes, one of these processes being activated by IL-6 which inter alia induces regeneration of the epithelium in the intestine [54].

Patients with IBD/ colitis have also, like patients with peritonitis, a higher risk of CAL and severe infectious complications postoperatively, especially when they still use high doses of corticosteroids.

In **Chapter 11** the same positive effect of anastomotic healing by application of cyanoacrylate glue was found, compared with TissuCol (fibrin glue) and Duraseal (polyethylene glycol adhesive) in an IBD-rat model. In this experimental study a switch from M1 macrophages to M2 macrophages was also observed.

In **Chapter 12** by performing a porcine study, the use of Histoacryl Flex® (cyanoacrylate) became more translational for human practice. A high risk porcine model was used with ischemic anstomosis and a leakage model to make sure that the risk of CAL was

high, even for the young pigs. In this porcine study the advantages of small amounts of cyanoacrylate applied at the anastomotic site, became once again very clear. Our study demonstrated that reinforcement of the colonic anastomosis with cyanoacrylate glue is feasible and prevents leakage in a high-risk colorectal anastomosis. No anastomotic dehiscence and only one small abscess occurred in the cyanoacrylate groups. Furthermore, no undesirable effects like stricture formation were observed as also shown in other porcine studies without negative results [55]. In our study there were no more negative signs of the foreign body response compared to anastomosis without glue.

Based on **Chapter 9-12** it was concluded that cyanoacrylate not only strengthens the colorectal anastomosis, but also seals the anastomosis to prevent micro-leakage. Furthermore it seals the anastomosis to prevent from getting infected by the envrionment, for example when peritonitis develops during operation and last but not least, cyanoacrylate modulates the desirable inflammatory part of wound healing response by shifting the macrophage phenotype from M1 (pro-inflammatory) to M2 (anti-inflammatory) [56-58].

Another approach, instead of using foreign body material like tissue adhesives, could be to use of own cells or biological materials.

In **Chapter 7, 8, 10 and 11,** a lower anastomotic leakage rate was associated with a higher number of M2 macrophages at the anastomotic site, compared to anastomosis without HBOT, stem cells, or application of cyanoacrylate glue.

Monocytes and macrophages are among the first responders to ischemic, traumatic or surgical injury and are required for successful tissue regeneration [59]. These monocytes differentiate into macrophages based on the environment. This innate immune response is highly dynamic. Macrophage functional diversity is described as a continuum from inflammatory (M1) to anti-inflammatory or pro-regenerative macrophages (M2), and plasticity is believed to be retained in order to rapidly respond to micro-environmental changes [60].

In our studies M2 macrophages or anti-inflammatory macrophages seem to play an important role in the healing of the anastomosis. M2 macrophages are protentially important in promotion of wound healing and tissue remodelling, taking part in dampening of inflammation, and angiogenisis [61-63]. These wound healing macrophages express high levels of arginase-1 in response to IL-4, allowing M2 to generate precursors for collagen and fibroblast stimulating factors with regard to extracellular matrix deposition. M2 macrophages also suppress inflammation though secretion of IL-10 [60].

The inflammatory response can also be influenced by biomaterials. Foreign materials unavoidably elicit a biological response when implanted. This response can be influences by physical properties such a topography, porosity, surface chemistry, and degradation mode [64]. By engineering biomaterial properties, the biological response can be tuned to maximally promote repair, while not prolonging the inflammatory response [60].

THE USE OF BIOMATERIALS

In surgery many varieties biomaterials are used to regenerate or replace tissue. Every biomaterial induces a foreign body response after implantation into the body.

In **Chapter 9-11** it was shown that the material can influence the macrophage phenotype, this earlier being shown in *in vitro* models by our group [65, 66]. Depending on the material, macrophages can differentiate towards cells promoting or preventing healing. Modulation of the inflammatory response is used in strategies to reduce inflammation resulting in a more favorable outcome, the extend to which a material is able to induce these favorable aspects being a measure for success of the biomaterial [56].

Each individual responds in a different way to biomaterials regarding the foreign body response and although medicine is more and more personalized still techniques and materials are used in surgery chosen based on what is the best for a general group of patients. Since the behavior of macrophages alters in reponse to biomaterials we investigated whether it is possible to predict the foreign body response to a biomaterial beforehand, defining an individual profile and then deciding which material to use in surgery for that individual.

In **Chapter 13** it was shown that there are many different materials used for regenerative medicine, cells responding in their own specific way to each material. Furthermore, every individual response is unique.

In **Chapter 14** the individual response to different commonly used polymers was shown in an *in vitro* model. First healthy and obese patients were compared. Obesity is known to negatively influence the immune reaction. The inflammatory response between healthy and obese patients on 4 different types of materials were compared. Monocyte-derived macrophages of obese patients responded more pro-inflammatory and less anti-inflammatory to biomaterials than macrophages from lean subjects the response depending on the type of biomaterial. This variation in cytokine production by the macrophages was associated by the percentages of monocyte subsets in the peripheral

blood, serum CRP levels, or BMI of the patient. Obesity is a growing healthcare issue and is associated with surgical complications including cutaneous wound helaing, wound failure, and fascial dehiscence [67]. Obesity initiates a low-grade inflammatory response and inflammatory mediators in the plasma increasing with adipose tissue mass including TNF-α, IL-6, and TGF-β [68]. In adipose tissue there is also a shift from anti-inflammatory M2 macrophages to M1 macrophages [69]. Besides the cellular alterations there is also a higher oxidative stress in subcutaneous adipose tissue, this relative hypoperfusion causing disfunctioning of fibroblasts to synthesize collagen which is essential for wound healing [70]. In our study the results showed that macrophages derived from monocytes from obese patients still respond pro-inflammatory, even when being not in an obese environment anymore. This is an important finding because this makes it possible to continue *in vitro* studies for future research studies aiming at personalized medicine, eventually leading to a model that can be used to test biomaterials for tissue repair and tissue engineering using patient's own cells prior to implantation of a biomaterial to decide which matierial is the best for that specific individual.

FUTURE PERSPECTIVES

The prevention and early detection of major postoperative complications of abdominal surgery is not simple. It is a multifactorial process determining whether a patient will develop a postoperative complication or not. In this thesis three important postoperative complications have been researched: incisional hernia (IH), prolonged postoperative ileus (PPOI), and colorectal anastomotic leakage (CAL).

The use of biomaterials e.g. meshes in medicine is well known to prevent from IH. However each biomaterial induces an inflammatory response, which can lead to complications like wound healing problems. Therefore It would be ideal if one can anticipate on the patients personal foreign body response prior to implanting the material. This can be done by using an *in vitro* model with patients' own cells cultured on the materials to be implanted. Monocytes of the individual who needs to undergo the operation can be isolated from periferal blood. These monocytes can be cultured on the most common used materials for that specific operation. Depending on the foreign body response of the cultured monocytes (macrophages) to the material, the 'best' material can be chosen. A more anti-inflammatory response of the macrophages is preferable to promote tissue healing. It would even be more ideal if from the monocyte subsets in the blood of the patient the foreign body response can be predicted. If this is possible, the process of culturing the material, a time-consuming proceeding, can be omitted. In this respect

personalized medicine, moreover: personalised surgery, is necessary to improve patient outcome in the future.

For prevention of CAL several targets can be defined in the future: 1) improvement of anastomotic healing by application of tissue adhesives, HBOT or stem cells, 2) reinforcement of the anastomosis by application of tissue adhesives, 3) protect the anastomosis from infection on the extraluminal site by tissue adhesives.

Based on the experimental research studies presented in this thesis, it is justified to perform a clinical study with application of cyanoacrylate glue on the colorectal anastomosis in a controlled manner. Application of the very liquid tissue adhesive on the colon anastomosis without spill and without spending a lot of time is a challenge. A technical device to assist in the application would be preferable in clinic. For example incorporation of the tissue adhesive in a circular stapler that is used for the anastomosis would be ideal, this will not cost not much extra operation time and no extra procedure is necessary. At the moment prototypes of such a device are in development.

Especially the colorectal anastomosis that is at high risk, for example in a patient who needs to be operated in an acute setting with peritonitis, extra prevention of the anastomosis is welcome therefore this patient group will be perfect to test cyanoacrylate glue in a clinical study.

Application of HBOT in the clinic will be more challenging especially starting the treatment preoperatively. However patients who would have a greater risk of ischemic anastomosis can be detected preoperatively e.g. smoker, patients with atherosclerosis, diabetic mellitus, cardiovascular disease. If HBOT can be applied and researched in this specific group and the clinal outcome will be improved, application of HBOT can be considerd in a larger group of patients.

The use of stemcell sheets to facilitate anastomotic healing is still experimental but also promising. These days bioscaffolds are also used to improve tissue healing. Preformed grafts, for example SIS grafts made of porcine small intestine submuca, showed amazing results in literature and have been rigorously tested to confirm biocompatibility. SIS are cell-free scaffolds with collagen matrix that demonstrated the ability for constructive remodeling of missing of damaged tissue, for example in esophageal defects or bladder augmentation [71, 72]. Hence, SIS seems to be an interesting candidate to be researched to prevent anastomotic leakage. The cost of these bioscaffolds being high, they are of shelf available. However, a major advantage of using patients own adipose tissue stem cells to form a sheet that can be applied on the anastomosis during colorectal surgery

would be ideal to avoid the foreign body response like with regard to other biomaterials. Of course the production process should be less time consuming and more efficient to become a cost-effective additional preventive therapy.

Future research regarding the relationship between abdominal atherosclerosis and the risk of CAL should concentrate on the relationship between abdominal calcifications and the severity of abdominal artery stenosis and the perfusion of the colon and/or rectum during surgery. Furthermore, visualization of the collateral network around the anastomosis may provide extra information on the onset of ischemia leading to CAL. According to the perfusion of the anastomosis, this miniaturized device can be further developed in a sensor performing realtime measurements of the anastomosis. This principle is ideal to use as an objective evalution monitoring the microvasularisation during and after performing the anastomosis and providing the clinician more information than only the symptoms of CAL. The mDLS device that was tested can be integrated in a smart sensor to observe the real-time status of the anastomosis postoperatively. Such sensor should be placed near the anastomosis sending information via Bluetooth to a computer device. There are also studies that monitor the anastomosis accurately by the use of a smart drain [11, 73]. In this respect measurements of cytokines and bacteria in drain fluid can indicate early CAL and PPOI [11, 74, 75].

For conclusion, nowadays it is extremely difficult to find any funding for research on the still far too frequently occurring major complications of abdominal surgery as themes like oncology, cardiovascular disease, transplantation or Alzheimer disease are far more prominent and better known in public. One of the reasons for this might be represented by a certain degree of reticence on complications in general, still existing within the professional field. In the age of transparency and quality of care efforts to improve surgical care via the avoidance of complications should be considered paramount by the surgical community overtly and also generously supported by the industry and government.

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Chapter 16

Summary
Samenvatting

SUMMARY

In this thesis the focus was on three major complications after abdominal surgery: incisional hernia (IH), prolonged postoperative ileus (PPOI), and colorectal anastomotic leakage (CAL). The results were summarized in three parts: **Part 1** focused on prediction and detection of these surgical complications; **Part 2** describes different methods to prevent complications; **Part 3** analyzed also prevention of major surgical complications with a focus on patients at risk.

Part 1

In **Chapter 2** it was investigated if the amount of calcification in the major abdominal arteries can be used as a prognostic factor for colorectal anastomotic leakage. In this case-control study it was demonstrated that the abdominal calcium score and calcium volume in the aortoiliac trajectory as determined by CT-scan analysis, are not correlated with anastomotic leakage for left-sided colon anastomosis.

In **Chapter 3** was the perioperative anastomotic perfusion measured with a miniaturized Dynamic Light Scattering (mDLS) device, aiming to determine whether anastomotic perfusion correlated with the anastomotic healing process in a rat colectomy model. This study demonstrated that postoperative perfusion-monitoring can assist the detection of anastomotic leakage earlier.

In **Chapter 4** cytokine levels in the blood from patients who underwent oncological colorectal surgery were studied to detect infectious complications earlier. Cytokine levels of patients with infectious complications were compared with patients without complications. This study showed that perioperative changes of IL-6 levels on postoperative day 1 and 3 had a better diagnostic value compared to leucocyte count and CRP changes. However, POI cannot be predicted with inflammatory cytokines.

In **Chapter 5** an overview of the literature about clinical endpoints, early detection, and differential diagnosis of POI was given. This overview showed that the best clinical endpoint of POI is defecation and eating of solid food. A CT-scan is the most valuable radiologic diagnostic method to differentiate between POI or other diagnosis.

Part 2

In **Chapter 6** the hypothesis regarding vagal nerve stimulating by chewing nictone gum to prevent PPOI was described in detail. For more than ten years several studies have been published investigating the use of chewing gum and reduction of POI. Chewing of chewing gum stimulates the vagal nerve. The vagal nerve is one of the twelve

cranial nerves. The vagal nerve is the most important nerve to stimulate the digestive tract stimulating gastric juice secretion and emptying of the stomach. Vagal nerve stimulation induces production of acetylcholine that stimulates production of gastric juices. Moreover, vagal nerve stimulation reduces the inflammatory response. Nicotine also stimulates the vagal nerve. Therefore it seemed logical to combine nicotine with chewing gum to gain a stronger stimulating effect on the vagal nerve to reduce POI. To research this hypothesis a human randomized controlled trial should performed.

In **Chapter 7** the use of hyperbaric oxygen therapy (HBOT) to improve the healing of the anastomosis was researched in a rat model. In this rat model a partial colon resection was combined with an ischemic anastomosis, half of the animals received perioperative HBOT compared with no treatment. No anastomotic dehiscence was seen in the HBOT groups, compared to 37.5% and 28.6% dehiscence in the control group on postoperative day 3 and 7, respectively. Histological research of the anastomosis showed significantly more type 2 macrophages (anti-inflammatory).

In **Chapter 8** another approach to prevent anastomotic leakage by means of stimulating the healing was investigated. This innovative study explored the therapeutic potential of adipose tissue derived stem cells (ASC) applied as a sheet on the anastomosis for preventing dehiscence of sutured colorectal anastomoses. ASC sheet application significantly enhanced healing of colorectal anastomoses with decreased incidence of dehiscence and abscess formation. The increased numbers of anti-inflammatory macrophages and T-cells might have contributed to promotion of the healing process. This preclinical study indicates that ASC sheet application may have a therapeutic role in promoting colorectal healing and prevention of anastomosis-related complications. Also in this study, the amount of type 2 macrophages around the anastomosis was increased compared to the control group,

In **Chapter 9** a systematic review of the literature was given regarding the use of cyanoacrylate glue to prevent anastomotic leakage in colorectal anastomosis. Although there were substantial heterogeneity and methodological bias in the experimental studies, the results were promising in this overview. Therefore, in the following chapters the focus was on prevention of colorectal anastomotic leakage with tissue adhesives.

Part 3

In **Chapter 10-12** different tissue adhesives were tested in anastomotic leakage animal models. Special attention was paid to anastomosis that are at high risk for leakage, respectively a contaminated environment, IBD (inflammatory bowel disease), and ischemic anastomosis.

In **Chapter 10** three different types of tissue adhesives were tested in a rat model to be investigated if these sealants would prevent from leakage in a peritonitis model. These tissue adhesives: fibrin glue, cyanoacrylate glue, and polyethylene glycol glue were circularly applied on a sufficient sutured anastomosis. These three 'glue groups' were compared to each other and to a control group without glue. As expected anastomotic leakage and comprised anastomotic strength were observed in the animals with peritonitis. All three adhesives prevented occurrence of anastomotic leakage. Again histologic research of the anastomosis showed more type 2 macrophages in the best healed anastomosis. In this study the main role of the glue was sealing the anastomosis to prevent from contact of the intraperitoneal bacteria.

The promising data of **Chapter 10** led to the next study presented in **Chapter 11**. In this chapter the focus was on another high risk patient group: patients with IBD like Crohns disease or colitis. When these patients have to undergo an operation they have an increased risk of anastomotic leakage.

In **Chapter 11** the same tissue adhesives were used as in the previous chapter, but now in a surgical IBD model. This model caused compromised anastomotic healing with more abscess formation and a lower anastomotic bursting pressure because of inflammation and ischemia. All the tissue adhesive groups yielded a higher bursting pressure. On postoperative day 7 there were only abscesses in the control group. Histoacryl Flex®, a cyanoacrylate glue, was the only tissue adhesive that showed significantly less intra-abdominal abscesses compared to the control group. Type 2 macrophages were significantly more present in the Histoacryl Flex® group.

In **Chapter 12** the application of Histoacryl Flex® on a high risk porcine model was investigated representing a more translational study for human surgical procedures. In this ischemic and anastomotic leakage model the strength of cyanoacrylate glue was once again proved. Cyanoacrylate was effective and safe to prevent from leakage and with less adhesions, no stricture of the anastomosis, and no foreign body response to the glue shown with histological analysis.

In **Chapter 13** the effect of different biomaterials to the macrophage response was described in a detailed overview of the literature. A wide range of biomaterials, for example tissue adhesives or meshes for hernia surgery, are used as medical devices. These materials have their own characteristics, and every material induce a foreign body response by activation of macrophages. This response can be influenced by the environment, but also by the material itself. This initial foreign body response to a material can be researched in an *in vitro* model. The used methods are very variable. We concluded

in this review that i*n vitro* culture models using macrophages on biomaterials are a valuable addition to the development of new biomaterials. However there is a need for standardized culture models and a systematic comparison to the *in vivo* response.

In **Chapter 14** the influence of obesity on macrophages and the acute response to biomaterials were investigated. This study showed that macrophages from patients with obesity respond more pro-inflammatory to biomaterials than macrophages from lean subjects. This response is biomaterial dependent. The results of this *in vitro* study offer possibilities and could stimulate future research towards personalized medicine, eventually leading to a model that can be used to test biomaterials for tissue repair and tissue engineering using patient's own cells prior to implantation of a biomaterial.

In **Chapter 15** the results of this thesis were discussed. Based on the described studies new perspectives were given for the prediction, detection, and prevention of three major abdominal surgical complications.

SAMENVATTING

Jaarlijks ondergaan duizenden patiënten in Nederland een buikoperatie. Helaas gaat dit vaak gepaard met complicaties. Drie ernstige complicaties die vaak optreden zijn: littekenbreuk, postoperatieve ileus (POI: tijdelijk functieverlies van de darm na de operatie) en naadlekkage, dit betekent dat een nieuwe verbinding (anastomose: naad) tussen de darmuiteinden die tijdens de operatie gecreëerd is niet goed geneest en er daardoor ontlasting in de buikholte lekt. Het optreden van een dergelijk complicatie heeft meestal meerdere oorzaken. Veel factoren zijn patiënt gerelateerd en kunnen niet beïnvloed worden, enkele voorbeelden zijn: leeftijd, geslacht, eerdere buik operaties, co-morbiditeit, immuundeficiëntie en acute operaties. Sommige factoren zijn wel te beïnvloeden zoals voedingsstatus, overgewicht, ervaring van de chirurg en de gebruikte operatietechniek. Echter, meestal is er sprake van een multifactoriële oorzaak.

In dit proefschrift is met name aandacht besteed aan deze drie ernstige complicaties. De resultaten worden besproken in drie verschillende onderdelen: **Deel 1** richt zich op de genoemde chirurgische complicaties en het voorspellen en detecteren daarvan. In **Deel 2** worden preventiemethoden besproken om complicaties te voorkomen. Tot slot zijn in **Deel 3** diverse onderzoeken naar preventie van complicaties bij patiënten die een verhoogd risico lopen uitgelicht.

Deel 1

In **Hoofdstuk 2** is onderzocht of de mate van calcificatie van de aorta-iliacale vaten van invloed is op het risico van naadlekkage. De hypothese was dat des te ernstiger de calcificaties zijn, des te meer de bloedvoorziening van de darm te wensen over laat en daarmee een hoger risico op naadlekkage zal ontstaan. Uit deze studie bleek dat de mate van de hoeveelheid calcium in de arteriën (slagaders) geen directe voorspellende waarde op het ontstaan van naadlekkage heeft.

In **Hoofdstuk 3** is de doorbloeding van de anastomose in een ratmodel gedurende en na de operatie van de dikke darm (colon) gemeten met behulp van een apparaat dat dynamische lichtverstrooiingen kan meten. Uit deze studie bleek dat met name ischemie (onvoldoende doorbloeding) van de anastomose gemeten ná de operatie kan voorspellen of er naadlekkage zal optreden.

In **Hoofdstuk 4** is onderzocht of het mogelijk was om aan de hand van 'biomarkers' (o.a. cytokines in bloed) POI en infectieuze complicaties eerder te detecteren dan aan de hand van de klinische presentatie van de patiënt. Dit is onderzocht bij patiënten die een operatie van het colon of de endeldarm (rectum) hebben ondergaan in verband met

kanker. Hierbij bleek dat POI met behulp van systemische cytokine levels niet eerder voorspeld kan worden. Echter, een stijging van interleukine-6 (IL-6) in het bloed vanaf postoperatieve dag 1 naar dag 3, gaf een betere voorspellende waarde voor bijvoorbeeld wondinfectie, longontsteking, naadlekkage en urineweginfectie in vergelijking met de voorspellende waarde van leukocyten (witte bloedcellen) of CRP.

In **Hoofdstuk 5** is een overzicht gegeven van de literatuur over de definitie, vroegdetectie en differentiaal diagnose van POI. Uit dit overzicht bleek dat POI het beste gedefinieerd kan worden aan de hand van de eerste ontlasting en het nuttigen van vast voedsel. Een CT-scan bleek het meest waardevolle onderzoek te zijn om te beoordelen of bij een patiënt inderdaad van POI sprake is.

Deel 2

In de afgelopen jaren zijn er vele studies verricht waaruit bleek dat het kauwen van kauwgom preventief werkt om POI te voorkomen dan wel te verkorten door stimulatie van de nervus vagus (één van de twaalf hersenzenuwen). De nervus vagus is de belangrijkste zenuw voor de aansturing van de maag en de regulatie van de productie van maagsappen. De nervus vagus produceert de stof acetylcholine (neurotransmitter) die ervoor zorgt dat de maagsapklieren actief zijn. Daarnaast is de inflammatoire respons verminderd door activatie van de nervus vagus. Nicotine zorgt ook voor stimulatie van de nervus vagus. Daarom ontstond het idee om nicotine met kauwgom te combineren om zo een versterkt stimulerend effect te verkrijgen. Deze hypothese is uitgebreid in **Hoofdstuk 6** beschreven. Deze hypothese moet nog wetenschappelijk onderzocht worden in klinische studies.

In **Hoofdstuk 7** zijn de effecten van hyperbare zuurstof therapie (HBOT) op de genezing van de darmanastomose in een ratmodel onderzocht. In dit model, waar een deel van het colon werd verwijderd en een ischemische anastomose werd aangelegd, ontving één gedeelte van de dieren wel HBOT en de andere groep niet. Het bleek dat bij de ratten die rond de operatie HBOT hadden ontvangen geen naadlekkage werd geconstateerd en de anastomose sterker was in vergelijking met de groep zonder HBOT. Bij histologisch onderzoek van de anastomose bleek dat type 2 macrofagen (anti-inflammatoire macrofagen) significant meer aanwezig waren.

In **Hoofdstuk 8** is een andere methode onderzocht om de genezing van de anastomose te stimuleren en daarmee naadlekkage te voorkomen. In deze innovatieve studie is voor het eerst onderzocht of het bedekken van een colonanastomose met een stamcel-sheet de genezing van de anastomose bevordert en naadlekkage voorkomt. Naadlekkage van de anastomose werd op dag 3 na de operatie in de stamcel groep in 14% van de geval-

len gezien tegenover 71% bij de ratten zonder stamcelapplicatie. Daarnaast werd ook in deze studie een verhoogd aantal type 2 macrofagen ter hoogte van de naad gezien ten opzicht van de controlegroep.

In **Hoofdstuk 9** is beschreven welke studies er tot nog toe in de literatuur bekend zijn met betrekking tot het gebruik van cyanoacrylaatlijm ter preventie van naadlekkage bij colorectale anastomosen. Dit overzicht laat zien dat er veel variatie in de methode van het gebruik van diermodellen en soorten weefsellijmen bestaat. In de volgende hoofstukken zijn verschillende weefsellijmen ter preventie van naadlekkage onderzocht.

Deel 3

In **Hoofdstuk 10-12** zijn verschillende weefsellijmen getest in naadlekkagediermodellen. In deze studies lag de focus op hoog risico-anastomosen, respectievelijk in een gecontamineerd milieu, bij IBD (inflammatoire darmziekte) en ischemische anastomosen.

In **Hoofdstuk 10** is het gebruik van 3 soorten weefsellijm getest in een rat model waarbij de colonanastomose in een gecontamineerd milieu was vervaardigd, het zogenaamde peritonitis model. Deze 3 weefsellijmen: fibrine lijm, PEG-lijm (polyethyleen glycol) en cyanoacrylaat lijm zijn uitgekozen op basis van eerdere studies. De lijm werd circulair op de anastomose aangebracht. De lijmgroepen zijn vergeleken met een controlegroep zonder lijm. Zoals verwacht bij dit peritonitis model bleek de anastomose minder sterk en een naadlekkage kwam vaker voor. Echter in de lijmgroepen kwam geen naadlekkage voor. Wederom werd er bij histologisch onderzoek een hoger aantal type 2 macrofagen gevonden bij de anastomosen zonder lekkage. In deze studie is aangetoond dat het sealen van de anastomose met lijm de anastomose beschermt tegen invloed van bacteriën van buitenaf. Het aanbrengen van lijm lijkt een veilige methode om naadlekkage te voorkomen in een gecontamineerd milieu.

De aansprekende resultaten uit het vorige hoofdstuk hebben er toe geleid tot een vervolgstudie waar de focus lag op een andere groep patiënten die ook een verhoogd risico op naadlekkage hebben: patiënten met IBD zoals de ziekte van Crohn of colitis ulcerosa.

In **Hoofdstuk 11** zijn dezelfde lijmen ter preventie van naadlekkage gebruikt als in Hoofdstuk 10, maar dan in een chirurgisch IBD-ratmodel. In dit model ontstaan er pathologische veranderingen in de darm die patiënten met IBD ook hebben, zoals ontstekingen en ischemie. In deze studie werd ook gezien dat de lijmen beschermend zijn en dus naadlekkage voorkomen. Histoacryl Flex®, een cyanoacrylaat lijm, liet als enige weefsellijm significant minder abcessen zien in vergelijking met de controlegroep. Ook

hier werd bij histologisch onderzoek een significant aantal meer type 2 macrofagen geconstateerd.

In **Hoofdstuk 12** is een varkensmodel beschreven dat de stap van proefdieronderzoek naar klinische studies verkleint. In dit ischemie- en naadlekkagemodel is wederom de werking van cyanoacrylaat lijm (Histoacryl Flex®) aangetoond. In de lijmgroepen werd geen naadlekkage geconstateerd, daarnaast werden er meer adhesies (verklevingen) in de groepen zonder lijm gevonden. Deze studie laat zien dat het gebruik van cyanoacrylaatlijm veilig en effectief is om naadlekkage te voorkomen.

In **Hoofdstuk 13** is een gedetailleerd overzicht gegeven van de literatuur over de macrofaag respons op verschillende biomaterialen. Bij het gebruik van biomaterialen, zoals lijm of een mesh (matje) voor bijvoorbeeld het herstel van een littekenbreuk ontstaat altijd een vreemdlichaamreactie. Deze reactie kan beïnvloed worden door de omgeving, maar ook door het materiaal zelf. Macrofagen zijn nauw betrokken bij deze respons. Deze initiële respons op een biomateriaal kan goed onderzocht worden in een *in vitro* model (celkweek). Echter een systematische aanpak voor dit soort studies ontbreekt en de voorkeur gaat dan ook sterk uit naar het ontwikkelen van een gestandaardiseerde aanpak voor dit soort studies om verschillende *in vitro* studies met elkaar te kunnen vergelijken.

In **Hoofdstuk 14** is de invloed van obesitas op de respons van macrofagen op verschillende biomaterialen onderzocht. In deze studie is aangetoond dat patiënten met obesitas een pro-inflammatoire respons hebben op biomaterialen in vergelijking met mensen zonder obesitas. Deze respons is biomateriaal afhankelijk. De macrofaag respons werd onderzocht door de productie van pro- en anti-inflammatoire cytokines te bepalen. Deze *in vitro* studie biedt mogelijkheden om een model te ontwikkelen om preoperatief te bepalen welk materiaal voor de individuele patiënt het meest geschikt is.

Hoofdstuk 15 bevat een algemene discussie over de belangrijkste bevindingen van dit proefschrift. Op basis van de beschreven studies worden nieuwe inzichten in voorspelling, preventie en detectie van de belangrijkste abdominale chirurgische complicaties gepresenteerd.

Chapter 17

Appendices

Acknowledgements/ dankwoord

List of Publications

PhD portfolio

Curriculum Vitae

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LIST OF PUBLICATIONS

This thesis: accepted

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This thesis: submitted

Systemic levels of the inflammatory cytokines predict the infectious complications but not prolonged postoperative ileus after colorectal surgery. **GSA Boersema**, Z Wu, AG Menon, GJ Kleinrensink, J Jeekel, JF Lange.

Other publications: accepted

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PHD PORTFOLIO

Name PhD fellow: G.S.A. Boersema	PhD period: Sep 2012 – Dec 2015
Erasmus MC Department: Surgery	Promotoren: prof. dr. J.F. Lange prof. dr G.J. Kleinrensink Supervisor: dr. Y.M. Bastiaansen - Jenniskens prof.dr. J. Jeekel

1. PhD training		
General courses	**Year**	**ECTS**
- Biomedical English Writing and Communication	2014	3.0
- Research Integrity	2014	0.3
- Laboratory animal science	2012	4.5
- BROK ('Basiscursus Regelgeving Klinisch Onderzoek')	2012	1.5
- CPO mini course	2013	0.3
- Biostatical methods	2013	2.0
Presentations at (inter)national conferences		
- ASGBI, Glasgow (poster)	2013	1.0
- Najaarsdag Heelkunde, Den Bosch (oral + poster)	2013	1.5
- Chirurgendagen, Veldhoven (oral)	2014	1.0
- EAES, Paris (oral)	2014	1.5
- Tripartite, Birmingham (oral + poster)	2014	2.5
- NBTE, Lunteren (oral)	2014-2015	1.0
- Wetenschapsdag Heelkunde Erasmus MC, R'dam (oral)	2014-2015	1.0
- Wetenschapsdag arts-assistenten vereniging (oral)	2015	1.0
(Inter)national conferences		
- Chirurgendagen, Veldhoven	2013/2015	1.0
- Najaarsdag Heelkunde, Rotterdam	2014	1.0
Other		
- Journal club	2014-2015	2.0
- REPAIR	2012-2015	2..0
2. Teaching/ supervising	**Year**	**ECTS**
- Education medical students	2013-2014	2.0
- Examination of Basic Life Support medical students	2013-2015	1.0
- Master theses	2013-2015	4.0
Other		
-		

CURRICULUM VITAE

Geesien **Simone** Anja ter Hoeve - Boersema werd geboren op 26 april 1985 te Groningen. Ze groeide op in Usquert, in het hoge noorden op het platteland van Groningen. Tijdens de gehele middelbare school periode tenniste ze op hoog nationaal niveau. In 2003 behaalde ze haar middelbare school diploma op Het Hogeland College te Warffum. Via decentrale selectie begon ze in datzelfde jaar de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam, waar ze in maart 2010 afstudeerde. Daarna werkte ze ruim twee jaar als ANIOS (arts assistent niet in opleiding tot specialist) chirurgie in het Amphia Ziekenhuis te Breda (dr. L. van der Laan). Vervolgens begon ze in september 2012 als arts-onderzoeker bij de REPAIR onderzoeksgroep (prof. dr. J. Jeekel, prof. dr. G.J. Kleinrensink, prof. dr. J.F. Lange en dr. A.G. Menon), sinds 2014 werd ze ook betrokken bij onderzoek onder leiding van dr. Y.M. Bastiaansen-Jenniskens. Onderzoek verricht in deze periode heeft geleid tot dit proefschrift. In 2016 was ze werkzaam als ANIOS chirurgie in het Reinier de Graaf Gasthuis, te Delft (dr. M. van der Elst). Per 1 januari 2017 is ze begonnen als AIOS (arts in opleiding tot specialist) chirurgie in het Maasstad Ziekenhuis, te Rotterdam (dr. R.A. Klaassen).