Postoperative ileus: Pathophysiology & treatment strategies
van Bree, S.H.W.

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Patients undergoing an abdominal surgical procedure develop a transient episode of impaired gastrointestinal motility, or postoperative ileus. Postoperative ileus, remains an almost inevitable consequence of surgery. Importantly, postoperative ileus is a major determinant of recovery after intestinal surgery and leads to increased morbidity and prolonged hospitalization. The aetiology of postoperative ileus is multifactorial, but activation of a local inflammatory response within the intestinal muscularis has become an accepted pathophysiological mechanism. Although a variety of strategies reduce postoperative ileus, none of these methods have been completely successful in shortening its duration. This thesis consist of a series of investigations into the mechanisms behind postoperative ileus and highlight new strategies to intervene in the postoperative inflammatory cascade and prevent postoperative ileus.

Sjoerd van Bree was born on July 30th, 1981 in The Netherlands. After graduating from high school at the Gemeentelijk Gymnasium in Hilversum, he obtained his propaedeutics at the medical school of the University of Antwerp in Belgium. In 2007, he received his medical degree Cum Laude from the University of Amsterdam and subsequently started his PhD project under the guidance of Prof. dr. G. Boeckxstaens. Meanwhile, he was certified as an International Medical Graduate by the Educational Commission for Foreign Medical Graduates of the United States. In January 2012 he started his training in Internal Medicine in Amsterdam.
Postoperative ileus

Pathophysiology & Treatment Strategies

Sjoerd van Bree
Postoperative ileus:

Pathophysiology & Treatment Strategies

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus

prof. dr. D.C. van den Boom

ten overstaan van een door het college voor promoties ingestelde

commissie, in het openbaar te verdedigen in de Agnietenkapel

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Sjoerd Hendrika Willem van Bree

goingen te ’s Hertogenbosch
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Faculteit der Geneeskunde
# Postoperative Ileus | Pathophysiology & Treatment Strategies

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**Scientific output**: 191

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Introduction

Postoperative ileus is an iatrogenic condition that occurs following abdominal surgery, characterized by a transient cessation of coordinated propulsive motility.\(^1\) The clinical manifestations include abdominal distention, nausea, vomiting, and inability to pass stools or tolerate a solid diet. Besides the discomfort experienced by patients, postoperative ileus is also an important risk factor for complications such as wound dehiscence and for pulmonary and thromboembolic complications. Current management strategies consist of perioperative anaesthetic and analgesic management, avoidance of nasogastric tube feeding and the use of supportive therapies.\(^2\) Although a variety of strategies have been proposed to reduce postoperative ileus, including feeding soon after surgery, early ambulation, epidural analgesia, fluid restriction, and minimally invasive surgery, none of these have been completely successful in preventing postoperative ileus.\(^3\) The treatments currently available are reviewed in Chapter 1.

Since the beginning of the 20th century, postoperative ileus has been recognized as a highly prevalent consequence of abdominal surgery. Initially, inhibition of gastrointestinal motility immediately after surgery was shown to primarily result from anaesthetics and opioid analgesics. In addition, evidence was provided that handling of the intestine during surgery activates inhibitory neuronal reflexes\(^4\) involving both adrenergic and non-adrenergic pathways\(^5\).\(^6\) and leads to intestinal oedema by excessive intravascular fluid loading.\(^7\) These events, however, fail to explain the prolonged inhibition of gastrointestinal motility seen during several days after abdominal surgery. At the end of the 20\(^{th}\) century, the inflammatory-mediated ileus hypothesis was introduced, derived from data illustrating that inflammation of the intestinal muscularis externa is the main mechanism underlying postoperative ileus.\(^10\) Handling of the intestine during abdominal surgery activates resident innate immune cells located within the muscularis externa, triggering the release of inflammatory cytokines and chemokines, as well as increased expression of adhesion molecules on endothelial cells, which causes circulating leukocytes (mainly neutrophils and monocytes) to invade the muscularis externa.\(^10\) Invading monocytes and activated resident macrophages produce nitric oxide and prostaglandins, compromising the contractile activity.\(^11\) This inflammatory response has also been confirmed in human intestinal surgical samples,\(^12\) \(^13\) and is now considered to mediate impaired contraction of handled and inflamed tissue.\(^14\) Postoperative ileus, however, is not restricted to the small intestine but involves the entire gastrointestinal tract.\(^15\) The mechanisms underlying the generalized inhibition of gastrointestinal motility in response to this local inflammation comprise a complex neuronal and immunological response.
involving activation of inhibitory neural pathways that affect the entire gut\textsuperscript{16} and production of inflammatory cytokines and nitric oxide by resident muscularis macrophages.\textsuperscript{14} In addition, it has recently been shown that also the adaptive immune system is triggered in response to bowel manipulation. This is mediated through the activation of dendritic cells with subsequent migration of T helper (T\textsubscript{H}) cells into the systemic circulation leading to inflammation to distant non-manipulated areas of the intestine.\textsuperscript{11}

Currently, intestinal manipulation of the intestine is generally used as a preclinical model of postoperative ileus. The technique used to manipulate the intestine is however highly variable and difficult to standardize, leading to large variations and inconsistent findings between different investigators. To overcome these problems, we decided to develop a new method for studying postoperative ileus in mice (Chapter 2). An important point of consideration was that the new technique of intestinal manipulation should be performed in a controlled manner, providing a reproducible model with small variation. To this end, we developed a device allowing application of a fixed pressure during intestinal manipulation. Using this device, we first examined the effect of graded manipulation on postoperative gastrointestinal transit by evaluating the intestinal distribution of orally gavaged fluorescein isothiocyanate (FITC)-labeled dextran postoperatively. Next, we compared this new technique to the widely used conventional manipulation technique, focusing on gastrointestinal transit, infiltration of myeloperoxidase positive cells and cytokine production in the muscularis externa of the intestine.

Despite the recent insights underlying the inhibition of gastrointestinal motility, the mechanisms involved in more severe postoperative ileus remain unclear.\textsuperscript{7, 17} Animal studies suggest that the severity of the ileus is linked to the extent of intestinal handling and the provoked tissue trauma.\textsuperscript{17-19} The faster clinical recovery observed after laparoscopic surgery compared with open surgery could be explained by decreased tissue trauma with concomitant decreased immune cell activation leading to attenuated intestinal inflammation and thus a quicker gastrointestinal recovery. Indeed, several studies have reported an increased postoperative inflammatory response related to increased operative trauma with systemic release of cytokines and systemic spread of the inflammatory response.\textsuperscript{18, 20, 21} Moreover, we found that the severity of ileus seems to be explained by an inflammatory response that is independent of the number of leukocytes infiltrating the small intestinal muscularis, indicating that other mechanism are responsible for the more severe cases of ileus. One possibility is that the severity of tissue trauma determines the intensity of the immune response and thus the severity ileus. Enhanced local inflammation will result in a more systemic inflammatory response with increased serum levels of pro-inflammatory cytokines. The latter will consequently affect distant regions of the gut and contribute to the generalized
aspect of postoperative ileus (Figure 1). Therefore our aim in Chapter 3 was to investigate the mechanism behind severe postoperative ileus by studying the local and systemic inflammatory response, including brain stem activation after different intensities of intestinal handling.

Figure 1 | Postoperative ileus caused by local surgical manipulation induces the influx of leukocytes into the intestinal muscularis. This inflammatory response activates visceral sensory afferents and brainstem nuclei such as the nucleus of the solitary tract (NTS). We hypothesized that Intense manipulation triggers tissue damage and release of systemic inflammatory mediators that activate the area postrema (AP) in the brainstem.

We previously reported that mast cell activation may play an important role in triggering the inflammatory process underlying postoperative ileus.\textsuperscript{13, 22, 23} One of the earliest observations in rodent models is indeed the activation of mast cells and the subsequent release of mediators such as β-hexosaminidase and mMCP-1 in the peritoneal cavity.\textsuperscript{22} Moreover, W/W\textsuperscript{v} mutant mice that lack mast cells, fail to develop an intestinal infiltrate following intestinal manipulation. Reconstitution with wild-type mast cells on the other hand restores the capacity of mutant animals to recruit leukocytes to the intestine after surgery.\textsuperscript{22} Also in man, mast cell mediators are detected in peritoneal lavage fluid very early during surgery. Even very gentle inspection of the intestines at the beginning of the abdominal procedure increased the level of peritoneal tryptase. In contrast, patients undergoing a laparoscopic or a vaginal hysterectomy hardly showed an increase in tryptase.\textsuperscript{13} However, it remains unclear whether local release of mast cell mediators may directly activate the inflammatory cascade, or alternatively, that mast cells increase mucosal permeability shortly after intestinal manipulation. The latter may lead to bacterial translocation activating intestinal leukocytes with subsequent inflammation of the muscularis externa. Therefore, in Chapter 4, we further investigated the role of
mast cells in intestinal manipulation-induced barrier disruption using mast cell deficient mouse strains.

Previously de Jonge et al. have shown that the mast cell stabilizers ketotifen and doxantrazole reduced muscular inflammation and shortened postoperative ileus in our mouse model. This observation has led to a pilot study in which 60 patients undergoing abdominal surgery were treated with 4 or 12 mg ketotifen for 6 days. Although gastric emptying was statistically significant improved by ketotifen, no improvement of colonic transit was observed. These data suggest that more potent mast cell stabilizers might be more effective. One potential approach resulting in more potent mast cell stabilization might be blockade of the intracellular spleen tyrosine kinase (Syk). Syk is one of the critical tyrosine kinases involved in mast cell degranulation induced by IgE crosslinking. Crosslinking of the FceRI receptor causes phosphorylation of Syk subsequently activating intracellular pro-inflammatory pathways. Therefore, Syk inhibitors will suppress the signaling cascades that normally lead to degranulation of mast cells. Interestingly, inhibition of Syk signaling also diminishes macrophage activation. Hence, modulation of the Syk pathway may be a potential new therapeutic strategy for postoperative ileus. Therefore, in Chapter 5 we evaluated the effect of the Syk-inhibitor GSKcompound143 (GSK143) as potential future treatment to shorten postoperative ileus in patients. To this end we evaluated the effect of GSK143 on cultured peritoneal mast cells and bone marrow derived macrophages and subsequently tested its effect in our postoperative ileus mouse model.

An alternative approach to reduce the inflammatory response evoked by intestinal handling is electrical stimulation of the vagus nerve. During the past decade, the importance of the vagus nerve in modulating the immune system has been repeatedly demonstrated. In a rat model of sepsis, electrical stimulation of the vagus nerve was shown to have anti-inflammatory properties: it reduced tumour necrosis factor (TNF) levels and improved survival, an effect mediated by neuronal acetylcholine α7 receptors. Recently our group demonstrated that electrical stimulation of the vagus nerve in mice improves intestinal transit and dampens intestinal muscular inflammation through alpha 7 nicotinic acetylcholine receptor (α7nAChRs) expressed on resident macrophages. The anti-inflammatory effect of the vagus nerve is part of a reflex by which the brain senses inflammatory information in the periphery through vagal afferents and subsequently creates an integrated anti-inflammatory response through vagal efferent fibres. This so-called cholinergic anti-inflammatory pathway is suggested to represent an additional system controlling the inflammatory response to a wide range of threats to the organism. Still, the presence of such a feedback loop (i.e. reflex) and its anatomical connections have not been demonstrated. Hence, in Chapter 6, we investigated whether the anti-inflammatory pathway is indeed a hard-wired neural circuit. As
such, we tested whether intestinal inflammation indeed triggers a vagus-nerve-mediated circuit leading to activation of vagal motor neurons in the brainstem connected to the inflamed intestine. To this end, a retrograde neuronal tracer was used to show neural connections from the intestine to the central nervous system.

Considerable progress has been made in understanding the mechanism behind postoperative ileus using experimental animals, and several translational studies show that the pathophysiological mechanisms described above also apply to humans. Hence, this new insight will ultimately lead to the development of new drugs to treat postoperative ileus. Obviously, these compounds will have to be tested in large clinical trials, obviating the need of validated outcome measures to assess their clinical efficacy. However, to date, parameters such as first defecation and flatus are often used as primary outcome parameters in clinical trials. Time to first flatus strongly depends on patient reporting whereas passage of stool might simply reflect rectal emptying and provides no reliable information on recovery of whole gut transit. Thus, with novel treatments for postoperative ileus in development, there is a definite need for more reliable outcome parameters in order to evaluate new treatments. In Chapter 7 we determined the relationship between clinical symptoms and gastrointestinal transit, assessed using scintigraphy, to identify clinical hallmarks associated with recovery of gastrointestinal transit in a large cohort of postoperative patients.

Postoperative ileus is a major determinant of recovery after colorectal surgery. Laparoscopic surgery and the implementation of an enhanced recovery after surgery program, also referred to as 'fast-track' perioperative care, are the two most important recent advances in modern surgical care. Both laparoscopic surgery and fast-track multimodal perioperative care have been reported to be safe and effective with earlier recovery of gastrointestinal function and less morbidity compared to open colorectal surgery and standard care. Clinical hallmarks of gastrointestinal function are less accurate and reliable to objectively evaluate the effect of different treatment strategies on postoperative ileus, as such clinical parameters not necessarily adequately reflect recovery of effective gastrointestinal motility. However, objective measures supporting faster gastrointestinal recovery are lacking. Up to date, scintigraphic recording of gastrointestinal transit is considered the gold standard. Therefore, in Chapter 8 we conducted a randomized double-blind study assessing gastrointestinal transit following open and laparoscopic colorectal surgery with or without fast-track care.
References

Chapter 1

New therapeutic strategies for postoperative ileus

Adapted from:

New therapeutic strategies for postoperative ileus.
Abstract

Patients undergoing an abdominal surgical procedure develop a transient episode of impaired gastrointestinal motility or postoperative ileus. Importantly, postoperative ileus is a major determinant of recovery after intestinal surgery and leads to increased morbidity and prolonged hospitalization, which is a great economic burden to health-care systems. Although a variety of strategies have been shown to reduce postoperative ileus, including multimodal postoperative rehabilitation (fast-track care) and minimally invasive surgery, none of these has been completely successful in shortening postoperative ileus. The etiology of postoperative ileus is multifactorial, but recent advances into the insight of the pathogenesis of postoperative ileus have identified intestinal inflammation triggered by surgical handling as the main mechanism. The importance of this inflammatory response in postoperative ileus is underscored by the beneficial effect of pharmacological interventions blocking the influx of leukocytes. New insights into the pathophysiology of postoperative ileus as the involvement of the innate- and the adaptive (T-helper type 1 cell-mediated immune response) immune system offer interesting and important new approaches to prevent postoperative ileus. In this review, we discuss the latest insights into the mechanisms behind postoperative ileus and new strategies to intervene in the postoperative inflammatory cascade.
Introduction

Postoperative ileus is an iatrogenic condition characterized by a transient cessation of coordinated propulsive motility. Postoperative ileus can occur after intestinal surgery and leads to increased morbidity and prolonged hospitalization. Postoperative ileus also generates a significant burden to healthcare cost. Two large prospective cohort studies in the USA and the UK have demonstrated that gastrointestinal dysfunction is the most common type of postoperative complication after major non-cardiac surgery.\(^1\), \(^2\) In a retrospective cohort study of patient records from >500 hospitals in the USA, ileus was found to be an important predictor of extended postoperative hospital stays and costs in patients undergoing colectomy.\(^3\) The economic burden of postoperative ileus has been estimated to exceed US $750 million per year and, interestingly, postoperative ileus was as expensive as managing severe postoperative complications (e.g. deep venous thrombosis, pulmonary embolism, surgical site infection) that might not lead to ileus.\(^4\) The possible benefits of improved and effective management of postoperative ileus include reduced use of resources, fewer complications and shortened hospital stay. In this Review, we discuss the latest insights in the mechanisms and treatment of postoperative ileus and focus on new therapeutic approaches that involve intervention in the inflammatory cascade.

Mechanisms of postoperative ileus

Postoperative ileus is immune-mediated

Since the beginning of the 20th century, postoperative ileus has been recognized as a highly prevalent consequence of abdominal surgery and the inhibition of gastrointestinal motility induced immediately after surgery was shown to primarily result from anaesthetics and opioid analgesics. Moreover, handling of the intestine during surgery activates inhibitory neuronal reflexes\(^5\)-\(^7\) involving adrenergic and non-adrenergic pathways\(^8\), \(^9\) and leads to intestinal oedema by excessive intravascular fluid loading.\(^10\) These events, however, fail to explain the prolonged inhibition of gastrointestinal motility seen during several days after abdominal surgery. At the end of the 20th century, the inflammatory-mediated ileus hypothesis was introduced, derived from data illustrating that inflammation of the intestinal muscularis externa is the main mechanism underlying postoperative ileus.\(^11\) Handling of the intestine during abdominal surgery activates resident innate immune cells located within the muscularis externa, triggering the release of inflammatory cytokines and chemokines, as well as increased expression of adhesion molecules on endothelial cells, which causes circulating leukocytes (mainly neutrophils and monocytes) to invade the muscularis externa.\(^11\) Invading monocytes
and activated resident macrophages produce nitric oxide and prostaglandins, compromising the contractile activity. This inflammatory response has also been confirmed in human intestinal surgical samples, and is now considered to mediate impaired contraction of handled and inflamed tissue. Postoperative ileus, however, is not restricted to the small intestine but involves the entire gastrointestinal tract. The mechanisms underlying the generalized inhibition of gastrointestinal motility in response to this local inflammation comprise a complex neuronal and immunological response involving leukocytic production of nitric oxide, panintestinal dissemination of inflammation mediated by T helper (T<sub>H</sub>) cells, and activation of inhibitory neural pathways that affect the entire gut.

**Innate and adaptive inflammatory mediators**

Although the influx of neutrophils and monocytes into the muscularis externa of the small bowel has been shown to underlie impaired gut motility after intestinal manipulation, the initial trigger of the inflammatory cascade is unclear and could involve dendritic cells, mast cells and/or macrophages. In mouse studies, peritoneal mast cells were activated, which caused a subsequent release of mast cell mediators and an inflammatory response in the intestine. In humans, intestinal manipulation during abdominal hysterectomy caused an immediate release of mast-cell activation marker tryptase in the peritoneal fluid followed by an increase of pro-inflammatory cytokines IL-6 and IL-8. Patients who underwent minimally invasive surgery had lower levels of mast cell activation compared with those who had intestinal contact during open surgery indicating that the degree of intestinal handling correlated with the level of mast cell activation and the subsequent inflammatory response. Moreover, mast-cell deficient Kit<sup>W</sup>/Kit<sup>W<sub>v</sub></sup> mice failed to develop inflammation in the intestinal muscularis externa after surgery and reconstitution of mast cells in those mice restored the handling-induced inflammation in the intestine.

An important feature of postoperative ileus is that it has a disseminated nature, whereby motility of the entire gastrointestinal tract is impaired even if only part of the intestine has been handled or is inflamed. Activation of inhibitory neural pathways by inflammatory mediators, such as cytokines and prostaglandins, has been proposed as the underlying mechanism. An alternative theory is that the inflammatory response is disseminated by memory T cells to unmanipulated areas of the gastrointestinal tract, which could underlie the panenteric nature of postoperative ileus. Intestinal manipulation could stimulate resident dendritic cells to release IL-12 and trigger T<sub>H<sub>1</sub></sub> memory cells to egress into the systemic circulation and migrate to non-manipulated areas of the intestine. These T<sub>H<sub>1</sub></sub> memory cells release IFN-γ, which results in stimulation of macrophages in the muscularis externa and dissemination of the inflammatory response.
The vagal anti-inflammatory pathway

During the past decade, the importance of the vagus nerve in regulation of intestinal immunity was established. In a rat model of sepsis, electrical stimulation of the vagus nerve was shown to reduce tumour necrosis factor (TNF) levels, indicating that inflammation had been decreased, and to improve survival. Neuronal acetylcholine receptor α7 signalling is involved with mediating the effects of vagus nerve stimulation. The anti-inflammatory effect of the vagus nerve is part of a reflex by which the brain senses inflammatory information in the periphery through vagal afferents and subsequently creates an integrated anti-inflammatory response through vagal efferent fibres. Our research group showed in a mouse model of postoperative ileus that electrical stimulation of the vagus nerve reduces macrophage activation, dampens intestinal muscular inflammation and improves postoperative ileus. Moreover, immune cells in the intestinal wall are in close proximity to cholinergic nerve fibres further demonstrating that interaction between the nervous system and immune system is an important mechanism that modulates intestinal inflammation. The cholinergic neuronal circuitry can also be centrally stimulated pharmacologically by intracerebroventricular injection of semapimod (tetravalent guanyl-hydrazone known as CNI-1493) or muscarinic agonist receptor (McN-A-343) or by intravenous injection of a ghrelin agonist or acetylcholinesterase inhibitor (galantamine). Moreover, the ‘cholinergic inflammatory reflex’ is also activated through enteral feeding of lipid-rich nutrition. These different interventions reduce manipulation-induced inflammation of the intestine and accelerate recovery of gastrointestinal motility in rodent models of postoperative ileus. Interestingly, jatrorrhizine, an alkaloid isolated from medicinal plants, dose-dependently increased gastrointestinal transit in a rat model of ileus by activation of the cholinergic pathway.

Our research group used a retrograde neuronal tracer, which travels along neurons and can be used to show neural connections from the periphery to the central nervous system, to show that intestinal inflammation triggers a vagus-nerve-mediated circuit leading to activation of vagal motor neurons in the brainstem that are connected to the inflamed intestine. These findings demonstrate that the anti-inflammatory pathway is indeed a hard-wired neural circuit.

From bench to bedside

Considerable progress has been made in understanding the mechanism behind postoperative ileus using experimental animals and several translational studies show that the pathophysiological mechanisms described above could be applicable to humans. However, it should be emphasized that data obtained in animal models might not necessarily translate to humans. For example, there are differences in mediators and receptor expression profiles between humans and
rodents; the presence of comorbidity, such as diabetes and hypertension is difficult to model in experimental animal studies; and the type of surgery studied. Moreover, there is a need for reliable outcome measures to evaluate clinical success in new drug trials. Parameters such as first defecation and flatus are often used as primary outcome parameters in clinical trials; however, these parameters are rather unreliable. Time to first flatus strongly depends on patient reporting, and passage of stool might simply reflect rectal emptying and provide no reliable information on recovery of whole gut transit. Thus, with novel treatments for postoperative ileus in development, there will be a definite need for more reliable outcome parameters in order to evaluate new treatments.

Current postoperative ileus therapies

Multimodal enhanced recovery programs

As postoperative ileus is a multifactorial disorder, a multimodal approach to shorten the duration of disease has been advocated. Enhanced recovery after surgery (ERAS) protocols or fast-track programmes have been introduced in several surgical centres in order to accelerate recovery of gastrointestinal function, improve clinical outcome and reduce hospital length of stay. In these programmes, several perioperative measures including improved perioperative fluid management, early ambulation and feeding and optimal analgesia are incorporated into patient management to reduce the rate of perioperative morbidity.

Laparoscopic surgery

Minimal invasive surgery using laparoscopy has many potential advantages over conventional open surgery, including smaller incisions, reduced pain and inflammation, earlier gastrointestinal recovery and shorter hospital stay. Several studies and a 2012 meta-analysis that included 4614 patients with colon cancer demonstrate that laparoscopic surgery significantly reduces time until recovery of bowel function (by 1 day on average) and duration of hospital stay compared with open colonic resections. Furthermore, postoperative ileus occurs more frequently after conventional laparotomy than mini laparotomy for the resection of colorectal cancer. Therefore, minimally invasive surgery and fast-track perioperative care are likely to decrease the risk and/or duration of postoperative ileus.

Prokinetics, local anaesthetics and laxatives

A Cochrane review that evaluated the benefits of prokinetic agents including cisapride, erythromycin, cholecystokinin and dopamine antagonists indicated that routine administration of prokinetics for prevention of postoperative ileus is not recommended. The effectiveness of these agents is probably reduced as
contraction of the inflamed gastrointestinal smooth muscle is strongly compromised by the inflammatory process. Metoclopramide is a dopamine D2 receptor antagonist with mixed 5-HT3 receptor antagonistic and 5-HT4 receptor agonistic properties. It is commonly used to treat nausea and vomiting and to promote gastric emptying, especially in patients with diabetes mellitus and gastroparesis. These prokinetic characteristics of metoclopramide have led to evaluations of the drug as potential treatment of postoperative ileus. Clinical studies have reported conflicting results, with some demonstrating a reduction in time until first bowel movement and resumption of oral soft diet and some showing no effect. Moreover, the number of patients included in these trials was low (only 16 per group), which makes it difficult to draw any solid conclusions and, therefore, further studies are required.

Epidural local anaesthetics, for postoperative analgesia, used in conjunction with fast-track care minimize systemic opioid use and shorten the duration of postoperative ileus. Hence, epidural analgesia is included in most published ERAS protocols, and has been advocated in a recent published consensus review. Laxatives such as bisacodyl or magnesium oxide are commonly used as part of a multimodal approach to manage postoperative ileus and preliminary studies have been positive. Laxatives are inexpensive treatments but further studies are required before general recommendations are made.

Alvimopan

Opioid agonists are often used for postoperative analgesia and, in combination with endogenously released opioids, contribute to postoperative ileus by decreasing intestinal motility through stimulation of μ-type opioid receptors in the gut. Alvimopan is a peripherally-acting μ-opioid-receptor-antagonist. It belongs to a new class of drugs designed to reverse opioid-induced gastrointestinal effects without affecting the centrally-mediated analgesic effects of opioids and, therefore, not compromise pain relief. A pooled, post-hoc analysis of four randomized, double-blind, placebo-controlled, phase III trials showed that alvimopan led to a reduction in the time until tolerance of solid food and bowel movement and a statistically significant reduction in the duration of hospital stay. Hence, FDA approval was granted in 2008. However, the use of alvimopan was recently associated with an increased rate of myocardial infarction, limiting its clinical application. Only one phase III trial was conducted outside North America and assessed the effect of alvimopan on postoperative bowel recovery after open abdominal surgery carried out at 70 hospitals in 11 countries, predominantly within the European Union. The study showed a potential benefit although it was not statistically significant, possibly as the opioid doses used in this trial were low. The drug was cost saving, although this has not yet been thoroughly assessed for treating patients undergoing laparoscopic surgery, but randomized, double-blind,
controlled trials are currently running in the USA, to determine its therapeutic and cost saving potential in patients undergoing laparoscopic colonic resection.

**Therapies currently in clinical trials**

Improved knowledge of the pathophysiology of postoperative ileus has led to development of new compounds to use as new therapies. A number of drugs and approaches are currently in clinical development.

**Methylnaltrexone**

Methylnaltrexone is a peripherally acting μ-type opioid receptor antagonist that does not readily cross the blood-brain barrier. Similarly to alvimopan, methylnaltrexone has been evaluated as potential treatment for postoperative ileus. In a phase II study, 65 patients who underwent segmental colectomy received 0.3 mg/kg methylnaltrexone or placebo intravenously every 6 hours starting at 90 minutes after surgery and continuing either for up to 24 hours after gastrointestinal recovery or for up to 7 days. Compared with placebo, methylnaltrexone led to a statistically significant reduction in the time until tolerance of solid food and bowel movement and significantly reduction in time to hospital discharge by one day. However, two recent, placebo-controlled phase III trials evaluating the use of intravenous methylnaltrexone at doses of 12 mg and 24 mg in 1048 patients undergoing segmental colectomy failed to show improvement of postoperative ileus and time to hospital discharge (Table 1).

**Lidocaine**

Local anesthetics such as lidocaine reduce pain perception and also decrease inflammation. Lidocaine can promote gut motility by blocking the afferent and/or efferent arms of the sympathetic inhibitory spinal and prevertebral reflexes, which are involved in ileus. Moreover, lidocaine decreases sympathetic nervous system activity and has a direct excitatory effect on intestinal smooth muscle. In six clinical studies (including 116 patients) intravenous administration of lidocaine (1–3 mg/min) during 4 or 24 hours after surgery shortened the time until the return of bowel function (1 day earlier than saline) and shortened the length of hospital stay. It should be emphasized though those results varied with the type of resection and surgical approach and that the exact mechanism of action remains unclear.
### Table 1 | Summary results of phase II and phase III trials of pharmacological compounds

<table>
<thead>
<tr>
<th>Study</th>
<th>Pharmacological Intervention (administration route)</th>
<th>Type of surgery (n)</th>
<th>Reduction in time to outcome compared with placebo (h)</th>
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<td>Passing 1st flatus</td>
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<tr>
<td>Harvey et al. (2009)</td>
<td>Lidocaine 1 mg/min (intravenous)</td>
<td>Open or laparoscopic colectomy (22)</td>
<td>NS</td>
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<tr>
<td>Popescu et al. (2010)</td>
<td>TZP-101 20–600 µg/kg (intravenous)</td>
<td>Open colectomy (236)</td>
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<tr>
<td>Bocchicchio et al. (2012)</td>
<td>TZP-101 20–600 µg/kg (intravenous)</td>
<td>Open colectomy (236)</td>
<td>NA</td>
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<td>Narita et al. (2008)</td>
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<td>Laparoscopic colectomy (40)</td>
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<td>Toyomasu et al. (2011)</td>
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<td>Laparoscopic colectomy (30)</td>
<td>46, P=0.02</td>
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<td>Galendriuk et al. (2008)</td>
<td>Pracalone 0.5, 2 and 4 mg (oral)</td>
<td>Laparoscopic colectomy (317)</td>
<td>10.8, P=0.03</td>
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<td>Open colectomy (141)</td>
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<td>12, P=0.003</td>
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<td>Viscusi et al. (2005)</td>
<td>Methylsalaziné 0.3 mg/kg (intravenous)</td>
<td>Open colectomy (65)</td>
<td>NA</td>
</tr>
<tr>
<td>Yu et al. (2011)</td>
<td>Methylsalaziné 12 mg (intravenous)</td>
<td>Open colectomy (1,048)</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Phase II trial. All other studies were phase II. Abbreviations: NA, not available; NS, no significant improvement.

### Ghrelin agonists

Ghrelin is an orexigenic hormone mainly produced in the fundus of the stomach and in the pancreas. Recent rat studies using ghrelin as treatment of sepsis provide evidence for the anti-inflammatory properties of ghrelin. Administration of ghrelin or a ghrelin agonist before surgery seems therefore a promising therapeutic strategy to prevent the onset of intestinal inflammation and thus ileus. Agonists of ghrelin such as TZP-101 (ulimorelin hydrochloride have powerful prokinetic properties and are being evaluated as potential therapies for postoperative ileus. However, ghrelin activates an anti-inflammatory pathway and improves inflammatory conditions such as colitis, ischaemia reperfusion injury and sepsis. It is not clear if the anti-inflammatory properties contribute to the beneficial effect of ghrelin agonists on postoperative ileus. Nevertheless, TZP-101 effectively prevented ileus in a rat model of postoperative ileus. Perioperative intravenous treatment with 0.03–1 mg/kg with TZP-101 at 0, 2 and 4 hours after surgery improved gut transit and increased faecal pellet output. Two phase IIb studies have assessed TZP-101 safety and efficacy in postoperative ileus management. Treatment with 20–600 µg/kg TZP-101 by 30-minute intravenous infusion within 1 hour of surgical closure, then daily for up to 7 days, decreased the time to first bowel movement and shortened hospital stay. In the other phase IIb study, the effect of TZP-101 treatment (480 µg/kg) was tested in 168 patients who
underwent colonic surgery and showed similar results. TZP-101 is currently being evaluated in a phase III clinical trial as a result of these promising findings.

5-HT$_4$ receptor agonists

5-hydroxytryptamine receptor 4 (5-HT$_4$) agonists such as cisapride, mosapride, and prucalopride are potent prokinetic agents that exert effects in the upper and lower gastrointestinal tract. Although several studies have demonstrated improvement of postoperative ileus with cisapride, this compound has been withdrawn from the market because it caused cardiovascular adverse events in some patients. However, treatment with 15 mg of mosapride citrate taken orally three times a day (starting on day 1 after surgery) reduced postoperative ileus in 15 patients who underwent colectomy. Interestingly, a recent preclinical study showed that mosapride and another 5-HT$_4$ agonist (CJ-033,466) improved postoperative ileus in rats by reducing the inflammatory response evoked by surgery. The improvement if postoperative ileus was mediated by activation of cholinergic myenteric neurons and resulted in suppression of resident muscular macrophages, but it is unclear whether the immunosuppressive effects would also occur in humans. Prucalopride is a selective, high-affinity 5-HT$_4$ agonist, which is used as a treatment for chronic idiopathic constipation. Prucalopride in combination with granisetron (a 5-HT$_3$ receptor antagonist) improved gastrointestinal transit in a rat model of postoperative ileus. In humans, prucalopride was safe and well tolerated in a phase II trial for postoperative ileus. In a study that included 317 patients who underwent partial colectomy, 0.5 mg, 2 mg or 4 mg prucalopride once daily until the third day after surgery led to faster recovery of gastrointestinal motility, and a 10% increase in the number of patients released from hospital in <6 days in the group taking 4 mg of prucalopride compared with the placebo group. Phase III studies evaluating this compound as treatment for postoperative ileus are planned.

COX-2 inhibitors

Non-steroidal anti-inflammatory drugs (NSAIDS) such as ibuprofen are inhibitors of cyclooxygenase 2 (COX-2), which is also known as prostaglandin G/H-2 synthase, and prevents arachidonic acid conversion to prostaglandin H$_2$. Interestingly, postoperative ibuprofen use was associated with decreased risk of postoperative ileus in women undergoing primary staging and debulking for ovarian carcinoma. Novel COX-2 inhibitors have been developed to achieve the analgesic, antipyretic, and anti-inflammatory activity effects of the non-selective COX inhibitor ibuprofen and other NSAIDS without gastrointestinal ulceration. These COX-2 inhibitors are commonly used in postoperative care for their analgesic properties and as part of multimodal early recovery protocols. As prostaglandins have been
proposed to have a crucial role in reducing gastrointestinal motility following surgery,\textsuperscript{90} selective COX-2 inhibitors administered prior to surgery should, theoretically, improve postoperative ileus. In animal models, COX-2 inhibition did indeed reduce the delay in gastrointestinal transit and diminished intestinal inflammation.\textsuperscript{19, 90} A clinical trial in which patients were given 100 mg of oral celecoxib failed to confirm these findings because it did not accelerate recovery of bowel motility, although the incidence of severe or very prolonged paralytic ileus was reduced from 13.4 \% in the placebo group to 1.3 \% in the active treatment group.\textsuperscript{93} More promising results were obtained with 40 mg oral valdecoxib in a study involving 80 patients undergoing elective colorectal resections. Valdecoxib was administered as close as possible to the start of surgery and each subsequent dose was given at 24 hour intervals up to a maximum of 120 hours. This treatment regimen resulted in a reduction of time to first bowel sound and movement, first passage of flatus and tolerance of solid diet together with a reduction in the discharge from hospital by two days.\textsuperscript{94}

**Gum chewing**

Four studies\textsuperscript{95-98} and a meta-analysis of nine prospective randomized trials investigated gum chewing as treatment for postoperative ileus.\textsuperscript{99} Daily gum chewing was started after colorectal surgery and led to a decrease of 1 day in the duration of postoperative ileus, with no adverse effects.\textsuperscript{99} However, no benefit was seen after laparoscopic gastrointestinal surgery and length of hospital stay was not significantly reduced.\textsuperscript{100} The exact mechanism of action remains unclear, but it is possible that sham stimulation of the vagus nerve could trigger the cholinergic anti-inflammatory pathway. Although chewing gum would be safe, simple and cheap strategy the therapeutic effect seems rather limited.

**Future therapeutic strategies**

**Mast cell stabilizers**

The mast cell stabilizers ketotifen and doxantrazole reduced muscular inflammation and shortened disease in a mouse model of postoperative ileus,\textsuperscript{18} which led to a pilot study in which 60 patients undergoing abdominal surgery were treated with 4 or 12 mg ketotifen for 6 days .\textsuperscript{101} Although gastric emptying was statistically significant improved by ketotifen, no improvement of colonic transit was observed. More potent mast cell stabilizers might be more effective. An alternative approach might be to block intracellular tyrosine-protein kinase Syk, one of the critical tyrosine kinases involved in mast cell degranulation.\textsuperscript{102, 103} Perioperative oral administration of a Syk inhibitor (1 mg/kg) statistically significant improved recovery in mouse model of postoperative ileus.\textsuperscript{104}
Chapter 1 | Therapeutic Strategies

Blocking adhesion molecules and integrins

One of the first events leading to extravasation of leukocytes into the manipulated intestine is the upregulation of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), which can be induced by a number of proinflammatory cytokines such as IL-1β, TNF, IFN-γ, and is expressed by vascular endothelial cells and subsets of leukocytes.105-107 In rats, increased ICAM-1 expression is found in the microvasculature and endothelial network in the intestinal muscularis 3 hours after abdominal surgery and raised levels are sustained up to 24 hours. Treatment before and after surgery (at 3, 6 and 12 hours) with a mixture of monoclonal antibodies against ICAM-1(1A29), β2-integrins (CD11a/CD18 and CD11b/CD8), which are expressed by leukocytes, prevents immune cell infiltration within the muscularis externa.11 Even more a single injection prior to surgery with a mixture of the two adhesion blocking molecules anti-ICAM 1 (anti-CD54) and anti-LFA-1 (CD11a) antibodies prevented leukocyte infiltration and ameliorated gastroparesis.17 The ICAM-1 antisense oligonucleotide ISIS 3082 also prevented manipulation-induced inflammation and delayed gastric emptying.108 Taken together, these data suggest that targeting adhesion molecules could be an useful approach to prevent postoperative ileus in humans.

Other potential new therapeutic strategies

Glycine has immunomodulatory effects in transplantation and sepsis,109 inhibiting the inflammatory reaction of macrophages and neutrophils by binding to specific glycine-gated chloride channels, subsequently modulating intracellular calcium concentrations. Glycine-gated chloride channels are localized to muscularis externa macrophages and infiltrating leukocytes. Moreover, in a rodent model of postoperative ileus, preoperative glycine treatment statistically significantly attenuated the inflammatory response and improved postoperative gastrointestinal transit. Thus, therapeutic modulation of resident macrophages by glycine could be a novel pharmacological strategy.109

Matrix metalloproteinase (MMP)-9, a member of the gelatinase family of MMPs, is upregulated following intestinal manipulation that leads to leukocyte migration into the intestinal muscularis externa. Inhibition of MMP-9 reduces the number of infiltrating inflammatory cells and prevents the surgically-induced reduction in contraction of bowel smooth muscle mice.110 Depleting DCs, the use of immunosuppressants such as anti-IL-12 antibodies or inhibiting Th1 cell migration by FTY-720 could reduce postoperative ileus, although these approaches should be used with caution because of the risk of increasing the risk of infection.44, 47
Conclusions

Postoperative ileus is a major contributor to increased length of hospital stay and health care costs for patients undergoing intestinal surgery. Its pathophysiology is multifactorial but activation of a local inflammatory response within the intestinal muscularis externa has become an accepted pathophysiological mechanism, opening a new avenue of potential targets for treatment. Inhibiting intestinal macrophage or mast cell function, or intervening in the adaptive immune response and systemic spread of inflammation might reduce the duration of postoperative ileus in patients following abdominal surgery. In addition, improvements in recovery time have been obtained since the introduction of laparoscopic surgery, and with perioperative strategies such as fast-track care.

Review criteria

PubMed was searched in March 2012 for full-text articles written in English using the terms “postoperative ileus”, “treatment”, "etiology", and "pathophysiology". Papers published since 2008 combined with previous extensive reviews published up to 2009 were included, as were additional references from the author’s files and studies on inflammation. Furthermore, we used the reference list of identified publications to select other relevant papers.

Acknowledgements

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References


Chapter 2

Novel method for studying postoperative ileus in mice

Abstract

Introduction: Postoperative ileus (POI) is characterized by a transient inhibition of coordinated motility of the gastrointestinal (GI) tract after abdominal surgery and leads to increased morbidity and prolonged hospitalization. Currently, intestinal manipulation of the intestine is widely used as a preclinical model of POI. The technique used to manipulate the intestine is however highly variable and difficult to standardize, leading to large variations and inconsistent findings between different investigators. Therefore, we developed a device by which a fixed and adjustable pressure can be applied during intestinal manipulation.

Methods: The standardized pressure manipulation method was developed using the purpose-designed device (Fig. 1D). First, the effect of graded manipulation was examined on postoperative GI transit. Next, this new technique was compared to the conventional manipulation technique used in previous studies. GI transit was measured by evaluating the intestinal distribution of orally gavaged fluorescein isothiocyanate (FITC)-labeled dextran. Infiltration of myeloperoxidase positive cells and cytokine production (ELISA) in the muscularis externa of the intestine were assessed.

Results: Increasing pressures resulted in a graded reduction of intestinal transit and was associated with intestinal inflammation as demonstrated by influx of leukocytes and increased levels of IL-6, IL-1β and MCP-1 compared to control mice. With an applied pressure of 9 grams a similar delay in intestinal transit could be obtained with a smaller standard deviation, leading to a reduced intra-individual variation.

Conclusions: This method provides a reproducible model with small variation to study the pathophysiology of POI and to evaluate new anti-inflammatory strategies.
### Introduction

Abdominal surgery commonly leads to a temporary inhibition of intestinal motility, known as postoperative ileus (POI) \(^1\), \(^2\). Recent evidence shows that POI is mediated by intestinal inflammation triggered by handling of the intestine \(^3\), with activation of resident muscularis externa macrophages as a crucial step \(^4\), \(^5\). These macrophages release pro-inflammatory cytokines and chemokines resulting in infiltration of leukocytes, in particular monocytes and neutrophils. This inflammatory response leads to increased release of nitric oxide and prostaglandins in the muscularis and impaired intestinal smooth muscle contractility, thereby leading to a delay in GI transit. The importance of this inflammatory response in POI is underscored by the beneficial effect of pharmacological interventions reducing the intestinal inflammation \(^6\).

Manual compression of the small intestine by means of two cotton applicators \(^7\) is currently widely used to induce POI \(^8\)-\(^11\). However, the amount of manual compression of the intestine is difficult to standardize and therefore may vary between experiments, animals studied and even investigators. In addition, accidental damage to the intestine, blood vessels and mesentery is very difficult to control, leading to a large inter- and intra-individual variation. This large variation has a major impact on the number of animals required to achieve statistical power and implicates a great ethical burden to animal research. Therefore, there is a large need for standardization and increased reproducibility of intestinal manipulation applied in models of POI. Here, we developed a novel method fulfilling these needs allowing us to better study the mechanisms involved in POI and to evaluate new compounds as potential treatment options for POI.

### Materials and Methods

**Animals**

Laboratory animals were kept under environmentally controlled conditions (light on from 8:00 AM to 8:00 PM with water and rodent non-purified diet ad libitum; 20°C–22°C, 55% humidity). Animals were acclimatized to the new laboratory environment. There was at least one week conventional acclimatization at the laboratory. Ten to twelve weeks old C57NL/BL6 mice were purchased from Charles River Laboratories (Maastricht, The Netherlands). Mice were maintained at the animal facility of the Academic Medical Centre in Amsterdam and were used at 11–14 weeks of age; weight 20-25 grams. Studies were performed according to the guidelines of the Dutch Central Committee for Animal Experiments. All experiments were approved by the Animal Care and Use Committee of the University of Amsterdam (Amsterdam, The Netherlands) and
the Animal Experiments Committee of the Medical Faculty of the Catholic University of Leuven (Leuven, Belgium).

**Experimental groups**
Eleven to fourteen weeks old mice underwent control surgery of only laparotomy (L), L followed by standardized pressure intestinal manipulation or L followed by conventional intestinal manipulation.

**Surgical procedures**
Mice were anesthetized by an intraperitoneal (i.p.) injection of a mixture of Ketamine (Ketalar 100 mg/kg) and Xylazine (Rompun 10 mg/kg). Surgery was performed under sterile conditions. Mice underwent control surgery of only laparotomy, L followed by gentle intestinal pressure manipulation or L followed by conventional intestinal manipulation. During and after the procedure, mice were positioned on a heating map (32 °C) until they recovered from anesthesia. The surgery was performed as follows: the abdomen was shaved using a shaving machine and sterilized with 70% ethanol. A 1-cm mid-line abdominal incision was made and the peritoneal cavity was entered via another incision along the linea alba using curved forceps and sterile small scissors. The opened abdominal cavity was covered with moist (0.9% saline solution) sterile gauze.12

**Standardized pressure manipulation:** The standardized pressure manipulation was performed by mounting the intestine on a plexiglas platform and manipulating the small intestine three times back and forth using a purpose-designed device. The device enables the application of a constant pressure to the intestine by a cotton applicator attached to its end (Figure 1D & Appendix). The cecum and the small intestine were carefully externalized onto the gauze using two saline-moistened cotton swabs. The stomach and the colon remained in the abdominal cavity and contact with or stretch of these parts of the gut was strictly avoided. The small intestine was wrapped up in the moistened gauze and pulled through a hole in the center of a plexiglas platform. After removal of the gauze, the small intestine was spread out onto the platform encircling the central hole of the platform: first the cecum was put at three o’clock (relative to the hole), then the second distal half of the small intestine was spread out in a circle around the hole. Starting at the cecum, the most distal part of the small intestine was manipulated first in a retrograde direction (to the proximal part) and after reaching the end of the circle (i.e. halfway ileum-jejunem), the small intestine was manipulated in the same way in an aboral direction (back to the cecum). The first round consisted of placing the tip of the large cotton swab every time on the intestine to flatten the surface of the intestine and its connecting mesenteric vasculature on the plexiglas platform. The cotton swab was attached to a device, which enabled the application of a constant
pressure to the surface of the small intestine (Figure 1D). The tip of the cotton swab was moved to an adjacent intestinal surface area continuing the flattening of the intestine in a retrograde direction (in steps of ± 20 mm$^2$) till the end of the spread out intestine was reached. The second and third round consisted of placing the tip of the cotton swab just at the mesenteric attachment of the small intestine and gently sliding it towards the anti-mesenteric side. The intestine was moistened with saline every round. The duodenum (i.e. the most proximal 2 cm of small intestine) was neither spread out nor manipulated. During manipulation, rubbing of the mesentery and especially the blood vessels entering the bowel wall from the mesenteric side was strictly avoided. After finishing the manipulation, the small intestine was carefully repositioned in the abdomen with two moist cotton swabs. The abdomen was closed by two continuous sutures (Mersilene 6-0 silk). All animals recovered rapidly after the surgical procedure.

Conventional manipulation: The cecum and the small intestine were carefully externalized onto the gauze using two saline-moistened cotton swabs. The stomach and the colon remained in the abdominal cavity and contact with or stretch of these parts of the gut was strictly avoided. The conventional intestinal manipulation was performed by compression of the small bowel using moist cotton applicators such that the luminal contents was moved aborally as previously described. After finishing the manipulation, first cecum and subsequently small intestine were carefully placed back into the abdomen with two moist cotton swabs. The abdomen was closed by two continuous sutures. All animals recovered rapidly after the surgical procedure.

After 24 hours animals were anesthetized and sacrificed by cervical dislocation, the complete GI tract was removed, flushed in ice-cold oxygenated KREBS solution, divided into several segments and stored for further analysis. Further analysis included gastrointestinal transit measurements, quantification of infiltration of leukocytes in the intestinal muscularis, and determination of cytokine levels in the intestinal muscularis.

Gastrointestinal transit measurements
GI transit was measured by evaluating the intestinal distribution of orally gavaged fluorescein isothiocyanate (FITC)-labeled dextran. Three hours before sacrifice, food pellets were removed from the cage. One and a half hour before sacrifice, 10 μL FITC-dextran (70,000 Da; Invitrogen, Paisley, UK) dissolved in 0.9% saline (6.25 mg/mL) was administered to the mouse via oral gavage and water was removed from the cage. Ninety minutes after administration, the animal was sacrificed, the abdomen was reopened and the complete gastrointestinal tract from stomach to distal colon was collected. The contents of the stomach, small bowel (divided into 10 segments of equal length), the cecum, and colon (3 segments of equal length) were collected and assayed in duplicate for the presence of fluorescent label (Synergy HT, BioTek Instruments Inc.,
VT, USA; excitation wavelength: 485 nm, emission wavelength: 528 nm) for quantification of the fluorescent signal in each bowel segment. The distribution of signal along the gastrointestinal tract was determined by calculating the geometric center (GC): \( \Sigma (\text{percent of total fluorescent signal in each segment} \times \text{the segment number}) / 100 \) for quantitative statistical comparison among experimental groups.\(^{15}\) Individual transit distribution histograms were plotted, and transits were statistically analyzed using the calculated GC.

**Whole mount preparation and histochemistry**
To quantify the degree of inflammation in whole mounts of the intestinal muscularis, ileal segments (approximately 12 cm proximal from the cecum) were quickly excised, and the mesenteric attachment was removed. Ileal segments were cut open along the mesentery border, fecal content was washed out in ice-cold modified Krebs solution, and segments were fixed with 100% ethanol for 10 minutes, transferred to ice-cold modified Krebs solution and pinned flat in a glass-dish. Mucosa and submucosa were removed, and the remaining full-thickness sheets of muscularis externa were stained for polymorphonuclear neutrophils with Hanker Yates reagent (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 10 minutes. To quantify the extent of intestinal muscle inflammation, the number of myeloperoxidase (MPO) positive cells in 10 randomly chosen representative high-power fields was counted at a 200-fold magnification and the average was calculated. Tissue sections were coded so that the observer was unaware of the surgical treatment of the specimens.

**Cytokine measurements**
For cytokine measurements, 3 cm long jejunal muscularis segments were added to 500 μL lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl₂, 2 mM CaCl₂, 1% Triton X-100, pepstatin A, leupeptin, and aprotinin (all 20 ng/mL; pH 7.4), homogenized, and incubated at 4°C for 30 minutes. Homogenates were centrifuged at 1500 x g at 4°C for 15 minutes and supernatants were stored at -20°C until assays were performed. IL-6, IL-1β, MCP-1 and TNF-α in supernatants were analyzed by mouse ELISA (R&D Systems, Abingdon, England) according to manufacturer’s instructions.

**Statistical analysis**
The results are expressed as mean ± SEM. Statistical analysis of cytokine levels was performed using the Mann Whitney U test. All other data were statistically analyzed by one-way analysis of variance (ANOVA) followed by Dunnett’s Multiple Comparison Test using Graph Pad Prism version 5.01. A probability level of \( P \) less than 0.05 was considered significant. Variances in GC between the groups were compared using the
Levene’s test with rank transformed values. The latter because of the non-normal distribution of the GC measurements.

Results & Discussion

We first examined the effect of graded manipulation on postoperative GI transit 24 hours after surgery. Mice were subjected to laparotomy (L) only, L followed by externalization of the small intestine and cecum without manipulation (L+E), or L followed by different degrees (2, 3.5, 5.5 or 9 grams) of standardized pressure manipulation of the small intestine. In control (L) mice, the fluorescent dye was rapidly transported to the distal ileum (mean GC ± SEM: 9.2 ± 0.7). As shown in Figure 1A, increased intestinal manipulation resulted in a pressure-dependent decrease in intestinal transit starting from a pressure > 3.5 grams and a pressure-dependent increase in the production of inflammatory cytokines (IL-6, IL-1β and MCP-1) 24 hours after surgery (Figure 2A-C). Interestingly, only externalization of the small intestine and cecum outside the abdominal cavity without manipulation already induced a significant influx of leukocytes into the intestinal muscularis to a similar level as the manipulated groups (Figure 1B-C). The activation and recruitment of these polymorphonuclear cells, known to be the primary constituents of the acute inflammatory response, can be influenced by numerous different chemo-attractants, including bacterial products, complement, and cytokines which may come into play after the exposure of the intestine to environmental air. Arriving at their designated site, they act as the first recruited wave of defense against invading pathogens. These data show that handling of the intestine leads to a delay in transit with influx of leukocytes in the muscularis, while the pressure-dependent decrease in transit is more likely explained by a local pro-inflammatory cytokine mediated inflammatory response that is independent of the number of leukocytes infiltrating the muscularis.

We next compared this new technique to the conventional manipulation technique used in previous studies. To this end, mice were subjected to L only, L followed by standardized pressure (9 grams) manipulation or L followed by conventional manipulation. In the conventional technique, manipulation of the small intestine is performed by compressing the small bowel with the tips of two cotton applicators such that the lumenal contents are moved aborally. Both the conventional- and standardized manipulation technique induced a significant delay in GI transit, but the intra-individual variability of GC was smaller for the standardized method compared to the conventional manipulation technique (standardized GC = 5.2 ± 1.00, conventional GC = 5.2 ± 2.19, n= 12 and 14 respectively; mean ± SD) (Figure 2D). The variances in GC in the group treated with the standardized method was significantly (p<0.013) smaller
Figure 1 | Different degrees of manipulation of the small intestine induced a dose-dependent delay in gastrointestinal (GI) transit. (A) Twenty-four hours after intestinal manipulation (IM), GI transit was determined by the calculation of the Geometric Center (GC). The GC was significantly decreased after manipulation with a pressure of 5.5 and 9 grams. (B & C) Twenty-four hours after IM, muscular inflammation was determined by counting the number of MPO positive cells in the muscularis of the small intestine (B) and colon (C). The number of MPO positive cells was significantly increased after manipulation, but no significant differences between the groups with different degrees of standardized pressure manipulation were found. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett’s Multiple Comparison Test; * P < 0.05 compared to laparotomy (L). Bars indicate mean ± SEM. Fig. 1 A & B: L: n = 4; L + externalization of small intestine and cecum (L+E): n = 5-6; IM: n = 5-8 mice per group. Fig. 1 C: L: n = 4; L+E: n = 5; IM: n = 2-4 mice per group.

Panel D | Construction drawing of the device to apply standardized manipulation of the small intestine.

than those in the group treated with the conventional method. This difference in standard deviation has a major impact on the number of animals required to achieve the desired statistical power. Taken together, these data indicate that conventional and standardized IM resulted in a similar delay in GI transit, while the standardized pressure method was more reproducible with a smaller intra-individual variability.

In summary, we have developed a new technique to manipulate the intestine in a more controlled manner that results in a pressure-dependent decrease in intestinal transit with small intra-individual variability. This model recapitulates important clinical
phenomena (e.g. an inflammatory response in the muscle layer of the small intestine) of the POI seen in surgical patients, suggesting that this novel method provides a methodologically convenient and useful model for investigation of the underlying mechanisms of POI. Additionally, this innovative model offers the capability to study the potential of new anti-inflammatory strategies in a reliable and adequately controlled manner.

**Figure 2** | Different degrees of manipulation of the small intestine induced a pressure-dependent production of pro-inflammatory cytokines.

(A-C) Twenty-four hours after intestinal manipulation (IM), cytokine production in the muscle layer of the small intestine was determined by ELISA. IL-6 (Panel A), IL-1β (Panel B) and MCP-1 (Panel C) levels were significantly increased after manipulation with a pressure of 5.5 grams. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett’s Multiple Comparison Test; * P < 0.05 compared to laparotomy (L). Bars indicate mean ± SEM. L: n = 3, L + externalization of small intestine and cecum (L+E): n = 5, IM: n = 3-6 per group.

Panel D | Both conventional intestinal manipulation (IM) and standardized pressure IM of the small intestine induced a delay in gastrointestinal (GI) transit. Twenty-four hours after IM, GI transit was determined by the calculation of the Geometric Center (GC). The GC was significantly decreased by both methods, but standardized pressure manipulation resulted in a smaller variation. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett’s Multiple Comparison Test; * P < 0.05 compared to laparotomy. Bars indicate mean ± SD. Laparotomy: n = 7; conventional IM: n = 14; standardized IM: n = 12.
Appendix

Materials
Equipment for the preparation of anesthetic (coagulation tube)
Equipment for the induction of anesthesia (25 gauge i.p. needles)
Heating map covered with blanket
Shaving machine (Wella)
Scissors, surgical forceps, straight forceps, curved forceps
Sterile cotton gauze (NW Drain compress 10x10 cm split compress 4 layers, Medeco b.v. REF 175051)
Plexiglas platform (self made)
Small cotton swabs (Stoelting 50975)
Large cotton swabs (MEDICA EUROPE BV, Oss, the Netherlands): cut off the wooden shaft, but leave 5 mm of the wooden shaft extending from the cotton applicator
Large cotton swab attached to a device (self made) (Figure 1D) with different weights: to apply a standardized pressure of 9 grams, mount the appropriate weight and check that the balance indicates 9 grams when the tip of the cotton applicator (attached to the device with the right weight) is resting on the balance
Needle holder
Suture material (6-0 soft silk, Mersilene)

Procedure for intestinal pressure manipulation

- Timing 30 minutes per animal
1. Induce anesthesia by an intraperitoneal injection of a mixture of ketamine (Ketalar 100 mg/kg), xylazine (Rompun 10 mg/kg) and dH2O.
2. Check the level of anesthesia by pitching the tail or toe and position the mouse on a heating map until the mouse recovers from anesthesia.
3. Shave the abdomen using a shaving machine, sterilize the abdomen with 70% ethanol and label the tail of the mouse.
4. Using a sterile small scissor, make a vertical 1 cm mid-line abdominal incision downwards distally from the xiphisternum. Enter the peritoneal cavity via a second incision in the peritoneum along the linea alba using curved forceps and sterile small scissors.
5. Place sterile moist cotton gauze around the incision and carefully externalize the cecum and the small intestine with two saline-moistened cotton swabs onto the sterile cotton gauze. Leave the stomach and the colon in the abdominal cavity and strictly avoid contact with or stretch of these parts of the gut.
6. Wrap up the small intestine in the moistened gauze and pull the gauze containing the small intestine through a hole in the center of a plexiglas platform.

7. Spread out the gauze and the small intestine onto the platform. First spread out the cecum on the right side and then spread out the second distal half of the small intestine in a circle around the hole (refer to troubleshooting below).

8. Starting at the cecum, first manipulate the most distal part of the small intestine using the cotton swab attached to a device (Figure 1D) in a retrograde direction (to the proximal part) and after reaching the end of the circle, manipulate the small intestine in the same way in an aboral direction (back to the cecum). The manipulation takes 15 minutes (approximately 6.5 minutes for the distal part, 2 minutes to switch from the distal part to the proximal part and 6.5 minutes for the proximal part). The first round consists of placing the tip of the cotton swab on an adjacent proximal area (in steps of ± 20 mm² intestinal surface area), only to smooth the surface of the intestine and its connecting mesenteric vasculature on the plexiglas platform. This cotton swab is attached to a device, which enables the application of a constant pressure to the surface of the small intestine with the tip of the cotton swab.

9. The second round consists of placing the tip of the cotton swab on the small intestine and rubbing the small intestine from the mesenteric towards the anti-mesenteric side. When the cotton swab does not touch the small intestine anymore, move the cotton swab upwards and manipulate the next adjacent area.

10. The third and last round is exactly the same as the second round. Moisten the small intestine with saline before every round to prevent dehydration.

11. After manipulating the distal half of the small intestine, replace the distal half by the proximal half of the small intestine by moving the distal half to the right with two saline-moistened cotton swabs and spread out the proximal half around the hole. Manipulate this part of the small intestine three times there and back in exactly the same manner as the distal part of the small intestine. Do not manipulate the last most proximal 2 cm of the small intestine. Strictly avoid rubbing of the mesentery (especially the blood vessels entering the bowel wall from the mesenteric site) during the manipulation (refer to troubleshooting below).

12. Carefully wrap up the small intestine in the moistened gauze and push the gauze, containing the small intestine, back through the hole in the platform on the abdomen of the mouse. Open the gauze and carefully place the cecum followed by the small intestine back into the abdomen with two moist cotton swabs.

13. Complete surgical closure of the abdomen by two continuous sutures using the needle holder, curved forceps and suture material.

14. Allow the animal to recover from the surgery positioned on a heating map (32°C).
Complications are rare but might include torsion of the intestine, local intestinal hematoma and postoperative infection of the laparotomy wound. The risk of bleeding and complications can be minimized by strictly avoiding rubbing the mesentery, especially the blood vessels entering the bowel wall from the mesenteric site.

Timing
The procedure of anesthesia, laparotomy, intestinal manipulation and wound closure (steps 1-13) takes approximately 30 minutes per animal.
- Step 8, 9 & 10: Pressure manipulation of the distal part of the small intestine takes approximately 6.5 minutes.
- Step 11: Changing from the distal part to the proximal part takes approximately 2 minutes.
- Step 11: Pressure manipulation of the proximal part of the small intestine takes approximately 6.5 minutes.

Troubleshooting
Step 7: Twisting of the intestine must be strictly avoided to prevent a mechanical obstruction.
Step 11: Damage to the intestinal blood vessels and mesentery must be strictly avoided.

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References


Chapter 3

Systemic inflammation and enhanced brain activation contribute to more severe delay in postoperative ileus

van Bree SH, Cailotto C, Di Giovangiulio M, van der Vliet J, Costes L, Depoortere I, Gomez-Pinilla PJ, Gianluca M, Boeckxstaens GE.

Submitted
Abstract

Objective: To investigate the mechanism behind severe postoperative ileus (POI) by studying the local and systemic inflammatory response, including brain stem activation after different intensities of intestinal handling.

Summary Background Data: The severity of POI has been reported to result from decreased contractility of the muscularis inversely related to the number of infiltrating leukocytes. However, we previously observed that the severity of POI is independent of the number of infiltrating leukocytes, indicating that different mechanisms must be involved. Here, we hypothesize that the degree of tissue damage in response to intestinal handling determines the upregulation of local cytokine production and correlates with the severity of POI.

Methods: Mice were subjected to gentle manipulation of the small intestine (gentle IM), more intense manipulation (intense IM), or only laparotomy. Postoperative intestinal transit, local and systemic inflammatory response, I-FABP (a marker for tissue damage) levels and brain activation were determined. Finally, in humans the duration of POI, plasma levels of I-FABP and inflammatory cytokines after open and laparoscopic segmental colectomy were measured.

Results: Intense IM induced a more pronounced ileus compared to gentle IM ($P=0.0001$). No difference in leukocytic infiltrates in the handled and non-handled parts of the gut was observed between the two IM procedures. However, intense IM resulted in significantly more severe tissue damage and was accompanied by a systemic inflammation with increased plasma levels of pro-inflammatory cytokines. In addition, intense but not gentle IM triggered enhanced c-Fos expression in the nucleus of the solitary tract and area postrema ($P=0.0014$). In patients, plasma levels of I-FABP and inflammatory cytokines were significantly higher after open compared to laparoscopic surgery, and were associated with more severe POI.

Conclusions: Not the influx of leukocytes, but rather the manipulation-induced tissue damage and subsequent inflammatory response determine the severity of POI. The release of tissue damage mediators and pro-inflammatory cytokines into the systemic circulation most likely contribute to the impaired motility of non-manipulated intestine.
Introduction

Postoperative ileus (POI) is characterized by a transient inhibition of gastrointestinal (GI) motility following surgery. Patients experience significant discomfort such as abdominal distention, nausea and inability to pass stool or tolerate food. Especially prolonged ileus leads to an increased risk for wound dehiscence, pulmonary and thromboembolic complications and a prolonged hospital stay and is associated with an enormous economic burden. During the last decade, evidence has accumulated that intestinal inflammation evoked by handling of the intestine is a key mechanism underlying impaired GI motility following surgery, both in humans and in animal models. These studies demonstrated that infiltrating leukocytes inhibit the contractile activity of the manipulated intestine by local release of pro-inflammatory mediators such as nitric oxide and prostaglandins.

It is becoming increasingly clear that POI mainly results from intestinal handling of the intestine during surgery. In rodents, Kalff et al. elegantly showed that manipulation of the intestine triggered the influx of leukocytes in the muscularis, starting from 3 hours onwards and further increasing up to 24 hours after surgery. Of note, the number of infiltrating leukocytes increased with the severity of intestinal manipulation with compression of the intestine yielding more influx than running along the intestine with cotton swaps. These infiltrating leukocytes, mainly monocytes, subsequently release inflammatory mediators such as prostaglandins and nitric oxide impairing the contractility of smooth muscle strips of the intestine. The latter has been proposed to underlie the delay in intestinal transit observed 24 hours after the abdominal surgical procedure. Recently, however, we observed that eventration of the small intestine and graded manipulation of the intestine up to 3.5 grams did not delay GI transit, but was associated with influx of leukocytes to the same level as more intense manipulation that did lead to POI. These data would imply that other mechanisms must be involved.

Based on the above, we reasoned that the degree of tissue damage evoked by intestinal handling may be an important determinant of the severity of POI. Several clinical studies indeed have reported an increased postoperative inflammatory response related to increased operative trauma with systemic release of cytokines and systemic spread of the inflammatory response. Tissue damage can trigger an innate immune response via the local release of damage-associated molecular pattern molecules, evoking an inflammatory response involving macrophages and/or mast cells. The resulting enhanced local inflammation may result in a more systemic inflammatory response with increased serum levels of pro-inflammatory mediators.
cytokines. The latter will consequently affect distant regions of the gut and contribute to the generalized aspect of POI.

In the present study, therefore, we investigated the mechanism behind severe POI by studying the local and systemic inflammatory response, including brain stem activation after different intensities of intestinal handling.

**Materials and Methods**

**Animals**
Laboratory animals were kept under environmentally controlled conditions (light on from 8:00 AM to 8:00 PM with water and food ad libitum; 20°C–22°C, 55% humidity). Ten to twelve weeks old C57NL/BL6 mice were purchased from Charles River Laboratories (Maastricht, The Netherlands). Mice were maintained at the animal facility of the Academic Medical Centre in Amsterdam and were used at 12–14 weeks of age. Studies were performed according to the guidelines of the Dutch Central Committee for Animal Experiments. All experiments were approved by the Animal Care and Use Committee of the University of Amsterdam (Amsterdam, The Netherlands).

**Patients**
Patients undergoing elective segmental colectomy for colonic cancer were invited to participate. The protocol was approved by the Medical Ethics Review Board of the Academic Medical Center in Amsterdam (The Netherlands) and was conducted in accordance with the principles of the Declaration of Helsinki and good clinical practice guidelines.

**Surgical procedures**
Anaesthesia was performed by an intraperitoneal (i.p.) injection of a mixture of Ketamine (Ketalar 100 mg/kg) and Xylazine (Rompun 10 mg/kg). Mice (5–8 per group) underwent a laparotomy alone, or a laparotomy followed by small intestinal manipulation (IM). Surgery was performed as follows: a midline abdominal incision was made and the peritoneum was opened over the linea alba and the small bowel was carefully layered on a sterile moist gauze pad. The small intestine was manipulated from the distal duodenum to the cecum and back for a total of three times. Contact with or stretch on stomach or colon was strictly avoided. Gentle standardized bowel manipulation (gentle IM) was constructed using a sterile moist cotton applicator attached to a device enabling the application of a constant pressure of 9 grams to the intestine. The more intense manipulation (intense IM) was performed by compression of the small bowel using moist cotton applicators.
such that the luminal content was moved abroad as previously described. After the surgical procedure, the abdomen was closed by a continuous 2-layer suture (Mersilene, 6-0 silk). After closure, mice were allowed to recover for 3 hours in a heated (32 °C) recovery cage.

**Gastrointestinal transit measurements**

Gastrointestinal function 24 hours postoperatively was determined in vivo by measurement of gastrointestinal transit of liquid non-absorbable fluorescein isothiocyanate–dextran (FITC-dextran) (70,000 Da; Invitrogen, Paisley, UK). 10μl of FITC-dextran dissolved in 0.9% saline (6,25 mg/mL) was administered via oral gavage. Ninety minutes later, animals were sacrificed and the entire bowel from stomach to distal colon was collected. The contents of the stomach, small bowel (divided into 10 segments of equal length), cecum, and colon (3 segments of equal length) were collected and assayed in duplicate (Synergy HT, BioTek Instruments Inc., VT, USA; excitation wavelength: 485 nm, emission wavelength: 528 nm) for quantification of the fluorescent signal in each bowel segment. The distribution of the fluorescent label along the gastrointestinal tract was determined by calculating the geometric center (GC): $\Sigma ($% of total fluorescent signal in each segment $\times$ the segment number)/100 for quantitative statistical comparison among experimental groups.

**Colonic transit**

Colon function was determined in vivo by measurement of colon transit of a glass ball. One and a half hour before sacrifice, mice were briefly anesthetized with isoflurane (Abbott). Patency of the colon was carefully checked by inserting a polished metal rod 3 cm into the colon. The rod was pulled out and a 2.2 mm plastic ball was transanally inserted with blunt surgical forceps and pushed forward for 3 cm into the colon with a polished metal rod. The time from insertion until excretion of the plastic ball was considered as colonic transit time.

**c-Fos expression in the brain**

Twenty four hours after surgery mice were sacrificed by transcardiac perfusion with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) (pH 7.4). Brains were collected, post-fixed for 4 h (4 °C) and cryo-protected by immersion in 30% sucrose in 0.2M PBS (pH 7.4) at 4 °C overnight. Coronal sections of 30 μm of the brainstem were collected. After rinsing in 0.05 M Tris-buffered saline TBS (pH 7.4), sections were incubated overnight at 4°C with goat anti-Fos (1:1500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) primary antibodies. Then, sections were incubated 1 hour with biotinylated secondary antibody and after with avidin-biotin complex (ABC, Vector, Burlingame, USA) for 1 hour. The reaction product was
visualized by incubation with 1% diaminobenzidine (DAB), 0.05% nickel ammonium sulphate and 0.01% hydrogen peroxide H$_2$O$_2$ for 5min. To count the number of c-Fos immunoreactive neurons, tiled images were captured by a computerized image analysis system consisting of an Axioskop 9811-Sony XC77 colour camera (Sony Corp., Tokyo, Japan). A minimum of 7 sections was used for c-Fos counting in the NTS (from Bregma -7.20 mm to -7.76 mm) and Area Postrema (Bregma -7.32 mm to -7.76 mm), and 9-11 sections for PVN (Bregma -0.58 mm to -1.22 mm).

**Immunohistochemistry**
To quantify the degree of inflammation in whole mounts of the intestinal muscularis, ileal segments were cut open and rinsed in ice-cold modified Krebs solution. The segments were fixed with 100% ethanol for 10 minutes, transferred to ice cold modified Krebs solution and pinned flat in a glass-dish. Mucosa and submucosa were removed, and the remaining full-thickness sheets of muscularis externa were stained for polymorphonuclear neutrophils with Hanker Yates reagent (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 10 minutes. To quantify the extent of intestinal muscle inflammation, the number of myeloperoxidase (MPO) positive cells in 10 randomly chosen representative high-power fields (HPF, 668.4 µm x 891.2 µm) was counted and the average was calculated. Tissue sections were coded so that the observer was unaware of the surgical treatment of the specimens.

**Blood analysis: tissue damage & plasma levels of inflammatory cytokines**
Mice: tissue damage was assessed by determining plasma levels of Intestinal fatty acid-binding protein (I-FABP). Levels of I-FABP in the plasma were determined using standard enzyme-linked immunosorbent assay (ELISA) for mouse I-FABP (Hycult Biotechnology (Hbt), Uden, the Netherlands). Interleukin (IL)-6, Interleukin (IL)-8, Monocyte Chemoattractant Protein-1 (MCP-1), Tumour Necrosis Factor (TNF-α) and Interleukin (IL)-1β plasma levels of venous blood retrieved 1, 6 and 24 hours after surgery were determined using cytometric bead array kits (CBA) according to the manufacturer's instructions (BD Biosciences, Erembodegem, Belgium). Flow cytometric analysis was performed using a FACSArray flow cytometer (BD Biosciences, Erembodegem, Belgium). Cytometric bead assay results were analyzed using the FCAP Array™ software (BD Biosciences, Erembodegem, Belgium).

Patients: IL-6, IL-8, MCP-1, TNF-α and IL-1β plasma levels of venous blood retrieved 2 hours after surgery were determined using CBA kits for human IL-6, IL-8, MCP-1, TNF-α (for human TNF-α and IL-1β the enhanced sensitivity flex set kits were used) according to the manufacturer's instructions (BD Biosciences, Erembodegem, Belgium). Flow cytometric analysis was performed using a FACSArray flow
cytometer (BD Biosciences, Erembodegem, Belgium). Cytometric bead assay results were analyzed using the FCAP Array™ software (BD Biosciences, Erembodegem, Belgium). Before determination of I-FABP in the human samples, blood was centrifuged two times and the obtained plasma was concentrated with the use of Vivaspin 23000 MW sample concentrators (GE Healthcare) by centrifuging at 3506 X g at 4 °C for 2 hours and further processed according to the manufacturer’s instructions. Levels of I-FABP in the concentrated plasma were determined using ELISA human I-FABP (Hycult Biotechnology (Hbt), Uden, the Netherlands).

**RNA extraction and inflammatory gene expression**

Total RNA was extracted from the intestinal muscularis externa of the distal stomach, jejunum, and distal colon at 6 and 24 after start surgery. The muscularis was microscopically dissected from the submucosa and immediately snap frozen in Tripure (Roche diagnostics, Mannheim, Germany) and stored at −80 °C. Tissue was homogenized by a Precellys 24 homogenizer (Bertin Technologies). RNA extraction was performed using RNeasy Mini Kit (Qiagen # 74104) according to manufacturer’s instructions. Total of RNA were transcribed into complementary cDNA by qScript cDNA SuperMix (Quanta Biosciences) according to manufacturer’s instructions. Quantitative real-time transcription polymerase chain reaction (RT-PCR) were performed with the LightCycler 480 SYBR Green I Master (Roche) on the Light Cycler 480, Roche (Roche). Results were quantified using the $2^{-\Delta\Delta C_T}$ method (PMID:11328886). The expression levels of the genes of interest were normalized to the expression levels of the reference gene (RPL32). PCR experiments were performed in triplicate, and standard deviations calculated and displayed as error bars. Primer sequences used are listed in supplementary table 1.

**Statistical Analyses**

The data on human plasma cytokine levels were not normally distributed. The Kruskal–Wallis test was performed to assess whether the cohort of data was statistically different. When variance of medians was statistically significant, the Mann–Whitney U test was used to identify the statistical differences within the cohort. For comparison of the time to recovery of GI function and plasma I-FABP levels between open and laparoscopy treated patients, the Mann-Whitney test was used and results were shown as median with interquartile ranges (IQR). All other data were statistically analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s Multiple Comparison analysis and are presented as means ± s.e.m. A probability level of P less than 0.05 was considered statistically significant. Graph Pad Prism version 5.01 software was used to perform statistical analysis and create graphs.
Results

GI transit and colonic transit

Twenty-four hours after surgery, the intestinal transit was significantly delayed by intestinal manipulation (IM) compared to laparotomy (GC: 8.9 ± 0.7). Notably, intense IM (GC: 3.8 ± 0.2) induced a more severe delay in intestinal transit compared to gentle IM (GC: 6.3 ± 0.8) (Fig. 1A). Colonic transit did not differ significantly between laparotomy and gentle IM, but was significantly delayed after intense IM compared to laparotomy (Fig. 1B).

![Figure 1](image1.png)  
*Figure 1 | Intense manipulation leads to a more pronounced delay of intestinal transit compared to gentle manipulation with similar leukocytic infiltrate in the manipulated small intestine. Mean geometrical center (GC) of orally administered FITC-dextran (Panel A), or colonic transit time of a 2-mm plastic ball (Panel B) after intestinal manipulation (IM) or sham operation (laparotomy) at 24 hours after surgery. Results are representative of three independent experiments in groups of 7-8 mice and data are means ± s.e.m.. * P < 0.05, one-way ANOVA followed by Tukey's Multiple Comparison analysis.

Intense handling leads to enhanced inflammation in the manipulated intestine

In the small intestine, IM but not laparotomy resulted in an influx of MPO-positive inflammatory cells in the muscle layer of the small intestine with similar leukocytic counts in the gentle and intense IM groups 24 hours after surgery (Fig. 2). In addition, IM resulted in the upregulation of the pro-inflammatory cytokines IL-6, IL-1β and TNFα at 6 and 24 hours compared to laparotomy. Importantly, intense manipulation induced significantly more upregulation of IL-6 and TNFα as compared to gentle manipulation (Fig. 3).
Figure 2 | Gentle and intense manipulation lead to a similar leukocytic infiltrate in the manipulated and non-handled parts of the intestinal tract. Leukocyte recruitment reflected as the number of myeloperoxidase (MPO) positive cells per high power field (HPF) in the muscularis externa in the different parts of the intestinal tract at 24 hours after surgery. However, the influx did not differ significantly (NS) between gentle IM (grey bars) and intense IM mice (black bars) in the small intestine or colon. Results are representative of three independent experiments in groups of 6-8 mice and data are means ± S.E.M. * P < 0.05, one-way ANOVA followed by Tukey's Multiple Comparison analysis.

In the non-handled colon leukocyte infiltration was significantly increased after IM compared to laparotomy. However, no difference in leukocyte infiltration in the colon was observed after intense and gentle IM (Fig. 3). In line, IL-6, IL-1β and TNFα mRNA levels were not significantly different after IM compared to laparotomy, indicating that there was no detectable inflammatory response (Fig. 3). Also in the stomach, manipulation of the intestine did not result in an influx of MPO positive cells in the muscularis (data not shown), or an increase in mRNA levels of IL-6, IL-1β and TNFα (Fig. 3).
Figure 3 | Expression of inflammatory cytokines in different parts of the gastrointestinal tract.
Quantitative PCR for IL-6 (Panel A), IL1β (Panel B) and TNFα (Panel C) in muscularis of the stomach, small intestine and colon at 6 and 24 hours after surgery. Results are representative of three independent experiments in groups of 4-7 mice and data are means ± s.e.m; * $P < 0.05$, one-way ANOVA followed by Tukey's Multiple Comparison analysis.

Intense IM results in tissue damage with release of cytokines into the circulation

Next, we investigated whether increased levels of tissue damage could be associated with a more pronounced upregulation of pro-inflammatory cytokines reflected in increased cytokine plasma levels. One hour after surgery, plasma levels of I-FABP, a marker of intestinal tissue damage, were significantly elevated in mice subjected to more intense IM (Fig. 4A). After 24 hours, I-FABP was no longer detectable.

To study whether intense IM results in a systemic inflammatory response, circulating pro-inflammatory cytokine levels were determined at 1, 6 and 24 hours following surgery. As shown in Figure 4, plasma levels of KC, MCP-1 and IL-6 were significantly increased following intense IM, but not after gentle IM or laparotomy, whereas manipulation of the intestine did not result in enhanced plasma levels of IL-1β and TNFα (data not shown for TNFα).
Figure 4 | Tissue damage and plasma levels of inflammatory cytokines after different intensities of surgical manipulation.

Plasma I-FABP (marker for tissue damage) levels 1 hour after surgery (Panel A). Plasma levels of IL-6 (Panel B), KC (CXCL-1) (Panel C), IL-1β (Panel D) and MCP-1 (Panel E) were determined in blood retrieved at 1, 6 and 24 hours after surgery. Results are representative of three independent experiments in groups of 6-8 mice and data are means ± s.e.m; * P < 0.05, one-way ANOVA followed by Tukey's Multiple Comparison analysis.

Intense IM induced tissue damage is associated with area postrema activation

Previous studies have reported activation of brain areas following abdominal surgery, a mechanism that was proposed to contribute to the development of POI. To investigate whether brain activation contributes to the severity of POI or is associated with increased plasma levels of cytokines, we investigated the expression of c-Fos to determine the amount of neuronal activation in the brainstem 24 hours after surgery. The neurons of the area postrema, which is exposed to systemic circulation, relay their signal to the nucleus of the solitary tract (NTS) and can thereby result in an enhanced activation of the NTS. The number of c-Fos positive neurons in the area postrema and NTS was significantly higher after intense compared to gentle IM (Fig. 5). c-Fos expression was also significantly higher after intense IM at higher levels of the neurocircuitry, namely in the hypothalamic paraventricular nucleus (PVN) (data not shown). In line, we observed
a positive correlation of c-Fos expression in the area postrema with plasma I-FABP levels (Spearman’s ρ correlation coefficient 0.65 (95% CI: 0.30 - 0.85; P = 0.0013)), suggesting that the degree of tissue damage is associated with activation of the area postrema (Fig. 5).

**Figure 5** | Intensity of IM and tissue damage are associated with enhanced brainstem activation.

Representative images of IM-induced c-Fos expression in brainstem nuclei 24 hours after surgery (Panel A). Panel B correspond to c-Fos expression in the Nucleus of the solitary tract (NTS). Panel C shows c-Fos expression in the area postrema (AP). Data are expressed as mean ± s.e.m. for 6-8 mice per group. Activation of the AP is associated with tissue damage (plasma I-FABP levels 1 hour after surgery); Spearman’s ρ correlation coefficient 0.65 (95% CI: 0.30 - 0.85; P = 0.0013 (Panel D).

* P < 0.05, one-way ANOVA followed by Tukey’s Multiple Comparison analysis.

Recovery of GI function, tissue damage and systemic inflammatory cytokines in humans after different intensities of surgical handling

In patients undergoing elective intestinal surgery, plasma levels of I-FABP and the inflammatory cytokines IL-6, IL-1β, MCP-1 and IL-8 were significantly higher after open compared to laparoscopic intestinal surgery (Fig. 6B-C). This was associated with a longer duration of POI (median time until tolerance of solid food and passing defecation: 72 hours after laparoscopic vs 96 hours after open colonic surgery (Fig. 6A), confirming that more intense manipulation of the intestine leads to an increase in plasma levels of pro-inflammatory cytokines and more severe POI.
Discussion

Inflammation of the intestinal muscularis is abundantly demonstrated to underlie POI. Here we demonstrated that not the number of infiltrating leukocytes, but that rather tissue damage and the release of inflammatory cytokines into the circulation are important factors determining the severity of POI. Concomitantly we found in humans that open abdominal surgery leads to more tissue damage and increased levels of circulating cytokines compared with minimally invasive laparoscopic surgery. Finally, increased tissue damage and plasma levels of cytokines lead to activation of the area postrema and PVN, possible contributing to
the development of more severe POI. Taken together, our findings indicate that
more severe upregulation of pro-inflammatory cytokines, in response to increased
tissue damage, with “leakage” of pro-inflammatory cytokines into the systemic
circulation significantly contribute to the severity of POI.

The pathophysiology of POI involves recruitment of leukocytes into the
intestine impairing smooth muscle contractility. Incremental degrees of
manipulation of the small intestine cause a progressive increase in leukocyte
infiltration. These infiltrating leukocytes subsequently release inflammatory
mediators such as prostaglandins and nitric oxide impairing the contractility of
smooth muscle strips of the muscularis and have been proposed to underlie the
delay in intestinal transit. Up to date, there is still a scarcity of information on the
influence of the severity and extent of surgery on the duration of POI. Graber et al.
subjected 6 monkeys to 3 operations varying in extent and site of dissection. In this
cross-over study the duration of postoperative dysmotility was independent of the
extent, and site of the operative procedure. However, years later Uemura et al.
showed in rats that the magnitude of the abdominal incision does affect the duration
of POI. We previously demonstrated that only externalization of the intestine
outside the abdominal cavity already induced a significant influx of leukocytes
without resulting in POI. Also in the present study, no significant difference in
leukocyte recruitment was observed in the small intestinal muscularis following
intense IM compared to gentle IM. As we failed to demonstrate that increased influx
of leukocytes is associated with prolonged POI, other mechanisms seem to
determine the severity of POI.

It is reasonable to speculate that more severe handling of the intestine will
result in more tissue damage. Veenhof et al. recently demonstrated a significant
increase in IL-6 in serum of patients undergoing open rectal procedures compared
to patients undergoing a laparoscopic procedure. In line, several studies have
reported an increased postoperative inflammatory response related to increased
operative trauma. Damaged tissue releases pro-inflammatory mediators
(also called pro-inflammatory damage-associated molecular patterns, or DAMPs)
such as heat shock proteins, uric acid, HMGB-1, SAP130, DNA and S100 proteins that
are normally intracellular. Mast cells and macrophages, two cell types known to be
involved in the pathogenesis of POI, may be activated by interaction with these
DAMPs. In the present study, we indeed recorded higher levels of I-FABP, a marker
for tissue damage, both in mice and patients undergoing more severe intestinal
handling. Moreover, intense IM was associated with more pronounced upregulation
of pro-inflammatory cytokines, associated with detection of these cytokines in the
systemic circulation. Clearly, this increased inflammatory response in the handled
intestine will impair smooth muscle function. A possible additional factor
contributing to more severe ileus may result from the increased levels of plasma
cytokines activating the hypothalamic-pituitary-adrenal axis. During tissue trauma, immune cells release the pro-inflammatory cytokines IL-1, IL-6 and TNFα into the general circulation. These cytokines result in enhanced activation of the hypothalamus triggering hypothalamic-pituitary-adrenal activity.15, 21-24 This results in an enhanced sympathetic inhibition of intestinal motility through stimulation of α2-adrenergic receptors on monocytes leading to an increased release of nitric oxide.25 Indeed, our preliminary brain histology data (unpublished) indicated enhanced activation of the hypothalamic PVN in the intense IM mice that still had detectable systemic IL-6 levels 24 hours after surgery. Finally, the more enhanced delay in transit might result from direct activation of residential macrophages by circulating cytokines, DAMPs and other tissue damage products, or even bacterial products. These muscularis-resident macrophages can induce nitric oxide synthase thereby further contributing to the postoperative impairment of GI motility.6, 26

As POI is characterized by impaired motility of the entire GI tract, including areas that have not been manipulated, other factors than local inflammation should be involved. Previously, evidence has been reported that the local inflammation, mainly via prostaglandins, activates afferent nerves triggering inhibitory neural pathways affecting motility of distant non-inflamed areas.2, 6, 26-28 More recently, Engel et al. showed that IM evokes local IL-12 production and thereby triggers TH1 memory cells to egress into the systemic circulation and migrate to non-manipulated areas of the intestine. There, these TH1 memory cells stimulate macrophages in the muscularis externa leading to dissemination of the inflammatory response.14 In previous experiments, however, we were unable to demonstrate increased levels of IL-12.29 Moreover, we showed that RAG1 -/- mice, devoid of T cells, developed POI to the same extent as wild type mice,30 suggesting that other mechanisms must be involved. In the present study, we observed that IM of the small intestine resulted in an influx of leukocytes into the colon, but the degree of influx was not related to impaired motility. Notably, colonic transit was only delayed after intense IM but not following gentle manipulation although the influx of leukocytes was comparable. Similarly, the upregulation of inflammatory cytokine levels after intestinal handling, both after intense and gentle IM, did not differ from laparotomy mice (data not shown), indicating that reduction in colonic motility does not result from disseminated inflammation. Based on the observation that delayed colonic transit is rather associated with increased systemic levels of pro-inflammatory cytokines, we speculate that impaired colonic motility rather results from the known inhibitory effects of pro-inflammatory cytokines on smooth muscle function. Pro-inflammatory cytokines such as TNFα, IL-1β, KC and MCP-1 may affect directly enteric neural coordination of motility or intestinal muscle contractility.15-18;19;15 In addition, we showed that increased plasma levels of inflammatory cytokines, only observed following intense IM, activate the area
postrema and NTS. This activation may subsequently trigger inhibitory neural motor pathways affecting distant regions of the gut with enhanced sympathetic inhibition of intestinal motility, and thereby further contribute to more severe POI.

In conclusion, our findings indicate that more severe upregulation of pro-inflammatory cytokines, in response to increased tissue damage, with “leakage” of pro-inflammatory cytokines into the systemic circulation significantly contribute to the severity of POI. Our observations indicate that identification of the host cell(s) that initiate release of inflammatory cytokines into the circulation may aid in the development of strategies to selectively block this response and reduce the severity of ileus. In addition, more insight into how tissue damage triggers the release of systemic cytokines may also lead to therapeutics to prevent this response.

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Supplementary Material

Supplementary table 1 | Primer sequences for qRT-PCR.

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References

Chapter 4

Mast cells trigger epithelial barrier dysfunction, bacterial translocation and postoperative ileus in a mouse model


Abstract

Background: Abdominal surgery involving bowel manipulation commonly results in inflammation of the bowel wall, which leads to impaired intestinal motility and postoperative ileus (POI). Mast cells have shown to play a key role in the pathogenesis of POI in mouse models and human studies. We studied whether mast cells contribute to the pathogenesis of POI by eliciting intestinal barrier dysfunction.

Methods: C57BL/6 mice, and two mast cell deficient mutant mice Kit<sup>W/W-v</sup>, and Kit<sup>W-sh/W-sh</sup> underwent laparotomy (L) or manipulation of the small bowel (IM). Postoperative inflammatory infiltrates and cytokine production were assessed. Epithelial barrier function was determined in Ussing chambers, by measuring transport of luminal particles to the vena mesenterica and by assessing bacterial translocation.

Results: In WT mice IM resulted in pro-inflammatory cytokine and chemokine production, and neutrophil extravasation to the manipulated bowel wall. This response to IM was reduced in mast cell deficient mice. IM caused epithelial barrier dysfunction in WT mice but not in the two mast cell deficient strains. IM resulted in a decrease in mean arterial pressure in both WT as well as mast cell deficient mice, indicating that impaired barrier function was not likely explained by tissue hypoperfusion but involved mast cell mediators.

Conclusions: mast cell activation during abdominal surgery causes epithelial barrier dysfunction and inflammation of the muscularis externa of the bowel. The impairment of the epithelial barrier likely contributes to the pathogenesis of POI. Our data further underscore that mast cells are bona fide cellular targets to ameliorate POI.
Introduction

Postoperative ileus (POI) is characterized by a transient cessation of intestinal motor activity following abdominal surgery, and as a result, patients suffer from complications and prolonged hospital stay \(^1\), \(^2\). The costs related to POI have been estimated to amount 1.47 billion dollars annually in the USA, illustrating its large socio-economical impact \(^1\). Regarding the pathogenesis of POI, it has become evident from animal and human studies that postoperative intestinal hypomotility in POI is the result of an influx of leukocytes into the manipulated muscularis externa \(^3\), \(^4\). Neutrophil infiltrates have been shown to inhibit local contractile activity, i.e. via the release of nitric oxide (NO) \(^5\)–\(^7\), or general motility via the activation of sympathetic inhibitory neural reflexes \(^8\). The importance of this inflammatory response in POI is underscored for instance by the success of therapeutic strategies aimed at blocking neutrophil recruitment to ameliorate POI \(^1\), \(^3\), \(^9\). However, the pathophysiological mechanisms behind the immune response to bowel manipulation remain to be clarified. In this respect, an important factor could be the reduced epithelial barrier function resulting from bowel handling that was previously observed in rodent models of POI \(^5\), \(^10\). This would be in line with previous observations that bowel wall mechanical stretch \(^11\) and manipulation \(^12\) augments inflammatory responses of bowel wall macrophage populations and local dys-contractility via TLR activation.

We have previously shown that mast cells are crucial players in the intestinal inflammation that mediates POI \(^13\) and that mast cell stabilizers and histamine receptor antagonists are instrumental in reducing POI in animal models \(^13\) and human POI \(^4\), \(^14\). Mast cells are implicated in barrier dysfunction in animal models of chronic stress \(^15\), \(^16\), allergic inflammation \(^17\), parasitic infection \(^18\) and endoxemia \(^19\). Thus, given the implication of mast cells in the pathogenesis of POI, and their potential to regulate intestinal barrier function, we assessed the role of mast cell-induced barrier dysfunction in the occurrence of POI by using two mast cell deficient mouse strains, Kit\(^{W/W-v}\) and Kit\(^{W-sh/W-sh}\). Here, we show that IM during abdominal surgery is associated with intestinal barrier dysfunction and inflammation of the manipulated bowel muscularis externa. Our data indicate that both inflammation and barrier dysfunction are mediated by mast cells and can be considered as factors contributing to POI pathogenesis.

Materials and Methods

Laboratory animals
Mice (C57BL/6, Harlan Nederland, Horst, The Netherlands) were kept under environmentally controlled conditions (light on from 8 AM to 8 PM; water and rodent
nonpurified diet ad libitum; 20°C–22°C, 55% humidity). Mast cell-deficient Kit^{W/W-\nu} (WBB6F1-W/W\nu), the mast cell-sufficient Kit controls (Kit^{+/+}) and Kit^{W-sh/W-sh} (B6.Cg-KitW-sh/HNijhrJaeBsmJ) and their C57BL/6 controls were from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were maintained at the animal facility of the Academic Medical Centre in Amsterdam and were used at 12–20 weeks of age. Animal experiments were performed in accordance with the guidelines of the Ethical Animal Research Committee of the University of Amsterdam.

Surgical procedures: abdominal surgery with intestinal manipulation
Mice were anesthetized by an intraperitoneal (IP) injection of a mixture of fentanyl citrate/fluanisone (Hypnorm; Janssen, Beerse, Belgium) and midazolam (Dormicum; Roche, Mijdrecht, The Netherlands). Surgery was performed under sterile conditions. Mice (8–11 per treatment group) underwent control surgery of only laparotomy (L) or laparotomy followed by intestinal manipulation (IM) as described earlier 13. After 24h mice were anesthetized and killed by cervical dislocation, mesenteric lymph nodes were harvested under aseptic conditions, subsequently the small intestine was removed, flushed in ice-cold saline, divided into several segments and stored for further analysis.

FACS analyses
Mesenteric lymph nodes (MLN) were isolated at indicated time points after surgery and cleared from fat. Tissue was digested for 15 minutes using collagenase IV and cell suspension was obtained after filtering and cells were resuspended, washed, and taken up in RPMI medium/10% FCS and incubated for 3h in BrefeldinA. Cells were then washed and resuspended in FACS buffer, and incubated with the appropriate antibodies. Cells were fixed with 2% PFA; for intracellular FACS, cells were treated with 0.5% saponin in FACS buffer.

Bacterial Translocation
Mesenteric lymph nodes (MLN) were weighed, placed in a tube containing 300 μL of ice-cold Luria-Bertani (LB) broth, homogenized with a sterile grinder, and plated onto blood agar plates under aerobic and anaerobic conditions. After 48 hours of incubation at 37°C, the number of colony forming units (CFU) per milligram lymph node was assessed.

Ussing Chamber Experiments
Segments of tissue of the distal small intestine were opened, cut, and immersed in Modified Meyler’s Buffer (128 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl_2·6H_2O, 20.2 mM NaHCO_3, 0.4 NaH_2PO_4·H_2O, 0.33mmol/L Na_2HPO_4, and 1.0 MgCl_2·6H_2O, 10 mM Hepes, 10 mM glucose at pH 7.4). Ileal segments were removed 45 minutes after surgery and 15
minutes thereafter the tissue was mounted in Ussing chambers (World Precision Instruments, Berlin, Germany). Serosal and mucosal areas were exposed to 2 mL of circulating oxygenated Modified Meyler Buffer maintained at 37°C. After 15 minutes, horseradish peroxidase (HRP, Sigma-Aldrich) was added to the luminal buffer at a final concentration of 10 μM. After 30, 60 and 90 minutes, samples (300 μl) were taken from the serosal side and replaced with fresh buffer. The enzymatic activity of HRP was measured using o-Phenylenediamine dihydrochloride (Sigma-Aldrich) as a substrate. The transepithelial flux of HRP was represented as pMol/h/cm².

In vivo intestinal permeability measurement
Mice (female C57BL/6 and Kit^{W-sh/W-sh}) were anaesthetized using mixture of fentanyl citrate/fluanisone (Hypnorm; Janssen) and midazolam (Dormicum; Janssen). Abdominal surgery with intestinal manipulation was performed as described 8. Mice (n = 5-7 per group) were assigned to the following two groups: laparotomy only (sham); laparotomy followed by intestinal manipulation (IM). At 1 hour after surgery, the mice were re-anaesthetised with Isoflurane, 1.2 – 2.5 % vol (Abott Laboratories, Kent, UK) and cannulation of the Superior Mesenteric Vein (SMV) was performed under sterile conditions. After ligating the ileum 5 cm from the ileocecal valve and 5 cm proximal from the initial ligation, 0.5 mL of saline solution containing fluorescent probe (Fitc-Dextran (3-5 kD, Sigma-Aldrich); Rhodamine Dextran (10 kD, Invitrogen, Carlsbad, CA); in saline solution) was gently injected into the lumen. Blood samples (with concomitant fluid replacement) were drawn at intervals of 10 mins until one hour from cannulation using Lithium heparin filled tubes (Greiner bio-one).

Antibiotic treatment
Gut decontamination was performed by administration of polymyxin B (75 mg/kg/d) and neomycin (225 mg/kg/d) as described earlier (turler et al 2007). The antibiotics were instilled into the stomach by a gastric tube for 6 days, once daily. Control animals received vehicle (normal saline) at appropriate time points. Mice underwent surgery with intestinal manipulation as described above on the sixth day of treatment and were killed on day 7.

Measurement of Mean Arterial Pressure
Mean arterial pressure (MAP) was recorded as previously reported 20. In short, after induction of anesthesia, the carotid artery was cannulated and blood pressure and heart rate were recorded using a heparinized saline-filled catheter. The catheter was connected to a pressure transducer (Truwave PX-600F; Baxter, Deerfield, IL, USA) and signals samples and stored using Labview applications (National Instruments, Austin, TX,
USA). Rectal temperature was monitored continuously and remained at 37°C throughout the experiment. Sodium Nitroprusside treatment: after being anesthetized using FFM, mice were injected with 50 µg/kg Sodium nitroprusside (SNP; Sigma-Aldrich) i.p. using an automatic infusion pump. The infusion rate was adjusted whenever necessary in order to maintain the desired MAP drop for the time indicated.

### Gastrointestinal transit

Gastrointestinal function was determined in vivo by measurement of gastrointestinal transit of liquid FITC-dextran (70,000 Da; Invitrogen, Paisley, UK). Ninety minutes after administration through oral gavage, the animal was killed and the entire bowel from stomach to distal colon was collected. The contents of the stomach, small bowel (divided into 10 segments of equal length), the cecum, and colon (3 segments of equal length) were collected and assayed in duplicate for the presence of fluorescent label (Synergy HT, BioTek Instruments Inc., VT, USA; excitation wavelength: 485 nm, emission wavelength: 528 nm) for quantification of the fluorescent signal in each bowel segment. The distribution of signal along the gastrointestinal tract was determined by calculating the geometric centre (GC): Σ (percent of total fluorescent signal in each segment X the segment number)/100 for quantitative statistical comparison among experimental groups. Individual transit distribution histograms were plotted, and transits were statistically analyzed using the calculated geometric centre (GC).

### Mast cell culture and Reconstitution of Kit^W-sh/W-sh^ mice

Kit^W-sh/W-sh^ mice were reconstituted by the injection of bone marrow–derived cultured mast cells into the peritoneal cavity, as described earlier. Sterile, endotoxin-free medium was flushed repeatedly through the bone shaft using a needle and syringe. The suspension of BM cells was centrifuged at 320 g for 10 min and cultured at a concentration of 0.5 × 10^6 nucleated cells/ml in RPMI 1640 with 10% FCS (Sigma-Aldrich) 100 units/ml penicillin, 100 µg/ml streptomycin (Life Technology, Breda, The Netherlands), 10 µg/ml gentamycine, 2 mM L-glutamine, and 0.1 mM nonessential amino acids (referred to as enriched medium) and a combination of IL-3 (5 ng/ml) and SCF (50 ng/ml) for 3 weeks at 37°C in a humidified atmosphere with 5% CO₂. Nonadherent cells were transferred to fresh medium at least once a week. After 3–4 weeks when a mast cell purity of ≥ 95% was achieved, as assessed by toluidine blue staining, the cells were harvested for experiments. Three weeks old Kit^W-sh/W-sh^ received 10 × 10^5 cells in 100 µL PBS through IP injection. Mice were used 10 weeks after adoptive transfer of mast cells. This procedure reconstitutes the mast cell population without systemic effects.
Immunohistochemistry and visualization of myeloperoxidase positive cells

Immunohistochemical staining for CD11a and CD3 was performed on acetone fixed transverse ileal segments. Endogenous peroxidase activity was eliminated by incubation of segments in methanol containing 0.3% H₂O₂. Nonspecific protein-binding sites were blocked by incubation in PBS, pH 7.4, containing 10% of normal goat serum for 10 min. Sections were incubated overnight with biotinylated hamster anti-mouse CD11a or CD3 antibodies (Pharmingen, San Diego, CA, U.S.A.) (dilution 1 : 1000). Next, sections were incubated with ABCComplex/HRP (DAKOCytomation, Glostrup, Denmark) for 30 min. HRP was visualised using SigmaFast DAB (Sigma-Aldrich), incubating 5 min, and contra-stained with haematoxilin/eosin. Visualization and quantification of myeloperoxidase (MPO) positive cells in the ileal muscularis externa was performed as described elsewhere 21.

Tissue cytokine production

For cytokine measurements, mucosa was separated from the muscle tissue using a glass slide, in icecold modified Meyler’s buffer. Six cm segments were added to 500 µl lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl₂, 2 mM CaCl₂, 1% Triton X-100; pepstatin A, leupeptin, and aprotinin (all 20 ng mL⁻¹; pH 7.4), homogenized, and incubated at 4°C for 30 min. Homogenates were centrifuged at 1500 x g at 4°C for 15 min, and supernatants were stored at -20°C until assays were performed. TNF, IL-6, MCP-1, RANTES, IL-12p70 and KC in supernatants were analyzed by mouse ELISA (R&D systems, Abingdon, United Kingdom) according to manufacturer’s instructions.

Statistical analysis

The data are expressed as mean ± SEM and were analyzed using the nonparametric Mann-Whitney U test. A P value less than 0.05 was considered significant. IM surgery groups were compared to L control groups where indicated.

Results

Intestinal manipulation results in an immune response localized at the muscularis externa

We first established in a mouse model for POI that IM resulted in the activation and infiltration of CD11a-positive leukocytes in the muscularis externa of the small intestine. The intestinal muscularis is known to contain a network of resident macrophage-like cells of which a proportion is F4/80 positive (Fig. 1A) 22. The activation of this cell population has been implicated in the leukocyte recruitment in POI 3, 13 leading to an extravasation of CD11a expressing cells that appeared in muscularis
externa of the small intestine 24h after IM (Fig. 1A and B). The population of CD11a positive cells is negative for CD3 as shown in consecutive sections (Fig. 1B) and comprises extravasated monocytes and neutrophils, as demonstrated earlier. We further assessed the IM induced cytokine production 1h, 3h, 6h and 24h after IM in the muscular layer and mucosa. IL-6 production and signaling is an important characteristic of POI induced inflammation so we choose to measure IL-6 along with chemokines MCP-1 (CCL-2) and KC (the mouse homologue of IL-8) as key parameters of localized inflammation. In 3- to 24 hours after IM we observed a significant increased production of the cytokines CCL-2, IL-6, and KC in the mice that had undergone IM, but not in control L mice (Fig. 1C, left panels); levels of IL-12p70 and TNF were not significantly induced within this time frame. In conjunction with previous reports, the IM procedure did not lead to significant cytokine production in the small intestinal mucosa (Fig. 1C, right panels). Notably, RANTES was constitutively expressed in the mucosa and unaffected by IM (Fig. 1C).

In a recent study intestinal surgery and manipulation was reported to elicit DC derived IL-12 production within 30 minutes after surgery, initiating a Th1 response leading to POI. However in our model, enhanced production of IL-12 at earlier time points after IM in the muscularis tissue was not apparent and IL-12 only increased in muscle tissue after 24 hours. (Fig 1C), i.e. at a time point were an inflammatory infiltrate was already established.

**IM induced inflammation is dependent on the presence of mast cells**

We have previously implicated mast cells in the pathogenesis of POI. Hence, we sought to establish that mast cell activation is involved in the muscularis inflammatory response and POI after intestinal surgery. Upon activation, connective tissue mast cells release chymases such as mouse mast cell protease 1 (mMCP-1). mMCP-1 release in peritoneal lavage fluid was increased one hour after intestinal manipulation compared to L (Fig. 2A). In C57BL/6 mice, mast cells were found in the muscularis externa, Peyer’s Patches and mesentery (Fig 2A). Next, we investigated the role of mast cells in the leukocyte recruitment to the ileum muscularis externa observed after IM. To this end we studied the response to IM in 2 mast cell deficient mice strains that carry different spontaneous mutations in the gene for ckit (White spotting (W) locus) Kit\(^{W/W-v}\) and Kit\(^{W-sh/W-sh}\) mice. Reduced c-kit tyrosine kinase-dependent signaling results in mast cell reduction in both mouse strains. In Kit\(^{W/W-sh}\) mice, a reduced inflammatory infiltrate was observed after IM if compared to their mast cell sufficient Kit\(^{+/+}\) cognate controls (Fig
Figure 1 | IM induced inflammation is localized to the muscularis externa. In animals that underwent laparotomy (L), CD11a-expression, indicating leukocyte activation, is absent from the muscularis externa (A, left panel). Resident macrophages are present in the muscularis externa of unmanipulated mice, as indicated by F4/80 staining (A, right panel). After intestinal manipulation (IM), CD11a expression in the muscularis externa was induced (B, right panel). T-cells (CD3+) do not infiltrate the muscularis externa 24h after IM (B, right panel). IM induced production of cytokines and chemokines in muscularis externa homogenates and in mucosa homogenates is shown at indicated time points after surgery (C). RANTES protein expression was unaltered and constitutively expressed in the small intestinal mucosa (C); * P < 0.05, ** P < 0.01 (compared to the respective L-groups). Bars indicate mean ± SEM. (n = 6 per group).

2B). In addition, the production of cytokines MCP-1 and IL-6 depends on the appropriate development of mast cells as both cytokines are significantly reduced in Kit<sup>W/W<sup>-v mice (Fig. 2B). These data were corroborated by a reduced cellular MPO positive infiltrate, and reduced levels of IL-6 and MCP-1 after IM in mice that were treated with mast cell stabilizers ketotifen or doxantrazole (13 and data not shown).

In addition to a mast cell deficiency, in Kit<sup>W/W<sup>v mice haematopoiesis is affected, they are anaemic and infertile26, 27. Therefore we additionally analyzed the alternative c-
kit mutant Kit\textsuperscript{W-sh/W-sh} and the respective WT strain C57Bl/6, that comprise a more selective mast cell deficient phenotype compared to the Kit\textsuperscript{W/W-v} strain 27.

In contrast to C57BL/6 controls, IM in the Kit\textsuperscript{W-sh/W-sh} mice did not lead to a significant increase in MPO positive cells compared to controls (Fig. 2C). The production of IL-6 and CCL-2 in their muscular compartment after IM surgery was significantly reduced compared to their C57BL/6 controls (Fig. 2D). To demonstrate that the observed phenotype was mast cell dependent, we reconstituted Kit\textsuperscript{W-sh/W-sh} mice with mast cells (MC) obtained from C57BL/6 bone marrow derived mast cell cultures, and measured the cytokine response to IM. Success of reconstitution was demonstrated by presence of mast cells in Kit\textsuperscript{W-sh/W-sh} ileum (see Fig 3C). From these analyses we concluded that the number, or tissue location, of ileal mast cells found in mast cell reconstituted Kit\textsuperscript{W-sh/W-sh} mice was not different from those found in C57Bl/6 mice (Fig 3C). In addition, earlier studies have shown that this treatment allows reconstitution to functionally active mast cells 13, 27. We next assessed whether mast cell reconstitution restores the inflammatory response to IM. In contrast to vehicle treated Kit\textsuperscript{W-sh/W-sh} mice, in Kit\textsuperscript{W-sh/W-sh} mice that were reconstituted with C57Bl/6 cultured mast cells the production of CCL-2 after IM was significantly increased, and was comparable to C57BL/6 WT levels. IM-induced IL-6 (Fig. 2D) was unaffected by reconstitution (Fig. 2D), indicating that mast cells do not play a role in the induction of IL-6 in the model.

**IM induced delay in gastrointestinal transit is dependent on the presence of mast cells**

We next examined the role of mast cells in POI by analyzing the functional changes in gastrointestinal transit induced by IM. We choose for Kit\textsuperscript{W-sh/W-sh} mouse strain to study the effect of mast cell activation on motility because the use of Kit\textsuperscript{W-sh/W-sh} mice allows the motility parameters to be compared to C57BL/6 mice as these mice are derived on a C57BL/6 background. In the L control group, 24h after surgery, gastrointestinal transit in Kit\textsuperscript{W-sh/W-sh} mice was delayed compared to C57BL/6 control mice. The majority of the fluorescent marker in Kit\textsuperscript{W-sh/W-sh} mice is present in segment (sg) 7 and sg8, whereas the marker in the C57BL/6 mice is mainly present in sg9, sg10, and the cecum (Fig. 3A, left panel). This is reflected in the average calculated geometric centre (GC) (Fig. 3B). After IM, in C57BL/6 mice, the gastrointestinal transit is
Figure 2 | Mast cells are crucial in muscularis externa inflammation following bowel handling. Mouse mast cell protease (mMCP-1), determined in peritoneal lavage 1h after surgery, was increased after intestinal manipulation (IM) compared to laparotomy (L) (A). Mast cells (purple) were visualized in C57BL/6 mice with Toluidin Blue staining and were present muscularis externa, serosa, Peyer’s patches and the mesentery (A). The number of myeloperoxidase (MPO) positive cells per mm$^2$ and production of proinflammatory cytokines MCP-1 and IL-6 in the muscularis externa was significantly reduced in mast cell deficient Kit$^{W/W}$ compared to control mice (Kit$^{+/+}$) (B). In Kit$^{W-sh/W-sh}$ mice, the number of MPO+ cells was not increased after IM compared to control (C57BL/6). Similarly, in Kit$^{W-sh/W-sh}$ reconstituted with C57BL/6 bone marrow derived mast cells, the number of MPO positive cells as unaffected after IM (C). CCL-2, and IL-6 production in Kit$^{W-sh/W-sh}$ and C57BL/6 and Kit$^{W-sh/W-sh}$ reconstituted with bone marrow derived mast cells (D). Asterisks indicate significant differences where indicated; ** P < 0.01, * P < 0.05. Bars indicate mean ± SEM, for all groups L: n = 5-7 IM: n = 10 mice per group.
significantly delayed as the fluorescence marker is mainly present in sg4 and sg5 (Fig. 3A, middle panel), also shown by the GC (Fig. 3B). However, the Kit$^{W-sh/W-sh}$ mice display only a minor delay in gastrointestinal transit after IM (Fig. 3A, middle panel), clearly reflected in the GC values shown in Fig. 4B. After Kit$^{W-sh/W-sh}$ mice were reconstituted with WT mast cells, the delay in gastrointestinal transit partially returned, indicated by the more widespread distribution of the fluorescent marker, in sg 2, 3, 5 and also 10 (Fig 3A, right panel). The IM induced delay is also reflected in decreased GC values (Fig 3B).

**Figure 3** | Role of mast cells in gastrointestinal transit after intestinal manipulation. Twenty four hours after laparotomy (L) or intestinal manipulation (IM), gastrointestinal transit was determined by the % distribution of the fluorescent marker and calculation of the geometric centre (GC) as indicated in materials and methods. St=stomach, cc=cecum. The fluorescent marker (dextran-FITC) in the L groups is located more proximally in the Kit$^{W-sh/W-sh}$ than in the C57BL/6 mice as depicted in (A, left panel). After IM, dextran is localized more proximally in C57BL/6 mice than in Kit$^{W-sh/W-sh}$ mice (A, middle panel). IM induced delay in gastrointestinal transit is partially rescued by reconstitution of Kit$^{W-sh/W-sh}$ with WT mast cells (A, right panel). GC values are shown in (B). * P < 0.05 (compared to the respective L group). Bars indicate mean ± SEM. (L: n = 4, IM: n = 4 - 7 mice per group). Panel C: Mast cell were visualized using toluidin blue staining in Kit$^{W-sh/W-sh}$ mice (upper panel), or Kit$^{W-sh/W-sh}$ mice that were reconstituted with C57BL/6 cultured mast cells (lower panel). D) the quantification of the number of mast cells recovered in the ileum after reconstitution. N=4 mice per treatment group. ND is not detectable.
A significant decrease in GC value was observed after IM in control C57BL/6 mice, but not in KitW-sh/W-sh. Although mast cell reconstitution in these mice partially protects from POI as judged by the transit data (Fig. 3A), the GC values were unaffected and were not reduced as seen in C57BL/6 WT mice.

**IM induces mast cell dependent bacterial translocation to MLN**

Given the implication of mast cells in epithelial barrier dysfunction in other models, we next assessed whether mast cells may contribute to the pathogenesis of POI by affecting the epithelial barrier function. To this end we analyzed barrier function after IM in 3 ways: by determining postoperative bacterial translocation to draining mesenteric lymph nodes (MLN), by measuring permeability to HRP in Ussing chambers, and by assessing postoperative particle leakage from the small intestinal lumen into the blood circulation. We first assessed whether IM led to bacterial translocation 24h after surgery by assessing the number of Colony forming units (CFU) in the MLN. In C57BL/6 mice that had undergone IM, the number of bacteria that had translocated to the MLN was significantly increased as compared to the L control group (Fig. 4A). The numbers of CFU cultured from MLN under aerobic and anaerobic culture conditions were comparable and therefore, in subsequent experiments we only cultured bacteria under aerobic conditions. We next assessed whether mast cells play a causal role in the impaired intestinal barrier function after IM. To this end we measured bacterial translocation in mast cell deficient KitW/Wv mice. Baseline and IM induced bacterial translocation did not differ in KitW/Wv mice, but this was not a characteristic of the lack of mast cells per se as the number of CFU was also not different in the Kit+/+ control mice (Fig. 4B). In the KitW-sh/W-sh mice the IM induced bacterial translocation was not increased after IM as compared to C57BL/6 control mice, although it should be noted that the actual number of translocating bacteria is generally low in these mice (Fig. 4C). Irrespectively, these data indicate that mast cells are likely to play a role in the bacterial translocation to the MLN after intestinal surgery.

**Intestinal manipulation leads to an impaired barrier function via mast cell activity**

The number of viable bacteria cultured from the MLN is dependent on the integrity of the epithelium, but also largely depends on processes such as uptake, and killing capacity CX3CR1-positive dendritic cells and macrophages. To specify the effect of IM on barrier function we therefore decided to additionally assess epithelial permeability for protein directly after the IM surgery in Ussing chambers. We measured flux of HRP in ileum segments mounted 1h after L or IM. In the IM group, the mucosal to serosal flux of HRP was enhanced as compared to the L group (Fig. 5A), reflecting an increase in epithelial permeability after IM. In contrast however, ileal permeability...
Figure 4 | Role of mast cells in IM induced bacterial translocation. Bacterial translocation to the mesenteric lymph nodes (MLN) was determined 24h after bowel handling. Following intestinal manipulation (IM), translocation of both aerobic and anaerobic cultured bacteria was significantly increased in C57BL/6 mice as compared to laparotomy (L) controls (L: n = 5, IM: n = 10 mice per group) (A). Bacterial translocation was also increased after IM in Kit\(^{+/+}\) mice and Kit\(^{W/W-v}\) mice compared to the L groups but did not differ between the two groups (B). The number of CFU cultured from mesenteric lymph nodes of Kit\(^{W-sh/W-sh}\) mice was not significantly different between L and IM groups (C). Kit\(^{W/W-v}\) and Kit\(^{+/+}\): L: n = 5, IM: n = 10; Kit\(^{W-sh/W-sh}\) and C57BL/6: L: n = 3, IM: n = 7 mice per group. Asterisks *P <0.05 (compared to L-group), bars indicate mean ± SEM.

measured in Ussing chambers was not significantly enhanced by IM in mast cell deficient mice Kit\(^{W-sh/W-sh}\) (Fig 5A), Kit\(^{W/W-v}\) and Kit\(^{+/+}\) (Fig 5B). To further substantiate these data we additionally measured intestinal permeability for different sized luminal particles in vivo1h after IM surgery, i.e. at a time point at which no neutrophil recruitment was observed yet. To this end we assessed transport of luminal dextran particles to the draining vena mesenteric vein. We observed a significant increase in the concentration of dextrans of 3-5 kD (Fig 5C, left panel) as well as 10kD (Fig. 5C, right panel) in mesenteric venous blood 1-2hrs after IM, but not after L. As unaffected tight junctions
Figure 5 | Mast cells regulate the transepithelial flux of small particles after intestinal manipulation. Mucosal to serosal transfer of horse radish peroxidase (HRP) was determined in Ussing chambers 1h after surgery in ileal tissue of C57BL/6 and mast cell deficient Kit^{W-sh/W-sh} and Kit^{W/W-v}. The mucosal to serosal flux of HRP was increased after intestinal manipulation (IM) compared to laparotomy (L) (A) in C57BL/6, but not in the mast cell deficient Kit^{W-sh/W-sh} (A) and Kit^{W/W-v} (B) (n = 5 mice per group). In C57BL/6, the flux of 3-5 kD (C, left panel) and 10 kD (C, right panel) particles from the small intestinal lumen to mesenteric blood is shown at indicated timepoints 1 hour after IM (open circles) as compared to sham (closed circles). In Kit^{W-sh/W-sh}, the IM induced increase in transepithelial flux particles was completely abolished as indicated in D (left panel: 3kD, right panel: 10 kD). Asterisks *P <0.05 (compared to L-group (A)) ** P < 0.01, ***P <0.001 (compared to sham-group (C)), bars indicate mean ± SEM. (n = 5 mice per group)
only allow transport of particles smaller than about 700D, leakage of particles of a bigger size suggest a disarrangement of tight junction proteins resulting from IM. In conjunction, in vivo measurement of intestinal permeability using luminal 3-5 kD and 10 kD dextran particles demonstrated that in Kit\textsuperscript{W-sh/W-sh} mice were completely protected against IM-induced epithelial permeability changes (Fig 5D). Together, these data indicate that mast cells are required for the pathological barrier dysfunction, associated with inflammatory response to the muscle layer and POI. We next assessed whether bacterial translocation would play a significant role in the pathogenesis of POI. To study this we decontaminated the gut using a 6-day regiment of antibiotic treatment and examined the effect of gut decontamination in our model of POI. As described earlier for colonic ileus\textsuperscript{12} decontamination reduced the number of infiltrating leukocytes significantly (Fig. 6A) and improved post-operative transit after IM (Fig. 6B). These data indicate a role for intestinal bacteria in the pathogenesis of POI and point towards to possibility that enhanced bacterial translocation could contribute to the inflammatory response and POI after IM.

Figure 6 | Antibiotics treatment reduce inflammatory parameters and improve postoperative transit. (A) Antibiotics treatment reduces IM-induced inflammatory infiltrates in the muscularis externa, and improves post-operative transit reflected by an enhanced GC value (B). N=4, data shown are average +/- SEM. Asterisks * P<0.05 (compared to saline treated-group (A) and **P <0.01 (compared to - L group (B).
Intestinal manipulation induced decrease in blood pressure does not account for barrier dysfunction

To assess whether mast cells contribute directly to the decreased barrier function in our POI model, or whether that involves alternative mechanisms, we next explored alternative mechanisms that lead to barrier dysfunction, such as intestinal hypoperfusion. To this end, we next assessed whether IM coincided with a decrease in mean arterial pressure (MAP), by monitoring MAP in arterial cannulated mice during abdominal surgery. As shown in Fig. 7, after opening of the abdomen the MAP was significantly reduced whereas the reduction in the L group was minimal (Fig. 7A). This effect was independent of mast cells as in the KitW-sh/W-sh, IM led to a similar decrease in blood pressure if compared to WT (Fig. 7B). For both mouse strains, the decreased MAP sustained until mice recovered from anesthesia after 40 min. (Fig. 7A/B right panels) while no difference in heart rate between L and IM was observed (results not shown). To validate whether the decrease in MAP alone could account for the impaired barrier dysfunction, we administered SNP, a NO donor, to achieve a decrease in MAP comparable that that seen after IM surgery. Intravenous rate-controlled perfusion of SNP allowed dosing SNP to lower MAP to mimic that observed after IM (Fig. 7C). SNP treatment did not trigger an inflammatory reaction 24h after administration (Fig 7D, nor did it lead to bacterial translocation to MLN (Fig 7E), or epithelial barrier dysfunction (Fig.7F). From these data it can be concluded that the reduced MAP during IM does not account for the impaired barrier function, or the inflammatory response after IM, and that mast cells are involved in the pathogenesis of POI via a mechanism that is independent of the reduction in MAP.
**Figure 7** | Blood pressure during IM procedure. Mean Arterial Blood pressure (MAP) was recorded during the surgical procedure. Surgery was started when blood pressure was stable (t=0). At t=5', the % of MAP as compared to MAP at t=0 (basal level) was significantly decreased during intestinal manipulation (IM) but not laparotomy (L) in both C57BL/6 (A, left panel) and mast cell deficient Kit\(^{W-sh/W-sh}\) mice (B, left panel). Representative MAP recording during surgery is shown in right panels of A (C57BL/6) and B (Kit\(^{W-sh/W-sh}\)) (L=grey line) and IM (black line). Absolute values are shown at the top of the graphs (A and B) (n = 3 mice per group). Administration of the blood pressure lowering agent Sodium nitroprusside (SNP) gives similar blood pressure reduction as compared to IM (C). SNP administration does not affect MPO count (D), bacterial translocation (E) 24h after IM or epithelial permeability (E) at indicated time points 1 hour after IM. Asterisks *P <0.05 (compared to L-group), bars indicate mean ± SEM. (control: n = 4; SNP: n = 3 mice per group).
Discussion

The prolonged impairment of gastrointestinal motility after intestinal manipulation is a significant confounding factor in postoperative recovery. Rodent models and human studies have demonstrated that surgical inspection and manipulation of the bowel leads to the activation of antigen presenting cells that reside in the intestinal muscularis layer. The general paralysis of the entire GI tract - including the unmanipulated segments - is a commonly seen characteristic of POI. This clinically important aspect of POI involves the activation of an inhibitory neural reflex arch by local inflammatory infiltrates, and was recently also shown to involve the production of IFN-γ by CCR9+T-cells that are activated at the site of manipulation. We have shown previously that the activation of mast cells resulting from local manipulation of the bowel is a pivotal factor in the pathogenesis of POI and the inflammatory response to local manipulation. Hence, we questioned in the current study whether mast cell derived mediators contribute to POI either as local activators of dendritic cells recruited to the gut wall or via epithelial permeability changes.

We show here that intestinal manipulation induces barrier dysfunction via a mechanism that is crucially dependent on mast cells. In patients, barrier dysfunction frequently occurs during abdominal surgery and has been associated with increased postoperative septic morbidity in surgical patients undergoing laparotomy. In addition to this model of POI, as well as in human ileus, mast cell activation has been associated with disturbed intestinal barrier function in ulcerative colitis and several disease entities such as stress-induced hypersensitivity of the bowel and endotoxemia. In these models, the rapid release of serine proteases following triggering of mast cells, possibly via release of Corticotropin Releasing Factor (CRF), is responsible for an increase in epithelial permeability. Likewise, the nature of the mast cell mediators that affect barrier function in our model involves similar rapid mechanisms and mediator release. This mechanism may involve the activation of protease activated receptor-2 (PAR-2) that is expressed on epithelial cells, although most studies have focused on PAR-2 activation by protease activity in the lumen.

Our data are in line with earlier observations in patients and rodent models implicating a disturbed intestinal barrier function after intestinal surgery involving bowel handling. It has been shown that in POI, bacterial products may reach the intestinal muscularis after IM of the small bowel and that antibiotic treatment decreases muscular inflammation after colon manipulation. The implications of this process for the pathogenesis of POI are incompletely understood, but irrespective thereof, the clinical impact of bacterial translocation during surgery is significant. A recent study which included 927 patients over 13 years showed that bacterial translocation was
associated with increased postoperative septic morbidity in surgical patients undergoing laparotomy.

In this study, we assessed barrier integrity by measuring bacterial translocation to the MLN. Most likely this process reflects dendritic cell (or CX3CR1 expressing macrophages) mediated uptake of bacteria that are still viable once transported by dendritic cells to the MLN. Thus the bacterial translocation is dependent on a number of immunological processes including phagocytosis, killing and bacterial cultures in the MLN may not reflect merely epithelial integrity. Therefore we performed measurements of barrier function in the small intestine in Ussing chambers, reflecting the para- and transepithelial transport of the 40kD HRP, as well as in vivo measurement of real time changes in epithelial leakage to the vena mesenteria. Using this combination of methods we demonstrated that IM in our model of POI led to a mast cell dependent epithelial barrier dysfunction. This mechanism may explain the important role of mast cells in the pathogenesis of POI and validate mast cells as a bona fide drug target to shorten POI and improve postoperative recovery and barrier function. We performed our experiments in two strains of mast cell deficient mice, Kit\(^{W/W-v}\) and Kit\(^{W-sh/W-sh}\) mice which both carry mutations in the Kit gene (White spotting (W) locus). C-kit is the receptor for Stem Cell Factor (SCF) and involved in regulation of hematopoiesis, proliferation and migration of primordial germ cells and melanoblasts during development. The Kit\(^{W/W-v}\) mice carry the W mutation, resulting in deletion of the transmembrane domain of the c-kit protein as well as the dominant negative Wv mutation, a point mutation that affects c-kit kinase activity. On the other hand, the mast cell deficient Kit\(^{W-sh/W-sh}\) mice carry a mutation that reflects an inversion in the kit locus spanning a 2.8 mb segment. Hence, the resulting phenotypes are different: Kit\(^{W-wv}\) mice have phenotypic abnormalities including sterility, anemia, lack of interstitial cells of Cajal (ICC), and have defects in hematopoiesis that lead to an absence of intra epithelial T-cells, neutropenia, and poor mobilization of blood neutrophils. In contrast, Kit\(^{W-sh/W-sh}\) mice, bearing the W-sash (W(sh)) inversion mutation, have mast cell deficiency but are not anemic nor sterile. Adult Kit\(^{W-sh/W-sh}\) mice have been shown to have a profound deficiency in tissue mast cells but normal levels of major classes of other differentiated hematopoietic and lymphoid cells.

As the pathogenesis of POI is mediated by neutrophil influx, we reasoned that the protection after IM in our POI model in these Kit\(^{W/W-v}\) mice might not be selectively dependent on the lack of mast cells. Therefore, we also carried out our experiments in the Kit\(^{W-sh/W-sh}\) mice for two reasons: first, in contrast to the Kit\(^{W/W-v}\), the Kit\(^{W-sh/W-sh}\) mice can be tested against C57BL/6 WT control mice. Second, Kit\(^{W-sh/W-sh}\) have normal neutrophil numbers, are fertile, contain normal number of intra epithelial lymphocytes (IELS), and are not anemic. The latter likely explains why the effects of IM induced
neutrophilic extravasation and inflammatory response were much less pronounced in the Kit<sup>W-sh/W-sh</sup> compared to the Kit<sup>W/W-v</sup>.

Interstitial cells of Cajal (ICC) require intact ckit signaling for proper development and are defective in both ckit mutant mice, although ICC subsets have been shown to develop in Kit<sup>W-sh/W-sh</sup> mice<sup>43</sup>. Because Kit<sup>W/W-v</sup> mice are hemizygous we assessed basic GI motility in Kit<sup>W-sh/W-sh</sup> that are on a C57BL/6 background. Indeed we observed a disturbed motility and delayed transit under normal conditions compared to C57BL/6 mice. But this difference did not reach significance so we decided to include Kit<sup>W-sh/W-sh</sup> in our motility analyses.

The observation that the bacterial translocation in Kit<sup>W/W-v</sup> was almost completely abolished in both affected Kit<sup>W/W-v</sup> as well as its control Kit<sup>+/+</sup> may reflect a defective representation of innate immune cells in the lamina propria in these mice, given the purported role of lamina propria APCs in bacterial sampling<sup>39, 44</sup>. In addition, we observed that inflammatory parameters, bacterial translocation, and permeability to HRP differed between the control groups: C57BL/6 and Kit<sup>+/+</sup>. This is probably due to differences in immune responses that generally exist between mouse strains: for example between Balb/c and C57BL/6<sup>45</sup>.

In this study we found that luminal bacteria were involved in IM induced inflammation and ileus by treating mice with antibiotics, indicating that bacterial translocation contributes to the POI. It has been described that the immune response is induced soon after IM, for example ICAM-1 mRNA is expressed in the muscularis within 15 minutes of manipulation<sup>3</sup>, while luminal products start to appear in the muscularis externa externa 6h after intestinal manipulation<sup>38</sup>, indicating that translocated bacterial antigens may not trigger muscularis immune responses, but may exacerbate immune responses, as shown by our data presented here. It is important to note that lamina propria intestinal macrophages that form the first line of defense generally do not produce high levels of cytokines upon bacterial challenge. Rather, dendritic cells in draining lymph are activated by luminal antigens which may not lead to mucosal inflammation but can contribute to the pathogenesis of POI in unmanipulated areas<sup>25</sup>.

We show in this study that IM is associated with a postoperative decrease in MAP. Hypoperfusion of intestinal tissue following abdominal as well as non-abdominal surgery has been associated with impaired barrier function preceded by hypotension, mesentery hypoperfusion and enterocyte damage<sup>29, 46</sup>. In addition, these studies show that aberrations in actin reorganization, cell proliferation and mitochondrial function are maximal at 60 min. after mechanical bowel manipulation (i.e. the same time point at which we measured intestinal barrier function in the current study) and was partially recovered after 24 hours<sup>46, 47</sup> (at which we measured inflammatory mediators). Although MAP decreases after IM, when MAP was pharmacological lowered, the
intestinal barrier function was not affected and inflammation did not occur to a similar extend. Circulating SNP-derived NO causes smooth muscle relaxation and subsequent microvascular vasodilatation. NO might affect inflammatory processes and intestinal barrier function independent of blood pressure alterations but we show that NO has no effect on these processes. Of note, MAP measured in the carotid artery in our study likely reflects the blood pressure of the internal organs, but we cannot exclude that perfusion in the small intestine is different from the carotid artery.

Concluding, we show that IM elicits a mast cell dependent inflammatory response and intestinal barrier disturbances that may contribute to the pathogenesis of POI. Our study further underscores the potential of mast cell stabilization in ameliorating postoperative recovery, warranting that this treatment strategy should be pursued in clinical setting.
References


Chapter 5

Inhibition of spleen tyrosine kinase as treatment of postoperative ileus


Abstract

Objective: Intestinal inflammation resulting from manipulation-induced mast cell activation is a crucial mechanism in the pathophysiology of postoperative ileus (POI). Recently it has been shown that spleen tyrosine kinase (Syk) is involved in mast cell degranulation. Therefore, we have evaluated the effect of the Syk-inhibitor GSKcompound143 (GSK143) as potential treatment to shorten POI.

Design: In vivo: In a mouse model of POI, the effect of the Syk inhibitor (GSK143) was evaluated on gastrointestinal transit, muscular inflammation and cytokine production. In vitro: The effect of GSK143 and doxantrazole were evaluated on cultured peritoneal mast cells and bone marrow derived macrophages.

Results: In vivo: Intestinal manipulation resulted in a delay of gastrointestinal transit at t=24h (Geometric Center (GC): 4.4±0.3). Doxantrazole and GSK143 significantly increased gastrointestinal transit (GC doxantrazole (10 mg/kg): 7.2±0.7, GSK143 (1mg/Kg): 7.6±0.6), reduced inflammation and prevented recruitment of immune cells in the intestinal muscularis. In vitro: In peritoneal mast cells, SP (0-90 μM) and trinitrophenyl (0-4 μg/mL) induced a concentration-dependent release of β-hexosaminidase. Pretreatment with doxantrazole and GSK143 (0.03-10 μM) concentration-dependently blocked SP and trinitrophenyl induced β-hexosaminidase release. In addition, GSK143 was able to reduce cytokine expression in endotoxin treated bone marrow derived macrophages in a concentration dependent manner.

Conclusions: The Syk-inhibitor GSK143 reduces macrophage activation and mast cell degranulation in vitro. In addition, it inhibits manipulation-induced intestinal muscular inflammation and restores intestinal transit in mice. These findings suggest that Syk inhibition may be a new tool to shorten POI.
Introduction

Postoperative ileus (POI) is characterized by generalized hypomotility of the gastrointestinal tract after abdominal surgery and leads to increased morbidity and prolonged hospitalization. Recent evidence shows that POI is mediated by infiltration of leukocytes in the intestinal muscle layer in response to surgical handling of the gut.(1-3) The importance of this inflammatory response in POI is underscored by the beneficial effect of pharmacological interventions blocking the influx of leukocytes.(1;2;4) We previously demonstrated that mast cells are involved in this inflammatory response: leukocyte recruitment was reduced in mast cell deficient mice whereas the mast cell stabilizers ketotifen and doxantrazole reduced muscular inflammation and shortened POI.(5) Based on these results, we performed a pilot study with ketotifen (4 or 12 mg for 6 days) in patients undergoing abdominal surgery.(6) Although gastric emptying was significantly improved by ketotifen, this compound has central side effects such as sleepiness and dizziness and has anticholinergic properties potentially counteracting the beneficial effect of mast cell stabilization on gastrointestinal motility.(7-9) A potential alternative approach to stabilize mast cells is blockade of intracellular spleen tyrosine kinase (Syk). Syk is one of the critical tyrosine kinases involved in mast cell degranulation induced by IgE crosslinking. Crosslinking of the FcεRI receptor causes phosphorylation of Syk subsequently activating intracellular pro-inflammatory pathways.(10) Therefore, Syk inhibitors suppress the signaling cascades that normally lead to degranulation of mast cells.(11) In addition to mast cells, activation of macrophages residing in the muscularis externa has been correlated with the intestinal inflammatory response resulting in POI.(12) Interestingly, Syk is also playing a crucial role in macrophage activation.(13-15) Indeed, inhibition of Syk signalling resulted in inhibition of the NF-kappaB (NF-kB) pathway and reduced cytokine production in lipopolysaccharide (LPS)-treated macrophages. Therefore, we hypothesized that modulation of the Syk pathway may be a potential new therapeutic strategy for POI.

GSK143 is a potent and highly selective Syk inhibitor with efficacy in a range of inflammatory models.(16) The aim of this study was to investigate the effect of GSK143 in preventing manipulation-induced intestinal inflammation thereby shortening POI.
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Materials and Methods

Animals
Laboratory animals were kept under environmentally controlled conditions (light on from 8:00 AM to 8:00 PM with standard mouse chow and water *ad libitum*; 20°C–22°C, 55% humidity). Ten to twelve weeks old wild type C57NL/BL6 mice were purchased from Charles River Laboratories (Maastricht, The Netherlands). Studies were performed according to the guidelines of the Dutch Central Committee for Animal Experiments. All experiments were approved by the Animal Care and Use Committee of the University of Amsterdam (Amsterdam, The Netherlands) and the Animal Experiments Committee of the Medical Faculty of the Catholic University of Leuven (Leuven, Belgium).

Primary culture of peritoneal mast cells
Peritoneal cells were harvested by abdominal lavage with phosphate buffered saline (PBS) in C57NL/BL6 mice. After centrifugation, peritoneal cells were resuspended in peritoneal mast cell (PMC) culture medium (RPMI 1640 (GIBCO) containing 10% fetal calf serum, 1% penicillin/streptomycin and 6% bone marrow mast cell supplement (containing 20% MEM non-essential amino acids, 1% L-glutamine, 0.22% Sodium Pyruvate, 0.005% β-mercapto-ethanol). Stem cell factor (SCF) 100 ng/mL was added to achieve enrichment of PMC. Cells were incubated in a 5% CO₂ humidified atmosphere at 37°C in 75 cm² tissue culture flasks for a minimum of 3 weeks. PMC cultured for 4-7 weeks were used for the in vitro experiments. FcεRI and CD117 expression was assessed by direct immunofluorescence. Cells were harvested and washed using ice-cold staining buffer (PBS supplemented with 0.5% BSA, 0.3mM EDTA and 0.01% NaN₃). Next, cells were incubated at 4°C with FITC-labeled anti-mouse CD117 (c-kit; 1:150) and PE labelled FcεRI-α (1:800) or corresponding isotype control and subsequently washed with staining buffer. Fluorescence was analyzed by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA) equipped with CellQuest software.

Activation of peritoneal mast cells
Cells were collected and centrifuged for 5 minutes at 370 g and resuspended in Tyrode’s buffer supplemented with 0.1% BSA at a density of 1 x 10⁶ cells/mL. Cells (50 μL/well; 5x10⁴ cells/well) were seeded in 96-well plates and activated by SP or TNP-Ovalbumin. To this end, cells were incubated with Tyrode’s buffer at 37 °C for 30 minutes and challenged with Tyrode’s buffer (control), SP (0-90 μM) or TNP-Ovalbumin (0-4 μg/mL) at 37°C for 10 or 30 minutes. Cells challenged with TNP-Ovalbumin were incubated overnight with mouse IgE anti-TNP (0.5 μg/μL) in PMC culture medium at a density of 2x10⁶ cells/mL. To stop this reaction, plates were centrifuged at 390 g at 4 °C for 5
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minutes and supernatant was collected. In a separate series of experiments, the effect of GSK143 and doxantrazole on SP and IgE crosslinking (TNP-Ovalbumin induced) mediated mast cell activation was evaluated. Cells were pretreated with 10 μL Tyrode’s buffer (placebo), GSK143 (0.03-10 μM) or the classic mast cell stabilizer doxantrazole (227 μM) at 37°C for 30 minutes. Subsequently, cells were activated with 50 μL SP (90 μM) or TNP (40 ng/mL) at 37°C for 10 minutes.

Primary Bone Marrow-Derived Macrophages
Bone marrow cells were isolated from C57NL/BL6 mice. One hundred and fifty thousand bone marrow cells were plated in 10 cm plates in 2 ml of BM-medium (DMEM supplemented with 20% low-endotoxin fetal bovine serum, 30% L929-cell conditioned medium, 1% L-glutamine, 1% Pen/Strep, 0.5% Na Pyruvate, 0.1% β-mercaptoethanol) and cultured for 10 days (17). At day 10, macrophages were incubated with different concentration of GSK143 or with doxantrazole as shown in figure 4 for 15 minutes before stimulation with LPS (100ng/ml, E. coli 055:B5) for 2 h. To assess purity at day 10, cells were harvested and washed using ice-cold staining buffer (PBS supplemented with 0.5% BSA, 0.3mM EDTA and 0.01% NaN3). Next, cells were incubated at 4 °C with Pe-Cy7-labeled anti-mouse CD11b (1:200) and APC labelled F4/80 (1:100) or corresponding isotype control for 40 minutes and subsequently washed with staining buffer. Fluorescence was analyzed by flow cytometry using a FACSCanto (BD Biosciences, San Jose, CA). Analysis was performed using FlowJo (version 4.6.2, Treestar).

Measurement of degranulation of peritoneal mast cells
To quantify mast cell activation, we measured the release of β-hexosaminidase in the supernatant. Supernatant was incubated during 2 h with a 4-methylumbelliferyl glucosaminide (4-MUG) substrate solution (3.79 mg MUG/mL DMSO) in 0.1 M citrate buffer (pH 4.5) at 37°C in a 5% CO₂ humidified atmosphere. The reaction was stopped by adding 0.2 M glycine buffer (pH 10.7). Fluorescence was measured using a multiwell plate reader at an emission wavelength (λ) of 360 nm and excitation wavelength (λ) of 460 nm. The percentage of degranulation was calculated as follows: ((a – b)/(t – b)) x 100, where a is the amount of β-hexosaminidase released from stimulated cells, b is the amount released from unstimulated cells (basal release by cells incubated with Tyrode’s buffer only), and t is total cellular β-hexosaminidase cellular content, determined by total lysis of cells by 1% Triton X-100.

Reagents
RPMI 1640 (containing 10% fetal calf serum, 1% penicillin/streptomycin and 6% bone marrow mast cell supplement (containing 20% MEM non-essential amino acids, 1% L-
glutamine, 0.22% Sodium Pyruvate, 0.005% β-mercapto-ethanol)) was purchased from Gibco. Tyrode’s buffer (5.6 mM glucose, 10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 1 mM CaCl₂, 0.4 mM Na₂HPO₄, pH 7.2) supplemented with bovine serum albumin (BSA) was used as stimulation medium. Recombinant Mouse SCF/C-KIT Ligand (1.0 mg Recombinant Mouse SCF in 1.8 mL 40 mM Tris Buffer) was purchased from Invitrogen. Doxantrazole (a kind gift of Agne’s François, Institut Gustave Roussy, Villejuif, France) was dissolved in dimethyl sulphoxide (DMSO). GSK143 (kindly provided by GlaxoSmithKline, Gunnels Wood Road, Stevenage, United Kingdom) was dissolved in DMSO at 10 mM and serial dilutions were prepared in DMSO. The final concentration of DMSO in cell suspensions was 0.5% and did not evoke more β-hexosaminidase release compared to basal release by cells incubated with Tyrode’s buffer only. Mouse IgE anti-TNP was obtained from BD Pharmingen. SP was purchased from Sigma-Aldrich and TNP-Ovalbumin from Biosearch Technologies. Lipopolysaccharide from Escherichia coli 055:B5 was obtained from Sigma-Aldrich.

Surgical procedure
Mice were anesthetized by intraperitoneal (i.p.) injection of a mixture of Ketamine (Ketalar 100 mg/kg) and Xylazine (Rompun 10 mg/kg). Anesthetized mice underwent a laparotomy alone or a laparotomy followed by small intestinal manipulation (IM). (18) Surgery was performed under sterile conditions and performed as follows: a midline abdominal incision was made, and the peritoneum was opened over the linea alba. The small bowel was carefully externalized and layered on a moist gauze pad. Contact with or stretch on stomach or colon was strictly avoided. The small intestine was manipulated from the cecum to the distal duodenum and back for a total of three times, using a sterile moist cotton applicator attached to a device enabling the application of a constant pressure with 9 grams of weight. After the surgical procedure, the abdomen was closed by a continuous 2-layer suture (Mersilene, 6-0 silk). After closure, mice were allowed to recover for 3 h in a heated (32°C) recovery cage without administration of analgesic agents as these can interact with intestinal motility and the postoperative inflammatory process.

Drug administration
Mice received doxantrazole (10 mg/kg in 5% NaHCO₃, pH 7.4) 16 h and 1 h before and 1 h after IM by i.p. injection. The other groups of animals received orally GSK143 (0.1 - 10 mg/kg in 0.5% methylcellulose solution in water) or vehicle (0.5% methylcellulose solution in water) also at 3 time points; 1.5 h before, and 1.5 and 6 h after IM (n= 5-8 per group). In another group of mice the effect of a single oral administration of GSK143 (1, 3 or 10 mg/kg in 0.5% methylcellulose solution in water) was given 1.5 h before
intestinal manipulation. The researcher performing the operations was blinded for the type of pharmacological treatment.

Gastrointestinal transit measurements
Gastrointestinal function 24 h postoperatively was determined in vivo by measurement of gastrointestinal transit of liquid non-absorbable fluorescein labeled dextran (FITC-dextran). Ten microliters of FITC-dextran (70,000 Da; Invitrogen, Paisley, UK) dissolved in 0.9% saline (6,25 mg/mL) were administered via oral gavage. Ninety minutes later, animals were killed by cervical dislocation and the entire bowel from stomach to distal colon was collected. The contents of the stomach, small bowel (divided into 10 segments of equal length), the cecum, and colon (3 segments of equal length) were collected and the amount of FITC in each bowel segment was determined in duplicate using a fluorimeter (Synergy HT, BioTek Instruments Inc., VT, USA) with an excitation wavelength (λ) of 485 nm and emission wavelength (λ) of 528 nm. The distribution of the fluorescent dextran along the gastrointestinal tract was determined by calculating the geometric center (GC): Σ (percent of total fluorescent signal in each segment × the segment number)/100 for quantitative statistical comparison among experimental groups.(19)

Whole mount preparation and histochemistry
To quantify the degree of inflammation in whole mounts of the intestinal muscularis, segment number 7 of the small bowel was cut open, fecal content was washed, and segments were fixed with 100% ethanol for 10 minutes, transferred to ice cold modified Krebs solution and pinned flat in a glass-dish. Mucosa and submucosa were removed and the remaining full-thickness sheets of muscularis externa were stained for polymorphonuclear neutrophils with Hanker Yates reagent (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 10 minutes. To quantify the extent of intestinal muscle inflammation, the number of myeloperoxidase (MPO) positive cells in 10 randomly chosen representative high-power fields (HPF, 668.4 µm x 891.2 µm) was counted and the average was calculated. Tissue sections were coded so that the researchers were unaware of the surgical and pharmacological treatment of the specimens when the number of MPO positive cells was determined.

Cytokine measurements
For cytokine measurements, 2 jejunal muscularis segments (segments 4 and 5) were added to 500 μL lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl₂, 2 mM CaCl₂, 1% Triton X-100, pepstatin A, leupeptin and aprotinin (all 20 ng/mL; pH 7.4), homogenized, and incubated at 4°C for 30 minutes. Homogenates were centrifuged at
1500 g at 4°C for 15 minutes and supernatants were stored at -20°C until assays were performed. Ccl2, IL-6, IL-1β and TNF-α in supernatants were analyzed by mouse ELISA (R&D Systems, Abingdon, England) according to the manufacturer’s instructions.

**RNA extraction and inflammatory gene expression**

Total RNA was extracted from bone marrow derived macrophages stimulated with LPS (100ng/ml, *E. coli* 055:B5) for 2 h. RNA extraction was performed using RNeasy Mini Kit (Qiagen) following the manufacturer’s instructions. Total RNA was transcribed into complementary cDNA by qScript cDNA SuperMix (Quanta Biosciences) according to the manufacturer’s instructions. Quantitative real-time transcription polymerase chain reactions (RT-PCR) were performed with the LightCycler 480 SYBR Green I Master (Roche) on the Light Cycler 480, (Roche). Results were quantified using the 2-ΔΔCT method (20). The expression levels of the genes of interest were normalized to the expression levels of the reference gene *rpl32*. PCR experiments were performed in triplicate. Primer sequences used are listed in supplementary table 1.

**Cell isolation from the intestinal muscularis for flow cytometry**

24 h after the surgery, muscularis externa from the small intestine was isolated and enzymatically digested in MEMα medium (Lonza) containing 100 µg/ml of Penicillin, 100 µg/ml of Streptomycin, 50 µM beta-mercaptoethanol, 5% FCS, 5mg/ml protease type I (Sigma-Aldrich), 20 mg/ml collagenase type II (Sigma-Aldrich) and 5U/ml DNase I for 15 minutes at 37 °C. Cell suspensions were filtered through a nylon mesh. Before staining, cells were pre-incubated with an anti-FcR antibody (clone 24G2; BD Biosciences). Cells were then stained with the following antibodies: CD45.2-FITC (104, BD Biosciences), CD11b-PeCy7 (M1/70, BD Biosciences), F4/80-APC (BM8, eBioscience), Ly6G-PercPCy5.5 (IA8, BD Biosciences), Ly6C-PE (AL-21, BD Biosciences) and analyzed by flow cytometry using a FACSCanto (BD Biosciences). Analysis was performed using FlowJo (version 4.6.2, Treestar). Immune cells were gated for their surface expression of CD45.2. Subsequently monocytes were identified for their surface expression of CD45.2, CD11b, F4/80 and Ly6C, while neutrophils were identified as CD45.2, CD11b and Ly6G positive but F4/80 negative cells (21).

**Blood quantification of GSK143**

GSK143 blood concentration levels were assessed in mice treated with a single oral dose of GSK143 (prepared in 0.5% Methylcellulose) at 1, 3 and 10 mg/kg or vehicle alone. Blood samples were taken at 1.5 h post dose (prior to surgery) and at 6 h and 25.5 h post dose. The blood concentration levels were determined by reverse phase liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) at the laboratories.
of the Quantitative Pharmacology group at GlaxoSmithKline, Stevenage, United Kingdom. In brief, 40 µL of the diluted samples (blood:water, 1:1, v/v) were assayed against matrix matched calibration standards prepared over the range 1 to 2000 ng/mL. Samples and calibration standards were extracted by protein precipitation with 200 µL of acetonitrile containing 50 ng/mL of a GSK proprietary internal standard and centrifuged at 3600 g. All prepared samples and standards were analyses on a LC gradient using a Phenomenex Kinetex C18, 2.6 µm, 50 x 2.1 mm column. GSK143 concentration data was generated and processed using Analyst 1.4.2 software.

Statistical analysis
The data on muscularis cytokine production were not normally distributed. The Kruskal–Wallis test was performed to assess whether the cohort of data was statistically different. When variance of medians was statistically significant, the Mann–Whitney U test was used to identify the statistical differences within the cohort. All other data were statistically analyzed by use of one-way analysis of variance (ANOVA) followed by Dunnett’s Multiple Comparison test (for in vitro data) or Bonferroni’s multiple comparison test (for in vivo data). A probability level of P less than 0.05 was considered statistically significant and results are shown as means ± SEM. Graph Pad Prism version 5.01 software was used to perform statistical analysis and create graphs.
Results

GSK143 prevented manipulation-induced influx of leukocytes and improved postoperative gastrointestinal transit

Intestinal manipulation (IM) resulted in an impairment of gastrointestinal transit shown by a reduction in the geometrical center (GC) (laparotomy + placebo GC = 9.5 ± 0.3, IM + placebo GC = 4.4 ± 0.3, P < 0.05, n= 5 and 7 respectively). Treatment with doxantrazole (10mg/kg) or GSK143 (0.1-10mg/kg) did not affect intestinal transit in mice that underwent laparotomy (supplementary fig. 1). In contrast, IM-induced delay in intestinal transit was significantly prevented in mice treated with three doses of doxantrazole (10 mg/kg) (fig. 1: GC: 7.2 ± 0.7, n=6) or GSK143 (1–3 mg/kg) (1 mg/kg): 7.6 ± 0.6, n=8; 3 mg/kg 7.3 ± 0.5, n=8). On the contrary GSK143 three doses of 10 mg/kg did not significantly protect against the development of POI (GC: 6.1 ± 0.6; GSK143: n = 9). In the same line, a single dose of 3 mg/kg of GSK143 administered 1.5 h before surgery was able to significantly improve intestinal transit (GC: 6.83 ± 0.6, n = 8) and to reduce MPO-positive cell recruitment in the muscularis externa 24 h after IM (supplementary fig. 2).

![Fig. 1](image)

Fig. 1 | GSK143 prevented manipulation-induced delayed gastrointestinal transit. (A) Effect of three times administration of placebo (vehicle of GSK143; black bar), GSK143 (0.1 mg/kg - 10 mg/kg; grey bars) or DOX (10 mg/kg; diagonally striped bar) on gastrointestinal transit 24 h after intestinal manipulation. Gastrointestinal transit was determined by the calculation of the geometric center (GC). The GC was significantly increased in the doxantrazole treated group, and GSK143 treated mice at a concentration of 1 and 3 mg/kg. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test; * P <0.05 for GSK143 or DOX versus Placebo; **P < 0.001 for GSK143 versus Placebo. Data are expressed as mean ± SEM. Placebo: n = 7, GSK143: n = 5-8 per group, DOX: n = 6.
Intestinal manipulation, but not laparotomy resulted in an influx of MPO-positive inflammatory cells in the muscle layer of the small intestine 24 h after surgery (fig. 2: 257 ± 45 MPO+ cells/HPF). As shown in figure 2, both doxantrazole (10 mg/kg) and GSK143 (1-10 mg/kg) significantly reduced the influx of MPO positive cells in a dose dependent manner.

Fig. 2 | GSK143 reduced manipulation-induced recruitment of myeloperoxidase positive cells in the muscularis externa. (A) Representative images of myeloperoxidase (MPO) positive cells recruited in the muscularis externa of mice 24 h after intestinal manipulation for the different experimental groups. Effect of three times administration of placebo, GSK143 (0.1 mg/kg - 10 mg/kg) or DOX (10 mg/kg) on the number of MPO positive cells recruited in the muscularis externa of mice 24 h after intestinal manipulation. Scale bar is 100 μm. (B) Effect of three times administration of placebo, GSK143 (0.1 mg/kg - 10 mg/kg) or DOX (10 mg/kg) on the number of MPO positive cells recruited in the muscularis externa of mice 24 h after intestinal manipulation. Scale bar is 100 μm. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test; * P < 0.05, ***P < 0.001 for GSK143 or DOX versus Placebo. Dots represent individual mice.
GSK143 reduced manipulation-induced cytokine production in the intestinal muscularis

Intestinal manipulation of the small intestine markedly increased IL-1β, CCL-2 and IL-6 protein expression, but not that of TNF-α, in the intestinal muscularis compared to laparotomy alone (data not shown). Interestingly, GSK143 (1 mg/kg) administered three times significantly reduced the levels of IL-1β and Ccl2 compared to placebo treated mice. IL-6 production was reduced by both treatments but this effect was not statistically significant (fig. 3A).

As Syk inhibition resulted in a reduced number of MPO-positive cells and a lower amount of cytokine secretion in the muscularis layer of manipulated mice, we addressed whether GSK143 may also affect recruitment of specific subsets of inflammatory cells. As shown in figure 3B, treatment with GSK143 (1mg/kg) administered three times resulted in a significant reduction of immune cell recruitment in the muscularis externa. Syk inhibition affected recruitment of both neutrophils and monocytes suggesting a potent and broad anti-inflammatory effect of this treatment (fig. 3B).
Fig. 3 | GSK143 reduced manipulation-induced production of inflammatory cytokines and recruitment of immune cells in the muscularis externa. (A) Cytokine production in the muscle layer of the small intestine 24 h after intestinal manipulation and treatment three times with placebo (black bars), GSK143 (1 mg/kg; grey bars) or doxantrazole (DOX 10 mg/kg; diagonally striped bars). IL-1β and CCL-2 levels were significantly ameliorated by GSK143. The Kruskal-Wallis test was performed to identify statistical differences between the groups. The Mann-Whitney U test was used to compare Placebo versus GSK143 and Placebo versus DOX. Data are expressed as mean ± SEM. * P < 0.05 (IL-1β: P = 0.0140; CCL-2: P = 0.0401), GSK143 versus Placebo. Placebo: n = 7, GSK143: n = 8 per group, DOX: n = 6.

(B) Immune cell recruitment in the muscle layer of the small intestine 24 h after intestinal manipulation and treatment three times with placebo or GSK143 (1 mg/kg). Cells were isolated from enzymatically digested intestinal muscularis and assessed by flow cytometry. Absolute number of CD45 positive immune cells, Ly6G & CD11b positive neutrophils and F4/80 & CD11b positive monocytes were significantly reduced by GSK143 treatment. The Mann-Whitney U test was used to compare Placebo versus GSK143. * P < 0.05 and ** P < 0.01, GSK143 versus Placebo. Dots represent individual mice.

GSK143 inhibited SP and TNP induced peritoneal mast cell degranulation and LPS-induced activation in macrophages

To define the concentration range of GSK143 to be tested in vitro, blood samples were collected from mice treated with a single dose of 1, 3 or 10 mg/kg GSK143 (Supplementary figure 3). Based on these data, the effect of GSK143 in the concentration range of 0.03-10 uM GSK143 on isolated mast cells and macrophages was further studied. Freshly isolated peritoneal cells from C57NL/BL6 mice were cultured for 4-7 weeks yielding a > 94% (FcεRI+, CD117+) pure PMC population resulting from an expansion of differentiated PMC in the presence of 100 ng/mL SCF (supplementary fig. 4A). Basal release of β-hexosaminidase by PMC incubated without stimulus (control) was below 7% of total cellular β-hexosaminidase content. Stimulation with SP (0-90 μM) or TNP (0-4 μg/mL) for 10 minutes resulted in a concentration-dependent response of β-hexosaminidase release with maximal release 54.7 ± 2.6% and 92.6 ± 2.2% of total cellular β-hexosaminidase content for SP and TNP respectively (supplementary fig. 5). Stimulation with SP or TNP for 30 minutes showed similar results (supplementary fig. 5).

Subsequently, the effect of GSK143, vehicle or doxantrazole was studied on PMC stimulated with 90 μM SP or 0.04 μg/mL TNP. Pretreatment with doxantrazole (227 μM) significantly reduced SP induced β-hexosaminidase release from 43.5 ± 1.0% of total cellular content to 2.9 ± 0.2%. β-hexosaminidase release by SP stimulated PMC was also inhibited significantly by ≥ 0.3 μM GSK143, but to a lesser extent than TNP-induced mast cell activation. (fig. 4A). The TNP induced β-hexosaminidase release was 70.3 ± 4.3% of total cellular content. Pretreatment with doxantrazole (227 μM) and GSK143 (≥ 0.3 μM) significantly reduced this release to a maximum of 2.8 and 8.7% respectively (fig. 4B).
Cultured bone marrow derived macrophages (purity > 95% (CD11b, F4/80; supplementary fig. 4B) from C57NL/BL6 mice were pretreated with GSK143 (0.1-10 μM) or doxantrazole (227 μM) for 30 minutes prior LPS stimulation (100 ng/ml). Two hours after stimulation, macrophages were harvested and cytokine expression was assessed by quantitative PCR. GSK143 significantly reduced expression of cytokines such as IL-6, TNF-a, IL-1β and CCL2 (fig. 5). Interestingly, pretreatment with doxantrazole (227 μM) did not affect macrophage activation.

**Fig. 4** | GSK143 inhibited substance P (SP) and TNP induced degranulation in a concentration dependent manner. Mast cells were incubated with Tyrode’s buffer (placebo, black bars), GSK143 (0.03 μM-10 μM; grey bars) or doxantrazole (DOX (227 μM); diagonally striped bar), followed by stimulation for 10 minutes with (A) 90 μM SP or (B) 0.04 μg/mL TNP. * P < 0.05, ** P < 0.01, *** P < 0.001 compared to vehicle, one-way analysis of variance (ANOVA) followed by Dunnett’s Multiple Comparison Test. Data are expressed as mean ± SEM of at least 3 independent experiments.
Fig. 5 | GSK143 reduced cytokine expression in macrophages in a concentration dependent manner. Bone marrow derived macrophages were incubated in cultured medium alone (placebo, black bar) or with GSK143 (0.1 μM-10 μM; grey bars) or doxantrazole (DOX (227 μM, diagonally striped bars). Thirty minutes later macrophages were stimulated for 2 h with LPS (100 ng/ml) and cells were harvested for mRNA expression analysis of IL-6 (A), TNF-α (B), IL-1β (C), and CCL-2 (D). *** P < 0.001 GSK143 compared to vehicle, one-way analysis of variance (ANOVA) followed by Dunnett’s Multiple Comparison Test. Data are expressed as mean ± SEM of at least 3 independent experiments.

Discussion

Postoperative ileus is mediated by intestinal inflammation resulting from manipulation-induced mast cell and macrophage activation. In the present study, we investigated the anti-inflammatory effect of a new Syk inhibitor and its ability to reduce POI. The Syk inhibitor GSK143 significantly reduced the inflammatory response to intestinal manipulation thereby preventing POI. In addition, we demonstrated that GSK143 inhibited both FcεRI and SP mediated degranulation of PMC and endotoxin-
induced macrophage activation. Taken together, these data strongly suggest that Syk inhibition may represent a new therapeutic approach for POI.

Syk is required for FcεRI signalling in mast cells and activates intracellular signaling cascades involved in the transcription and translation of inflammatory mediators.(22;23) Syk inhibitors suppress the signaling cascades that normally lead to degranulation of mast cells.(11;22-24) In animal models Syk inhibitors have successfully prevented mast cell mediated inflammatory diseases such as rheumatoid arthritis and allergic rhinitis. (25;26) Moreover, Syk has been reported to play a crucial role in macrophage activation.(13-15) As both mast cells and macrophages have been implicated in the pathophysiology of POI, the present study was designed to establish whether blockade of intracellular Syk could represent an alternative approach to inhibit immune cell activation evoked by intestinal manipulation and thus represent a new tool to shorten POI.

At first, we evaluated the effect of GSK143 on primary cultured PMC. We have chosen to study this subset of mast cells as they functionally resemble connective tissue mast cells,(27) the subpopulation of mast cells most likely involved in POI.(1;27) In addition to IgE crosslinking, mast cells can also be activated by SP released by visceral afferent nerves with subsequent activation of inflammatory cells, a mechanism referred to as neurogenic inflammation.(5;27;27;28) In our in vitro experiments, we indeed showed concentration-dependent activation of PMC by SP and IgE crosslinking. In addition, we demonstrated that GSK143 significantly blocked the FcεRI and SP mediated degranulation of PMC. Of note, the concentrations of GSK143 inhibiting the activation of mast cells and macrophages in vitro were in the range of serum levels obtained following single administration of the compound. Interestingly, the inhibitory effect of GSK143 was more potent for β-hexosaminidase release induced by IgE crosslinking compared with SP. This finding suggests that the signaling pathways involved in SP-induced mast cell activation are less dependent on spleen tyrosine phosphorylation compared to the FcεRI induced degranulation. Several lines of evidence indicate that SP can stimulate mast cells not only via its NK1 receptor, but also by NK1 receptor-independent pathways. Notably, at high concentrations SP induces mast cell degranulation by receptor-independent pathways,(29) which is mediated by G protein(s), protein kinase C, calcium, and phospholipase C.(29-35) These data would suggest that in addition to NK1 receptor mediated activation of Syk, other mechanisms may be involved explaining why GSK143 markedly inhibited FcεRI mediated and, to a lesser degree at higher drug concentrations, SP mediated degranulation. Alternatively, if SP-induced mast cell activation does not involve Syk, our data would indicate that GSK143 interacts with different intracellular signaling pathways. Potential mechanism could be competitive inhibition at the SP receptor level by membrane incorporation of
GSK143 resulting in the competition of SP binding to the cell membrane surface proteoglycans.\(^{29;36;37}\) Secondly, GSK143 may interfere with intracellular inositol triphosphate and subsequent increase intracellular calcium ions ultimately inhibiting degranulation.\(^{38;39}\) An in-depth analysis of these signaling pathways was beyond the scope of this article, and further study is needed to fully understand the mechanisms underlying Syk-inhibition of SP induced mast cell degranulation.

Syk inhibition has been proposed as an important therapeutic strategy for the treatment of mast cell mediated upper airway diseases, such as allergic rhinitis.\(^{40}\) Furthermore, Weinblatt et al. detected mast cells and Syk expression in the synovium of rheumatoid arthritis patients and demonstrated a significant clinical improvement after treatment with an oral Syk inhibitor.\(^{41}\) As a consequence, inhibition of Syk has received increasing attention as new therapeutic approach for a variety of disorders. Previously, we have shown in mice that mast cells are important players in triggering the local intestinal inflammatory response leading to POI.\(^{42-44}\) In patients undergoing surgery, even gentle inspection of the intestine at the very beginning of the surgical procedure triggers the release of mast cell mediators.\(^{43}\) In accordance with these data, recent in vitro work demonstrated that Syk-deficient mast cells fail to release mast cell mediators.\(^{10}\) In the study reported here, GSK143 significantly reduced the upregulation of pro-inflammatory cytokines in the intestinal muscularis 24 h after intestinal manipulation. Moreover, GSK143 and the mast cell stabilizer doxantrazole attenuated the leukocytic influx and in addition improved gastrointestinal transit. This confirms the beneficial effect of Syk inhibition on the postoperative inflammatory phase within the intestine.\(^{24}\)

Syk has been recently reported to also represent a key player in the regulation of the NF-kB pathway in LPS-treated macrophages. Indeed, inhibition of Syk signalling in rat alveolar as well as in peritoneal macrophages and in the monocytic cell line THP-1 resulted in reduction of pro-inflammatory cytokine secretion.\(^{14}\) In the present study, we evaluated the effect of GSK143 on cultured macrophages treated with endotoxin. In line with previous reports, we showed that GSK143 reduced the expression of IL-6, TNF-\(\alpha\), IL-1\(\beta\) and CCL2 in the intestinal muscularis from intestinal manipulated mice. As macrophages are key players in orchestrating the leukocytic influx in the manipulated intestine (12), these data suggest that interaction of GSK143 with macrophages in the intestinal muscularis also contributes to the beneficial effect of GSK143 reducing muscular inflammation. Similar findings were previously reported by Moore et al. as treatment of mice with the inhibitor of protein tyrosine kinase tyrphostin reduced inflammatory influx and upregulation of pro-inflammatory cytokines, and significantly inhibited activation of NF-kB.\(^{3}\) Taken together, our in vitro data indicate that GSK143 may inhibit at the same time mast cells and macrophages exerting a broad anti-
inflammatory effect in vivo. It should be emphasized though that Syk also affects the adaptive immune system. (45) Hence, we cannot exclude that the beneficial effects of GSK143 on POI reported here may extend beyond its interaction with mast cells and macrophages. (24)

To date, the exact triggers activating mast cells and macrophages during abdominal surgery are still unclear but besides neuropeptides (such as SP, VIP and CGRP) and specific antigens (via IgE crosslinking) (28) a variety of stimuli, including bacterial components and several physical stimuli could be involved. Physical stimuli may be perioperative temperature changes, or the inevitable surgical induced tissue damage that may activate mast cells via the local release of Damage Associated Molecular Pattern Molecules, IL-1, reactive oxygen species and complement fragments such as C3a and C5a. (46) Interestingly, Pamuk et al. recently investigated the ability of a Syk inhibitor to protect mice against mesenteric ischemia-reperfusion induced injury and found that both local and remote lung injury were reduced with a significant reduction of leukocytic infiltration, (47) suggesting the use of Syk inhibitors in the suppression of tissue damage evoked inflammatory response. The effect of GSK143 on resident macrophages or peritoneal mast cells should be further evaluated by studying the levels of Syk phosphorylation. Nonetheless, our data indicate that GSK143 inhibits mast cell and macrophage activation, and consequently prevents the inflammatory cascade leading to POI. (12; 42-44)

Clinical studies have shown that Syk inhibitors are efficient in allergy, (48) immune thrombocytopenic purpura, (49) B-cell lineage malignancies and autoimmune diseases like rheumatoid arthritis. (41; 50) Importantly, these compounds were mostly well tolerated, (48) and not associated with serious side effects. (41; 47; 49-51) Syk inhibition in patients with rheumatoid arthritis for 6 months was reported to be associated with the development of elevated blood pressure, mild neutropenia and gastrointestinal adverse effects such as gastritis and nausea. (41) Although these side effects may develop after prolonged use of Syk inhibitors, it is suspected that even milder or no side effects will develop in postoperative patients who require shorter treatment. This is in line with our experimental model where one single dose of GSK143 prior surgery was able to prevent delay in gastrointestinal transit and to significantly reduce intestinal inflammation.

In conclusion, our study showed that GSK143 inhibited degranulation of peritoneal mast cells and reduced the expression of inflammatory cytokines by macrophages. In addition, Syk inhibition improved intestinal inflammation and intestinal transit, suggesting that GSK143 can be a useful tool to treat POI.
Supplementary Materials

**Supplementary fig. 1**: Intestinal transit is not affected by GSK143 and doxantrazole treatment in mice that underwent laparotomy.

A. Effect of three times administration of placebo (black bar), GSK143 (0.1 mg/kg - 10 mg/kg; grey bars) or DOX (10 mg/kg; diagonally striped bar) on gastrointestinal transit 24 h after laparotomy. Gastrointestinal transit was determined by the calculation of the geometric center (GC). The GC was not significantly altered in doxantrazole and GSK143 treated mice. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test. Data are expressed as mean ± SEM. Placebo: n = 5, GSK143: n = 5 per group, DOX: n = 5.
Supplementary fig. 2: Single dose of GSK143 prevented manipulation-induced delayed gastrointestinal transit and recruitment of inflammatory cells. (A) Single dose treatment of GSK143 (1-10 mg/kg; grey bars) or placebo 1.5 h before surgery on gastrointestinal transit 24 h after IM. Gastrointestinal transit was determined by the calculation of the geometric center (GC). The GC was significantly increased in the GSK143 treated mice at a concentration of 3 mg/kg. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test; **P < 0.001 for GSK143 versus Placebo. Data are expressed as mean ± SEM. Placebo: n = 7, GSK143: n = 7 per group. (B) Effect of placebo, or single dose of GSK143 (1 mg/kg - 10 mg/kg) on the number of MPO positive cells recruited in the muscularis externa of mice 24 h after intestinal manipulation. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test; * P < 0.05, **P < 0.01 for GSK143 versus Placebo. Dots represent individual mice.
Supplementary fig. 3: Blood concentration levels of GSK143 after single oral dose treatment. GSK143 blood concentration levels were assessed in mice treated with a single oral dose of GSK143 (prepared in 0.5 % Methylcellulose) at 1, 3 and 10 mg/kg or vehicle alone. Blood samples were taken at 1.5 h (prior to surgery) and at 6 h and 25.5 h post dose. The blood concentration levels were determined by reverse phase liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). (A) Graph presenting the blood concentration levels obtained after a single oral dose of GSK143. (B) Data (mean ± sd) from each group of animals surgically treated during this study at 1.5, 6 and 25.5 h post-dose.
Supplementary fig. 4: FACS plot of cultured mouse peritoneal mast cells and bone marrow derived macrophages. (A) FcεRI and CD117 expression was assessed by flow cytometry in cultured mouse peritoneal mast cells. Only more than 94% pure PMC (FcεRI+, CD117+) populations were used for the experiments. Data are representative of 3 experiments. (B) CD11b and F4/80 expression was assessed by flow cytometry mouse bone marrow derived macrophages (BMDMs) after 10 days of culture. Only more than 94% pure BMDMs (CD11b+, F4/80+) populations were used for the experiments. Data are representative of 3 independent experiments.
Supplementary fig. 5: Substance P and TNP induced concentration-dependent peritoneal mast cell degranulation. Mast cells were incubated with (A, C) substance P; 0-90 μM and (B, D) trinitrophenyl (TNP); 0-4 μg/mL. The β-hexosaminidase release was significantly increased by SP at a concentration of ≥ 30 μM and by TNP at a concentration of ≥ 0.004 μg/mL. Cells incubated with vehicle (Control) reflect the basal release. *** P < 0.001 compared to control, one-way analysis of variance (ANOVA) followed by Dunnett’s Multiple Comparison Test. Data are expressed as mean ± SEM of at least 3 independent experiments.

Supplementary table S1. Primer sequences used for qRT-PCR.

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References

Chapter 5 | Inhibition of Syk

Chapter 6

Neuroanatomical evidence demonstrating the existence of the vagal anti-inflammatory reflex in the intestine


*Neurogastroenterology and Motility. 2012 Feb.*
Abstract

**Background:** The cholinergic anti-inflammatory pathway is proposed to be part of the so-called vago-vagal “inflammatory reflex”. The aim of this study is to provide neuro-anatomical evidence to support the existence of a functional neuronal circuit and its activation in response to intestinal inflammation.

**Methods:** The expression of c-Fos was evaluated at different levels of the neurocircuitry in the course of postoperative ileus (POI) in a mouse model. Specific activation of the motor neurons innervating the inflamed intestine and the spleen was monitored by retrograde tracing using cholera toxin-b. The role of the vagal afferent pathway nerve was evaluated by selective vagal denervation of the intestine.

**Results:** Abdominal surgery resulted in subtle inflammation of the manipulated intestine at 24hrs (late phase), but not after 2 and 6 hrs (early) after surgery. This local inflammation was associated with activation of neurons in the nucleus of the solitary tract and in the dorsal nucleus of the vagus. The vagal output mainly targeted the inflamed zone: 42% of motor neurons innervating the intestine expressed cFos IR in contrast to 7% of those innervating the spleen. Vagal denervation of the intestine abolished cFos expression in the brain nuclei involved in the neuronal network activated by intestinal inflammation.

**Conclusions:** Our data demonstrate that intestinal inflammation triggers a vagally mediated circuit leading mainly to activation of vagal motor neurons connected to the inflamed intestine. These findings for the first time provide neuro-anatomical evidence for the existence of the endogenous “inflammatory reflex” and its activation during inflammation.
Introduction

Vago-vagal reflexes coordinate gastric and intestinal digestive functions and motility. These functions are controlled through functionally distinct pathways, called neurocircuitries, connecting the intestine with the brain stem nuclei, i.e., the nucleus of the solitary tract (NTS) and the dorsal motor nucleus of the vagus (DMV). Mechanical (i.e., contraction, distention) and chemical (i.e., nutrients) signals are transmitted to the NTS via the vagal ascending fibers. After integration of the incoming information and through neuronal communication between the NTS and the DMV, vagal efferent output is triggered to adjust gastrointestinal (GI) functions such as secretion, absorption and motility.

Recently, it was suggested that also the immune system is modulated by the vagus nerve. Tracey and coworkers demonstrated that electrical stimulation of the vagus nerve prevents the development of endotoxin-induced shock by reduction of pro-inflammatory cytokine production, in particular TNF. This anti-inflammatory effect is mediated by activation of nicotinic receptors located on immune cells (in particular macrophages) in response to acetylcholine released by vagal efferent nerve terminals. The discovery of the cholinergic anti-inflammatory vagal efferent pathway introduced the concept of the “inflammatory reflex” by which the central nervous system is capable of sensing inflammation and subsequently modulating the immune response. To date, however no anatomical evidence has been reported supporting the existence of this neurocircuit.

Recently, we extended the anti-inflammatory role of the vagal efferent route in a confined/subtle model of inflammation, i.e. post-operative ileus (POI). POI is characterized by a generalized hypomotility of the GI tract, and occurs after almost every abdominal surgical procedure. The prolonged paralytic phase of POI is mediated by an inflammatory response in the muscular layer of the intestine triggered by activation of resident macrophages following intestinal handling. Previously, we demonstrated that electrical stimulation of the vagus nerve inhibits the production of pro-inflammatory cytokines, reduces intestinal inflammation and shortens POI. This effect was blocked by incubation of the intestine with the nicotinic receptor antagonist hexamethonium, indicating vagal modulation of the intestinal immune response within the intestinal wall. To what extent this anti-inflammatory mechanism is also endogenously activated during inflammatory conditions, such as POI, and is part of a vago-vagal neurocircuitry remains however unclear.

The expression of the nucleoprotein Fos, a product of the c-fos immediate-early gene, is widely considered a marker of neuronal activity and has been used repeatedly to map functional brainstem pathways in response to various stimuli. The
distribution of cFos expression induced by abdominal surgery enlightened specific neural circuits including brainstem nuclei (NTS, locus coeruleus, caudal ventral medulla and cuneate, lateral parabrachial nuclei) but also hypothalamic ones such as the paraventricular nucleus PVN. In the current study, we focused on the expression of cFos at different levels of the neuronal pathway proposed to mediate the vagal anti-inflammatory mechanism. Finally, selective vagal denervation of the small intestine was performed to further evaluate the role of the vagus nerve in the modulation of peripheral inflammation.

**Materials and Methods**

**Animals**

Mice (female BALB/c; Harlan Nederland, Horst, The Netherlands) were kept in 12h light/12h dark cycle (lights on at 8:00 AM to 8:00 PM) under constant conditions of temperature (20± 2°C) and humidity (55% humidity) with water and food *ad libitum*. Mice were studied at 10 -12 weeks of age. All experiments were conducted in accordance with the guidelines of the Ethical Animal Research Committee of the Academic Medical Center.

**CTB injection in peripheral organs: small intestine and spleen**

The cholera toxin-B conjugated with Alexa Fluor 555 (1%; CTB-Alexa Fluor 555) (Molecular Probes, USA) was used to label the neuronal innervation of the small intestine and the spleen. 0.5µl of the retrograde tracer was injected (flow of 0.5µl/min) at different spots (5) along the ileum. 3µl of the tracer was injected in both ends of the spleen. The injection was performed with a fused silica tubing (40µm i.d., 105µm o.d.) (Aurora Borealis control, Schoonebeek, The Netherlands) protected at its end by a 30-gauge½ needle. The probe was connected to an injection pump via a guide PEEK tube (PK005-02, Aurora Borealis control). The time period required for a retrograde tracer to reach the brainstem was estimated to be 7 days. However, to avoid possible interference of inflammation triggered by tracer injection, we expanded the period of recovery to 15 days. To validate the specificity of the tracer injection within the small intestine, we performed injections of CTB-fluorophore 555 and 647 in the proximal and distal part of the ileum. Moreover injection of the same volume of the tracer was applied in the peritoneal cavity.

To further validate the specificity of the tracer protocol evaluating the vagal innervation of the spleen, we added an additional control in which tracer was applied on the top of the splenic hilum (i.e., the point of insertion of the splenic artery and vein). Finally, we included a group of mice that underwent vagal denervation of the spleen followed by
CTb injection at the tip of the organ. Loss of CTb positive neurons in the DMV indicated successful (i.e., complete) vagal denervation of the spleen.

Experimental protocols

Protocol 1: Neuronal circuitry and cFos expression at different time points post-surgery

Control animals (no treatment) and animals that underwent laparotomy (L) or intestinal manipulation (IM) were examined at different time points after surgery: L and IM at 2, 6 and 24hrs (n = 24) and controls at 2hrs (n = 12). The controls mice were divided into two groups: one group underwent standard anesthesia (Control group) whereas the other was left untouched to estimate the baseline expression of cFos proteins (Baseline (BL) group). Mice were sacrificed by transcardiac perfusion with Phosphate buffered saline (PBS) followed by 4% paraformaldehyde PFA (pH 7.4). Brains and nodose ganglia were collected, post-fixed for 4hrs (4°C) and cryo-protected by immersion with 30% sucrose in 0.2M PBS (pH 7.4) at 4°C overnight. Intestinal tissue was collected prior to PFA perfusion. Intestinal tissue was cut along the mesentery border, washed in cold saline and directly fixed into 100% ethanol for 30min and then kept in 70% ethanol at 4°C until analysis.

Protocol 2: The spleen as target of the endogenous vagal anti-inflammatory pathway

Intestinal inflammation leads to a consistent cFos expression in the DMV that is not restricted to the small intestine (Figure 3A, B). Recent data identified the spleen as an important player in the cholinergic anti-inflammatory mechanism. Since a direct vagal innervation of the spleen has been recently identified, we decided to investigate whether the vagal motor neurons that innervate directly the spleen express cFos IR upon intestinal inflammation, i.e. 24hrs after IM. Mice (n =12) were injected with CTB in the spleen. Two weeks after recovery, mice underwent either laparotomy (n =5) or IM (n =7). 24hrs later, mice were sacrificed with transcardiac perfusion, brain and intestinal tissue was collected.

Protocol 3: Selective vagal denervation of the small intestine: analysis 24hrs post-surgery

To evaluate the role of the afferent vagal pathway in the activation of the anti-inflammatory efferent, the small intestine was selectively denervated (i.e., disruption of the sensory and motor nerves fibers of the vagus, IntX) and the effect on cFos expression in the brain stem nuclei involved in the “inflammatory reflex” was studied. Four groups were examined: L, IM, L+IntX and IM+IntX. In short, vagal denervation (n = 24) or sham-operation (n = 16) mice received intestinal CTB injection. Two weeks later, mice underwent IM or L and sacrificed 24hrs after surgery with transcardiac perfusion, as described above. Brain and intestinal tissue were collected.
Surgical procedures
Surgical experiments started at 8:30 am. Mice were operated at 11-13 weeks of age after one week of adaptation in the animal facilities. For all surgical procedure (tracer injection, denervation, intact, L or IM), mice were anesthetized by an intraperitoneal (i.p.) injection of a mixture of fentanyl/citrate/fluanisone (Hypnorm; Janssen, Beerse, Belgium) and midozolam (Dormicum; Roche, Mijdrecht, The Netherlands). The anesthetic mixture was in a ratio of 1:1:2 of hypnorm, midazolam and water respectively. Subcutaneous injection of fynadine (0,03ml/100g, from a 10x diluted stock solution, 50mg/ml) was performed after the first surgery (i.e., denervation or injection of the tracer). The same length of the midline incision was performed in all surgical protocols (i.e. sham-operated vs denervated, L vs IM).

CTB injection: In protocols 1 & 3, mice were injected with the neuronal tracer in the small bowel. In brief, the animals were anesthetized and the abdomen was opened by a midline incision. The small bowel was carefully eviscerated and placed on a moist gauze pad to allow CTB injections. In protocol 2, mice underwent CTB injection in the spleen.

Vagal intestinal denervation (IntX): The right celiac branch of the vagus nerve supplies the jejunum, ileum and caecum and is embedded in fat/connective tissue that lies above the superior mesenteric artery. After removal of this fat/connective tissue, the nerve was cut. Vagal denervation was combined with CTB injection to evaluate the success of the denervation procedure. The absence of CTB labeled motor neurons in the DMV is considered indicative for complete vagal denervation of the bowel.

Intestinal manipulation (IM) or laparotomy (L): Mice underwent L or L followed by IM, as previously described. In brief, a midline abdominal incision was performed along the linea alba and the peritoneum was opened. The small bowel was carefully removed from the peritoneal cavity and positioned on a moist gauze pad. The entire small bowel was manipulated from the distal duodenum to the cecum with moist cotton applicators for 5 minutes. Contact or stretch on the stomach or colon was strictly avoided. The surgical procedures were performed under sterile conditions. At the end of the surgery, the abdomen was closed with Mersilene, 6-0 silk.

Histological techniques
Coronal sections of 30μm for brain/brainstem and 16μm for nodose ganglia were collected. After rinsing in 0.05M Tris-buffered saline TBS (pH 7.4), sections were incubated overnight at 4°C with goat anti-Fos (1:1500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and/or rabbit anti-CTB (1:10 000; C3062, Sigma, USA, to recognize the CTb-conjugated alexa fluorophore 555) primary antibodies.

For cFos staining, sections were first incubated 1hr in biotinylated secondary antibody and then in avidin-biotin complex (ABC, Vector) for 1hr. The reaction product was
visualized by incubation with 1% diaminobenzidine (DAB), 0.05% nickel ammonium sulfate and 0.01% hydrogen peroxide H₂O₂ for 5min. For cFos/CTB double staining, sections underwent once more 1 hr incubation in secondary antibody followed by 1hr incubation with ABC and ended with 7 min incubation in 1% DAB and 0.01% H₂O₂.

To count CTB and/or cFos immunoreactive neurons, tiled images were captured by a computerized image analysis system consisting of an Axioskop 9811-Sony XC77 color camera (Sony Corp., Tokyo, Japan). Brainstem sections, from bregma -7.20 to -7.76mm, were used for cFos and CTB counting in the NTS/DMV counted the brainstem section for the experiments. The counting of cFos in the PVN was performed on hypothalamic sections collected from Bregma -0.58 mm to -1.22 mm. The counting of cFos + cells was performed bilaterally for each nucleus. Data are represented as a mean of the relative density of c-fos positive cells counts on 9-10 and 9-11 sections (non adjacent section, at least separated 90μm) for the NTS/DMV and PVN, respectively.

Immunohistochemical staining for leukocyte infiltration of the small intestinal muscularis

Whole mounts of the small intestinal muscularis were used to determine the degree of inflammation. Myeloperoxidase staining was performed with 3- amino-9-ethly carbazole (Sigma, St Louis, MO), 0.01% H2O2 in Sodium Acetate buffer (pH = 5) for 20 minutes, as previously described 21, 22.

Random counting of whole mount sections

MPO⁺ cells in the whole mount of the intestinal muscularis were counted using an image analysis system (ImagePro v4.5, Media Cybernetics, Silver Spring, USA) connected to a color camera (JVC KY-F55 3CCD) and a plain objective microscope (Zeiss Axioskop with Plan-NEOFLUAR Zeiss objectives). For each section, an image covering the entire sample (2.5× objective) was loaded into the IBAS and displayed on the computer monitor. The region of interest, i.e. the muscularis devoid of Peyer’s patch and damaged areas, was identified and a grid of rectangular areas (representing 20X magnification) was superimposed on the image. From this grid, 5% of the fields were selected randomly and from the selected images (20X), MPO⁺ cells were counted.

Statistical analysis

Data from all experiments are presented as a mean ± S.E.M. Kolmogorov-Smirnov Test was used to determine whether the data set followed a normal distribution (SPSS package v 16.0). Square root transformation was applied to the non normal data set. Two way ANOVA analysis was performed to evaluate the two factors interaction: time (i.e. 2, 6 and 24hrs) and treatment (L vs IM) or vagotomy (i.e., intact vs. denervation)
and treatment (i.e., L vs. IM). When the ANOVA analysis indicated difference, One way ANOVA was performed to demonstrate a statistical significant difference among the different time point per treatment group (i.e., L vs. IM) followed by post hoc (LSD) analysis. The student T test (un-paired) was applied to estimate a significant difference between L vs. IM at a specific time point or between intact vs. denervated. Repeated-measure analysis of variance (ANOVA) was conducted to test the effect of vagal denervation on the mice body weight during the recovery period. All statistical analysis was performed with a significance set at 0.05.

Results

Distribution of CTb labeled neurons after intestinal/splenic injection

**Intestine:** The retrograde labeling was limited to the circumscribed region of the DMV. CTb labeled neurons were localized in the lateral part of the DMV observed from -7.32 to -7.76mm Bregma, as expected from previous anatomical studies. Given the distribution of the vagal sensory innervation of the gastrointestinal tract and the distribution of the CTb positive neurons, brainstem sections from bregma -7.20 to -7.76mm were used for cFos and CTB counting in the NTS/DMV.

Injections of CTB-fluorophore 555 and 647 in the proximal and distal part of the ileum respectively labeled 2 distinct set of neuronal population (Figure 1B). No CTB+ cells were observed in the DMV after injection of the same volume of tracer in the peritoneal cavity. This observation shows a restricted uptake of the neuronal tracer by the small intestine. Moreover the remarkable differentiation of innervation of the ileum by DMV neurons illustrates the specific uptake by defined sites of the ileum.

**Spleen:** The retrograde tracer labeled a restricted neuronal population within the DMV (bregma -7.32mm to -7.76mm; Figure 1C), as previously reported. Application of the tracer on splenic vessels at the level of the hilum led to faint limited amount of labeled neurons (1 or 2) that were located in the DMV more rostrally of the brainstem from bregma -6.96mm to -7.08mm (Figure 1B). These CTb positive neurons, however, were not found after injection of the CTb at the extremity of the spleen. Vagal denervation of the spleen followed by CTb injection at the tip of the organ led to a loss of CTb positive cells in the DMV (Figure 1E) excluding the possibility that CTb positivity results from leakage of tracer into the peritoneal cavity. On the other hand, sympathetic post-ganglionic neurons exhibit CTb stained cell bodies in the mesenteric ganglion (Figure 1F). We, therefore, used the brainstem sections from bregma -7.32mm to -7.76mm for cFos counting in NTS/DMV in the following experiments.
Figure 1 | Distribution of CTb labeled cells in the brainstem nuclei after tracer injection in the intestine (A). Injection of Cholera toxin b (fluorophore 555, red and 647, blue) into the proximal and distal part of the ileum labeled distinct motor neurons in the DMV (B). The distribution of the CTb+ cells in the brainstem nuclei after CTb application on splenic blood vessels differ from the CTb labeled cells (C) found in the DMV after tracer injection at the tip of the spleen (D). Vagal denervation of the spleen prevents CTb labeling of neurons in the DMV. Here we show an example of complete denervation in which no CTb positive cell body was found in the DMV (E). CTb labeled neuron population in the mesenteric ganglion (i.e., sympathetic post-ganglionic neurons) was found in denervated mice (F). The scale bars represent 100µm and 0.50mm for (A,B, F) and (C,D,E), respectively. Green: primary cFos antibody and streptavidine alexa-fluo 488. Sx: vagal denervation of the spleen.
Early and late phase of postoperative ileus: role of inflammation

Intestinal inflammation was monitored by the degree of leukocyte infiltration at the different time points after surgery. MPO$^+$ cells were occasionally observed in the muscle layers of both L and IM mice early after surgery (2hrs L, 1±1 cells/mm$^2$ vs. IM, 2±1 cells/mm$^2$; 6 hrs L, 1±1 cells/mm$^2$ vs. IM, 3±1 cells/mm$^2$). In contrast, a clear infiltration of leukocytes was observed in the late phase of postoperative ileus (24hrs after surgery) (Figure 2A-B) and was significantly higher in IM mice (276 ± 64 cells/mm$^2$) compared to the L mice (1 ± 1 cells/mm$^2$, p=0.007).

Neuronal circuitry: cFos expression at the early phase (2 and 6hrs)

Two hours after surgery, all experimental groups (control, L and IM) exhibited a significant increase of cFos IR in the NTS and PVN compared to the baseline (Figure 2). Even the control mice that only received anesthesia had a comparable number of cFos + cells as L or IM, suggesting NTS/PVN activation induced by anesthesia and/or perioperative stress. In the same line, no difference was observed in cFos IR in nodose ganglia cells bodies (i.e., vagal afferent nerve) (Figure 2D) between L and IM mice, 2 hrs after surgery.

In animals receiving L only, the number of cFos + cells in the NTS decreased in time (one way ANOVA, p =0.002) and reached basal levels at 24hrs post-surgery (L 14.5±5.5 vs BL 15.44±2.7, p=0.689), suggesting a transient effect of the surgical intervention on cFos expression (Figure 2C). In animals that underwent IM, the level of cFos expression remained high (one way ANOVA, p = 0,028) and was significantly different from L at 6 hrs post-surgery.

In the PVN (Figure 2E), cFos IR in both L and IM groups significantly decreased with time after surgery (Two way ANOVA: time effect p < 0.05; treatment or treatment*time interaction, p > 0.05), suggesting a role of the PVN only at the early phase of the POI.

The number of cFos IR motor neurons in the DMV was significantly increased in both L and IM compared to the control (anesthesia only) or basal level (p < 0.05; Figure 3A) at 2 and 6hrs post-surgery, suggesting that additional sensory afferent activation triggered by skin incision and/or intestinal handling generates vagal output. In the L group, the number of cFos + neurons returned to baseline 24hrs after surgery.
Figure 2 | MPO+ cells in whole mount preparations at 24hrs post-surgery (A,B). C-Fos expression in the NTS (C), nodose ganglia (D) and PVN (E) at the early and late phase of POI. Data are expressed as mean ± S.E.M. for n=5-7 mice (A, C & E). The baseline expression of cFos expression in the PVN at 24hrs is similar in L or IM (data not shown). In the figure D, the upper and lower panel illustrated cFos expression in the nodose ganglia (after L or IM) at 2hrs and 24hrs, respectively. The scale bars represent 100µm and 50µm for (B) and (C), respectively. # indicates significant differences (#, p <0.05; ##, p <0.01) compared to t=2hrs. Asterisks indicate significant differences: *, p < 0.05; **, p <0.01), L vs. IM.

cFos IR in CTB labeled motor neurons innervating the small intestine

To evaluate whether trauma/inflammation activates neurons in the DMV targeting the inflamed intestine, we counted the amount of cFos+ cells in the CTB labeled neurons, i.e., motor neurons that specifically innervate the small intestine. The quantitative cFos analysis within the CTB labeled neuronal population showed that 2hrs
and 6hrs after surgery a low percentage (below 10%) of CTB+ neurons co-express cFos IR in both L and IM mice (Figure 3C).

**Neuronal circuitry: cFos expression at the late phase of POI (24hrs)**

At the late phase of the POI (t=24 hrs, inflammatory phase characterized by leukocyte infiltrates in the gut muscularis), activation of the NTS and nodose ganglia after IM was still significantly increased compared to L and baseline: IM 73 ±35 vs. L 14±4 or BL 15±3, p = 0.005) (Figure 2C and D). However, no significant difference in cFos expression in the PVN was observed between L and IM (Figure 2E). In contrast, the number of cFos + neurons in the DMV further increased after IM at 24hrs (IM 24.3±8 vs L 7.7±1.2, p = 0.001) while the number of cFos + neurons after L returned to basal level (Figure 3A-B).

**cFos IR in CTB labeled motor neurons innervating the small intestine**

Nearly half of the CTB neurons that project to the manipulated intestine exhibited positive IR for c-Fos (p < 0.05), i.e., 42% in IM vs. 4% in L (Figure 3C-D). These data provide evidence that the late inflammatory phase is associated with cFos expression in NTS, DMV. This vagal neuronal circuit activation is based on a vagal output targeting specifically the inflamed zone, i.e., the intestine.

**cFos IR in CTB labeled motor neurons innervating the spleen**

Previous studies of Tracey et al., provided strong evidence that the spleen is involved in the vagal anti-inflammatory pathways 5, 16. Since the spleen is directly innervated by the vagus nerve 17, 28, we investigated whether the vagal output triggered by intestinal inflammation also targeted the spleen. To this end, we retrogradely labeled the vagal motor neurons innervating the spleen prior to intestinal manipulation. As previously reported 17, injection of the CTB in both ends of the spleen labeled a neuronal population in the DMV (-7.20 to -7.76mm Bregma) (Figure 3E). The average mean of cFos + cells counted in NTS and DMV from control mice (n = 2; injection of CTb only) was not significantly different from the mice that underwent laparotomy and euthanized 24hrs after surgery. However, twenty four hours after surgery, IM triggered an increase of cFos expression in the NTS and DMV compared to L (NTS: 82±10 vs 5±1; DMV: 15±2 vs. 1±0). Seven percent of the CTB + neurons that project to the spleen exhibited c-Fos IR (p < 0.05) in IM mice while no cFos+ CTB+ neurons were observed in L mice (Figure 3C).
**Figure 3** IM induced cFos expression at different time points after surgery in the DMV (A, B) and in a subgroup of motor neurons innervating the small intestine (C). In panel D, part of the cFos + neurons (black nucleus) is also stained with the retrograde tracer (cytoplasm), identifying vagal motor neurons innervating the intestine (black arrow). cFos IR was also detected in the motor neurons that innervate the spleen at t= 24hrs post-surgery (C, E). Data are expressed as mean ± S.E.M. for n= 5-7. The scale bars represent 200µm (B, E) and 50µm (D). # indicates significant differences (#, p <0.05; ##, p <0.01) compared to t=2hrs. Asterisks indicate significant differences: *, p < 0.05; **, p <0.01), L vs. IM. AP, Area postrema; NTS, nucleus of the solitary tract; DMV, dorsal motor nucleus of the vagus; PVN, paraventricular nucleus of the hypothalamus; IM, intestinal manipulation.
Selective vagal denervation: analysis 24 hrs post-surgery

The neuro-anatomical proof of an endogenous vagal response triggered at the late inflammatory phase of POI and restricted to the inflamed intestine suggests the existence of a vago-vagal inflammatory reflex. To confirm our hypothesis, we selectively denervated the vagal innervation of the small bowel to prove that the afferent limb of the “reflex” is indeed of vagal origin.

In the set up of the denervation procedure, we monitored the mice with sham-operation and denervation during their recovery period (sham-operated mice n = 5, denervated mice n = 7). Mice started with an average body weight of 22.7±0.2g prior surgery. At the end of the recovery period, we did not observe a difference between sham-operated and denervated mice body weight (23.7±1.5g and 23.9±0.96g, respectively). ANOVA with repeated measurement indicated a time effect (i.e., body weight measurement over days, p < 0.05) but no interaction time*denervation effect (p = 0.740) or denervation effect (p = 0.432). At the time of euthanasia, the control groups (laparotomy) did not exhibit distended stomach in sham-operated or denervated mice. Selective vagal denervation (IntX) completely abolished the presence of CTB+ cells in the DMV indicating that the vagal denervation was successful. Among the 20 mice (out of 24) successfully denervated, 11 underwent IM and the 9 left underwent laparotomy.

In denervated mice, IM tended to increase the degree of intestinal inflammation compared sham operated mice, but this difference did not reach statistical significance (336±79 vs. 254±43, respectively; p = 0.188). In both IM groups, inflammation of the intestinal muscularis 24hrs after surgery (Figure 4A) was significantly more pronounced compared to laparotomy mice (7±6 vs. 9±4 for denervated and sham respectively). Even in the presence of inflammation, cFos expression in both the NTS and DMV was significantly reduced by selective vagal denervation (Figure 4B, C and D). IM mice displayed the same levels of cFos expression as the control group (IM IntX vs L IntX; p = 0.289 for NTS and p = 0.043 for DMV). Selective vagal denervation did not affect cFos expression in laparotomy animals.

These data indicate that the vagal sensory pathway indeed transmits the inflammatory signal to the brain stem activating the vagal output to modulate the inflammatory response.
Discussion

Recently, Tracey and coworkers introduced the concept of the vagal “inflammatory reflex” as a protective mechanism to restore immune homeostasis after an immunological challenge. Although it is well established that pro-inflammatory cytokines and endotoxin stimulate vagal afferents leading to brain stem activation, data supporting activation of motor neurons of the vagal nerve closing the anti-inflammatory loop are lacking. Here, we demonstrate that subtle intestinal inflammation leads to NTS and DMV activation that is abolished by selective intestinal vagotomy. Importantly, more than 40% of the activated DMV neurons targeted the inflamed intestine, supporting the existence of an endogenous vagal “inflammatory reflex” modulating intestinal inflammation.
During abdominal surgery, multi-synaptic neuronal pathways are activated involving mainly the NTS and the PVN. All experiments started early morning to avoid any circadian influence on cFos expression. The effect of intestinal manipulation was compared to laparotomy group to avoid any influence of the circadian rhythm while intact mice control groups performed at ZT =2 were compared with 2 and 24hrs (ZT=2).

The NTS is the brain stem nucleus receiving somato/visceral sensory information whereas the PVN is located in the hypothalamus known to regulate stress-related events and endocrine response. The PVN, most specifically the parvocellular part, plays an essential role in mediating the immediate and early (up to 3hrs) hypomotility of the GI tract after abdominal surgery, most likely in response to surgery-induced stress and activation of visceral sensory afferents by skin incision and surgery-induced noxious stimuli. In the present study, we confirmed activation of the PVN in the peri-operative phase (2hrs post-surgery). Interestingly, cFos expression was similar in animals that underwent anesthesia, laparotomy only or intestinal handling suggesting that factors such as stress induced by pain, anesthesia and others largely contribute to PVN activation and even appear to overrule the effect of intestinal handling. Alternatively, the PVN is maximally activated by anesthesia and peri-operative stress thereby obscuring the effect of adding intestinal handling to the surgical protocol. The fact that PVN activation returned to baseline levels at 6 hrs in control and laparotomy animals suggests that peri-operative stress is indeed a major trigger in the early phase. In contrast, the degree of activation in NTS and PVN remained elevated in animals that underwent IM up to 6 hours after surgery. As the effect of peri-operative stress and anesthesia has disappeared by then, as shown in the L mice, these data suggest that NTS and PVN activation at this stage is rather related to the procedure of surgery and intestinal manipulation. As shown in Figure 3, both L and IM activated the DMV, but this activation was not specifically directed towards the intestine as less than 10% these neurons were labeled with the retrograde tracer injected in the intestine. These data thus suggest that in the early phase of postoperative ileus, activation of the brain stem nuclei is triggered by a generalized stress response combined with activation of mechano/nociceptive pathways, most likely to control systemic homeostasis of the organism.

In the second inflammatory stage of postoperative ileus, however, different mechanisms come into play. It is well recognized that intestinal handling during surgery triggers the influx of inflammatory cells, mainly neutrophils and monocytes. This inflammatory response is observed from 6 hours post-surgery onwards and is well established by 24 hours. Twenty four hours after surgery, infiltration of leukocytes within the muscle layers of the small intestine was observed in mice that underwent IM, associated with activation of the NTS and a subset of vagal motor neurons in the DMV.
targeting the inflamed zone. Indeed, we showed a significant increase of cFos IR in those neurons specifically innervating the intestine in IM mice (42%) compared to laparotomy mice (3.8%). Subsequently, we demonstrated that these motor neurons were activated by inflammation detected by vagal afferents. IM, but not laparotomy, resulted in cFos IR in the nodose ganglia. We are aware that the illustration of the presence of cFos + cells in IM in contrast to laparotomy is not sufficient to draw such statement. However the selective vagal denervation of the inflamed intestine abolished both NTS and DMV activation, confirming the importance of the vagal afferent activation in mediating the motor vagal neurons activation in response to IM. Although the latter could result from neuronal degeneration triggered by axotomy, studies demonstrate that neuronal degeneration is rather induced by vagotomy performed at the mid cervical level, i.e. close to the cell body of the neurons. In our study, vagotomy was performed distally close to the nerve endings, reported to lead to neuronal regeneration rather than degeneration. This makes the explanation of neuronal degeneration as explanation for the loss of cFos expression in the DMV/NTS after vagotomy less likely. Therefore, our data indicate that vagal afferents and not splanchnic afferent nerves (through the spino-solitary tract) triggered NTS activation, subsequently generating DMV activation and thereby closing the inflammatory reflex. Most likely, the vagal sensory limb of the inflammatory reflex is triggered by the release of pro-inflammatory cytokines.

Vagal afferents indeed express IL1 and PGE2 receptors, two pro-inflammatory mediators known to be elevated 24hrs after intestinal manipulation. However, further studies are needed to identify the exact underlying mechanisms. Nevertheless, our data clearly demonstrate that intestinal inflammation triggers vagal afferents resulting in the activation of a vagal efferent feedback loop targeting the inflamed area.

Although our data suggest a direct input of the vagal anti-inflammatory pathway to the gut, recent studies identified the spleen as a major player in the cholinergic anti-inflammatory effect in a model of sepsis but also in a model of local inflammation such as the carrageenan air pouch model. To evaluate whether intestinal inflammation would lead to increased vagal output to the spleen, CTB was injected in both ends of the spleen to retrogradely label the DMV neurons innervating the spleen. Only seven percent of the labeled motor neurons expressed cFos IR after IM, indicating that the inflammatory reflex is mainly targeted to the inflamed area. Nevertheless, we cannot exclude that the spleen may contribute, albeit partially to the vagally mediated modulation of the inflammatory response.

In our previous studies, we demonstrated that perioperative electrical stimulation of the vagus nerve decreases intestinal inflammation most likely by inhibiting macrophage activation. Here we provide evidence that this cholinergic inflammatory pathway (i.e. DMV activation) mainly occurs 24hrs after surgery, i.e. once
leukocytes have infiltrated the gut muscularis, but not at the earlier stage where chemokines and cytokines are secreted by the resident immune cells. This would indicate that the endogenous anti-inflammatory pathway is most likely involved in restoring homeostasis once the inflammatory response is established and has fulfilled its task of attacking micro-organisms and clearing tissue damage. Compatible with this hypothesis is the observation that vagal denervation of the small intestine did not significantly enhance the inflammatory response (nor prostaglandin E2 release, data not shown) 24hrs after surgery. Further studies are however required to confirm this assumption.

In summary, we provide the first neuro-anatomical evidence illustrating the existence of an “inflammatory reflex” triggered by inflammation. Subtle intestinal inflammation is detected by vagal afferents triggering NTS activation and generating a specific vagal outflow previously shown to modulate the inflammatory response. These data provide solid evidence to accept the immune-modulatory role of the vagus nerve providing new opportunities to identify targets for the development of new anti-inflammatory strategies.

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Chapter 7

Identification of clinical outcome measures for recovery of gastrointestinal motility in postoperative ileus

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Abstract

Objective: To identify clinical hallmarks associated with recovery of gastrointestinal transit.

Summary Background Data: Impaired gastrointestinal transit or postoperative ileus largely determines clinical recovery after abdominal surgery. However, validated clinical hallmarks of gastrointestinal recovery to evaluate new treatments and readiness for discharge from the hospital are lacking.

Methods: Gastric emptying and colonic transit were scintigraphically assessed from postoperative day one to three in 84 patients requiring elective colonic surgery, and were compared with clinical parameters. The clinical hallmark that best reflected recovery of gastrointestinal transit was validated using data from a multicenter trial of 320 segmental colectomy patients.

Results: Seven out of 84 patients developed a major complication with paralytic ileus characterized by total inhibition of gastrointestinal motility and were excluded from further analysis. In the remaining patients, recovery of colonic transit (defined as geometric center of radioactivity ≥2 on day 3), but not gastric emptying, was significantly correlated with clinical recovery (ρ=-0.59, P<0.001). Conversely, the combined outcome measure of tolerance of solid food and having had defecation (SF+D) (area under the curve 0.9, SE 0.04, 95% CI= 0.79-0.95, P<0.001), but not time to first flatus, best indicated recovery of gastrointestinal transit with a positive predictive value of 93 (95% CI = 78-99)%. Also in the main clinical trial, multiple regression analysis revealed that SF+D best predicted the duration of hospital stay.

Conclusions: Our data indicate that the time to SF+D best reflects recovery of gastrointestinal transit and therefore should be considered as primary outcome measure in future clinical trials on postoperative ileus.
**Introduction**

Each patient undergoing an abdominal surgical procedure will develop an episode of impaired gastrointestinal (GI) motility or postoperative ileus (POI). POI largely determines clinical recovery and hospital stay after abdominal surgery [1-3], thereby significantly contributing to postoperative morbidity and hospitalization costs.[1, 2]

Postoperative inhibition of GI motility results in impaired intestinal transit and stasis of intraluminal contents, leading to symptoms and signs such as nausea, vomiting, bloating, delayed passage of flatus and stool, and the inability to tolerate solid food.[4, 5] As improvement of these parameters is believed to represent recovery of GI transit and resolution of POI, especially time to return of bowel sounds, first flatus and defecation are often used as primary and/or secondary outcome measures in clinical trials.[1, 6] It should be emphasized though that these parameters are difficult to assess accurately. Moreover, passing stool or flatus may rather mirror rectal emptying and therefore not necessarily adequately reflect recovery of effective GI motility. Therefore, it is of great importance for future studies to determine the relationship between objectively demonstrated recovery of GI transit and clinical parameters to identify potential clinical outcome measures. Subsequently, these parameters should be validated in a separate cohort before accepting one of these as outcome measure in future clinical trials.

Scintigraphic recording of GI transit is considered the gold standard to assess GI motility. This technique allows accurate evaluation of motility throughout the entire GI tract in a safe and non-invasive way.[7-9] It has been successfully used to evaluate pharmacological interventions in healthy subjects and patients with colonic motility disorders.[9-11] In the current study, we used this technique to study the relationship between GI transit and clinical symptoms in order to identify the most reliable clinical markers of bowel function recovery.

**Materials and Methods**

*Study I: Identification of clinical outcome measures for recovery of transit based on scintigraphic assessment*

**Patients**

This study was performed in the Academic Medical Center of Amsterdam. Patients enrolled in the LAFA (LAsparoscopy and/or FAsst track multimodal management versus standard care) multicenter trial were eligible for this study.[12] Between 2005 and 2009, patients were invited to participate if they were to undergo elective segmental
colectomy for colonic cancer without evidence of metastatic disease. This study is registered with Netherlands National Trial Register, number NTR1884. The study was conducted in accordance with the principles of the Declaration of Helsinki. The protocol was approved by the Medical Ethics Review Board of the Academic Medical Center in Amsterdam (The Netherlands).

**Study protocol**

Patients were randomized to laparoscopic or open surgery combined with fast-track or standard care. Immediately after surgery, the nasogastric tube was removed. Twenty-four hours after surgery, patients underwent a solid gastric emptying test (technetium-99 (99mTc) labeled pancake) directly followed by the ingestion of 60 ml of indium-111 (111In) labeled water to assess colonic transit on postoperative day two and three as previously described. [3] Calculation of gastric retention and colonic transit was performed blinded by two researchers independently (SvB & RJB) on a Hermes workstation. In addition, clinical signs and symptoms of upper- and lower GI motility were assessed on a daily basis by a trial nurse and/or a research physician using a standardized questionnaire (Fig. 1a). Patients were assisted to fill in this self-assessment sheet daily from the time of scintigraphy until discharge. Times to first occurrence of these events (passing flatus, passing stool, bowel sounds and consumption of solid food) and time to discharge were recorded. In addition, nursing staff reported daily on the patient’s progress, i.e. food intake, passage of flatus and stool and predefined discharge criteria were checked. The time until ready for discharge was defined as the time until a patient was without complications, tolerated solid food, and pain was adequately controlled with oral analgetics. Time to tolerance of solid food was defined as the time to solid food intake with no significant nausea or vomiting. Clinical parameter assessments were obtained daily by the principal investigators via interviewing the patients, followed by chart review.[13] The time to the clinical end points (e.g. toleration of solid food, defecation and flatus) were measured in hours, starting at completion of abdominal incision closure. A patient was considered to have met the endpoint when (s)he had first tolerated solid food and either passed flatus or experienced his/her first bowel movement. All patients were discharged according to the same predefined discharge criteria as patients from the LAFA multicenter trial, i.e. the nine-center randomized trial comparing fast-track or standard care in patients undergoing conventional and laparoscopic segmental resection for colon cancer (registration NTR number: NTR222). In this trial, patients were discharged if they complied with the following predefined discharge criteria: (1) adequate pain control with paracetamol and/or nonsteroidal anti-inflammatory drugs (2) ability to tolerate solid food (3) absence...
of nausea (4) passage of first flatus and/or first stool (5) mobilization as preoperative, and (6) acceptance of discharge by the patient.

**Study II: Validation of composite outcome for colonic transit recovery**

The clinical parameters identified in study I were validated in a second study, i.e. the main LAFA-trial that contained patients of study I. This was a nine-center (9 Dutch hospitals: 3 University hospitals and 6 teaching hospitals) randomized trial comparing fast-track or standard care in patients undergoing conventional and laparoscopic segmental resection for colon cancer (registration NTRnumber: NTR222).[14] In study II, after excluding the patients that participated in study I, we evaluated whether the clinical parameters identified in study I indeed best detected differences between the four treatment arms. Patient and baseline characteristics were similar to the patients of study I and are shown in supplementary Table 1. Subsequently, we merged all patients (n=320) and analyzed whether the clinical hallmarks independently predicted enhanced recovery (i.e. shorter length of hospital stay).

**Statistical analysis**

Categorical variables are expressed as number and/or percentage of patients. Continuous data are estimated as mean (SD) or as median and inter-quartile range (IQR) for non-normally distributed data. Differences between groups were assessed using the two-tailed Fisher exact test or the Chi-square test for categorical variables and using the Students’ t-test for continuous data with normal distribution. For non-normally distributed data the Mann-Whitney U test was used. Kaplan-Meier survivorship curves were generated for the GC ≥ 2 and the GC < 2 subgroup with the end point “ready for discharge”. The positive predictive values (PPV) of the clinical outcome parameters to predict recovery of GI transit was calculated as the number of patients with recovery of transit and the clinical parameter divided by the number of patients presenting with the clinical parameter. To evaluate the discriminatory ability of the different clinical parameters to correctly pick up patients with and without recovery of colonic transit, receiver operating characteristic (ROC) curves were utilized to display the trade-off between the true positive rate (sensitivity) and false positive rate (1- specificity) and assess the area under the ROC curve as a measure of inherent validity of the different parameters. See supplementary data for the statistical methods of study II.

A two tailed *P*-value < 0.05 was considered to be statistically significant. Statistical analysis was performed using SPSS for Windows version 17 (SPSS Inc. Chicago, USA).
Results

Study I: Identification of clinical outcome measures indicative of recovery of GI transit

Patient characteristics

Of the 95 patients enrolled, 11 patients discontinued, leaving 84 patients for analysis (Fig. 2). Gastric emptying was analyzed in 75 patients on postoperative day 1, as 3 patients were not able to ingest the pancake because of nausea and vomiting. In addition, gastric emptying could not be measured in another 6 patients because of technical or logistic problems (e.g. no availability of the gamma cameras to scan the patient). Data on colonic transit at day 2 or 3 were available in respectively 77 and 76 patients. The mean patient age was 64.3 years, and the majority of patients (58%) were male (Table 1).

GI transit in patients with paralytic ileus

In 7 of the 84 patients, the radiolabel had not reached the colon on day 3 (GC=0). These patients had developed a paralytic ileus resulting from a major surgical complication (internal herniations, adhesions, anastomotic leaks and intraperitoneal bleeding). There was no clear relationship between bowel sounds and the development of paralytic ileus, as in 6 of the 7 patients bowel sounds were still recorded during the first 3 days. In contrast, all patients required reinsertion of a nasogastric tube during the first 3 days (because of abdominal distension, nausea and emesis after having started a liquid diet). In these patients there was an increased length of stay (median 21.8 days (IQR, 17.9-23.9) compared with patients that not developed a paralytic ileus (5.8 (4.0-7.0))). Moreover, only 5 of the 77 patients in which the tracer did reach the colon required reinsertion of a nasogastric tube on day 3. Thus, the reinsertion of a nasogastric tube during the early postoperative period is strongly associated with the development of paralytic ileus and postoperative complications.

GI transit in patients without paralytic ileus

Gastric emptying was analyzed in 69 out of 77 patients on postoperative day 1. The median residual gastric content 2 hours after ingestion of the pancake was 59.4% (IQR, 30.8-93.0), varying from almost complete emptying to complete gastric stasis.

The total amount of $^{111}$In-DTPA tracer ingested had reached the colon in all but 3 patients on day 3. The mean calculated GC of activity on day 2 was 1.6 (SD, 0.6; n=71) and increased to 1.9 (SD, 0.7; n=69) on day 3. As shown in Figure 1b, patients with a GC between 0 and 1 at day 2 are at risk to have a prolonged hospital stay. Moreover, if GC on day 3 is below 1.5, these patients can only be discharged after more than 7 days, compared to before day 4 if GC is above 1.5. Calculation of the correlation between GC...
Table 1. Baseline Characteristics and Surgical Aspects

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and time to ready for discharge indeed shows a significant correlation of -0.59 (Spearman’s rank correlation (ρ), P<0.001, n=69).

Defecation of radiolabeled material was also assessed as indirect marker of recovery of transit. By day 2, 27% of the patients had defecated radioactivity whereas at day 3, 57% of patients had defecated radiolabeled material. At day 3 the median percentage of ¹¹¹In-DTPA tracer defecated in all 67 patients was 19% (IQR, 0-64) of the total ingested radioactive tracer at day 3. Similar to GC, the amount of Indium tracer defecated correlated significantly with time to ready for discharge (ρ=-0.45, P<0.001, n=67). In contrast, the residual gastric content of the radiolabeled pancake did not correlate with time until ready for discharge (ρ=0.12, P=0.300, n=75).
Chapter 7 | Clinical Outcome Measures

Figure 1a | Study protocol. Twenty-four hours after surgery, patients underwent a solid gastric emptying test ($^{99m}$Tc labeled pancake) directly followed by the ingestion of 60 ml of $^{111}$In-DTPA labeled water to assess colonic transit on postoperative day two and three. In addition, clinical signs and symptoms were assessed on a daily basis using a symptom questionnaire (Q).

Figure 1b | Time until ready for discharge versus colonic transit on day 2 and 3. Median time until ready for discharge versus colonic transit on day 2 (n=77) and 3 (n=76). Data are shown as median, IQR.
Scintigraphic definition of “recovery” of GI transit

In order to identify symptoms associated with recovery of GI transit, we first objectively defined “recovery” of GI transit. To this end, we determined the most optimal cut-off value of colonic transit for predicting whether patients had evacuated radiolabeled material at day 3. The cut-off level was determined using a receiver operating characteristic curve (ROC) that differentiated patients with and without defecation of radioactivity at day 3. As shown in supplementary Figure 1, a cut-off GC value of 2 at day 3 was most optimal in predicting whether patients had evacuated radioactivity (sensitivity 88%, specificity 86%) and thus indirectly recovery of GI transit. To evaluate the reliability of GC $\geq 2$ as marker of recovery of GI transit, evacuation of radiolabeled material was compared in patients with a GC $< 2$ or $\geq 2$ at day 3. Only 8 out of 37 patients with a GC $< 2$ had evacuated radioactivity. In contrast, 31 out of the 32 patients with GC $\geq 2$ had passed 66% (IQR, 39-91) of the radiolabel into the stool at day 3.

In the same line, if GC $\geq 2$ is a good marker of recovery of GI transit, patients with a GC $\geq 2$ should have better clinical outcome. Indeed, the median time until ready for
discharge (Fig. 3a), the time until first defecation (D), tolerance of solid food (SF) and time until patients had had tolerance of solid food and had defecation (SF+D) were all significantly shorter in patients with GC ≥2 or “recovery of GI transit” at day 3 (Fig. 3b). In contrast, time to first flatus was not different between the 2 groups.

**Figure 3a** | (Left panel) Kaplan-Meier curve of time until ready for discharge. Kaplan-Meier estimate of the time until ready for discharge for patient with GC ≥ 2 and for patients with GC < 2 at day 3. Patients with GC ≥ 2 (n=32) were significantly faster ready for discharge (median of 2.0 days) compared to patients with a GC < 2 (n=37).

**Figure 3b** | (Right panel) Clinical parameters versus recovery colonic transit. Time to first flatus, first defecation (D), first tolerance of solid food (SF), and time until patients tolerate solid food and passed stool (SF+D) in patients with GC ≥ 2 (n=32) and patients with GC <2 (n=37). Data are shown as median, IQR. The Mann-Whitney U test was used to compare the two groups. P-value < 0.05 was considered statistically significant. NS, non-significant.

**Identification of clinical parameters indicative of recovery of colonic transit**

Subsequently, we assessed which clinical parameters best identified patients with recovery of GI transit and thus represent the most optimal clinical outcome measure. To this end, ROC curves of clinical outcome measures were calculated for discriminating patients with a GC < or ≥ 2. The diagnostic capacity of a clinical outcome parameter to identify recovery of GI transit was determined by calculating the corresponding area under the ROC curve (AUC values). Due to missing data on colonic transit, the overall analysis of data for identification of clinical parameters indicative of recovery of colonic transit was conducted in 72 patients. Results on colonic transit were available in 71 and 69 patients at day two and three postoperatively.

As shown in Figure 4, the time to passing stool, or the time to tolerance of solid food had high accuracy for identifying patients with a GC ≥2 at day 3 (AUC respectively 0.77 and 0.81). The AUC for the time until patients had had SF+D was 0.87 (SE 0.04, 95% CI= 0.79-0.95, P<0.001) (Fig. 4). The time to first flatus was not a significant discriminator to identify recovery (P=0.467). Similar to day 3, the largest AUC discriminating patients
with recovery of GI transit at day 2 was provided by the model incorporating SF+D (AUC 0.85±0.05 (95% CI=0.75-0.95, P<0.001) (Fig. 4).

In addition, we evaluated whether the composite outcome SF+D was useful to indicate if a patient had recovery of GI transit on the ward. Therefore, we calculated the positive predictive values (PPV) of the clinical outcome parameters to predict recovery of GI transit. Passing flatus on or before day 2 had a PPV of 48% (95% CI=34-62). In contrast, the PPV of defecation or of tolerating solid food on or before day 2 was 71% (95% CI=52-86) and 58% (95% CI=39-57) respectively. If patients had had SF+D on day 2, GI transit was recovered in 88% (95% CI=64-99). The PPVs of the clinical outcome measures reported on day 3 increased to 76%, 85%, and 89% for respectively flatus, stool and tolerance of solid food, compared to 93% (95% CI =78-99) for SF+D.

Figure 4 | ROC curves of the clinical parameters: passing flatus, stool, tolerance solid food and the composite of stool and tolerance solid food. Identification of recovery of GI transit at day 2 (n=71) and day 3 (n=69) by clinical parameters in patients requiring elective colonic surgery. The largest area under the curve for discriminating the recovery from the non-recovery GI transit group at day 3 is provided by the model incorporating time until tolerance solid food and defecation (SF+D).

Study II: Validation of composite outcome for colonic transit recovery

We validated the composite outcome SF+D in 320 patients who participated in a large randomized surgical trial (LAFA trial) in which laparoscopic and open surgery were compared in patients undergoing standard or fast-track perioperative care.[12] To this end, we evaluated SF+D as outcome parameter for detecting differences between the 4 treatment arms. In line with previous reports on GI motility and transit [3, 13, 15, 16], the time to SF+D was significantly shorter after laparoscopy and fast-track care (Fig. 5). In contrast, as published by Vlug et al. [12] no statistically significant difference between the laparoscopy and open groups was detected by the traditional indicators of return of
bowel activity: time to first flatus, time to first defecation or time to tolerance of solid food. Moreover, when all 320 patients were merged multiple regression analysis revealed that achieving SF+D lead to a reduction of 43% (95% CI=35-48) in length of stay and best predicted hospital stay (supplementary Table 2).

**Figure 5** | The discriminatory ability of the clinical endpoint SF+D as outcome parameter for detecting differences between the 4 treatment groups of the LAFA trial, after excluding the patients of study I. The clinical outcome measure time until defecation and tolerated solid food (SF+D). All data are expressed as the median ± interquartile range. Data were analyzed by use of a Kruskal–Wallis test followed by Dunn’s Multiple Comparison test comparing all groups. * P < 0.05; # Significant difference (P < 0.05): # between Laparo/FT & Laparo/Standard; ## between Laparo/FT & Open/Standard; ^^^ between Open/FT & Open/Standard. Analysis of 320 patients; (Laparo+FT: laparoscopy & Fast-track care (n=79), Laparo+Standard: laparoscopy & Standard care (n=90), Open+FT: Open colectomy & Fast-track care (n=74), Open+Standard: Open & Standard care (n=77).

**Discussion**

To date, there is no consensus on the most clinically meaningful hallmark assessing recovery of GI motility following abdominal surgery.[2, 17, 18] The time to return of bowel sounds and time to first passage of flatus have been commonly referred to as indicators of bowel activity, notwithstanding the fact that these parameters are subjective and time dependent measures.[19, 20] Here, we objectively determined colonic transit using scintigraphy and showed that clinical recovery following abdominal surgery is indeed associated with recovery of colonic transit. Using the latter as objective criterion for clinical improvement, we showed that tolerance of solid food and having had defecation (SF+D) best predicted clinical recovery and length of hospital stay. In
contrast, time to first flatus was not associated with recovery of colonic transit or time to discharge. Based on these findings, we propose that the time to SF+D is the best objective parameter to assess clinical recovery and readiness to be discharged and should preferentially be used to determine the duration of POI in future clinical trials.

In the first part of this study, we aimed to study the relationship between clinical symptoms and recovery of GI transit. To this end, 84 patients ingested a $^{99m}$Tc labeled pancake and 100 ml of $^{111}$In labeled water 24 hours after intestinal surgery to assess gastric emptying and colonic transit. Gastric emptying measured on day 1 largely varied from complete gastric stasis to almost complete emptying and most importantly was not associated with clinical recovery, defined as time until ready for discharge. On the other hand, colonic transit measured as the geometric center of radioactivity correlated with the time to defecation of radiolabeled material and was significantly correlated with time to ready for discharge, indicating that recovery of colonic transit is indeed an important determinant of clinical recovery following abdominal surgery. In a next step, we used “recovery of colonic transit”, defined as a GC ≥2, to identify the clinical symptoms that best correlated with clinical recovery. Using ROC curve analysis, we observed that the clinical parameters passing stool and tolerance of solid food had a high accuracy for identifying patients with recovery of GI transit with an AUC of respectively 0.77 and 0.81 and a positive predictive value of 85 and 89% respectively. Moreover, when both parameters were present, i.e. patients tolerated solid food and had defecated, the AUC further increased to 0.87 with a positive predictive value of 93%. The positive predictive value for passing flatus was only 76%, indicating that this clinical parameter is a poor predictor of clinical recovery. In line with our findings, manometric studies evaluating the relationship between clinical parameters and recovery of GI motility also reported that the restoration of intestinal motility does not coincide with the first passage of flatus. In fact, bowel sounds and the first passage of flatus occurred later than the restoration of migrating motor complexes in the proximal jejunum. [21, 22] This contradiction between the resolution of small intestinal motility disturbances and the first passage of flatus and stool may be related to the more prolonged ileus of the colon in the postoperative period.[22] Recovery of small bowel motility indeed takes place within hours of operation, followed by return of gastric function within 1 to 2 days whereas colonic function may require 2 to 5 days to recover.[23, 24]

Taken together, our data indicate that the presence SF+D is the most reliable parameter indicating recovery of colonic transit, and thus could be useful to assess clinical recovery following abdominal surgery. Correspondingly, using the outcome parameter SF+D we were able to detect significant differences between the laparoscopy and open groups in the multicenter LAFA trial. In this large randomized trial a
significantly shorter hospital stay has been reported after laparoscopy and fast-track care, with no difference between the laparoscopy and open groups for traditional indicators of return of bowel function such as time to first flatus.[12] Moreover, when all 320 patients were merged multiple regression analysis revealed that SF+D best predicted hospital stay. These data confirm that SF+D indeed best reflects GI transit as well as clinical recovery, and are in line with a recent proposal promoting the composite end point SF+D as a more robust and appropriate endpoint because it is subject to considerable less variability compared to the composite endpoint incorporating time to first flatus.[25] Ludwig et al. used a composite assessment that measured upper (toleration of solid food) and lower (first defecation) GI tract recovery, with time to resolution of POI based on the last event to occur. As the time to first flatus may be a less objective end point than time to first defecation because a patient must be conscious and willing to report it,[20] the composite end point SF+D was advocated to be a more appropriate measure of GI tract recovery in the bowel resection population.[25] However, in contrast to our study, this conclusion was not supported by objective observational data of recovery of GI motility, but proposed after studying the variability of different endpoints. The implication of this composite endpoint for clinical routine is that it may help the clinician to decide which patients are ready for discharge.

The current study had several limitations. The analyses examining SF+D as an independent variable in predicting readiness for hospital discharge could be confounded by the fact that these same clinical parameters (ability to tolerate solid food, passage of stool) were being considered in the decision to regard the patient as suitable for discharge. Another limitation is that gastric emptying of the radiolabeled pancake was only assessed on day 1. At this time point, motility in general may still be significantly affected by the anesthetics and analgesics used, and it is therefore of no surprise that gastric emptying on day 1 is not associated with clinical recovery. In addition, an extended gastric emptying scintigraphy test from two to four hours may have been more sensitive in detecting patients with gastroparesis.[26] Nevertheless, the strength of our study is the objective measurement of motility by assessment of GI transit in a large cohort of segmental colectomy patients, and the subsequent validation of the clinical hallmark indicating recovery of bowel function in the patients of the main multicenter trial. To what extent our findings can be extrapolated to all types of surgical procedures warrants further investigation.

In conclusion, our study demonstrates that the presence of SF+D best reflects recovery of GI transit and indicates readiness for discharge. These data provide objective evidence that the presence of both clinical parameters is the best clinical marker of gut recovery and is to be preferred as primary outcome measure in future clinical trials on POI.
Acknowledgements

The authors thank J.W. de Jong, M. Spaeth and the Nuclear Medicine technicians and staff for the preparation of the scintigraphically labeled test meals. We are grateful to all investigators of the collaborative LAFA study group and all patients that participated in the LAFA-trial.

Collaborative LAFA study group

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Supplementary Material

Supplementary figure 1

Evacuation of radiolabeled material at day 3

- Sensitivity: 88%
- Specificity: 86%

AUC 0.93

P<0.001
### Supplementary Table 1: Baseline Characteristics and Surgical Aspects of Included Patients in LAFA-multicenter trial

<table>
<thead>
<tr>
<th>LAFA-multicenter trial (n = 320)</th>
<th></th>
</tr>
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<tbody>
<tr>
<td>Age, mean (SD), year</td>
<td>67.0 ± 8.5</td>
</tr>
<tr>
<td>Male sex, %</td>
<td>58</td>
</tr>
<tr>
<td>Body mass index, mean (SD), kg/m²</td>
<td>26.5 ± 4.3</td>
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<tr>
<td>ASA , %</td>
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<tr>
<td>Grade I or II</td>
<td>77</td>
</tr>
<tr>
<td>Co-morbidity, %</td>
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<tr>
<td>T stage, %</td>
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</tr>
<tr>
<td>T0</td>
<td>7</td>
</tr>
<tr>
<td>T1</td>
<td>5</td>
</tr>
<tr>
<td>T2</td>
<td>26</td>
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<tr>
<td>T3</td>
<td>58</td>
</tr>
<tr>
<td>T4</td>
<td>5</td>
</tr>
<tr>
<td>N stage, %</td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>59</td>
</tr>
<tr>
<td>N1</td>
<td>33</td>
</tr>
<tr>
<td>N2</td>
<td>9</td>
</tr>
<tr>
<td>M stage, %</td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>91</td>
</tr>
<tr>
<td>M1</td>
<td>9</td>
</tr>
<tr>
<td>Laparoscopic surgery, %</td>
<td>45</td>
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<tr>
<td>Fast track care, %</td>
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</tr>
<tr>
<td>Type of colectomy, %</td>
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<td>Right-sided</td>
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</tr>
<tr>
<td>Left-sided</td>
<td>54</td>
</tr>
<tr>
<td>Duration of surgery, median (IQR), minutes</td>
<td>145 (115 – 180)</td>
</tr>
<tr>
<td>Blood loss, median (IQR), milliliter</td>
<td>100 (3 – 300)</td>
</tr>
</tbody>
</table>

Data are expressed by mean ± SD, unless otherwise noted, BMI = body mass index. ASA = American Society of Anesthesiologists / IQR = interquartile range

### Supplementary Table 2: Results of Multiple Linear Regression Analysis Length of Postoperative hospital stay

<table>
<thead>
<tr>
<th>Postoperative hospital stay (n=320)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical hallmarks day 3</td>
<td>Factor (Fl/B)</td>
</tr>
<tr>
<td>Passing stool</td>
<td>0.84</td>
</tr>
<tr>
<td>Intake solid food</td>
<td>0.70</td>
</tr>
<tr>
<td>Absence nausea</td>
<td>0.69</td>
</tr>
</tbody>
</table>

Multiple linear regression analysis of achieved symptoms for recovery GI motility before day 4 on postoperative hospital stay, i.e. days until discharge (after log-transformation). The sequential multiple linear regression model
incorporated the various clinical parameters (passing flatus on/before day 3, passing stool on/before day 3, intake solid food on day 3, absence of nausea on day 3, after controlling for the baseline characteristics age (≤ 65 years), laparoscopic resection and Fast Track care. The presented factors (Fold Increase) and Confidence Intervals (CI) represent the back-transformed (antilog) parameter estimates, which may be interpreted as the fold increase of the geometric means of the postoperative hospital stay of the passing stool group versus non passing stool group, respectively. Forward sequential regression analysis identified for day 3 the following independent predictors of postoperative hospital stay: passing stool [B=0.84; 95% confidence interval (CI) 0.75 to 0.94; \( P=0.003 \), i.e. reduction of 16% (CI: 6 -25%) in postoperative hospital stay], intake solid food [B=0.70; 95% confidence interval (CI) 0.60 to 0.81; \( P<0.001 \), i.e. reduction of 30% (CI: 19 -40%) in postoperative hospital stay], and absence of nausea [B=0.69; 95% confidence interval (CI) 0.60 to 0.79; \( P<0.001 \), i.e. reduction of 31% (CI: 21 -40%) in postoperative hospital stay]. Passing flatus was not a significant independent predictor of postoperative hospital stay (\( P=0.878 \)).

**Supplementary statistical methods**

In study II, we validated the results with data from the LAFA-trial database. The sample size of the LAFA trial was calculated based on hospital stay as the primary efficacy parameter. Using a 5% significance level, a total sample size of 400 had a power of > 95% to detect a minimum reduction in total hospital stay of 1 day between laparoscopic and open surgery, 1 day reduction in total hospital stay between fast track and standard care, and a power of 80% to detect the same difference between the combination of fast track with laparoscopic surgery and open surgery with standard care.[14] The Kruskal-Wallis test followed by Dunn’s Multiple Comparison test was used to compare the 4 groups of study II. Postoperative hospital stay was calculated from the day of surgery until the day of discharge. As postoperative hospital stay was not normally distributed, these data were log-transformed. The covariates intake of solid food day 3, absence nausea on day 3, flatus before day 4 and defecation before day 4, and 7 baseline characteristics (sex, age (≤65 years), American Society of Anesthesiologists (ASA) grade (I/II), body mass index (BMI), type of perioperative care program (standard, fast track care), type of surgery (open, laparoscopic), and type of resection (right-sided, left-sided)) were tested by univariate linear regression analysis. All variables with \( P<0.100 \) were then entered in a sequential multiple linear regression analysis. The variables were: intake solid food day 3, absence nausea on day 3, flatus on/before day 3, passing stool on/before day 3, sex, age, ASA grade, type of perioperative care program, and type of surgery. Forward sequential elimination was used to create a final multivariate model retaining only variables with \( P<0.05 \), as this was considered to be statistically significant. A new variable was created for those patients that had achieved tolerance of solid food (i.e. intake solid food without nausea) and passed stool before day 4. This new variable, named ‘passing stool and tolerance solid food on day 3’ and the predictive baseline characteristics (age, type of perioperative care surgery and type of surgery) were entered in a new hierarchical multivariable linear regression model. B-values of significant predictive parameters were converted into percentages difference in postoperative hospital stay they would result in if present, with their 95% confidence intervals (CI).
References

Chapter 8

Faster recovery of gastrointestinal transit after laparoscopy and fast-track care in patients undergoing colonic surgery

Abstract

Background & Aims: Postoperative ileus is characterized by delayed gastrointestinal (GI) transit and is a major determinant of recovery after colorectal surgery. Both laparoscopic surgery and fast-track multimodal perioperative care have been reported to improve clinical recovery. However, objective measures supporting faster GI recovery are lacking. Therefore, GI transit was measured following open and laparoscopic colorectal surgery with or without fast-track care.

Methods: Patients (n=93) requiring elective colonic surgery were randomized to laparoscopic or conventional surgery with fast track multimodal management or standard care, resulting in four treatment arms. Gastric emptying and colonic transit were scintigraphically assessed from day 1 to 3 in 78 patients and compared to clinical parameters such as time to tolerance of solid food and/or bowel movement, and time until (ready for) discharge.

Results: 71 patients without mechanical bowel obstructions or surgical complications requiring intervention were available for analysis. No differences in gastric emptying 24 hours after surgery between the different groups were observed (P = 0.61). However, the median colonic transit of patients undergoing laparoscopic/fast-track care was significantly faster, compared to the laparoscopic/standard, open/fast-track, and open/standard care group. Multiple linear regression analysis showed that both laparoscopic surgery and fast-track care were significant independent predictive factors of improved colonic transit. Both were associated with significantly faster clinical recovery and shorter time until tolerance of solid food & first bowel movement.

Conclusions: These data demonstrate that colonic transit recovers significantly faster after laparoscopic surgery and the fast-track program, providing objective data that laparoscopy and fast-track care lead to faster recovery of GI motility and concomitant enhanced clinical recovery.
**Introduction**

Each patient undergoing an abdominal surgical procedure will develop a transient episode of impaired gastrointestinal (GI) motility or postoperative ileus (POI). Importantly, POI is the single most important determinant of hospital stay after abdominal surgery and consequently significantly contributes to postoperative morbidity and hospitalization costs.\(^1\)\(^,\)\(^2\) Laparoscopic surgery and the implementation of an enhanced recovery after surgery (ERAS) program, also referred to as ‘fast-track’ (FT) perioperative care, are the two most important recent advances in modern surgical care. Both have been reported to be safe and effective and to result in a shorter hospital stay with earlier recovery of GI function\(^3\)-\(^6\) and less morbidity compared to open colorectal surgery and standard care.\(^7\)-\(^10\)

FT programs in colonic surgery were introduced to reduce surgical stress response, organ dysfunction and morbidity, thereby promoting a faster recovery after surgery.\(^11\),\(^12\) This multimodal perioperative care strategy constitutes a multidisciplinary approach involving dieticians, nurses, surgeons, and anesthesiologists. It mostly focuses on adequate perioperative fluid management, optimized analgesia, early oral nutrition and early mobilization.\(^6\),\(^13\),\(^14\)

It should be emphasized though that most studies assessing POI have used rough clinical parameters such as return of bowel sounds and time to first flatus or defecation as primary outcomes. These parameters are however not only difficult to assess accurately, but it is also questionable to what extent they reflect restoration of GI motility. For example, the occurrence of first flatus may rather reflect emptying of rectal gas than recovery of colonic transit. To date, only two clinical studies comparing laparoscopic and open colectomies objectively measured postoperative GI motility and at best showed only a modest increase in the recovery of GI motility.\(^15\),\(^16\) Studies comparing the effect of FT care with standard postoperative care on GI transit however are lacking.

Although several methods have been employed, scintigraphic recording of GI transit is considered the gold standard. This technique is not only widely used to measure gastric emptying, it also allows accurate evaluation of motility throughout the entire GI tract in a safe and noninvasive way.\(^17\)-\(^19\) In addition, it has been successfully used to evaluate therapeutic interventions in healthy subjects and patients with colonic motility disorders.\(^19\)-\(^21\) Therefore, in the present study, scintigraphy was selected to objectively evaluate whether laparoscopy, FT care, or the combination of both result in faster recovery of GI transit after colonic surgery.
Materials and Methods

Patients and Eligibility
Patients enrolled in the Academic Medical Center Amsterdam as part of the LAFA (LAparoscopy and/or FAst track multimodal management versus standard care) multicenter trial were eligible for this study. Patients between 40 and 80 years of age, with an American Society of Anesthesiologists (ASA) status < IV, were invited to participate if they were to undergo elective segmental colectomy for histologically confirmed adenocarcinoma or adenoma without evidence of metastatic disease. Exclusion criteria were neoadjuvant radiotherapy, prior midline laparotomy, unavailability of a laparoscopic surgeon, emergency surgery or a planned stoma. The protocol was approved by the medical ethics review board of the Academic Medical Center Amsterdam. The study was conducted in accordance with the principles of the Declaration of Helsinki and good clinical practice guidelines.

Study design
A 2 x 2 balanced factorial block design was used to randomize patients to laparoscopic or open colectomy, and to the FT program or standard care by means of an internet randomization module. This resulted in four treatment groups: (I) laparoscopic colectomy with FT care (Lap/FT), (II) open colectomy with FT care (Open/FT), (III) laparoscopic colectomy with standard care (Lap/Standard), and (IV) open colectomy with standard care (Open/Standard).

24 hours after surgery, patients underwent a solid gastric emptying test ($^{99m}$Tc labeled pancake, 115 Kcal) directly followed by the ingestion of 60 ml of indium-111 labeled water to assess colonic transit on postoperative day 2 and 3 (see section Gastrointestinal Transit Studies for details). During hospital admission, patients were visited at least once daily by a trial nurse and/or a research physician for clinical evaluation. Clinical symptoms of upper- and lower GI motility were evaluated daily via self-designed questionnaires. Patients were assisted to fill in this self-assessment sheet daily at the time of scintigraphy until discharge. Until discharge, nursing staff reported daily on the patient’s progress, i.e. intake, passage of stool, and predefined discharge criteria were checked.

In order to avoid cross-over treatment by the nursing staff, patients were admitted either to a ward providing standard care or a ward providing FT care. The treatment protocols are described in detail in supplementary Table 1. Open surgery was performed through a midline laparotomy. The laparoscopic approach was a laparoscopic assisted technique comprising a medial to lateral approach with intracorporeal devascularization and mobilization. In right colectomies, the anastomosis was done.
extracorporeal, in left colectomies intracorporeal after extracorporeal insertion of the anvil in the afferent colon loop. All anastomoses were done using mechanical staplers. At the end of the surgery the abdomen was covered with a large dressing to hide abdominal incisions in order to blind the patients and nurses on the ward. This dressing was kept in place until the day of discharge, unless wound complaints or complications were suspected.

Outcomes
The primary outcome of efficacy was colonic transit on day 3, depicted as GC of intracolonic mass 48 hours postprandially of $^{111}$In-DTPA labeled water.

The secondary endpoints of this study were: (I) gastric retention at 24 hours after surgery, formulated as the percentage of $^{99m}$Tc labeled pancake present in the stomach two hours after ingestion; (II) time until first bowel movement; (III) time until first tolerance of solid food; (IV) the composite outcome of time to tolerate solid food and bowel movement. (V) length of hospital stay; (VI) time until ready for hospital discharge.

Gastrointestinal transit studies
On postoperative day 1, patients underwent a solid gastric emptying test. Two hours after ingestion of a $^{99m}$Tc labeled pancake (115 Kcal), a 5-minute acquisition was performed in a 128 matrix with the patient in a supine position using a single head gamma camera (Siemens Orbiter, or Diacam, Siemens, Hoffman Estates, IL) fitted with a medium energy collimator. The relative gastric content of the pancake 2 hours after ingestion was calculated as previously described. Briefly, to depict the percentage of activity present in the stomach compared with the total activity in the abdominal region of interest, the counts in the stomach were divided by the counts in the complete abdominal region, corrected for background. Directly after completion of the gastric emptying test patients were asked to drink 60 ml of tap water labeled with 4 MBq $^{111}$In-DTPA (DiethyleneTriaminePentaAcetate, Covidien, Petten, the Netherlands). A cobalt marker was placed on the iliac crest for anatomical reference and a baseline scan was performed to determine total amount of indium activity present in the abdomen 5 minutes after ingestion. To determine colonic transit, two 5-minute acquisitions were performed 24 and 48 hours after ingestion of the radiolabeled water using the same single head gamma camera. To enable calculation of colonic transit, the gut was subdivided into three segments (i.e., 0 = small intestine; 1 = proximal colon; 2 = distal colon; 3 = stool). The center of mass model was applied expressing colonic transit as 24 and 48 hours postprandial geometrical center (GC) of activity. The primary variable of interest in overall colonic transit was the GC at 48 hours. The GC is the weighted average of counts in the different colonic regions: proximal-, distal colon and stool.
Quantification of the counts in each region was performed using a Hermes Gold software program (Hermes Medical Solutions, Stockholm, Sweden). At any time, the portion of colonic counts in each colonic region (corrected for background activity and isotope decay) was multiplied by the corresponding weighting factors as follows: GC = (% proximal colon x 1 + % distal colon x 2 + % stool x 3)/100. Thus, a high GC implies faster colonic transit. A GC of 0 implies that none of the isotope has reached the colon, and a GC of 3 implies that all isotope is in the stool. The amount of $^{111}$In-DTPA tracer defecated before day 4 was computed by subtraction of decay corrected abdominal counts on day 3 from total abdominal counts on day 1. Interpretation and calculation of gastric retention and colonic transit was performed blinded by two researchers independently (SvB & RJB) on a Hermes workstation.

Sample Size Calculation and Statistical Analysis
Since resumption of colonic motility occurs on postoperative days 3 through 5 and typically is the rate-limiting factor for the resolution of ileus (reviewed in 25), the colonic transit on day 3 was used as the primary efficacy parameter. Sample size calculation was based on earlier studies on GI transit after abdominal surgery15, 23 and indicated that 18 patients per group were required to detect a significant (P <0.05) difference in the median colonic transit between groups, corresponding to a probability of 0.22 (or less) that the colonic transit of a patient in the open surgery group is slower than the colonic transit of a patient in the laparoscopy group (using a Wilcoxon (Mann-Whitney) rank-sum test, providing 80% power. A similar effect on colonic transit day 3 was expected for FT care compared to standard care and a total sample size of 2x2x18=72 was expected to provide sufficient power for both comparisons. Per-protocol analysis was applied on all data as this study was designed and powered to detect a difference in postoperative GI transit between patients who actually received the treatment of interest and had no major surgical complications of the intestinal tract. Data were non-normally distributed and were expressed as median values and inter-quartile range (IQR). A Kruskal-Wallis test was performed to identify statistical differences between the four treatment groups. A two-sided $P$-value < 0.05 was considered to be statistically significant. If a significant difference was detected, the Mann-Whitney U test was used to conduct the five pairwise comparisons among the four treatment groups: i.e. Lap/Standard vs Open/Standard; Open/FT vs Open/Standard and Lap/FT to each of the other three groups. The Holm's sequential Bonferroni method was used to control for Type I error at the 0.05 level across the comparisons.
For categorical variables, treatment groups were compared by means of the Chi-square test or Fisher’s Exact test. For continuous normally distributed data, the ANOVA test was used and data were presented as means ± standard deviations (SD).
Multiple linear regression analysis was used to assess the effect of laparoscopy, FT care and the combination of both on the primary and secondary endpoints. As the measurements of the endpoints were skewed, these data were log-transformed. First the two main effects (laparoscopy and FT care) were incorporated in the multiple linear regression model, and subsequently the interaction term (laparoscopy * FT care) was added. The interaction term was not incorporated in the final model if no significant contribution occurred. As we log-transformed the endpoints the antilog of the parameters of the linear regression model can be interpreted as the fold increase between the geometric means of the laparoscopic versus open surgery groups, and FT versus standard care groups. Statistical analysis was performed using SPSS for Windows version 17 (SPSS Inc. Chicago, Ill., USA).

Results

Patients

Between October 2005 and August 2009 a total of 93 patients were randomly assigned to one of the four treatment groups. Fifteen patients (n=1 Lap/FT; n=3 Lap/Standard; n=6 Open/FT; n=5 Open/Standard) were withdrawn for various reasons: resection of the small intestine (n=2), delirium (n=1), fascial dehiscence (n=1), metastasis at time of admission (n=4), protocol violation (n=5) and withdrawal informed consent (n=2). GI transit was assessed in 78 patients. Seven of these had mechanical bowel obstructions or surgical complications requiring intervention within the first five days: three Lap/FT patients (internal herniation n=2 & adhesion n=1); one Lap/Standard patient (anastomotic leak); one Open/FT patient (anastomotic leak); two Open/Standard patients (intraperitoneal bleed & adhesion). Therefore, data of 71 patients without major surgical complications was available for per protocol analysis (supplementary Figure 1). In one patient laparoscopy had to be converted to open colectomy because of the extent of tumor invasion. Baseline characteristics and surgical aspects did not differ significantly between the four treatment groups, except duration of surgery ($P < .001$) (Table 1).
Gastric retention day 1

The residual gastric content two hours after ingestion of the pancake varied from almost complete emptying to gastric stasis with more than 99% of the radiolabeled pancake still present in the stomach. Multiple linear regression analysis showed no effect of laparoscopy or FT care on gastric retention (Table 2). The median gastric retention did not differ between groups ($P = .61$, Kruskal-Wallis test) (Figure 1a). Gastric retention was 70% (IQR: 36-94), 81% (34-95), 58% (26-71), 58% (31-100) in patients treated with Lap/FT, Lap/Standard, Open/FT and Open/Standard respectively.

| Table 1. Baseline Characteristics and Surgical Aspects of the Included Patients per Group |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
|                                             | Lap/FT (n = 18)                               | Lap/Standard (n = 17)                          | Open/FT (n = 18)                              | Open/Standard (n = 18)                        |
| Age (y)$^a$                                  | 65 ± 9.4                                      | 66 ± 10.2                                     | 64 ± 10.1                                     | 66 ± 6.9                                      |
| Male sex (%)                                 | 50                                            | 53                                            | 61                                            | 61                                            |
| Body mass index (kg/m$^2$)$^b$                | 26.5 ± 4.4                                    | 24.0 ± 3.9                                    | 24.6 ± 3.5                                    | 25.0 ± 3.6                                    |
| ASA grade I or II (%)                         | 89                                            | 88                                            | 89                                            | 89                                            |
| Comorbidity (%)                              | 72                                            | 53                                            | 56                                            | 50                                            |
| Type of colectomy (%)                        | 28                                            | 55                                            | 28                                            | 39                                            |
| Right-sided (%)                              | 6                                             | 14                                            | 17                                            | 22                                            |
| Left-sided (%)                               | 67                                            | 32                                            | 56                                            | 39                                            |
| Duration of surgery, median (IQR)            | 191 (176-205)                                 | 179 (157-202)                                 | 146 (122-180)                                 | 139 (122-165)                                 |

ASA, American Society of Anesthesiologists.
$^a$Values are mean ± SD.
$^b$Analysis of variance, $^c$$\chi^2$ test, and $^d$Kruskal-Wallis test. Per-protocol analysis of 71 patients.

<table>
<thead>
<tr>
<th>Colonic transit</th>
</tr>
</thead>
</table>
| The median calculated GCs of activity at day 2 are shown in Figure 1b. Colonic transit on day 2 was significantly faster in the Lap/FT group (2.2 (1.8-2.6) compared to the Lap/Standard (1.3 (1.2-2.0), $P = .015$), Open/FT (1.5 (1.2-1.9), $P = .008$), and Open/Standard group (1.1 (1.0-1.4), $P = .001$).
These data indicate that most of the radio-labeled material was still located in the proximal part of the colon at day 2 in the latter three groups, while in the Lap/FT group it had already moved to the distal part of the colon. On postoperative day 3, most of the radiolabel was still present in the proximal colon in the Open/Standard group, but had moved into the distal colon in the other three groups. The median GC for patients in the Lap/FT (2.6 (2.0-2.9) was significantly higher, compared to the Lap/Standard (2.2 (1.6-2.5), $P = .044$), Open/FT (2.0 (1.6-2.4), $P = .020$), and those in the Open/Standard care group (1.3 (1.0-1.5), $P < .001$) (Figure 1b).

Multiple linear regression analysis indicates that colonic transit on day 3 was significantly influenced by both laparoscopy (fold increase of 1.48; CI: 1.24-1.76, $P < .001$; i.e. the geometric mean colonic transit increased by 48% (CI: 24-76%) and FT care (fold increase of 1.38; CI: 1.16-1.65, $P = .001$; i.e. geometric mean colonic transit increased by 38% (CI: 16-65%) compared with patients receiving standard care. However, the combination of both (i.e. the interaction of laparoscopy with FT care) was not identified to add any benefit (Table 2).

Defecation of radiolabeled feces

In addition to assessment of colonic transit using GC calculation, we also measured the amount of radiolabeled feces evacuated 48 hours after intake of the $^{111}$In-DTPA tracer. The median percentage of $^{111}$In-DTPA tracer defecated was 70% (19-94) in the Lap/FT treated group compared to 37% (0-58), 26% (0-57) and 0% (0-0) in the Lap/Standard and Open/FT and Open/Standard treated groups respectively (Figure 1c).
Figure 1 | Postoperative gastrointestinal transit. Scintigraphic evaluation of gastrointestinal transit of an In-111 labeled non-caloric liquid test-meal and gastric emptying of a 99mTc labeled pancake 24 h after surgery. All data are expressed as the median ± interquartile range in corresponding treatment groups. The Kruskal-Wallis test was performed to identify statistical differences between the four treatment groups. The Mann-Whitney U test was used to compare the different treatment groups. P-values were calculated using Holm’s sequential Bonferroni adjustment for multiple (n=5) comparisons. P-value < 0.05 was considered statistically significant. NS, non-significant. Per protocol analysis of 71 patients; (Lap+FT: laparoscopy & Fast-track care (n=18), Lap+Stand: laparoscopy & Standard care (n=17), Open+FT: Open colectomy & Fast-track care (n=18), Open+Stand: Open & Standard care (n=18). (A) Scintigraphic evaluation of gastric emptying of a 99mTc labeled pancake 24 h after surgery. Gastric retention was determined 2 h after ingestion of a radiolabeled pancake, and is depicted as percentage in stomach compared to total abdominal region of interest. Bars represent percentage of gastric retention in the corresponding treatment group. Note that Kruskal-Wallis test identified no statistical difference between the four treatment groups. (B) Scintigraphic evaluation of colonic transit on postoperative day 2 and day 3. Colonic transit was determined 24 h (postoperative day 2) and 48 h (postoperative day 3) after ingestion of 60 ml radiolabeled tap water, and depicted as geometrical center (GC) of colonic contents. Bars represent the GC in the corresponding treatment group. Significant differences (Mann-Whitney U test) were observed for colonic transit day 2 between Lap+FT & Lap+Standard (0.015); Lap+FT & Open+FT (0.008); Lap+FT & Open+Stand (0.001); Lap+Stand & Open+Standard (0.032); Open+FT & Open+Standard (0.032). P-values calculated using Holm’s sequential Bonferroni adjustment for multiple (n=5) comparisons. (C) Amount of 111In-DTPA tracer defecated within 48 h (on postoperative day 3) after ingestion of 60 ml radiolabeled tap water. Data are depicted as percentage of the total amount of 111In-DTPA tracer ingested on day 1. Mann-Whitney U test was used for statistical analysis.

Clinical signs of recovery and clinical evaluation

The median time until the composite outcome of time until first defecation & tolerance of solid food was significantly shorter for patients who underwent the Lap/FT treatment compared to the other groups (Figure 2). Laparoscopic resection and FT care independently resulted in a significantly shorter time until tolerance of solid
food, and the composite of first defecation and tolerance of solid food (Table 2). The combination (i.e. the interaction) of both was not identified to add any benefit.

There was no significant difference on a per protocol basis between the groups in re-admission rate (\(P = .850\)) and overall morbidity (\(P = .217\)) until 30 days after surgery. A list of the complications categorized by grade and treatment group is available in supplementary Table 2.

Individualized perioperative fluid management was based on dynamic preload optimization and led to similar (\(P = .092\), Kruskal-Wallis test) intraoperative fluid loading in all four treatment groups with a median of 2.0 (IQR: 1.3-3.0) L. Overall compliance of surgical, anesthesiological and nursing care personnel with the multimodal perioperative rehabilitation pathway was very good. Fifteen FT items were evaluated per patient. The following items were scored if successfully applied; preoperative counseling, omission of bowel preparation, intake of carbohydrate-loaded drinks at the day before surgery, intake of carbohydrate-loaded drinks at the morning before surgery, no preoperative fasting since midnight, omission of premedication, thoracic epidural analgesia, prevention of hypothermia, adequate perioperative fluid loading, removal of nasogastric tube before extubation, omission of abdominal drains, suprapubic catheter or no catheter, more than 500 ml of intake at postoperative day (POD) 0 including 200 ml carbohydrate-loaded drink, mobilization for at least 15 min at POD 0, and starting with laxative at POD 1. In the Lap/FT group 12.5 ± 1.5 out of the 15 items and in the Open/FT group 10.9 ± 2.6 items were successfully applied per patient (supplementary Table 3). Some FT items have also been implemented in the standard care groups; in the Lap/Standard 5.2 ± 1.1 items and in the Open/Standard 4.8 ± 0.7 items per patient (mean ± sd). Other items (not mentioned in supplementary Table 3) applied in all groups were; prevention of hypothermia in 100% of the patients, removal of the nasogastric tube before extubation in 89%, and omission of abdominal drains in 87%.

Relationship between colonic transit and clinical recovery

Colonic transit is considered to mainly determine clinical recovery. The latter (i.e. time until ready for discharge) correlated significantly with colonic GC on day 3 (\(r = -0.56, P < .001\); Spearman’s rank correlation). The time until patients were ready for discharge was significantly shorter in patients who underwent the Lap/FT treatment compared to the Open/FT and Open/standard care group. Similarly, length of stay for patients in the Lap/FT (median 3.9 days) was significantly shorter, compared to the Open/FT (5.9 days, \(P = .002\)), and those in the Open/Standard care group (6.0 days, \(P = .001\)) (Figure 2D&E). Multiple linear regression analysis showed that laparoscopic resection and FT care resulted in a significantly shorter time until ready for discharge...
and hospitalization (Table 2). However, the combination (i.e. the interaction) of both was not identified to add any benefit.

**Figure 2** | Clinical (secondary) endpoints. Time until first defecation (panel A), tolerance of solid food (panel B), first defecation + tolerance of solid food (panel C), discharge (panel D) and ready for discharge (panel E) for Lap+FT, Lap+Standard, Open+FT and Open+Standard. All data are expressed as the median ± interquartile range in corresponding treatment group. The Kruskal-Wallis test was performed to identify statistical differences between the four treatment groups. The Mann-Whitney U test was used to compare the different treatment groups. P-values
calculated using Holm’s sequential Bonferroni adjustment for multiple (n=5) comparisons. P-value < 0.05 was considered statistically significant. NS, non-significant. Per protocol analysis of 71 patients; (Lap+FT: laparoscopy & Fast-track care (n=18), Lap+Standard: laparoscopy & Standard care (n=17), Open+FT: Open colectomy & Fast-track care (n=18), Open+Standard: Open & Standard care (n=18).

Discussion

This study provides objective data indicating that laparoscopic surgery and FT care improve recovery of GI transit compared to open colectomy and standard care, contributing to faster clinical recovery. In a multiple linear regression analysis, both laparoscopic surgery and FT care were found to be significant predictors of improved colonic transit, and reduced time to tolerance of solid food & bowel movement. These data suggest that laparoscopic resection and FT care lead to faster recovery of GI transit after colorectal surgery.

Previously published clinical trials have shown a beneficial effect of laparoscopic procedures and FT care on the duration of POI.6,11-13 In our study clinical hallmarks were not the primary outcome measures for POI as these are less accurate and reliable to objectively evaluate the effect of different treatment strategies. Parameters such as nausea, vomiting and tolerance of solid food strongly depend on patient reporting, whereas first passage of stool or flatus may simply reflect rectal emptying and therefore not necessarily adequately reflect recovery of effective GI motility. In the present study, we therefore assessed GI transit, i.e. gastric retention and colonic transit, after surgery using scintigraphy. We observed major differences in colonic transit between the 4 different groups. Especially patients in the Lap/FT group had a significantly higher GC measured at 24 and 48 hours after ingestion of indium-111 labeled water. On the other hand, colonic transit was slowest in patients who underwent conventional surgery and postoperative care. In line with these data, 70% of the ingested 111In-tracer was defecated 48 hours after ingestion in Lap/FT patients, whereas no 111In-tracer had been excreted in the Open/Standard group. Multiple linear regression analysis indeed indicated laparoscopic surgery and FT care as predictive factors of faster colonic transit. Our scintigraphic data support the outcome of the multi-centre LAFA trial, i.e. the main trial of which this study was a substudy. This study demonstrated a quicker passage of first stool and tolerance of solid food after laparoscopy and FT care, and showed that the combination of laparoscopic surgery with FT care resulted in a significantly faster recovery than all other treatment combinations.26 Moreover, we also observed shorter time until the composite of first defecation & tolerance of solid food.

Our study is the first providing objective scintigraphic data supporting improved clinical recovery after laparoscopic surgery and FT care. Only one study has previously
evaluated GI transit after open and laparoscopic colectomy within a FT care program. This study however did not compare FT care with conventional perioperative care. Similar to our study, Basse et al. detected a significantly higher colonic transit in the laparoscopic group. However, no significant difference in the amount of $^{111}$In-tracer defecated was reported. It should be emphasized though that the time window of 48 hours after surgery may have been too short to detect differences in fecal $^{111}$In-tracer since colonic motility remains impaired until 48-72 hours after surgery.

In contrast to improvement in colonic transit, no significant effect of laparoscopy or FT was detected on gastric emptying. Gastric emptying of a radiolabeled pancake was determined 24 hours after surgery and varied considerably; some patients had emptied the stomach almost completely, whereas in others more than 99% of the pancake was still present in their stomach. Our data are in line with the study by Gelpi et al. that also failed to demonstrate differences in gastric emptying between the open- and laparoscopic treated patients. A possible explanation for the discrepancy between gastric and colonic transit data could be the fact that gastric emptying was assessed earlier after surgery. 24 hours after surgery, one may anticipate that motility is still largely impaired irrespective of the type of surgery or perioperative care. In other words, the effect of postoperative stress, pain, anesthesia and other factors related to the surgical procedure in the early postoperative period most likely have a major impact on GI function overriding the potential benefit of laparoscopy or FT care. Alternatively, differences in dosage of opioids or anti-emetics in the perioperative period may have obscured the beneficial effect of laparoscopy and FT care on gastric emptying. However, the amount of analgesics and anti-emetics did not differ between the different groups making the latter explanation less likely (supplementary Table 4).

The pathophysiological mechanisms involved in POI are still not completely understood, but recent studies have stressed the importance of inflammation of the intestinal muscularis resulting from surgical handling. The faster clinical recovery observed after laparoscopic surgery compared with open surgery could be explained by decreased tissue trauma with concomitant decreased mast cell activation leading to attenuated intestinal inflammation and thus a quicker GI recovery. The mechanisms behind the beneficial effect of the FT program remain unclear, but in a rat model enteral feeding was shown to improve postoperative GI transit through activation of the vagus nerve mediated anti-inflammatory pathway. To what extent vagal activation in response to early feeding may explain improved transit in the FT group in our study remains to be studied.

A limitation of our study is the omission of baseline GI transit measurements. This design would have allowed comparison before and after surgery in each individual patient, possibly reducing the variability and increasing the power of the study. However
such a design would imply an even larger burden to our patients, and moreover, would have required earlier hospitalization of at least 24 hours to perform the baseline recording and avoid interference with the postoperative scintigraphy. Nevertheless, our study is the first to assess GI transit in a large cohort of postoperative patients. Moreover, in contrast to previous studies, early postoperative gastric emptying was assessed after intake of a solid meal, which is superior to liquid emptying to demonstrate diminished gastric motility. Ideally, to compare both gastric emptying and colonic transit, gastric emptying should have been measured at the same time colonic transit was determined. In practice, performing additional gastric emptying tests on the clinically more relevant days 2 and 3 after surgery is not possible because of technical and ethical reasons. Secondly, compared to previous studies, our re-operation rate was relatively high. One explanation might be that surgery was performed in an academic hospital where residents/fellows perform the procedure assisted by a (laparoscopic) colorectal surgeon. However, we do not expect that the relatively high re-operation rate has biased the comparisons on intestinal transit between the four groups as there was no significant difference in the re-operation rate between the FT care and standard care group, or the laparoscopic and open groups. Thirdly, although all measures were installed to prevent that the treating physicians and nurses on the ward were informed on the type of surgery, it remains difficult to obtain a truly blinded study design. It should be emphasized though that the discharge criteria were strictly defined and the perioperative care protocols were protocolized in great detail. The adherence to the treatment protocols was high thus limiting the influence of the attending physicians and nurses on postoperative care.

In conclusion, our study demonstrates that laparoscopic surgery and the FT care program lead to a significantly faster recovery of GI transit. These data provide objective evidence confirming the faster clinical recovery with this perioperative strategy.

Acknowledgements

The authors thank F.J. Slors †, M. van Berge Henegouwen, D.J. Gouma, the GI surgery fellows, and the nursing staff for their support and enthusiasm in realizing this study in the department of Surgery. We are grateful to the department of anesthesiology for guarding a standardized perioperative care and postoperative analgesia protocol. We thank B. Braak, J. Wind, A. lei, O. Ayubi, S. El Temna for the assistance, and N. van Geloven for the advice during the statistical analysis. We acknowledge J.W. de Jong, M. Spaeth and the Nuclear Medicine technicians and staff for the preparation of the scintigraphically labeled test meals.

Trial Registration: http://www.trialregister.nl; identifier: NTR1884
### Supplementary Table 1. Treatment Protocols

<table>
<thead>
<tr>
<th></th>
<th>Standard care</th>
<th>FT care</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preoperative phase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outpatient department of surgery</td>
<td>Scheduling of operation, informed consent</td>
<td>Scheduling of operation, informed consent plus information about the FT program</td>
</tr>
<tr>
<td>Outpatient department of anesthesia</td>
<td>Preassessment for risk adjustment, open discussion about different possibilities for management of perioperative analgesia</td>
<td>Preassessment for risk adjustment, discussion focusing on placement of thoracic epidural catheter for management of perioperative analgesia plus discussion of the essence of the FT program, yes</td>
</tr>
<tr>
<td>Preadmission counseling and guided tour on surgical ward</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Day of admission</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intake</td>
<td>Routine</td>
<td>Additional FT information</td>
</tr>
<tr>
<td>Bowel preparation</td>
<td>Only enema</td>
<td>Only enema</td>
</tr>
<tr>
<td>Preoperative carbohydrate-loaded liquids</td>
<td>No</td>
<td>4 units (pre-Op)</td>
</tr>
<tr>
<td>Diet</td>
<td>Last meal until midnight</td>
<td>Last meal 6 h before operation</td>
</tr>
<tr>
<td>Anesthetic evening medication</td>
<td>Lorazepam 1 mg or temazepam 1.0 or 20 mg</td>
<td>Lorazepam 1 mg the evening before operation, if necessary</td>
</tr>
<tr>
<td>Day of surgery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preoperative fasting</td>
<td>Yes</td>
<td>No, 2 units CHL 2 h before surgery</td>
</tr>
<tr>
<td>Preanesthetic medication</td>
<td>Yes, lorazepam 1 mg or midazolam 7.0 mg</td>
<td>No, 7.0 mg</td>
</tr>
<tr>
<td>Anesthetic management</td>
<td>Placement thoracic epidural conform IT group, or lower level or PCA pump</td>
<td>Placement thoracic epidural (IT8-T10 depending on the surgical resection), test dose (bupivacaine 0.25% with adrenaline 1:200,000), topup dose (bupivacaine 0.25% [± 10 mL] with sufentanil 25 μg, followed by continuous infusion (bupivacaine 0.125% with fentanyl 2.5 μg × mL⁻¹) until day 2 postoperative, yes</td>
</tr>
<tr>
<td>Surgical management</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laparoscopy approach/laparotomy</td>
<td>Combined with balanced general anesthesia</td>
<td>Combined with balanced general anesthesia</td>
</tr>
<tr>
<td>Urine catheter according to attending surgeon</td>
<td>Standard preoperative fluid infusion regimen (Ringer’s lactate 20 mL × kg⁻¹ in the first hour followed by 10–12 mL × kg⁻¹ × h⁻¹)</td>
<td>Restricted preoperative fluid infusion regimen (Ringer’s lactate 20 mL × kg⁻¹ in the first hour followed by 6 mL × kg⁻¹ × h⁻¹)</td>
</tr>
<tr>
<td>No infiltration of surgical wounds with local anesthetic drugs</td>
<td>Use of extra fluid challenge as first choice for management of mean blood pressure drop &gt; 20% below baseline</td>
<td>Use of vasopressors as first choice for management of mean blood pressure drop &gt; 20% below baseline</td>
</tr>
<tr>
<td>No standard use of abdominal drains</td>
<td>Forced body heating (_ulong hugger system and warmed IV fluids)</td>
<td>Forced body heating (铢 hung system and warmed IV fluids)</td>
</tr>
<tr>
<td>Early postoperative management</td>
<td>Removal of nasogastric tube before extubation</td>
<td>Removal of nasogastric tube before extubation</td>
</tr>
<tr>
<td>Epidural or PCA morphine to which paracetamol 4 × 1 g × day⁻¹ and/or dextrose 3 × 50 mg × day⁻¹ are added</td>
<td>Use of epidural catheter as mentioned before to which paracetamol 4 × 1 g × day⁻¹ is added</td>
<td>Prophylactic use of ondansetron (4 mg) to prevent PONV</td>
</tr>
<tr>
<td>Small amount of water orally</td>
<td>IV Infusion of Ringer’s lactate 2.5 L × day⁻¹</td>
<td>Infusion of water orally</td>
</tr>
<tr>
<td>IV fluid administration (2.5 L × day⁻¹) is continued until adequate oral fluid intake</td>
<td>No mobilization scheme</td>
<td>Mobilization in the evening (&gt;2 h out of bed)</td>
</tr>
<tr>
<td>Day 1 after surgery Postoperative management</td>
<td>Oral intake &gt; 2 L (including 4 units CHL liquids)</td>
<td>First semi-solid food intake in the evening</td>
</tr>
<tr>
<td>Diet increased on daily basis</td>
<td>Normal diet</td>
<td>Std IV fluid administration (leave canula)</td>
</tr>
<tr>
<td>IV fluid administration (2.5 L × day⁻¹) is continued until adequate oral fluid intake</td>
<td>Stop IV canula</td>
<td>Start laxative (MgO, 2 × 1 g × day⁻¹)</td>
</tr>
<tr>
<td>Mobilization according to attending surgeon</td>
<td>Mobilization in the evening (&gt;2 h out of bed)</td>
<td>Clean suprapubic urethral catheter and remove when residue &lt; 50 mL</td>
</tr>
<tr>
<td>Day 2 after surgery Postoperative management</td>
<td>Epidural removed according to attending anesthesiologist</td>
<td>Expanding mobility (&gt;6 h out of bed)</td>
</tr>
<tr>
<td>Epidural removed according to attending anesthesiologist</td>
<td>Continue as on day 1 until discharge criteria fulfilled</td>
<td>Remove epidural; add dextrose 3 × 50 mg × day⁻¹</td>
</tr>
<tr>
<td>Continue as on day 2 until discharge criteria are fulfilled</td>
<td>Remove IV canula</td>
<td>Remove IV canula</td>
</tr>
<tr>
<td>Day 3 after surgery Postoperative management</td>
<td>Continue as on day 2 until discharge criteria are fulfilled</td>
<td>Continue as on day 2 until discharge criteria are fulfilled</td>
</tr>
</tbody>
</table>

CHL, carbohydrate loaded; IV, intravenous; MgO, magnesium oxide; PCA, patient-controlled anesthetics; PONV, postoperative nausea and vomiting.
### Supplementary Table 2. Postoperative Complications and Readmissions Within 30 Days After Surgery

<table>
<thead>
<tr>
<th>End point</th>
<th>Lap/FT (n = 18)</th>
<th>Lap/Standard (n = 17)</th>
<th>Open/FT (n = 18)</th>
<th>Open/Standard (n = 18)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rehospitalization</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.850</td>
</tr>
<tr>
<td>Patients with a complication (%)a</td>
<td>1 (11)</td>
<td>2 (29)</td>
<td>1 (39)</td>
<td>2 (39)</td>
<td>.217</td>
</tr>
<tr>
<td>Grade I</td>
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<td>1</td>
<td>3</td>
<td>3</td>
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<tr>
<td>Grade II</td>
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<td>2</td>
<td>2</td>
<td></td>
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<tr>
<td>Grade III</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>Grade IV</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Surgical complicationsb</td>
<td>1</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>General complicationsb</td>
<td>2</td>
<td>7</td>
<td>7</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE.** Total complications occurring until 30 days after surgery.

a The severity of complications was graded I to V, counting the severest complication per patient. Fisher exact test was used for statistical analysis to compare the different treatment arms. Per-protocol analysis of 71 patients.

b More than 1 complication was counted per patient.

### Supplementary Table 3. Protocol Compliance

<table>
<thead>
<tr>
<th>Preoperative phase, yes (%)</th>
<th>Lap/FT (n = 18)</th>
<th>Lap/Standard (n = 17)</th>
<th>P value for Lap/FT vs Lap/Standard</th>
<th>Open/FT vs Open/Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preoperative counseling</td>
<td>100 (100)</td>
<td>0 (0)</td>
<td>&lt;.001a</td>
<td>&lt;.001b</td>
</tr>
<tr>
<td>Intake of CHL, day before surgery (l), median (QR)</td>
<td>0.80 (0.40-0.80)</td>
<td>0.00 (0.00-0.00)</td>
<td>&lt;.001a</td>
<td>0.80 (0.00-0.80)</td>
</tr>
<tr>
<td>Day of surgery, yes (%)</td>
<td>Pad (1500-1000)</td>
<td>78 (13)</td>
<td>&lt;.001a</td>
<td>67 (31)</td>
</tr>
<tr>
<td>Effective thoracic epidural analgesia</td>
<td>49 (6)</td>
<td>49 (6)</td>
<td>&lt;.001a</td>
<td>49 (6)</td>
</tr>
<tr>
<td>Intake of CHL 2 hours before surgery (l), median (QR)</td>
<td>0.3 (0.30-0.40)</td>
<td>0.00 (0.00-0.00)</td>
<td>&lt;.001a</td>
<td>0.4 (0.00-0.40)</td>
</tr>
</tbody>
</table>

**Preoperative phase, yes (%)**

a Separate consultation before admission and/or FT trial nurse to discuss the essence of the FT program.

b Calculated by Fisher exact test, t-test, and Mann-Whitney U test.

### Supplementary Table 4. Consumption of Analgesics and Antiemetics

<table>
<thead>
<tr>
<th>Cumulative antiemetic consumption on POD 0 (mg)</th>
<th>Lap/FT (n = 18)</th>
<th>Lap/Standard (n = 17)</th>
<th>Open/FT (n = 18)</th>
<th>Open/Standard (n = 18)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cumulative morphine sulfate consumption during intraoperative surgery (mg)</td>
<td>9.8 ± 11.2</td>
<td>12.1 ± 9.6</td>
<td>9.9 ± 9.1</td>
<td>19.9 ± 24.2</td>
<td>.174</td>
</tr>
<tr>
<td>Cumulative morphine equivalent consumption until POD 5 (mg)</td>
<td>108.1 ± 32.4</td>
<td>93.7 ± 54.0</td>
<td>135.2 ± 67.9</td>
<td>119.4 ± 46.9</td>
<td>.165</td>
</tr>
</tbody>
</table>

**NOTE.** Values are expressed as mean ± SD unless otherwise noted. The antiemetic doses include the ondansetron equivalent doses of droperidol (0.625 mg of droperidol equals 4.0 mg of ondansetron). The morphine doses include the parenteral morphine equivalent doses of fentanyl, sufentanil, and tramadol (equianalgesic doses according to Foley19). Per-protocol analysis of 71 patients. The analysis of variance test was used for statistical analysis.
References

Chapter 9

Summary and Conclusions
Patients undergoing an abdominal surgical procedure develop a transient episode of impaired gastrointestinal motility or non-obstructive ileus. Postoperative ileus, characterized by impairment of coordinated propulsive intestinal motility, remains an almost inevitable consequence of surgery. Importantly, postoperative ileus is a major determinant of recovery after intestinal surgery and leads to increased morbidity and prolonged hospitalization, which is a great economic burden to health-care systems. Although a variety of strategies reduce postoperative ileus, none of these methods have been completely successful in shortening the duration of postoperative ileus. The aetiology of postoperative ileus is multifactorial, but activation of a local inflammatory response within the intestinal muscularis externa has become an accepted pathophysiological mechanism. The importance of this inflammatory response in postoperative ileus is underscored by the beneficial effect of pharmacological interventions that block the influx of leukocytes. In this thesis, we describe our latest insights into the mechanisms behind postoperative ileus and highlight new strategies to intervene in the postoperative inflammatory cascade.

Postoperative ileus is characterized by a transient inhibition of transit of gastrointestinal content after abdominal surgery. Currently, intestinal manipulation of the intestine in rodents is widely used as a preclinical model of postoperative ileus. In Chapter 2 we developed a new technique, using a purpose-designed device, to manipulate the intestine in a more controlled manner (Figure 1). Gastrointestinal transit, measured by evaluating the intestinal distribution of orally gavaged fluorescent labeled dextran, was used as a read-out to determine the degree of postoperative ileus. Our standardized manipulation technique resulted in a pressure-dependent decrease in intestinal transit with small variation and was associated with inflammation of the intestinal muscularis. This novel method provided a methodologically convenient and useful model to study the potential of new anti-inflammatory strategies (Chapter 5) in a reliable and adequately controlled manner.

The exact underlying molecular and cellular mechanisms of postoperative ileus are still under investigation. Animal models suggest that both neuronal and local inflammatory responses within the intestinal muscularis mechanismically contribute. The neuronal mechanism appears to involve the enhanced release of nitric oxide from inhibitory motor neurons. Likewise, nitric oxide and prostaglandins are released from inflammatory cells (macrophages and monocytes) via the induction of nitric oxide synthase (iNOS) and cyclooxygenase-2. The influx of leukocytes is not limited to the handled region, i.e. the small intestine, but is also present in the colon. Interestingly, we found that the severity of ileus results from an inflammatory response that is independent of the number of leukocytes infiltrating the small intestinal muscularis (Chapter 2), indicating that other mechanism are responsible for the more severe ileus.
These data bring forward the hypothesis that tissue trauma induced immune response via damage-associated molecular patterns may be involved in the pathophysiology of more severe ileus. In this line, enhanced local inflammation may result in a systemic inflammatory response and consequently trigger enhanced brain stem activation. To elucidate the contribution of tissue damage in the severe ileus, we analysed the local intestinal and systemic inflammatory response along with brain stem nuclei activation after different intensities of intestinal handling (Chapter 3). We showed that the manipulation-induced tissue damage led to not only an enhanced intestinal inflammatory response but also the release of pro-inflammatory molecules in the bloodstream. This tissue damage induced enhanced inflammatory response was also associated with enhanced brain activation in mice and correlated with the severity of postoperative ileus in both humans and mice. Together, our data provide evidence of an additional mechanism by which tissue damage mediators and pro-inflammatory cytokines released into the systemic circulation contribute to the impaired motility of non-manipulated intestine. Secondly, we demonstrated that severe ileus and tissue damage results in activation of brain stem areas such as the area postrema, most likely explaining the sickness behavior associated with postoperative ileus. To what extent this further contributes to the general hypomotility in postoperative ileus deserves further attention in future studies. Insights into how tissue damage triggers the release of systemic cytokines should aid in the development of therapeutics to prevent this response.

Both in humans and animals abdominal surgery has shown to affect the integrity of the intestinal epithelial barrier and may potentially influence postoperative recovery. Studies have enlightened the importance of mast cells in the regulation of the epithelial barrier in diverse intestinal inflammatory settings. Whether the integrity of the epithelial barrier is relevant or not in the pathogenesis of postoperative ileus is not fully proven, but irrespective thereof, the clinical impact of bacterial translocation during surgery is significant. In fact, in the course of abdominal surgery, barrier dysfunction has been associated with increased postoperative septic morbidity in surgical patients undergoing laparotomy. In Chapter 4 we describe that activated mast cells evoke a disturbance of intestinal barrier function. To this end we performed our experiments in two mast cell deficient mouse strains. We observed that intestinal manipulation during abdominal surgery in mice resulted in a mast cell dependent inflammation and barrier dysfunction. These data underscore the importance of mast cells in the pathogenesis of postoperative ileus and the potential of mast cell stabilizers to shorten the duration of postoperative ileus. In addition to activation of mast cells, the pathogenesis of postoperative ileus involves the activation of macrophages and dendritic cells that reside in the muscularis externa (Figure 2). The influx of bacteria and their antigens
across the epithelial barrier following intestinal manipulation may activate these cells. Alternatively, IL-1 that is quickly released as a response to tissue damage may be of importance in intestinal manipulation induced inflammation. Whether recognition of translocated bacteria is crucial in the initiation of surgically induced ileus or only plays a role in the perpetuation of ileus is currently under investigation.

Figure 2 | CD68 staining for macrophages and infiltrating monocytes in the muscle layer of small intestine. Whole mounts of small intestinal muscularis stained with anti-CD68 antibody at 200X magnification. Control laparotomy mouse (a); Laparotomy plus eventration small intestine and caecum without standardized pressure manipulation (b); Mouse with postoperative ileus after 9 grams standardized pressure manipulation resulting in post (c).

In the gut, cholinergic fibers are located in close proximity to immune cells and is therefore the ideal site for neuro-immune modulation. In the last decade, vagus nerve stimulation has been shown to dampen immune responses in a number of disease models; this is referred to as the ‘cholinergic anti-inflammatory pathway’. Recently, our group demonstrated that electrical stimulation of the vagus nerve in mice improves intestinal transit and dampens intestinal muscular inflammation through alpha 7 nicotinic acetylcholine receptor (α7nAChRs) expressed on resident macrophages. The cholinergic anti-inflammatory pathway is proposed to be part of the so-called vago-vagal ‘inflammatory reflex’. Inflammation is sensed by afferent nerve fibers and is subsequently relayed to the brain. After integration of afferent information, the motor neurons of the vagus nerve are activated and an integrated anti-inflammatory signal is sent back to the inflamed area. We provided the neuro-anatomical evidence that the
Figure 3 | The vagal anti-inflammatory pathway and postoperative ileus. The cholinergic anti-inflammatory reflex relies on activation of the vagal sensory afferents that subsequently creates an integrated anti-inflammatory response through vagal efferent fibres. In postoperative ileus, activation of the vagal sensory afferent is triggered by pro-inflammatory cytokines released within the muscle layers. Vagal afferents will trigger neuronal activity in the NTS (brainstem nuclei that receive visceral information) which results in the activation of motor neurons of the vagus nerve (located in the DMV). Once activated, the vagal efferent fibres release acetylcholine at the level of the myenteric plexus stimulating cholinergic enteric neurons. These neurons amplify vagal signalling by releasing acetylcholine in the muscle layer where the macrophages reside. Binding of acetylcholine to α7 nicotinic receptors on macrophages suppresses the release of pro-inflammatory cytokines. Stimulation of the sensory afferent limb of the neuronal circuit by enteral high fat diet through cholecystokinin release or electrical stimulation of the motor efferent limb of the vagus nerve prevent intestinal inflammation and ileus. Intra-cerebroventricular injection of semapimod (CNI 1493), acetylcholinesterase inhibitor (galantamine), ghrelin and muscarinic receptor 1 agonists (McN-A 343) also activate vagal efferents thereby suppressing inflammation. Abbreviations: Ach: acetylcholine; DMV: dorsal motor nucleus of the vagus; NTS: nucleus of the solitary tract VNS: vagal nerve stimulation.

vagal feedback loop is activated in course of local intestinal inflammation (Chapter 6), offering new approaches to modulate undesired inflammatory processes (Figure 3). Currently, we are studying whether intra-operative electrical stimulation of the intra-abdominal vagus nerve reduces the inflammatory response to abdominal surgery and can shorten the duration of postoperative ileus in patients undergoing open rectal resections. In addition, a multicenter trial is currently investigating if stimulating the vagus nerve in the neck of patients with rheumatoid arthritis can decrease joint inflammation.
In the second part of this thesis, we evaluated potential therapeutic strategies for postoperative ileus and its clinical aspects. With novel treatments for postoperative ileus in development, there is a definite need for reliable outcome measures to evaluate clinical success in new drug trials. Up to date validated clinical hallmarks of gastrointestinal recovery to evaluate new treatments and readiness for discharge from the hospital were lacking. In Chapter 7, we established what clinical hallmarks best identify recovery of gastrointestinal transit after intestinal surgery. We objectively determined colonic transit using scintigraphy and established that clinical recovery following abdominal surgery is indeed associated with recovery of colonic transit. Using the latter as objective criterion for clinical improvement, we showed that the combined hallmark of tolerance of solid food and having had defecation best predicted clinical recovery and indicates readiness for discharge. In contrast, time to first flatus was not associated with recovery of colonic transit or time to discharge. Our next step was to validate the outcome parameter tolerance of solid food and having had defecation in the large cohort of patients of the multicenter LAFA trial. These analyses confirmed that the presence of both clinical parameters is the best clinical marker of gut recovery and should be preferred as primary outcome measure in future clinical trials on postoperative ileus.

Postoperative ileus is a major determinant of recovery after colorectal surgery. Recent studies suggest substantial improvements in perioperative care (namely multimodal enhanced recovery programs and laparoscopic surgery), resulting in faster recovery of the gut and shorter hospital stay during the last decade. However, objective measures supporting faster gastrointestinal recovery are lacking. In Chapter 8 we demonstrated faster recovery of gastrointestinal transit after laparoscopic surgery and the fast-track program, providing objective data that laparoscopy and fast-track care lead to faster recovery of motility and concomitant enhanced clinical recovery. The faster clinical recovery observed after laparoscopic surgery compared with open surgery could be explained by decreased tissue trauma with concomitant decreased immune cell activation leading to attenuated intestinal inflammation and thus a quicker gastrointestinal recovery. The mechanisms behind the beneficial effect of the fast-track program remain unclear, but in a rat model enteral feeding was shown to improve postoperative gastrointestinal transit through activation of the vagus nerve-mediated anti-inflammatory pathway. Correspondingly, within the fast-track program patients are not only mobilized faster but also earlier resume oral feeding. This last observation leads us to speculate that feeding may activate the vagal anti-inflammatory pathway contributing to faster recovery in these patients. To what extent vagal activation in response to early feeding may explain improved transit in the fast-track groups in this randomized double-blind study remains to be studied. Despite the apparent
effectiveness of the fast-track approaches, this approach has not been fully implemented in the majority of surgical wards. Strategies to increase the implementation of these fast-track approaches should be encouraged. If this implementation can be achieved, it will markedly affect the study design of clinical trials as new therapeutic strategies being tested will have to show clinical benefit in the setting of a fast-track programme.

Finally, in Chapter 5 we describe a novel anti-inflammatory strategy to improve postoperative ileus in mice. We evaluated whether Spleen tyrosine kinase (Syk), one of the critical intracellular tyrosine kinases involved in mast cell degranulation and macrophage activation, can be a potential target for intestinal inflammation. As both mast cells and macrophages are involved in the pathophysiology of postoperative ileus, we established whether blockade of Syk could represent an alternative approach to inhibit immune cell activation evoked by intestinal manipulation and thus represent a new tool to shorten postoperative ileus. We showed here that the potent and highly selective Syk inhibitor GSK143 prevented FceRI and Substance P induced mast cell degranulation and endotoxin-induced macrophage activation in vitro. Secondly, in vivo GSK143 dampened the inflammatory cascade leading to postoperative ileus, without exerting a direct effect on gastrointestinal motility as it did not affect intestinal transit in mice that underwent laparotomy. Taken together, inhibition of Syk significantly reduced the inflammatory response to intestinal manipulation thereby preventing postoperative ileus. This suggests that Syk can be a potential target for intestinal inflammatory diseases and may be a new tool to shorten postoperative ileus.

**Future Perspectives**

In this thesis, new insights into the pathogenesis of postoperative ileus were revealed, which has led to the identification of new targets for treatment and novel therapeutic approaches. This includes Syk-inhibitors as novel anti-inflammatory strategy, and the implementation of multimodal postoperative rehabilitation (fast-track care) and minimally invasive surgery.

A clear implication of the data presented here is that it is of utmost importance to limit the amount of tissue damage. The identification of exact trigger that initiates the inflammatory response (mast cell activation, tissue damage, bacterial translocation) and more insight into how this inflammatory response spreads to the rest of the (unmanipulated) intestine should aid in the development and evaluation of therapeutics. Beyond this, we do not understand what controls the postoperative systemic inflammatory response and how this modulates the central nervous system and motility. Given the medical importance of the inflammatory response in postoperative ileus, these are key issues that must be addressed.
Recently, the group of Kalff discovered that besides the innate, also the adaptive immune system is triggered in response to bowel manipulation. It is suggested that this is mediated through the activation of dendritic cells that secrete IL-12 at the site of intestinal manipulation thereby stimulating memory Th1 cells in the inflamed muscularis, which subsequently egress into the systemic circulation to migrate to distant non-manipulated areas of the intestine. There, they release IFN-γ thereby inducing resident muscularis macrophages to produce NO and inflammatory cytokines. Depleting dendritic cells, the use of immunosuppressants such as anti-IL-12 antibodies or inhibiting Th1 cell migration by FTY-720 could reduce postoperative ileus, although the efficacy of these approaches still first have to be investigated in humans.

Taken together, a new avenue of potential targets for treatment has opened. Inhibiting intestinal macrophage or mast cell function by Syk modulation, or intervening in the adaptive immune response and systemic spread of inflammation might reduce the duration of postoperative ileus in patients following abdominal surgery. In addition interventions that activate the cholinergic anti-inflammatory pathway might also embody an attractive therapy.

From our work, it is becoming increasingly clear that new treatments of postoperative ileus should try to reduce the inflammatory response evoked by intestinal handling. Such anti-inflammatory strategies may however interfere with wound healing, the defense against micro-organism and ultimately with the clinical recovery of patients. Therefore it is important that before introduction of promising anti-inflammatory therapies in the clinic, their effect on wound healing in animal models is investigated. Another aspect that has to be taken into account when studying postoperative ileus is that the susceptibility to postoperative ileus following abdominal surgery increases with advancing age. There is both an age-dependent increase in the pro-inflammatory mediator expression and an age-dependent decrease in anti-inflammatory mediator expression following minor insult to the bowel in rodents. To what extent such imbalances between pro- and anti-inflammatory mechanisms may form the basis for increased susceptibility to ileus and for the increased severity and duration of ileus observed in the elderly is unknown. As more and more elderly will need abdominal surgery in the future, hopefully our insight on the role of altered inflammatory gene expression with advancing age in postoperative ileus will increase.

In summary, the data presented in this thesis have provided considerable new insight into the pathogenesis of postoperative ileus and have identified new therapeutic targets and strategies. Our work is also a direct plea for minimal invasive surgery as our data clearly indicate that intestinal handling and tissue injury during surgery should be avoided as much as possible.
Samenvatting en Conclusies
Patiënten die een abdominale chirurgische procedure ondergaan, ontwikkelen een episode van verminderde peristaltiek van het maag-, darmstelsel. Postoperatieve ileus wordt gekenmerkt door vermindering van gecoördineerde peristaltiek van de darmen en is een bijna onvermijdelijk gevolg van operaties. Het is zelfs een van de belangrijkste determinanten voor de duur tot herstel na een operatie aan de darmen. Dit leidt tot een toename van de morbiditeit en het verblijf in het ziekenhuis en leidt daarmee tot een aanzienlijke stijging van de kosten van de gezondheidszorg. Hoewel er verschillende strategieën zijn die de duur van postoperatieve ileus verkorten, is geen van deze strategieën volledig succesvol in het voorkomen van postoperatieve ileus.

De etiologie van postoperatieve ileus is multifactorieel, waarbij de lokale ontstekingsreactie in de spierlaag van de darmen inmiddels algemeen is geaccepteerd als belangrijk mechanisme in de pathofysiologie van postoperatieve ileus. De enorme impact van deze ontstekingsreactie wordt benadrukt door het gunstige effect van farmacologische interventies die de influx van leukocyten (witte bloedcellen) blokkeren.

Dit proefschrift beschrijft de nieuwste inzichten in de mechanismen van postoperatieve ileus en nieuwe strategieën om in te grijpen in de postoperatieve ontstekingscascade. Postoperatieve ileus wordt gekenmerkt door een tijdelijke remming van de maag-, darmfunctie na een buikoperatie. Momenteel wordt manipulatie van de darmen bij knaagdieren veel gebruikt als een preklinisch model voor postoperatieve ileus. In Hoofdstuk 2 beschrijven we een nieuwe techniek om de darmen op een meer gecontroleerde manier te manipuleren. Hierbij hebben we gebruik gemaakt van een speciaal ontworpen apparaat (Figuur 1). Voor bestudering van de transportfunctie van het maag-, darmkanaal wordt fluorescent gelabelde dextran na de operatie oraal toegediend en dient zodoende als parameter om de mate van postoperatieve ileus te bepalen. Deze gestandaardiseerde manipulatietechniek resulteerde in een controleerbare drukafhankelijke afname van de darmtransit (voortstuwen van de darminhoud) en leidde tot ontsteking van de intestinale muscularis (de spierlaag in de darmwand). Daarmee verschafte deze nieuwe methode een methodologisch geschikt en nuttig model om het potentiële effect van nieuwe anti-inflammatoire strategieën op een betrouwbare en voldoende gecontroleerde manier te bestuderen.

De laatste jaren is er veel onderzoek gedaan naar de onderliggende moleculaire en cellulaire mechanismen van postoperatieve ileus. Diermodellen suggereren dat zowel neuronale als lokale ontstekingsreacties in de intestinale muscularis van belang zijn. De verhoogde afgifte van stikstofmonoxide door motorische neuronen heeft een remmende werking op de maag- en darm- peristaltiek. Ook draagt de productie van stikstofmonoxide en prostaglandinen door ontstekingscellen zoals macrofagen en monocytten bij aan de verstoring van de peristaltiek. De influx van leukocyten is niet beperkt tot het gemanipuleerde gebied, d.w.z. de dunne darm, maar is ook aanwezig in
de dikke darm. Onze data illustreren dat de ernst van ileus het gevolg is van een ontstekingsreactie die onafhankelijk is van het aantal leukocyten dat in de spierlaag van de dunne darm wordt gevonden (Hoofdstuk 2). Dit geeft aan dat andere mechanismen verantwoordelijk zijn voor de meer ernstige ileus. Deze gegevens hebben geleid tot de hypothese dat weefselschade een ontstekingsreactie veroorzaakt middels schadegeassocieerde moleculen en zodoende leidt tot een meer ernstige ileus. Bovendien kan verhoogde lokale ontsteking resulteren in een systemische ontstekingsreactie en derhalve verhoogde activatie van neuronen in de hersenstam geven. Om de bijdrage van weefselschade in ernstige ileus te onderzoeken, hebben we de lokale ontstekingsreactie in de darmen, de systemische ontstekingsreactie en activatie van de hersenstam na verschillende intensiteiten van darmmanipulatie onderzocht (Hoofdstuk 3). We toonden aan dat manipulatie-geïnduceerde weefselschade niet alleen leidde tot een verhoogde intestinale ontstekingsreactie maar ook tot de afgifte van pro-inflammatoire moleculen in de bloedstroom. Deze weefselschade-geïnduceerde verhoogde ontstekingsreactie was tevens geassocieerd met verhoogde hersenactivatie in muizen. Daarbij correelde de mate van weefselschade en systemische ontsteking in zowel mensen als muizen met de ernst van postoperatieve ileus. Deze bevindingen leveren het bewijs dat de afgifte van weefselschade mediatoren en pro-inflammatoire cytokines in de systemische circulatie bijdragen aan de verminderde motilitie van de niet-gemanipuleerde darm. Daarnaast resulteren een ernstige ileus en weefselschade in de activatie van hersenstamgebieden zoals de area postrema, wat hoogstwaarschijnlijk het ziektegedrag, geassocieerd met postoperatieve ileus verklart. In hoeverre dit verder bijdraagt aan de verminderde motilitie van het gehele maag-, darmkanaal in postoperatieve ileus verdient nadere aandacht in toekomstige studies. Meer inzicht in hoe weefselschade de afgifte van systemische cytokines veroorzaakt, kan helpen bij de ontwikkeling van geneesmiddelen om deze systemische ontstekingsreactie te voorkomen.

Zowel bij mensen als bij dieren blijkt een buikoperatie de integriteit van de intestinale epitheliale barrière te beïnvloeden en mogelijk het postoperatieve herstel te verlengen. Diverse intestinale ontstekingsmodellen hebben de laatste jaren het belang van mestcellen in de regulatie van de epitheliale barrière aangetoond. De rol van de epitheliale barrière in de pathogenese van postoperatieve ileus is nog niet volledig opgehelderd, al is wel duidelijk dat de klinische gevolgen van bacteriële translocatie tijdens operaties significant zijn. Zo is bij patiënten die een buikoperatie ondergaan barrière dysfunctie geassocieerd met een verhoogde postoperatieve septische morbiditeit. In Hoofdstuk 4 hebben we onderzocht of geactiveerde mestcellen bijdragen aan een verstoring van de intestinale barrièrefunctie. Daartoe hebben we onze experimenten uitgevoerd in twee mestcel-deficiënte muizenstammen. We namen waar dat darmmanipulatie tijdens een open buikoperatie resulteerde in een mestcel
afhankelijke ontsteking en barrière dysfunctie. Deze gegevens benadrukken het belang van mestcellen in de pathogenese van postoperatieve ileus en het potentieel van mestcel stabilisatoren om de duur van postoperatieve ileus te verkorten. Naast activatie van mestcellen, spelen ook geactiveerde macrofagen en dendritische cellen in de spierlaag van de darm een rol in het ontstaan van postoperatieve ileus (Figuur 2). De influx van bacteriën en hun antigenen over de epitheliale barrière na darmmanipulatie kan deze cellen activeren. Als alternatief kan het ontstekingseiwit Interleukine-1, dat wordt afgegeven in reactie op weefselschade van belang zijn bij de door darmmanipulatie geïnduceerde ontsteking. De vraag of herkenning van getransloceerde bacteriën cruciaal is in de initiatie van chirurgisch geïnduceerde ileus of alleen een rol speelt in het handhaven van ileus wordt momenteel onderzocht.

In de darm bevinden cholinerge vezels zich in nabijheid van afweercellen. Dit maakt de darm tot een ideale plek voor neuro-immuun modulatie. In het afgelopen decennium is in een aantal diermodellen aangetoond dat stimulatie van de nervus vagus immuun reacties dempt; dit wordt aangeduid als de ‘cholinergie anti-inflammatoire route’. Onlangs heeft onze groep aangetoond dat elektrische stimulatie van de nervus vagus bij muizen de darmperistaltiek verbetert en de ontstekingsreactie in de spierlaag van de dunne darm dempt door stimulatie van alfa 7 nicotine acetylcholine receptoren (α7nAChRs). Deze komen op residerende macrofagen tot expressie. De cholinergie anti-inflammatoire route wordt beschouwd als onderdeel van de zogenaamde vago-vagale 'inflammatoire reflex'. Ontsteking wordt waargenomen door afferente zenuwvezels en vervolgens doorgegeven aan de hersenen. Na integratie van deze afferente informatie worden de motorische neuronen van de nervus vagus geactiveerd en een geïntegreerd anti-inflammatoir signaal wordt teruggestuurd naar het ontstoken gebied. In Hoofdstuk 6 leveren wij het neuro-anatomische bewijs dat de vagale terugkoppeling wordt geactiveerd in respons op de lokale ontsteking in de darm. Dit biedt nieuwe benaderingen om ongewenste ontstekingsprocessen te moduleren (Figuur 3). Momenteel zijn we aan het onderzoeken of intra-operatieve elektrische stimulatie van de intra-abdominale nervus vagus de ontstekingsreactie vermindert en de duur van postoperatieve ileus kan verkorten bij patiënten die open rectale resecties ondergaan. Daarnaast wordt momenteel middels een multicenter trial onderzocht of bij patiënten met reumatoïde artritis het stimuleren van de nervus vagus in de hals de gewrichtsontsteking kan verminderen.

In het tweede deel van dit proefschrift evalueren we klinische karakteristieken van postoperatieve ileus en potentiële therapeutische strategieën. Tot op heden zijn er geen gevalidateerde klinische kenmerken om het herstel van het maag-, darmkanaal te beoordelen en zodoende te evalueren of een patiënt kan worden ontslagen uit het
ziekenhuis. Gezien de ontwikkeling van nieuwe behandelingen voor postoperatieve ileus is er een duidelijke behoefte aan betrouwbare uitkomstmaten. Zodoende kan dan in toekomstige patiënten studies het effect van nieuwe medicijnen op postoperatieve ileus betrouwbaar worden geëvalueerd. In Hoofdstuk 7 hebben we vastgesteld welke klinische kenmerken het beste het herstel van de transportfunctie van het maag-, darmkanaal na een darmoperatie identificeren. Voor het objectief bepalen van de transportfunctie van de dikke darm werd een scintigrafische techniek gebruikt. Dit toonde aan dat het klinisch herstel na een buikoperatie inderdaad geassocieerd is met herstel van de transportfunctie van de dikke darm. Met behulp van het laatstgenoemde als objectief criterium voor klinische verbetering hebben we aangetoond dat de gecombineerde klinische uitkomstmaat “tolerantie van vast voedsel en het hebben van ontlasting” het beste klinisch herstel voorspelt en aangeeft of een patiënt klaar is voor ontslag uit het ziekenhuis. Daarentegen is de tijd tot de eerste flatus (windje) niet significant geassocieerd met het herstel van de dikke darmfunctie of de tijd tot het ontslag. De volgende stap was om de uitkomstparameter “tolerantie van vast voedsel en het hebben van ontlasting” in een groter cohort van patiënten (de multicenter LAFA trial) te valideren. Deze analyses bevestigen dat de aanwezigheid van beide klinische parameters het beste klinische teken is van herstel van de darm en impliceert dat dit de beste primaire uitkomstmaat is voor toekomstige klinische trials op het gebied van postoperatieve ileus.

Postoperatieve ileus is een belangrijke determinant voor het herstel na colorectale chirurgie. Recente studies suggereren aanzienlijke verbeteringen in de periope ratieve zorg, met name de multimodale versnelde herstelprogramma’s en laparoscopische chirurgie hebben geresulteerd in sneller herstel van de darm en kortere ziekenhuisopnames in het afgelopen decennium. Echter, objectieve bewijzen voor een sneller herstel van het maag-, darmkanaal ten gevolge van deze interventies ontbreken. Hoofdstuk 8 beschrijft het effect van laparoscopische chirurgie en het fast-track programma op de scintigrafische bepaalde transportfunctie van het maagdarmkanaal. Deze data tonen op objectieve wijze aan dat laparoscopie en fast-track zorg leiden tot een vlotter herstel van de motilitie van de darm en het klinisch herstel versnellen. Het versnelde klinisch herstel na laparoscopische chirurgie in vergelijking met open chirurgie is te verklaren door minder weefselbschade met een gereduceerde immuunrespons. Dit leidt tot een minder heftige ontsteking van de darm en zodoende tot minder remming van de darmperistaltiek. De mechanismen achter het gunstige effect van het fast-track programma blijven onduidelijk, maar in een postoperatief ileus model met ratten is aangetoond dat enterale voeding de darmfunctie verbetert door activatie van de nervus vagus gemedieerde anti-inflammatoire route. Overeenkomstig worden binnen het fast-track programma patiënten niet alleen sneller gemobiliseerd maar hervatten ook eerder
orale voeding. Deze laatste observatie suggereert dat voeding de vagale anti-inflammatoire route kan activeren wat bijdraagt aan sneller herstel bij deze patiënten. In hoeverre vagale activatie in reactie op vroege voeding de verbeterde darmfunctie in de fast-track groepen kan verklaren in deze gerandomiseerde dubbelblinde studie dient nog te worden onderzocht. Ondanks de schijnbare effectiviteit van het fast-track programma wordt deze strategie nog niet op alle chirurgische afdelingen uitgevoerd. Strategieën om de uitvoering van het fast-track programma te verbeteren dienen daarom te worden gestimuleerd. Indien dit in de toekomst succesvol is geïmplementeerd, zal dit duidelijk de onderzoeksoptzet van klinische trials beïnvloeden aangezien toekomstige geneesmiddelen dan een klinisch voordeel moeten laten zien bij patiënten die worden behandeld in het kader van een fast-track programma.

Tenslotte wordt in Hoofdstuk 5 een nieuwe anti-inflammatoire strategie beschreven om postoperatieve ileus tegen te gaan. Er werd geëvalueerd of Spleen tyrosine kinase (Syk), een van de belangrijkste intracellulaire tyrosine kinasen betrokken bij mestcel degranulatie en macrofaag activatie, een aangrijpingspunt kan zijn om de deontsteking in de darm te verminderen. Aangezien zowel mestcellen als macrofagen betrokken zijn bij de pathofysiologie van postoperatieve ileus werd geanalyseerd of blokkade van Syk een alternatieve benadering biedt om de manipulatie geïnduceerde activatie van witte bloedcellen af te remmen en de duur van postoperatieve ileus te verkorten. Ten eerste tonen wij aan dat de krachtige en zeer selectieve Syk remmer GSK143 zowel FcεRI- als Substantie P geïnduceerde mestcel degranulatie en endotoxine geïnduceerde macrofaag activatie in vitro weet te voorkomen. Ten tweede dempt GSK143 in vivo de ontstekingscascade die leidt tot postoperatieve ileus zonder een direct effect op darmmotilititeit uit te oefenen. GSK143 heeft namelijk geen stimulerende werking op de darmfunctie bij muizen die enkel een laparotomie (open buik operatie zonder manipulatie van de darmen) ondergaan. Deze bevindingen tonen aan dat remming van Syk de ontstekingsreactie in de darm significant vermindert en daarmee postoperatieve ileus voorkomt. Dit impliceert dat Syk als een belangrijk aangrijpingspunt in intestinale ontstekingsziekten kan dienen, en dat inhibtitie van Syk als een nieuwe behandeling voor postoperatieve ileus verder dient te worden onderzocht.

Toekomstperspectieven

In dit proefschrift zijn nieuwe inzichten in de pathogenese van postoperatieve ileus beschreven. Dit heeft geleid tot de identificatie van nieuwe aangrijpingspunten in de behandeling van postoperatieve ileus. Dit omvat Syk-remmers als nieuwe strategie om de ontstekingsreactie in de darmwand te remmen, en de uitvoering van multimodale versnelde herstel programma’s (fast-track zorg) en minimaal invasieve chirurgie.
Een duidelijke implicatie van de hier gepresenteerde gegevens is dat het zeer belangrijk is om de hoeveelheid weefselschade te beperken. De identificatie van de precieze oorzaak van de postoperatieve ontstekingsreactie (mestcel activatie, weefselschade, bacteriële translocatie) en meer inzicht in hoe deze ontstekingsreactie zich uitbreidt naar de rest van de (niet-gemanipuleerde) darm zou moeten helpen bij de ontwikkeling en evaluatie van geneesmiddelen. Daarnaast begrijpen we nog niet wat de postoperatieve systemische ontstekingsreactie controleert en hoe dit het centrale zenuwstelsel en de darmperistaltiek moduleert. Gezien het medische belang van de ontstekingsreactie in postoperatieve ileus zijn dit belangrijke kwesties die moeten worden aangepakt.

Onlangs heeft de groep van Kalff ontdekt dat naast het angeboren- ook het adaptieve immuunsysteem wordt geactiveerd in reactie op darmmanipulatie. Er wordt gesuggereerd dat dit wordt gemedieerd door de afgifte van IL-12 door geactiveerde dendritische cellen in de gemanipuleerde darm. Dit leidt tot activatie van Th1-cellen in de ontstoken spierlaag van de dunne darm, die vervolgens uittreden naar de systemische circulatie om naar verder afgelegen (niet-gemanipuleerde) gebieden in de darm te migreren. Daar geven ze IFN-γ af waardoor residente muscularis macrofagen worden gestimuleerd om stikstofmonoxide en inflammatoire cytokines te produceren. Het inactiveren van dendritische cellen, het gebruik van immunosuppressiva zoals anti-IL-12 moleculen of het remmen van Th1-celmigratie door FTY-720 zou postoperatieve ileus kunnen verminderen. De effectiviteit en veiligheid van deze benaderingen zal echter eerst nog moeten worden onderzocht bij mensen.

Concluderend is een nieuw vat met potentiële aangrijpingspunten voor de behandeling van postoperatieve ileus geopend. Het remmen van de intestinale macrofaag of de functie van mesteccellen door Syk modulatie, of het ingrijpen in de adaptieve immuunreactie en systemische verspreiding van ontsteking kan de duur van postoperatieve ileus verminderen. Daarnaast kunnen interventies die de cholinerge anti-inflammatoire route activeren ook een aantrekkelijke therapie voor de toekomst betekenen. De resultaten in dit proefschrift impliceren dat nieuwe behandelingen van postoperatieve ileus de doorgedragen manipulatie geïnduceerde postoperatieve ontstekingsreactie dienen te verminderen. Zulke anti-inflammatoire strategieën kunnen echter interfereren met de wondgenezing, de verdediging tegen micro-organismen en uiteindelijk met het klinisch herstel van patiënten. Daarom is het belangrijk dat vóór klinische toepassing van veelbelovende anti-inflammatoire therapieën eerst hun effect op de postoperatieve wondgenezing in diermodellen wordt onderzocht. Een ander aspect waar rekening mee dient te worden gehouden bij het bestuderen van postoperatieve ileus is dat de kans op het ontwikkelen van een significante ileus toe neemt met het stijgen van de leeftijd. Er is zowel een leeftijdsafhankelijke toename van
de pro-inflammatoire mediatorexpressie als een leeftijdsafhankelijke afname in anti-inflammatoire mediator expressie na lichte manipulatie van de darm in knaagdieren. In hoeverre dit verstoorde evenwicht tussen pro- en anti-inflammatoire cytokines ten grondslag ligt aan de verhoogde gevoeligheid, ernst en duur van ileus onder ouderen is onbekend. Hopelijk zal ons inzicht over de rol van veranderde inflammatoire genexpressie met een stijgende leeftijd in postoperatieve ileus snel toenemen, aangezien steeds meer oudere patiënten buikoperaties zullen ondergaan met het toenemen van de gemiddelde levensduur en de uitbreiding van screening op dikke darmkanker.

Concluderend verschaffen de bevindingen beschreven in dit proefschrift aanzienlijk nieuwe inzichten in de pathogenese van postoperatieve ileus en nieuwe therapeutische strategieën. Deze bevindingen zijn tevens direct een pleidooi voor minimaal invasieve ingrepen aangezien onze resultaten duidelijk tonen dat darmmanipulatie en weefselschade tijdens een operatie zoveel mogelijk dienen te worden vermeden.

Legenda bij de figuren te vinden in het vorige hoofdstuk “Summary and conclusions”

Figuur 1. Gestandaardiseerde manipulatie in experimenteel muizenmodel voor postoperatieve ileus.

Figuur 2. CD68 kleuring voor macrofagen en infiltrerende monocyten in de spierlaag van de dunne darm. Coupes van de buitenste spierlaag van de dunne darm gekleurd met antilichamen tegen CD68 bij 200X vergroting. Enkel laparotomie als controle muis (a); Laparotomie en het uit de buik halen van de dunne- en blindedarm zonder gestandaardiseerde drukmanipulatie (b); Muis met postoperatieve ileus na 9 gram gestandaardiseerde drukmanipulatie van de dunne darm (c).

Figuur 3. De vagale anti-inflammatoire route en postoperatieve ileus. De cholinerge anti-inflammatoire reflex is gebaseerd op activatie van de vagale sensorische afferente zenuwvezels die vervolgens een geïntegreerde anti-inflammatoire reactie creëren via vagale efferente vezels. In postoperatieve ileus wordt activatie van de vagale sensorische afferente teweeggebracht door pro-inflammatoire cytokines die afgegeven worden in de spierlaag van de darm. De vagale afferente zenuwvezels mediëren de neuronale activiteit naar de NTS (hersenstamkernen die viscerale informatie ontvangen). Dit resulteert in de activatie van motorische neuronen van de nervus vagus (die zich in de DMV bevinden). Eenmaal geactiveerd geven de vagale efferente vezels acetylcholine af op het niveau van de myenterische plexus en dit stimuleert de cholinergische enterische neuronen. Deze neuronen versterken de vagale signalering door het afgeven van acetylcholine in de spierlaag waar de macrofagen zich bevinden. Binding van acetylcholine aan α7 nicotine receptoren op macrofagen onderdrukt de afgifte van pro-inflammatoire cytokines. Stimulatie van het sensorische afferente deel van het neuronale circuit door een dieet van enterale vetrijke voeding, door cholecystokinine afgifte of elektrische stimulatie van het motorische efferente onderdeel van de nervus vagus voorkomen darmontsteking en ileus. Intracerebroventriculaire injectie van semapimod (CNI 1493), acetylcholinesterase remmer (galantamine), ghreline en muscarine receptor 1 agonisten (McN-A 343) activeren ook vagale afferente zenuwvezels waardoor ontsteking wordt onderdrukt. Afkortingen: Ach: acetylcholine; DMV: dorsale motorische nucleus van de vagus; NTS: nucleus tractus solitarius; VNS: nervus vagus stimulatie.
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van Bree SH, Cailotto C, Di Giovangiulio M, van der Vliet J, Costes L; Depoortere I, Gomez-Pinilla PJ, Gianluca M, Boeckxstaens GE. Systemic inflammation and enhanced brain activation contribute to more severe delay in postoperative ileus. Annals of Surgery (under review)


Stoffels B, Hupa KJ, Snoek SA, van Bree SH, Stein K, Schwandt T, Vilz TO, Lysson M, van’t Veer C, Hornung V, Kalff JC, de Jonge WJ, Wehner S. Interleukin-1 receptor signaling mediates postoperative ileus by targeting enteric glial cells. Submitted


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About the author

Sjoerd H. W. van Bree was born on July 30th, 1981 in ’s-Hertogenbosch, The Netherlands. After graduating from high school at the Gemeentelijk Gymnasium in Hilversum (The Netherlands) in 2000, he obtained his propaedeutics at the medical school of the University of Antwerp (Belgium). He received six more years of medical training at the Academic Medical Center. During this period he taught physiology, anatomy and counseled first year medical students. Subsequently he worked on a research project on idiopathic hyperCKemia of the Department of Neurology and Internal medicine, which fuelled his interest in research. In 2007, he received his medical degree Cum Laude from the University of Amsterdam (The Netherlands) and successively started his PhD project under the guidance of Professor Boeckxstaens at the Department of Gastroenterology and Hepatology at the Academic Medical Center of Amsterdam. The main focus of his research is the inflammatory pathway in postoperative ileus: investigating the key neural pathways and immune cells involved and exploring new anti-inflammatory approaches in humans. In 2009 he was certified as an International Medical Graduate by the Educational Commission for Foreign Medical Graduates of the United States. In January 2012 he started his training in Internal Medicine in Amsterdam.
AMC Graduate School for Medical Sciences PhD Portfolio

Summary of PhD training, teaching and parameters of esteem

Name PhD student: S. H.W. van Bree
PhD period: July 2007 - December 2012
Name PhD supervisors: Prof. G.E.E. Boeckxstaens and dr. C. Cailotto

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<tr>
<td>- AMC World of Science</td>
<td>2007</td>
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<td>- Clinical Data Management</td>
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<td>- Basiscursus Regelgeving Klinisch Onderzoek &amp; GCP</td>
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<td>- Basic Laboratory Safety</td>
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<td>- Practical Bio-statistics</td>
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<td>- Advanced Biostatistics</td>
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<td>- Handling the Media</td>
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<td><strong>Specific courses</strong></td>
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<tr>
<td>- Laboratory Animals and Article 9 approved according to the Dutch law on animal experiments</td>
<td>2007</td>
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<td>- Radiation Protection: Training in radiation and dealing with radioactive isotopes. Level 5B</td>
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<td>- Advanced Immunology</td>
<td>2009</td>
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<tr>
<td><strong>Seminars, Workshops and Masterclasses</strong></td>
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<tr>
<td>- Young investigators Meeting, Association of National European and Mediterranean Societies of Gastroenterology (ASNEMGE), Stockholm, Sweden</td>
<td>2011</td>
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<tr>
<td>- United European Gastroenterology Federation (UEGF) Teaching Activity on Basic Science: Innate immunity and the Gut, Murray Edwards College, Cambridge</td>
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<td><strong>Presentations</strong></td>
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<td><strong>Oral presentations:</strong></td>
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<tr>
<td>- Which clinical symptoms reflect postoperative recovery of gastrointestinal motility?</td>
<td>2010</td>
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<tr>
<td>- Annual Spring Conference Dutch Society for Gastroenterology, Veldhoven</td>
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<tr>
<td>- Digestive Disease Week, New Orleans, USA</td>
<td>2010</td>
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<tr>
<td>- United European Gastroenterology Week, Stockholm, Sweden</td>
<td>2011</td>
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</table>
- The effect of laparoscopy and/or fast track multimodal management on postoperative GI motility after colonic surgery: Annual Spring Conference Dutch Society for Gastroenterology

- Tissue damage and brain activation in postoperative ileus:
  - Annual Spring Conference Dutch Society for Gastroenterology
  - Digestive Disease Week, Chicago, USA
  - Mast cell inhibition by a new spleen tyrosine kinase inhibitor in the treatment of postoperative ileus: United European Gastroenterology Week, Stockholm, Sweden

**Poster presentations:**
- The effect of laparoscopy and/or fast track multimodal management on postoperative GI motility after colonic surgery: Digestive Disease Week, New Orleans, USA

- Novel mast cell stabilizers in the treatment of postoperative ileus: Digestive Disease Week, Chicago, USA

- Activation of the area postrema and systemic proinflammatory mediator release by intense surgical handling of the intestine: United European Gastroenterology Week, Stockholm, Sweden

- Pathophysiological mechanisms of severe postoperative ileus: United European Gastroenterology Week, Amsterdam

**(Inter)national conferences**
- Annual Spring Conference Dutch Society for Gastroenterology, Veldhoven
- Multidisciplinary Battles in GI surgery
- Hot Topics in Neurogastroenterology ‘Recent advances in management of constipation in adults’, Amsterdam
- Het Gastroenterologisch jaar 2010, Rotterdam

<table>
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<tr>
<th>Event</th>
<th>Year</th>
<th>Credits</th>
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<tr>
<td>The effect of laparoscopy and/or fast track multimodal management on postoperative GI motility after colonic surgery: Annual Spring Conference Dutch Society for Gastroenterology</td>
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<tr>
<td>Tissue damage and brain activation in postoperative ileus:</td>
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<tr>
<td>Annual Spring Conference Dutch Society for Gastroenterology</td>
<td>2011</td>
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<tr>
<td>Digestive Disease Week, Chicago, USA</td>
<td>2011</td>
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<tr>
<td>Mast cell inhibition by a new spleen tyrosine kinase inhibitor in the treatment of postoperative ileus: United European Gastroenterology Week, Stockholm, Sweden</td>
<td>2011</td>
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<tr>
<td>The effect of laparoscopy and/or fast track multimodal management on postoperative GI motility after colonic surgery: Digestive Disease Week, New Orleans, USA</td>
<td>2010</td>
<td>0.5</td>
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<tr>
<td>Novel mast cell stabilizers in the treatment of postoperative ileus: Digestive Disease Week, Chicago, USA</td>
<td>2011</td>
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<tr>
<td>Activation of the area postrema and systemic proinflammatory mediator release by intense surgical handling of the intestine: United European Gastroenterology Week, Stockholm, Sweden</td>
<td>2011</td>
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<tr>
<td>Pathophysiological mechanisms of severe postoperative ileus: United European Gastroenterology Week, Amsterdam</td>
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<tr>
<td>Annual Spring Conference Dutch Society for Gastroenterology, Veldhoven</td>
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<td>Multidisciplinary Battles in GI surgery</td>
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<tr>
<td>Hot Topics in Neurogastroenterology ‘Recent advances in management of constipation in adults’, Amsterdam</td>
<td>2010</td>
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<tr>
<td>Het Gastroenterologisch jaar 2010, Rotterdam</td>
<td>2010</td>
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- Digestive Disease Week, American Gastroenterology Association, San Diego/New Orleans/Chicago, USA
- United European Gastroenterology Week (UEGW), Stockholm, Sweden
- United European Gastroenterology Week (UEGW), Amsterdam

<table>
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<th>Year</th>
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### 2. Teaching

<table>
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<tr>
<th>Lecturing</th>
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<tr>
<td>Teaching sessions on postoperative ileus third and fourth year medical students</td>
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<table>
<thead>
<tr>
<th>Tutoring, Mentoring</th>
<th>Year</th>
<th>Workload</th>
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<tbody>
<tr>
<td>Mentoring students on Neurogastroenterology &amp; Motility</td>
<td>2009-2011</td>
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<table>
<thead>
<tr>
<th>Supervising</th>
<th>Year</th>
<th>Workload</th>
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<tbody>
<tr>
<td>Supervising Research internship students of Biomedical Sciences and Medicine and (5 students each 4-6 months). Projects involving basic and clinical research on postoperative ileus.</td>
<td>2008-2011</td>
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### 3. Parameters of Esteem

<table>
<thead>
<tr>
<th>Grants</th>
<th>Year</th>
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<tr>
<td>Travel Grant Dutch Gastroenterology Federation (NVGE)</td>
<td>2010</td>
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<tr>
<td>Travel Award United European Gastroenterology Week (UEGW), Stockholm, Sweden</td>
<td>2011</td>
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<tr>
<td>Travel Grant Dutch Gastroenterology Federation (NVGE)</td>
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<table>
<thead>
<tr>
<th>Awards and Prizes</th>
<th>Year</th>
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<tbody>
<tr>
<td>Poster of distinction Digestive Disease Week 2010, New Orleans, USA</td>
<td>2010</td>
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<tr>
<td>Poster of distinction Digestive Disease Week 2011, Chicago, USA</td>
<td>2011</td>
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