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Implication of mast cells, nerve growth factor and splanchnic nerves in postoperative ileus. Study in patients undergoing abdominal surgery and in a rat experimental model

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We hereby certify that the thesis entitled ‘‘Implication of mast cells, nerve growth factor and splanchnic nerves in postoperative ileus. Study in patients undergoing abdominal surgery and in a rat experimental model’’ submitted by SERGIO BERDÚN MARIN in partial fulfillment of the requirements for the degree of Doctor of Philosophy was carried out under our supervision and we authorize it submission for oral defense.

Bellatera, April 2015

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Cover image: Tryptase-immunostained mast cells in the outer muscular layer of human colon collected after colorectal surgery. Immunofluorescence technique. 100x.

‘’Knowledge is the death of research’’

Walther Hermann Nernst

German chemist and physicist

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Parece mentira que esté sentado delante del ordenador escribiendo estas palabras después de estos cinco años. Ahora me acuerdo de aquellos momentos en los que pensé en tirar la toalla y no puedo evitar hacer mención a todas aquellas personas que directa o indirectamente (aunque no seáis consciente de ello), fuera o dentro de la facultad, me habéis ayudado a recorrer este largo camino.

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ABBREVIATIONS

5-HT	5 hydroxytryptamine (Serotonin)
ACh	Acetylcholine
ANS	Autonomous nervous system
BMMC	Bone marrow-derived mast cells
CCL5	Chemokine ligand 5
CCK	Cholecystokinin
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
EEC	Entero-encodrine cells
ENS	Enteric nervous system
FcƐRI	High-affinity receptor for immunoglobulin E
GI	Gastrointestinal
GLP-1	Glucagon like peptide-1
GM CSF	Granulocyte macrophage colony stimulating factor
H_2	Hydrogen
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IL	Interleukin
IPANs	Intrinsic primary afferent neurons
MC	Mast cell
MC_{CT}	Chymase-tryptase subtype mast cells
MC_T	Tryptase subtype mast cells
MCP-1	Monocyte chemotactic protein 1
MIP	Macrophage inflammatory protein
mMCP	Mouse mast cell protease
MMC	Migrating motor complex

NANC	Non-adrenergic non-cholinergic
NGF	Nerve growth factor
NTS	Nucleus tractus solitary
PMC	Peritoneal mast cells
PYY	Peptide YY
POI	Postoperative ileus
RMCP	Rat mast cell protease
SP	Substance P
SMC	Smooth muscle cell
TNFα	Tumor necrosis factor α
TGFβ	Transforming growth factor β
TrkA	Tropomyosin kynase receptor A
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide

SUMMARY

Postoperative ileus (POI) is defined as a temporal cessation of propulsive gastrointestinal (GI) motility in patients undergoing abdominal surgery, especially those subjected to intestinal resection. Mast cell (MC) degranulation in peritoneal lavage has been reported in patients undergoing abdominal surgery and in experimental models of POI in rodents. Preventive treatments with MC stabilizers or the use of MC-deficient mutant models point towards a role for MCs in POI, but the exact mechanisms involved remains unclear. Interactions between MCs and nerve endings or inflammatory mediators such as the nerve growth factor (NGF) may represent a key factor in POI pathogenesis. The aim of this work was to study POI in human patients by characterizing the activation of peritoneal mast cells (PMCs) in colorectal surgery. A part from that, we also aimed to explore the role of NGF antagonists on MCs and MC-nerve interactions in a rat model of POI.

We firstly studied human POI. We evaluated MC protease release in peritoneal lavage (by means of ELISA) collected from patients undergoing laparoscopic and open colorectal surgery. In these patients we also studied MC density in colonic samples (by immunohistochemistry) and postoperative clinical recovery. Secondly, we set up a model of POI in rat to further explore the role of MCs. We evaluated the effect of pre-treatment with NGF receptor antagonist, K252a. *In vitro* effects of K252a were also evaluated on rat PMCs. Activation of dorsal root ganglia (DRG) in POI rat model was also characterized in a separate experiment in which the role of MCs was investigated using ketotifen (MC stabilizer) and compound 48/80 (C48/80, MC degranulator).

Our study demonstrated release of MC proteases into peritoneal cavity after colorectal surgery for both laparoscopic and open techniques. This protease release was observed only in patients with a subsequent delay of clinical recovery (those who developed POI) regardless of the surgical technique employed. MC density in colonic samples was unaltered by abdominal surgery. In our animal study we demonstrated that induction of POI by intestinal manipulation immediately evokes release of MC protease RMCP-6 in the peritoneal cavity. At 24h after intestinal manipulation, our model also presented delayed GI transit and increased expression of interleukin-6 (IL-6, RT-qPCR) and myeloperoxidase activity (MPO) in ileum samples. In contrast, density of intestinal MCs (immunohistochemistry and toluidine blue staining) and RMCP-2 and 6 gene expressions (RT-qPCR) in the ileum were unaltered in the animal POI model. K252a prevented PMC degranulation *in vitro* (β-hexosaminidase assay) and the release of RMCP-6 in the POI model. In addition, K252a attenuated IL-6 expression after intestinal manipulation and decreased basal peritoneal release of RMCP-2 and TrkA (NGF receptor) gene expression However, GI transit was not ameliorated after K252a treatment. Intestinal manipulation in the POI model also increased gene expression of calcitonin gene-related peptide, NGF, TrkA and protease-activated receptor-2 in somas of DRGs but these changes were not modulated by ketotifen or C48/80. In contrast, C48/80 did delay GI transit and induced up-regulation of IL-6 and MPO activity. Ketotifen also prevented delayed gastric emptying and the postoperative decrease of fecal output.

To sum up, our results indicate that intestinal manipulation is associated with a local response of MCs in the peritoneal cavity. Intestinal manipulation delayed GI motility *in vivo*, induced intestinal inflammation and activated DRGs. K252a stabilized MCs and down-regulated IL-6 expression in the inflammatory response leading to POI. Our data also showed that MCs are

involved in GI motility alteration and inflammation after intestinal manipulation. In contrast, activation of DRGs seems to be independent of MC activation based on our assessment using a pharmacological approach. We conclude that MCs participate in POI and that interactions between NGF, TrkA and PMCs may represent a target for treatment of POI or other MC-mediated diseases.

SUMMARY (SPANISH)

El íleo postoperatorio (IPO) se define como el cese temporal de la motilidad propulsiva gastrointestinal (GI) en pacientes sometidos a cirugía intestinal. En pacientes y en modelos experimentales de IPO, se ha observado degranulación de mastocitos (MCs) en lavado peritoneal. Estudios con fármacos estabilizadores de MCs o en modelos en roedores carentes de mastocitos apoyan la participación de los MCs en el IPO. No obstante, los mecanismos involucrados aún están por definir. La interacción nervio-mastocito, así como la interacción con el factor de crecimiento nervioso (NGF), podrían representar un factor clave en el desarrollo del IPO. El objetivo del presente trabajo ha sido estudiar el IPO humano caracterizando la activación de los mastocitos peritoneales (PMCs) durante la cirugía colorectal. A parte, también nos propusimos explorar el papel del NGF sobre los MCs y la interacción nervio-mastocito en un modelo experimental de IPO en rata.

Primero se estudió el IPO humano. Evaluamos la liberación de proteasas mastocitarias (mediante la técnica de ELISA) en el lavado peritoneal de pacientes sometidos a cirugía colorectal por laparoscopia y laparotomía, así como también la densidad de MCs (por inmunohistoquímica) y la recuperación clínica postoperatoria. Seguidamente se puso a punto un modelo de IPO en rata para profundizar en el papel de los MCs. En él, evaluamos el efecto del antagonista del receptor del NGF, K252a. Previamente habíamos demostrado el efecto *in vitro* del K252a sobre PMCs. Asimismo, hemos evaluado la participación de los ganglios de la raíz dorsal (DRG) en el IPO además de evaluar las consecuencias funcionales de la activación mastocitaria en animales tratados con el estabilizador de mastocitos ketotifeno o mediante la exposición del intestino al degranulador de mastocitos compuesto 48/80.

Los resultados de este trabajo demuestran que la cirugía colorectal induce la liberación de proteasas mastocitarias en la cavidad peritoneal en aquellos pacientes que desarrollaron un IPO. Además este hecho apareció independientemente de la técnica de resección empleada. La densidad de MCs en la pared del intestino no se vio alterada tras la cirugía. En la rata, inmediatamente tras la inducción del modelo, se produjo un aumento en la liberación de la proteasa mastocitaria RMCP-6 (inmunoensayo) en la cavidad peritoneal. A las 24h, se observó un retraso en el tránsito GI y un aumento en la expresión de la interleuquina-6 (IL-6, RT-qPCR) y en la actividad mieloperoxidasa (MPO) en las muestras de íleon. En cambio, la inducción del modelo no conllevó cambios ni en la densidad de MCs (visualizados mediante inmunohistoquímica y tinción con azul de toluidina) ni en la expresión de las proteasas mastocitarias RMCP-2 y 6. El K252a inhibió la degranulación *in vitro* de los PMCs (valorada mediante el ensayo de la β-hexosaminidasa) así como también atenuó la liberación de RMCP-6 y la expresión génica tanto de IL-6, como de RMCP-2 y TrkA (receptor del NGF) en el modelo. Sin embargo, este antagonista no mejoró la motilidad GI después de manipulación del intestino. Por otro lado, la expresión de mediadores calcitonin gene-related peptide (CGRP), NGF, TrkA and protease-activated receptor-2 en los DRGs aumentó tras la inducción del modelo. No obstante, estos cambios no se vieron afectados tras el tratamiento con ketotifeno o la exposición a C48/80. En cambio, el C48/80 si produjo un retraso en el vaciamiento gástrico/tránsito intestinal así como también aumentó la expresión de IL-6 y actividad MPO. El uso de ketotifeno, previno el retraso en el vaciamiento gástrico y la disminución post-operatoria del output fecal.

En resumen, nuestros resultados indican que la manipulación del intestino está asociada a una respuesta local mastocitaria en la cavidad peritoneal. La manipulación del intestino, induce retraso en la motilidad GI *in vivo*, inflamación intestinal y activación de los DRGs. El K252a estabilizó los MCs y disminuyó la expresión de IL-6 de la respuesta inflamatoria que lleva al IPO. Nuestros datos además demuestran que los MCs están involucrados en la alteración de la motilidad y la inflamación después de la manipulación del intestino. No obstante, la activación de los DRGs parece ser independiente de la activación mastocitaria, al menos con nuestra aproximación farmacológica.

Este trabajo no permite concluir que los MCs participan en el IPO y que la interacción entre el NGF, TrkA y PMCs puede representar una diana para el tratamiento del IPO u otras alteraciones mediadas por mastocitos.

INTRODUCTION

1. The gastrointestinal tract

The gastrointestinal (GI) tract allows us to consume and digest food, absorb nutrients and finally to excrete the remaining waste matter. In order to do this, the intestine integrates diverse functions such as motility, secretion, absorption and barrier function. These functions do not only permit the absorption of food to maintain the body but also protects the organism from external antigens and pathogenic agents.

1.1. Anatomy of the gastrointestinal tract

Major anatomical components of the GI tract are the stomach and the small and large intestine. The stomach connects with the small intestine through the pylorus, and both constitute the upper GI tract. The ileocecal valve or sphincter separates the small intestine from the large intestine, which composes the lower GI tract. The small intestine is divided, from oral to aboral, into the duodenum, jejunum and ileum, while the large intestine is divided into the cecum, colon and the rectum.

1.2. Histological organization of the intestinal wall

The understanding of the histological organization of the intestinal wall is of relevance in order to define the components that control and integrate the gastrointestinal function. The intestinal wall is mainly composed by four layers: Mucosa, submucosa, external muscular layer and serosa (Figure 1).

The **mucosa** is mainly dedicated to digestion and absorption as well as providing a first line of defense against food borne pathogens. In the small bowel, the mucosa forms large folds *(plicae circularis*) and small projections (villi) which increase the absorptive area. The intestinal mucosa is composed of a lining and an external epithelium which is in contact with luminal content. A layer of connective tissue, the *lamina propria*, underlies the epithelium and contains blood and lymphatic vessels, immune cells, and nerve fibers. The mucosa is also provided with a thin smooth muscle layer, *the muscularis mucosae*, which is located beneath the mucosal surface and

which regulates movements of the mucosa. Underlying the mucosa, we find the **submucosa**, a dense connective tissue layer that gives structural support to the mucosa. It contains the submucous or Meissner's plexus which mainly controls mucosal blood flow and secretomotor functions[1]. The **external muscular layer** (o *muscularis externa*) is composed by two layers of smooth muscle: the inner muscular layer (or circular layer) and the outer muscular layer (or longitudinal layer). Both are responsible for the contractile movements of the intestinal wall. Between these two layers, we find the myenteric or Aurbach's plexus which consists of a neuronal network and which controls the contraction of both muscular layers. The most external intestinal layer is the **serosa** which is composed of connective tissue covered by a monolayer of flat epithelial cells.

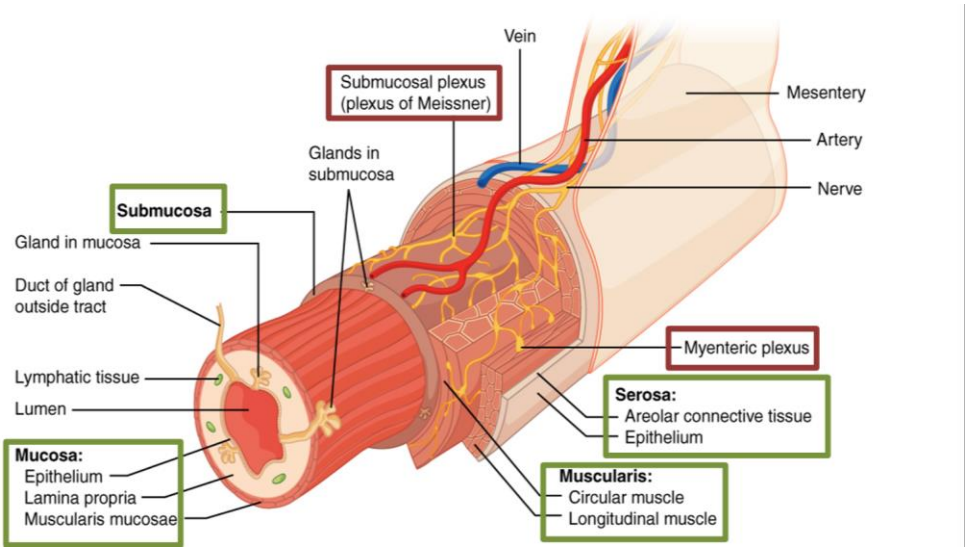

Figure 1. Organization of intestinal wall. Modified from http://philschatz.com/anatomy-book/contents/m46506.html.

2. Control of gastrointestinal motility

GI motility is finely regulated by the enteric nervous system (ENS) and the autonomous nervous system (ANS). While the ENS represents the intrinsic component within the intestinal wall, the ANS is referred as to the extrinsic component of GI regulation and a link to the central nervous system (CNS) [2]**.** In most of the cases, the ENS can initiate a motor program independently of the central neural connections. For instance, the peristaltic reflex, is a motor pattern exclusively elicited by ENS activity [3]. In contrast, ENS and ANS work together to regulate gastric emptying[4,5].

2.1 The enteric nervous system.

The ENS is composed of a neuronal network embedded within the intestinal wall. This so-called "*brain of the gut"* contains more than 10^8 neurons organised in two major ganglionated plexuses; the submucous or Meissner's plexus and the myenteric or Aurbach plexus[6]. Enteric neurons can be classified according to their function in motor neurons[7,8], interneurons[8,9] and sensory neurons[6,10], also called intrinsic primary afferent neurons (IPAN's).

The peristaltic reflex is an example of how enteric neurons work co-ordinately to allow contraction of the circular muscular layer oral to the point of stimulation while producing an aboral relaxation (Figure 2). The peristaltic reflex is initiated by mechanical stimulation of the mucosa and by presence of luminal nutrients which induces release of 5-hydroxytryptamine (5-HT) from mucosal enter endocrine cells. Subsequently, 5-HT activates IPANs which release calcitonin gene-related peptide (CGRP), to activate two types of interneurons 1) Ascending interneurons which activate excitatory, cholinergic and tachykininergic motor-neurons. This subsequent release of acetylcholine (ACh) and substance P (SP) induces contractions of circular muscle orally to the stimulation. 2) Descending interneurons which activate inhibitory motor-neurons that, through release of nitric oxide (NO), vasoactive intestinal peptide (VIP), and other potential neurotransmitters, such as ATP, induce the relaxation of the circular muscle aboral[11,12].

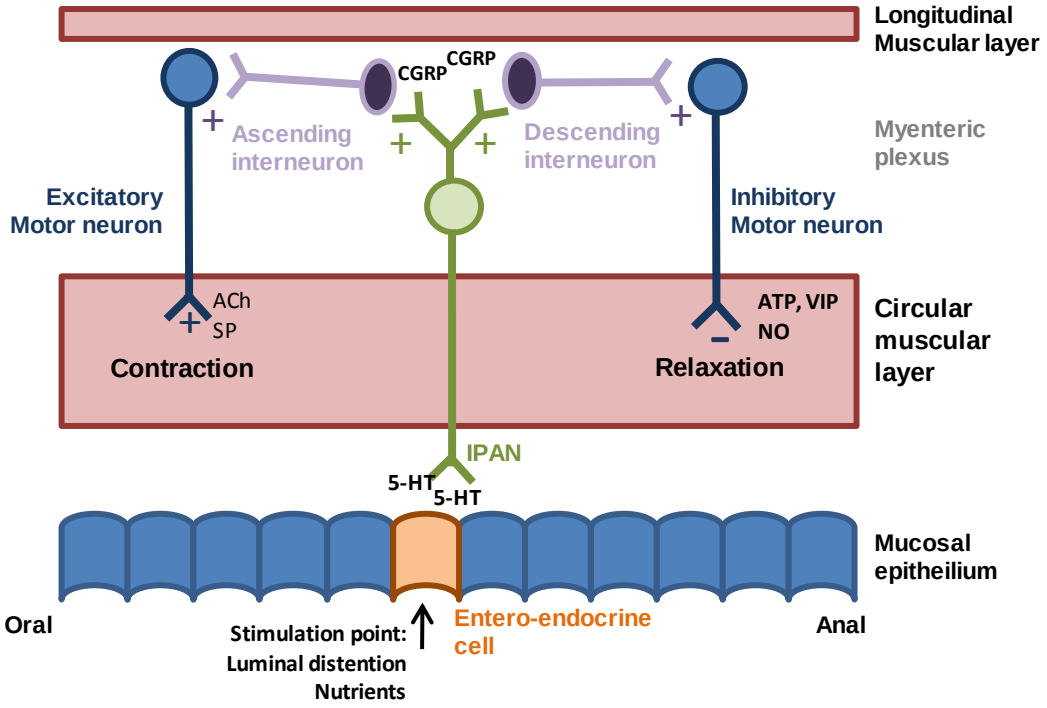

Figure 2. Schematic representation of the ENS regulating the peristaltic reflex. 5-HT, hydroxytryptamine (Serotonin). ACh, Acetylcholine. ATP, Adenosine triphosphate. CGRP, Calcitonin gene-related peptide. IPAN, Intrinsic primary afferent neuron. NO, Nitric oxide. SP, Substance P. VIP, Vasoactive intestinal peptide. Modified from Grider JR 2003

2.2 Extrinsic innervation of the gastrointestinal tract

The GI tract is innervated by more than 50.000 extrinsic fibers that also participate in the regulation of intestinal function. These fibers are mainly provided by two distinct anatomic components: The **vagus nerve** and the **spinal nerves,** which can be further subdivided into **splanchnic** (originating from thoraco-lumbar spinal cord) **and lumbo-sacral nerves** (originating from lumbo-sacral spinal cord). Fibers within the vagus nerve innervate the upper and middle GI tract (including proximal and mid colon) while pelvic fibers innervate the distal or descending colon and rectum[13] (Figure 3, left). In contrast, splanchnic nerves innervate the whole GI tract (Figure 3, right). Both anatomic systems belong to the ANS which comprises efferent and afferent fibres to viscera as well as spinal and supraspinal integrative centres that permit the CNS to sensor the GI microenvironment and perceive visceral sensation, thus, executing reflexes or behavioural/emotional responses in the so-called brain-gut axis[10].

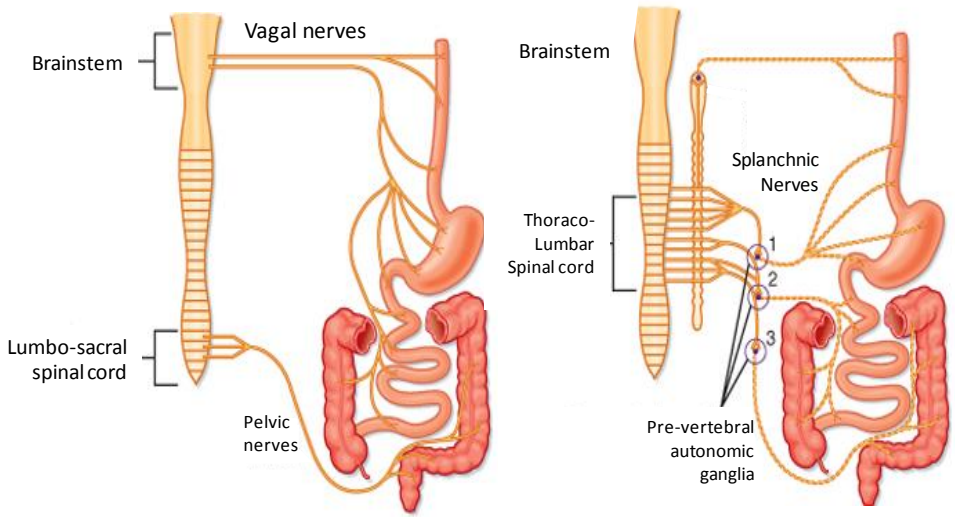

Figure 3. Dual extrinsic innervation of the gastrointestinal tract. Modified from Koeppen and Stanton, 2008.

2.2.1. Efferent fibers: parasympathetic and sympathetic innervation

Efferent fibers supplying the GI tract emerge from the brainstem and spinal segments and are organized in the parasympathetic nervous (rest and digest) system and sympathetic nervous system (fight and flight). These fibers are composed by preganglionic and postganglionic neurons that synapse in ganglia. In the parasympathetic system, synapses take place in enteric ganglia (thus, belonging to the ENS). In contrast, synapses of the sympathetic system are located in paravertebral and pre-vertebral ganglia (outside the intestinal wall)[13,14]. The parasympathetic innervation to the gut is provided by the vagus nerve and pelvic nerves. Vagal fibers participate in gastric emptying or regulation of migratory motor complex (MMC) while stimulation of parasympathetic pelvic pathways increase colorectal contractions and colonic transit and initiate defecation[15,16]. Sympathetic fibers are located in the splanchnic nerves. Postganglionic fibers mainly target neurons in the myenteric and submucous plexus. Activation of sympathetic fibers decreases gastric emptying and intestinal transit[14].

2.2.2. Afferent innervation

Afferent neurons within the extrinsic nerves provide an anatomical connection between the GI tract and the CNS. Therefore, these neurons convey sensorial information to the brain allowing individuals to have visceral perception (discomfort, pain perception, satiety, etc.).

The GI tract receives dual afferent innervation (Figure 3): the vagal afferent system (vagus nerve) and the spinal afferent system that divides into splanchnic nerves and pelvic nerves. Afferent extrinsic neurons have their cell bodies outside of the intestinal wall as described below and mainly, axons are small-diameter-sized and unmyelinated C fibers that conduce at a slow velocity (2m/s) [17,18]. Anatomical and functional differences between both systems are well characterized [17,19–23] and are summarized in table 1.

Table 1. Distinctive features of visceral afferent systems.

	Vagal afferent	**Splanchnic afferents**	**Pelvic afferents**
Location of cell bodies	Nodose and jugular ganglia	Thoraco-lumbar DRG	Lumbo-sacral DRG
CNS integration	NTS	Spinal dorsal horn	Spinal dorsal horn
CNS projections	Amygdale or hypothalamus from DMN/nucleus ambiguous	Thalamic nuclei through spinothalamic, spinoreticular or dorsal column pathways	
Stimuli	-Physiological -Non-painful -Low-threshold mechanical stimuli (10-20mmHg) -5-HT	-Non-physiological -Painful/ inflamm. -High threshold mechanical stimuli (>30mmHg) -Bradykinin	-Physiological -Non-painful -Low-threshold mechanical stimuli (10-20mmHg)
% afferent representation	90%	10-20%	30-40%
CGRP/SP content in nerve endings	Low	High	-

5-HT hydroxytryptamine (Serotonin), CGRP Calcitonin gene-related peptide, CNS Central nervous system, DMN Dorsal motor nucleus, DRG Dorsal root ganglia, NTS Nucleus tractus solitary, SP Substance P

Non-physiological and painful stimuli are mainly collected by splanchnic afferent fibers that convey into the thoraco-lumbar dorsal root ganglia (figure 4). The receptors involved are mesenteric/serosal and submucosal mechanonociceptors typically associated to intestinal arterioles. These specialized nerve endings branch extensively into varicose type endings responding to movements of the mesentery or intestinal walls. They do also respond to inflammatory mediators, NGF, mast cell mediators or hypoxia/ischemia, therefore, they can also act as chemonociceptors [18]. These mechanonociceptors are also involved in vasodilatation of arterioles during neurogenic inflammation by releasing CGRP and SP[24].

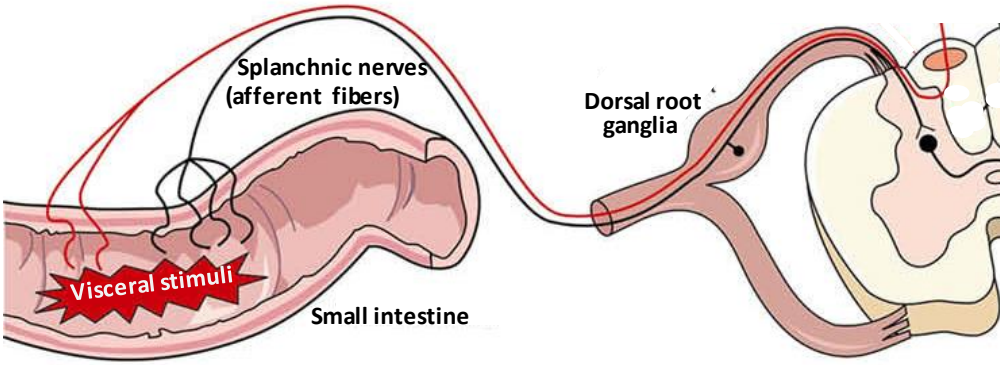

Figure 4. Schematic representation of splanchnic extrinsic afferent connections between small intestine and dorsal root ganglia. Modified from Knowles et al. 2009

2.3 Other mechanisms of gastrointestinal motility control

The GI tract, with a wide variety of entero-endocrine cells (EEC) and up to 20 identified GI hormones, is considered the largest endocrine organ. EECs are scatter-distributed in the mucosal epithelium [25,26] and release hormones in response to mechanical or luminal chemical stimuli[27]. These hormones can activate IPAN's and mucosal vagal afferent endings or directly act on smooth muscles cells, thus influencing gut motility[28–33]. In addition, the GI tract contains 70% of the body's immune cells [10], which protects the gut from external antigens. As an example, lymphocytes, dendritic cells, macrophages, mast cells or neutrophils[34–36] located in the GI tract

modulate GI motility by releasing mediators that act on smooth muscles[37–39] or afferent extrinsic pathways [40].

3. Gastrointestinal motility: patterns and assessment

GI motility is defined as the spatial and temporal coordinated contractions and relaxations of GI smooth muscle which allows the propulsion of ingested food along the GI tract. Motility patterns also promote mixing of ingested food with exocrine secretions and maximize the contact with the intestinal mucosa [5]. The propulsive activity also protects from accumulation of invasive pathogens or bacterial overgrowth which could endanger gut homeostasis [41].

3.1 Motility patterns

3.1.1 Gastric motility

Following ingestion of food, distinct motility patterns are produced in the proximal and the distal part of the stomach. In the proximal stomach, the muscular layer relaxes thus allowing accommodation and storage of the ingested food, while the muscular layer in the distal stomach contracts forming peristaltic waves that grind and propels the food bolus into the duodenum. In both patterns of motility the efferent pathway of the reflex is carried by fibers of the vagus nerve. In the proximal stomach, these fibers innervate inhibitory non-cholinergic non-adrenergic (NANC) neurons of the myenteric plexus. In contrast, in the distal portion, efferent fibers innervate excitatory cholinergic neurons in the myenteric plexus, thus, contracting the gastric musculature[5,42]. In addition, gastric emptying is also modulated by presence of nutrients in the GI tract that induces EEC to release GLP-1(Glucagon like peptide-1), PYY (Peptide YY) and CCK (Cholecystokinin). These hormones act on vagal afferent fibers that activate central efferent neural pathways that inhibit antral gastric contractions, thus, delaying gastric emptying[4,29,43–45].

3.1.2 Small intestine motility

The wall of the small intestine can execute two different motility programs: digestive and interdigestive patterns. During feeding, **peristaltic movements** allows aboral transport of the chyme bolus. Distention of the lumen and presence of nutrients trigger the peristaltic reflex which consists of coordinated contraction of the circular muscularis oral to the bolus and an aboral relaxation, thus allowing the chyme bolus to be propulsed gradually along the GI tract. The peristaltic reflex propagates aborally in a short distance of the GI tract. Peristaltic movements are alternated by **segmentation movements** which consist of regular and repeated contractions of the circular muscle layer simultaneously at different points of the digestive tube. As these contractions occur simultaneously the bolus is not propelled forward. Instead, it is subjected to back-and-forth movement thus, enabling segmentation and mixing with intestinal secretions[5,46,47].

During fasting or interdigestive state, the predominant motor pattern in the small intestine is the **migrating motor complex (MMC).** The function of the MMC is to expulse the remaining indigestible luminal content from the digestive tract. This also prevents bacterial overgrowth. The MMC propagates aborally and usually traverses the total length of the intestine or a long part of it (in contrast to the peristaltic reflex) [48].

3.1.3 Large intestine motility

Colonic motility accomplishes mixing the luminal content to facilitate water and electrolyte absorption and compression of food residues for defecation. We find two main motor patterns; a) rhythmic phasic contractions, that mix colonic content and b) giant migrating complexes that produce movement of faecal material along the colon and defecation[49].

3.2 Gastrointestinal motility measurement

The measurement of GI motility is of major importance to obtain information on physiological processes and pathologies related to the GI tract. The most common techniques used in the GI research and clinical field are summarized in table 2.

Table 2. Techniques to assess gastro-intestinal motility

		Parameter	
In vivo techniques			
	Distribution of oral markers	% GE	Mice[50]
		Geometric centre (intestinal transit)	Mice[51]
	Scintography	Time to half GE	Mice[52]
		Intestinal transit time	Human[53] Rat[54]
	Ultrasonography	Time to half GE	Human[55]
	Wireless motility capsule	GE and intestinal transit time	Human[56]
	Charcoal head	Intestinal transit	Rat[57]
	Lactulose breath test	Intestinal transit time	Human[53]
	Radiopaque markers	Intestinal transit time	Human[58] Cat[59]
	Strain gauge	Isometric tension (muscle contractibility)	Rat[60]
	Radio-telemetry	Muscle electrical activity	Rat[61]
	Manometry	Intraluminal pressure	Human[62]
	Food intake Defecation	Estimation of GE and colonic transit	Rat[63] Human[64]
In vitro techniques			
	Organ bath	Isometric tension (muscle contractibility)	Rat[40,65]
	Spatio-temporal motility mapping	Luminal diameter	Mice[66]

GE Gastric emptying; GC Geometric center

For the development of this thesis, *in vivo* **small intestine transit** has been used as a primary endpoint for motility assessment in rodents. Intestinal transit can be defined as the passage of ingested bolus through the GI time in a defined time. Because intestinal transit is subjected to various regulatory neural and hormonal mechanisms, it reflects a complete physiological process,

including feedback from other GI areas or the CNS. This provides a much more complete and integrated view of the GI transport function compared to the measurement of, for instance, isolated smooth muscle cells or muscular strips ex-vivo, which provide information on only intrinsic neuromuscular function [67]. In this work, the Geometric center (GC) technique has been used for the evaluation of intestinal transit in rodents for being more accurate than other methods.

Other methods commonly used include the charcoal head technique. In this method transit is measured by analyzing the time required for a suspension of non-absorbable charcoal to travel throughout the length of the GI tract. Intestinal transit is expressed as a percentage of the total intestinal length. While this method does not require much time it does not provide an accurate transit estimation since it only focuses on the head of the charcoal bolus [57]. On the other hand, the CG technique used in this work involves the analysis of the distribution of an oral-ingested marker along the GI tract over a period of time. Mostly, marker distribution is assessed post-mortem after dividing the GI tract in equally-sized segments. The calculated CG value is based on the percentage of the marker in the individual segments and indicates in which segment the marker has accumulated predominantly. Therefore, CG technique, compared to charcoal, results in a more accurate estimation of intestinal transit as it takes into consideration the distribution of the whole ingested bolus, not only the head of it, which may lead to intestinal transit overestimation. Higher CG values indicate a quicker intestinal transit[68]. Non-absorbable and inert colorimetric (phenol red), fluorescent (fluorescein-labelled dextran 70kDa) or radiolabeled markers (^{54}Cr) are routinely used [69–71].

For patients undergoing surgery, intestinal transit in this work was evaluated using the lactulose breath test. This technique measures oro-cecal transit time by means of ingestion of a carbohydrate which is only metabolized by cecal bacteria. Hydrogen (H_2) which is produced exclusively by fermentation of lactulose in the cecum passes from the blood to lungs and is finally exhaled. The exhaled H_2 is monitored by sampling the breath and the arrival of the lactulose into

the cecum can be registered as a sharp rise in breath H_2. The orocecal transit time is then given as the time between ingestion of lactulose and a specified threshold concentration of breath H_2[53,72].

Finally, intestinal motility was indirectly assessed in animal and patients studies using clinical parameters. For instance, fecal output and food intake have been previosly used as endopoints to define gastric emptying and colonic propulsion activity in animal models. Likewise, solid oral intake and defecation has been demonstrated to correlate with colonic transit in patients after colorectal surgery [64,73–75].

4. Mast cells

4.1 Activation and degranulation

In general terms, mast cells (MCs) are heterogeneous inflammatory cells widely distributed in the organism carrying out defense and surveillance functions. MC are predominantly located in strategic positions in tissues exposed to the external environment such as the as the respiratory epithelium, the mucosa and submucosa of digestive and urinary tract, and the skin [76,77]. This location allows MCs to be a first line of defense against noxious stimuli and pathogens and to orchestrate the innate and the adaptive immune response[76,77]. MCs are also involved in many inflammatory conditions such as asthma, atopic dermatitis, arthritis, atherosclerosis or interstitial cystitis [78–82]. Likewise, MCs are involved in diseases of the gastrointestinal tract (GI) such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) or postoperative ileus (POI) [83,84].

First reported by Paul Ehrlich over a century ago, MCs are easily recognized under light microscopy by cytoplasmatic granules that stain purple with basic aniline dyes such as toluidine blue, a phenomenon known as metachromasia[85]. These granules contain mediators that are released when the MC is stimulated. These mediators can be found already synthesized (and

therefore pre-stored in the granules) or be newly synthesized (de *novo* synthesis) after activation (table 3). Therefore, upon stimulation, mediators are released at two different times generating a quick response (seconds) by releasing pre-stored mediators, and/or synthesizing (*de novo)* new mediators that will be released long after the stimuli (hours). Mast cell mediators can increase vascular permeability and stimulate up-regulation of endothelial adhesion molecules thereby promoting infiltration of inflammatory cells to damaged or infected sites[86,87]. MCs are also involved in tissue remodeling by releasing proteases that alter the extracellular matrix, or stimulate proliferation of connective tissue[88,89]. Mast cells have thus important protective functions. However, unregulated release of MC mediators can alter homeostasis and under pathological conditions lead to inflammatory disease.

Table 3 Overview of main mast cell-derived mediators [91,92]

Pre-formed mediators	Newly generated mediators
Biological amines	*Lipid mediators (from arachidonic acid)*
Histamine	Leukotriene B_4 and C_4
5 hydroxytryptamine (5-HT)	Prostaglandin D_2
Vascular Endothelial Growth Factor	Platelet Activating Factor
Proteases	
Tryptase	*Cytokines*
Chymase	TNFα
Carboxypeptidase A	IL-1, IL-3 IL-4, IL-5, IL-6, IL-8, IL-1β
β- hexosaminidase	GM-CSF, MCP-1, MIPα
Proteolgycans	Transforming growth factor β (TGFβ)
Heparin	
Chondroitin sulfate	Nitric Oxide (NO)
Cytokines	
Tumor necrosis factor-α (TNFα)	
Interleukin(IL)-8	
Monocyte chemoattractant protein (MCP-1)	
Chemokine ligand 5 (CCL-5)	

Granulocyte Macrophage Colony Stimulating Factor, GM-CSF; MIP, Macrophage Inflammatory Protein. Modified from *Theoharis C Theoharides et al. 2012*

Degranulation and activation of MCs are induced as a response to a wide variety of stimuli orchestrated by different receptors expressed on MC. These stimuli include:

1) IgE antibody: IgE production, as a response to a specific antigen, binds to the high-affinity receptor for immunoglobulin E (FcƐRI) on MC membrane. When the antigen binds specifically to this complex, MC undergo intracellular signaling that leads to release of MC mediators (early response)[93]. This activation leads to immediate-type hypersensitivity reactions described in allergic responses.
2) Pathogenic agents: MCs express toll like receptors which recognize conserved components of pathogens such as lipopolysaccharide or peptidoglycans[94]. Likewise, recognition of M. tuberculosis by MC-expressed CD48 receptor leads to mast cell degranulation[95].
3) Endogenous inflammatory mediators (some examples are summarized in table 4).

Table 4 Endogenous inflammatory factors involved in mast cell degranulation

Stimuli	Type of MC	Action
Substance P (SP)	Human MC	β- hexosaminidase release, IL-8 and MCP-1 expression. TNF and IL-3 production[96]
Vasointestinal peptide (VIP)	Human MC	β- hexosaminidase release IL-8 and MCP-1 expression[96]
Calcitonin gene related peptide (CGRP)	Mice BMMC	Release of mMCP-1[97] Increased intracellular Ca^{++} concentration[98]
Complement 3a	Human MC	β- hexosaminidase release Expression of MCP-1 and CCL-5[99,100]
Complement 5a	Human MC	Expression MCP-1[100]
IL-1	Human MC	Secretion of IL-6[101]
Nerve Growth Factor (NGF)	Rat PMC	Histamine[102] Expression of IL-3,4,10 and TNF-α[103]

BMMC, Bone marrow-derived Mast cell; CCL-5, Chemokine ligand-5; IL, Interleukin; MC, Mast cell; mMCP-1, mouse mast cell protease-1; MCP-1, Monocyte chemoattractant protein-1; PMC, Peritoneal Mast Cell; Tumor necrosis factor-α, TNF- α.

4.2 Types of mast cells in the gastrointestinal tract

MCs derive from pluripotent hematopoietic myeloid precursors cells in bone marrow which migrate in the blood to peripheral tissues where they differentiate and mature. Along the GI tract, mature MCs are present in a relatively low number and are distributed throughout the mucosa, the connective tissue layers and the mesentery[103,104]. However, the number of MCs at a specific

location can increase dramatically as result of inflammatory response. Local environmental factors (Stem cell factor, IL-3, Th2 inflammation) drive the differentiation of MC precursors and ultimately lead to different phenotypes of MC[104,105]. Mature MC classification generally falls into two different subtypes. In the GI tract, we find mucosal mast cells (MMC) and Connective tissue mast cells (CTMC).

The most predominant feature that differentiates both subtypes of MC is the localization and the differential expression of MC specific serine-proteases pre-stored in cytoplasmatic granules.
In the rat, while MMC reside in the mucosa's *lamina propria*, CTMC can be found in the submucosa, muscular layers, serosa and peritoneum. Likewise rat MMC mainly express Rat Mast Cell Protease-2 (RMCP-2), a soluble chymase[106]. While rat CTMC mainly express the tryptase called RMCP-6[107].

In humans, the nomenclature differs from that on the rat. MCs expressing only tryptase are referred as tryptase subtype mast cells (MC_T) and reside predominantly in the mucosa. On the other hand, chymase-tryptase subtype mast cells (MC_{CT}) are those expressing both tryptase and chymase. MC_{CT} are confined to the submucosa, muscular layers, serosa and peritoneum[108,109]. Other morphological and functional differences between both subtypes of MCs are detailed in table 5. Each subtype has different roles in disease. Therefore, characterization and understanding of the differences of MC subtypes is of major relevance. In addition, it allows to specifically study the subtype of interest. For the development of this thesis RMCP-2, 6 and human tryptase and chymase have been selected as specific markers for identification of the different subtypes of MCs.

Table 5 Differences between mast cells subsets in the gastrointestinal tract in rat and human

	MMC/ MC_T	CTMC/MC_{CT}	Reference
Localization	Intestinal mucosa (*lamina propria*)	Intestinal submucosa Muscularis externa Serosa Peritoneal cavity	103,104
Specific protease expression			
Rat	RMCP-2,3,4,8,9,10	RMCP-1,5,6,7 Carboxypeptidase A	110 106,107
Human	Tryptase α,β	Tryptase α,β Chymase Carboxypeptidase A	111
Staining properties			
Toluidine blue	Weak if fixed with formol fixative	Good staining with formol fixative	112,113
Safranin/Alcian Blue	Safranin-/Alcian Blue +	Safranin+/Alcian Blue+	114,115
Proteoglycan content	Chondroitin sulfate	Heparin	116–118
Histamine content	Low	high	119

MMC, Mucosal mast cells; CTMC, Connective tissue mast cells; MC_{CT}, Chymase-tryptase subtype mast cells; $MC_{T,}$ Tryptase subtype mast cells; RMCP, Rat mast cell protease.

4.3 Mast cell-nerve interactions: Nerve endings and Nerve Growth Factor

Various studies have revealed MCs in close contact to extrinsic nerve endings supplying the gut and to enteric neurons, thus, presumably enabling communication [120–124]. Upon degranulation, mast cell mediators can activate both extrinsic and intrinsic neurons, thus suggesting that this communication is functional [125–129]. Furthermore, in the clinical setting, mast cell stabilization has been shown to decrease visceral hypersensitivity associated with IBS[130]. Thus, MCs are likely to play a role in activation of extrinsic afferent fibers resulting in fiber sensitization. Such activation is mediated by different mast cell mediator receptors expressed on extrinsic neurons. Spinal afferent neurons express Protease-activated receptor-2 (PAR-2) and activation by specific agonists mediates neuronal signaling [131,132]. Likewise, serotonin and histamine receptors are

expressed by sensory spinal neurons and their activation induces neuronal sensitization and enhances visceral hypersensitivity *in vivo* [133–136]. Conversely, CGRP and SP released by activated extrinsic afferent fibers can induce mast cell degranulation, thus mediating a bi-directional relationship between mast cells and extrinsic nerve fibers [98,126,132] (Figure 5). This reciprocal modulation can lead to a positive feed-back that may have implication in regulation of GI motility and functional disease [84].

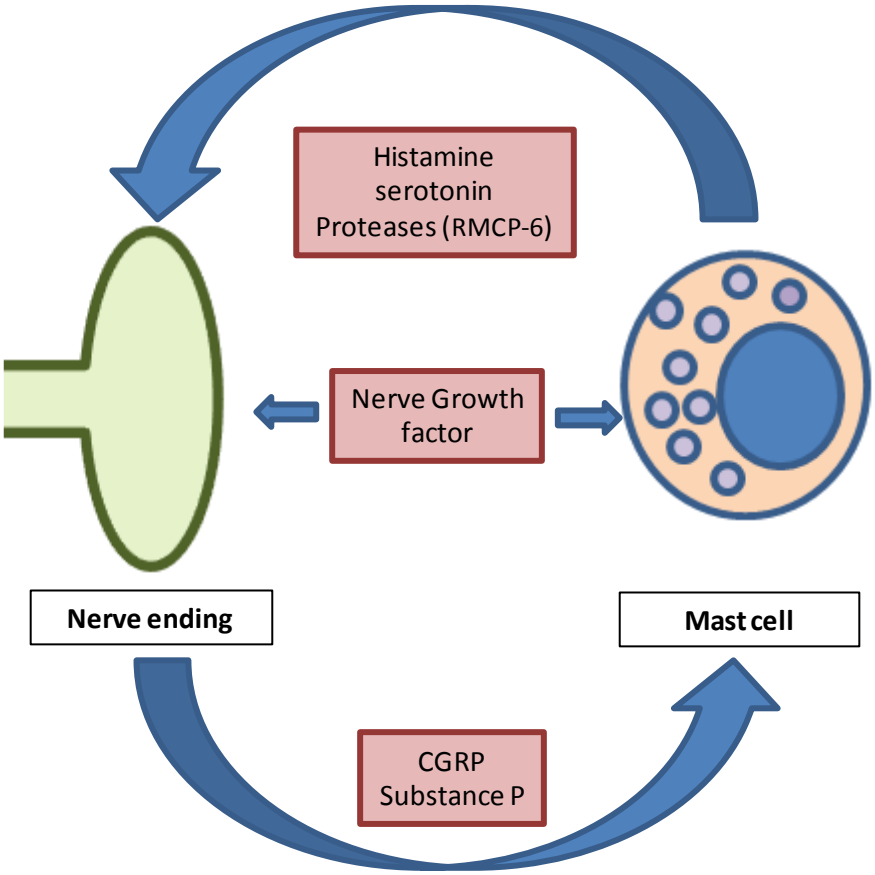

Figure 5. Schematic representation of bi-directional mast cell-nerve communication. CGRP, Calcitonin gene-related peptide, SP Substance P, RMCP-6, rat mast cell protease-6.

Nerve growth factor (NGF) is a member of the neurotrophin family involved in maintenance, differentiation and survival of developing sympathetic neurons and cholinergic neurons in the brain. However, NGF can also exert its actions on immune cells, including mast cells[137]. Mast

cells express the high affinity tropomyosin kynase receptor A (TrkA) through which NGF-induced responses are mediated [138–140]. NGF acts as an mast cell-chemoattractant *in vitro,* plays a role in differentiation and proliferation of MCs and serves as a trigger for mast cell degranulation and mast cell hyperplasia [98,102,126,141–145]. Furthermore, NGF contributes to the sensitization of spinal afferent neurons by up-regulating expression of neuropeptides CGRP and SP in dorsal root ganglia. This could lead to a NGF-dependent visceral hypersensitivity observed in a rat model of colitis[146–149]. Various studies reported a role for NGF in the IBS model, in which MC activation and impaired intestinal motility are prevented by using anti-NGF or NGF-blocking treatment[138,150]. Therefore, NGF-MC-nerve interactions (Figure 5) could provide a new therapeutic target to treat functional GI-disorders.

5. Postoperative ileus

5.1 Definition and clinical symptoms

Postoperative ileus (POI) is defined as a temporal cessation of bowel motility after major abdominal surgery leading to impaired propulsive activity of the GI tract[151]. POI occurs as consequence of opening of the peritoneum and handling or resection of the viscera especially the intestines[151]. The most common surgical procedures leading to POI are the resection of the small intestine (19.2% of small intestine resection patients) and large (14.9%) intestine [152]. Clinical symptoms of POI include abdominal pain and distention, nausea and vomiting, delayed passage of stool and the inability to ingest fluids and solids. Although POI is not life-threatening, it contributes to prolonged discomfort, morbidity and hospitalization of patients, thus increasing health care expenses. The economic burden is estimated to be 600 million € per year[153]. POI is therefore considered an important health care issue.

5.2 Pathophysiology of POI

The major factor underlying POI is the inhibition of intestinal motility. Neural pathways, intestinal inflammation and opioid drugs have been identified as major components in the pathophysiology of POI. Interplay of these factors is thought to result in the general hypomotility of the GI tract. Hypomotility in POI is a biphasic process, starting with an early, neural-mediated, phase of inhibition immediately after surgery, followed by a longer-lasting period of hypomotility evoked by an inflammatory response of the gut [154] (Figure 6). Intestinal manipulation thus triggers two major mechanisms impairing motility: 1) activation of spinal inhibitory neural reflexes; and 2) a local inflammatory response. The former lasts 2-3h and determines the first peak of motor impairment. The inflammatory phase lasts up to 3-4 days and determines the clinical course of POI [154].

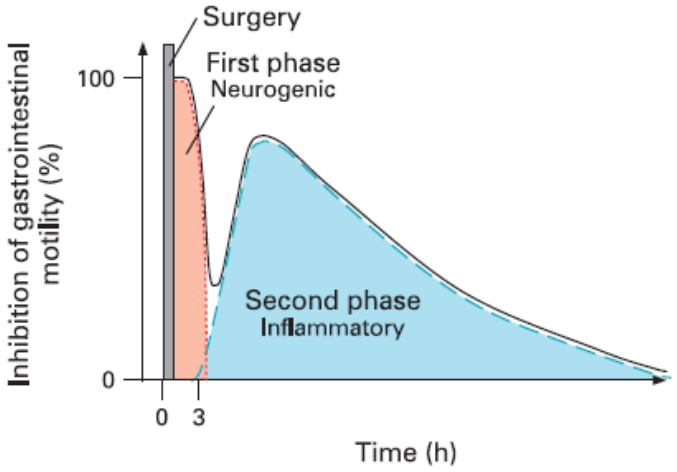

Figure 6. Schematic representation of postoperative ileus pathogenesis showing the two phases of hypomotility triggered by neural and inflammatory mechanisms. Adapted from GE Boeckxstaens, 2009.

5.2.1 Neural mechanisms

During the initial phase of POI, intestinal manipulation triggers afferent fibers of splanchnic and vagal nerves and activates the nucleus tractus solitary (NTS), the supraoptic and paraventricular hypothalamic nuclei and other CNS structures which play a pivotal role in regulation of GI

motility [155,156]. Adrenergic sympathetic pathways have been postulated as the efferent arm which inhibits intestinal motility following surgical handling of the bowel. These inhibitory pathways may arise from spinal ventral horn closing an entero-enteric inhibitory reflex or from descending pathways travelling back from the activated structures in the CNS[157]. A part from adrenergic pathways, inhibition of the intestine is also mediated by non-adrenergic non-cholinergic (NANC) efferent pathways[158,159] which could be mediated by the vagus nerve[160].

During the late phase of POI, inhibition of intestinal motility is also mediated by a spinal reflex through activation of splanchnic afferent and sympathetic fibers. This reflex depends on inflammatory leukocyte infiltrate in the muscular layer [155,157,161,162]. Simultaneously, the inflammatory infiltrate of the muscular layer, mobilized by the intestinal manipulation and tissue damage, activates the afferent and efferent arm of the vagus nerve thereby activating the anti-inflammatory vago-vagal reflex [163].

5.2.2 Inflammatory mechanisms

Intestinal manipulation of the bowel results in an inflammatory response of the muscular layer which leads to the dysfunction of smooth muscle contractibility. This was demonstrated by Kalff *et al.* who identified an inflammatory infiltrate composed of macrophages, neutrophils, natural killer cells, T-lymphocytes and mast cells in the manipulated jejunum which correlated with impairment of smooth muscle contractibility *in vitro* in rodents and human [164–167]. The infiltrating leucocytes, mostly macrophages and T_H1 lymphocytes, release inflammatory mediators such as IL-1 [168], IL-6 [169], IL-8[167], IL-12, IFN-γ [170], prostanoids and NO [39,171] which are up-regulated upon gut manipulation and contribute to dysmotility in POI. Intercellular adhesion molecule 1 (ICAM-1) and lymphocyte function-associated antigen 1 (LFA-1) participate in leukocyte recruitment and also play a role in the development of inflammatory POI[172,173].

5.2.3 Pharmacological mechanisms

Opiates given in the perioperative period and endogenous-opioids released during surgery also contribute to POI. Opioid-based pain management impairs intestinal smooth muscle

contractibility by interfering with enteric μ–opioid receptors which inhibits the peristaltic reflex[174] and delays intestinal transit[175]. Likewise, endogenous enkephalins, endorphins and dynorphins released during surgery can impair intestinal motility contributing to POI[176].

5.3 Strategies for prevention of postoperative ileus

Despite new findings on the factors that drive POI, its pathophysiology is not fully understood and there is still a lack of successful etiologic treatments. The strategies to prevent POI in the clinical setting focus on perioperative management of the patient. Multimodal programs are currently being implanted for major abdominal surgery and are based on the integration of enhanced surgical techniques, anesthesia and pain management and care program. These programs include the use of minimally invasive surgery and thoracic-epidural local anesthetics-based analgesia (opioid-spared pain management) along with anti-inflammatory drugs. Early oral feeding and mobilization, restriction of fluid therapy or elimination of nasogastric tubes have been also proved to shorten POI[177]. These multimodal programs, in combination with laparoscopic techniques, significantly improve the gastrointestinal recovery and therefore decrease the length of hospitalization following colorectal surgery[178,179]. As for pharmacological approach, the use of peripherally acting μ-opioid receptors antagonists, alvimopan, and other drugs, have shown preliminary positive effects but further clinical trials are requested [180–183]. Although, these approaches have contributed to reduce duration of POI, more basic research is needed in order to obtain more specific strategies to take to clinical trials.

5.4 Experimental models of POI

Experimental models of POI using rodents have been used over the last years to gain insight into the pathophysiological factors of this disorder and to evaluate new therapeutic targets. A classical model of POI is induced by manipulation of the GI tract exposed through a midline laparotomy using cotton swabs. Despite the difficulty to standardize the manual pressure applied to the bowels, intestinal manipulation is the most common model to induce POI. However, in order to reduce experimental variability, Van Bree et al, reported a device that allows standardization of

applied pressure onto the intestine[184]. Although these models are intended to resemble the surgical handling of the intestine during colorectal surgery, they do not always reflect the clinical setting. Such is the case with the intestinal anastomotic model, which although better reflecting the condition in patients, is not used routinely as a model of POI. This may be due to post-surgical complications – stenosis, dehiscence or ischemia - invalidating animals to remain in the experiment or interfering with functional outcome [185]. Finally, another model recently reported in POI research is the use of minimally invasive surgery in which intestinal manipulation is performed by laparoscopy, thus allowing comparison with open procedure[186].

5.5 Postoperative ileus and mast cells

Over the last ten years many studies have focused on the role of mast cells (MCs) in the pathogenesis of POI. The first evidence of MCs involvement in POI was the observation of mast cell protease release in peritoneal lavage soon after intestinal manipulation[187]. In addition, the use of mast cell stabilizer ketotifen improved symptoms of POI in animal models. Mast cell-deficient KO-mice also suffered from a less severe POI than wild type controls[187]. These observations suggested that MCs may be involved in the pathophysiology[52]. This was supported by the observation that degranulation of human peritoneal MCs (PMC) was related to the intensity of gut manipulation in patients undergoing abdominal surgery[167]. In addition, a clinical trial using ketotifen as pre-treatment demonstrated improvement of gastric emptying in patients undergoing gynecological surgery[188]. These data suggest an important role for MC in POI and has provided new potential therapeutic options for treatment.

In addition it has been proposed that locally released MC mediators, increase intestinal permeability[189]. This facilitates entrance of luminal, bacteria-derived products which additionally activates intestinal macrophages[154] following intestinal manipulation[164]. Upon activation, these macrophages secrete pro-inflammatory cytokines and chemokines that induce leukocyte recruitment and prostanoids and nitric oxide production that impair enteric muscular function[154]. Therefore, MCs have been considered as major contributors of the inflammatory cascade leading

to POI. However the exact mechanisms involved in the activation of MCs following abdominal surgery are not known. There is also a lack of knowledge on the mediators released by MCs during abdominal surgery and their effector mechanisms contributing to POI. Thus the specific role of MCs in the pathogenesis of POI remains unclear and requires further investigation.

HYPOTHESIS AND OBJECTIVES

Previous studies referred in the introduction suggest that MCs activation and impaired GI motility must be considered as potential effectors for POI. In addition, intestinal manipulation leads to activation of MCs located in the peritoneum and inhibition of such events prevents POI. On the other hand, MCs can interact with intestinal nerve endings. Such interactions are mainly mediated by neuropeptides or NGF, and may suppose one of the potential mechanisms leading to POI.

On this basis, our working hypothesis was that MC degranulation would be mediated by NGF and would trigger extrinsic neural activation in POI. MCs mediators (including or not NGF) would act on splanchnic afferents that would activate inhibitory mechanisms on GI motility. The ultimate aim of this thesis was to gain insight on mechanisms by which MCs contribute to POI.

For this purpose, we first studied patients undergoing colorectal surgery for being the most susceptible to POI development. And secondly, we set up a POI experimental model in the rat to further study and characterize GI motor function and morphological and molecular changes for the protocols proposed.

In order to verify our working hypothesis, the objectives for this work were the following:

1. To characterize and associate mast cell activation and clinical recovery in colorectal surgery.
2. To characterize intestinal motility, inflammation and mast cell activation in POI using a rat model.
3. To study if mast cells contribute to POI rat experimental model.
4. To analyze gene expression of nociceptors in thoraco-lumbar dorsal root ganglia with the aim to determine splanchnic activation in experimental POI.
5. To determine the involvement of mast cells in dorsal root ganglia activation during POI using both mast cell degranulators and stabilizing drugs.
6. To determine if the NGF-antagonist, K252a, inhibits mast cell degranulation *in vitro.*

7. To study the effect of K252a *in vivo on* mast cell degranulation and in an experimental model of POI.

CHAPTER 1

PERITONEAL MAST CELL DEGRANULATION AND GASTROINTESTINAL RECOVERY IN PATIENTS UNDERGOING COLORECTAL SURGERY

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Key words: Clinical recovery, intestinal surgery, mast cells, postoperative ileus

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ABSTRACT

Background: Degranulation of peritoneal mast cells (MCs) induced by intestinal manipulation has been proposed as a pathophysiological factor in postoperative ileus (POI). We aimed to explore the relationship between peritoneal and colonic mast cell degranulation and gastrointestinal recovery following colectomy.

Methods: Patients undergoing elective laparoscopic cholecystectomy (using a laparoscope and small abdominal incisions, n=14), and elective laparoscopic (n=32) or open partial colectomy (through a large abdominal incision, n=10) were studied. MC protease tryptase and chymase were studied in peritoneal fluid at the beginning, middle and end of each surgical intervention. Density of MCs in colectomy samples were examined as well as oro-caecal transit time by breath test, gastrointestinal function recovery by clinical composite endpoint GI-2 and association between mast cell proteases and clinical recovery.

Key Results: Open and laparoscopic colectomy caused greater peritoneal release of tryptase and chymase (323.0 ng mL^{-1} [IQR 53.05-381.4] and 118.6 ng mL^{-1} [IQR 53.60-240.3]), than cholecystectomy (41.64 ng mL^{-1} [IQR 11.17-90.93]) at the end of the surgical intervention. However, there were no differences between laparoscopic and open colectomy. Increased peritoneal protease release during surgery was observed in patients who developed POI after colectomy.

Conclusions & inferences: Colorectal surgery causes protease release from peritoneal MCs. Protease release does not differ between both types of colectomy (laparoscopy vs. laparotomy). However, MC activation is increased in colectomy patients developing POI. Therefore, degranulation of peritoneal MCs as a factor contributing to human POI after colectomy might be considered in future studies as a target to avoid POI.

ABBREVIATIONS

ASA-PS	American Society of Anaesthesiologists Physical-Health Status
GI	Gastrointestinal
GI-2	Gastrointestinal index recovery 2 composite endpoint
GI-3	Gastrointestinal index recovery 3 composite endpoint
LHBT	Lactulose hydrogen breath test
MC	Mast cells
MC_T	Tryptase subtype of mast cell
MC_{CT}	Chymase-tryptase subtype of mast cells
OCTT	Oro-caecal transit time
POI	Postoperative ileus

KEY MESSAGES

-Peritoneal mast cell activation and postoperative clinical recovery were evaluated in patients undergoing cholecystectomy and laparoscopic and open colectomies in a prospective and non-randomized study.

- We demonstrate for the first time an increased release of mast cell mediators into the peritoneal cavity during intestinal surgery.

- Colectomy patients with prolonged gastrointestinal clinical recovery presented higher levels of peritoneal mast cell mediators.

INTRODUCTION

Postoperative ileus (POI) is a prevalent motility disorder defined as the transient cessation of coordinated bowel motility after surgical intervention, which prevents effective transit of intestinal contents and/or tolerance of oral intake [1]. Primary POI occurs in almost all patients recovering from gastrointestinal surgery and is usually resolved spontaneously by the second or third day after laparoscopic surgery and by the fourth or fifth day after open laparotomy[2–6]. POI increases postoperative morbidity, prolongs hospitalization and increases healthcare costs[7]. Although postsurgical recovery of gastrointestinal (GI) functions has improved with minimally invasive surgery and multimodal perioperative care[8,9], the mechanisms underlying POI are still not fully understood and specific and effective treatments are needed[10].

Over the last decade, mast cells (MC) have been thought to have a major role in the pathophysiology of POI and could provide a mechanical connection between severity of intestinal manipulation during abdominal surgery and clinical duration of POI. Human MCs are classified according to their specific protease content. The tryptase subtype of mast cells, $MC_{T,}$ are located within the intestinal mucosa while the tryptase-chymase subtype, $MC_{CT,}$ are mainly confined to the submucosa, inner and outer muscularis and serosa/peritoneal cavity[11]. MC_{CT} located in the peritoneum release a wide range of inflammatory mediators when activated by intestinal manipulation[12–14], and this activation has been related to the intensity of bowel manipulation and POI[15] after major gynaecological surgery. In addition, De Jonge et al showed that stabilization of peritoneal mast cells prevented POI in rodents[14]. Studies with mast-cell-deficient mice, $kit^{W/Wv}$ and $kit^{W\text{-}sh/W\text{-}sh}$, further corroborate mast cell implication in POI dysmotility[12,14]. The *et al* reported that the MC stabilizer, ketotifen, improved gastric emptying after hysterectomy in humans[16]. However, results with a new model of mutant mast-cell-deficient mice[17] questioned the role of MC in POI.

The aim of the present study was to explore MC activation and release of MC mediators after colorectal surgery and to assess whether the intensity of this release was related to delay in

patients' intestinal motility and clinical recovery. To our knowledge this the first study to compare mast cell activation between laparoscopic and open colectomy. We focused on colorectal surgery as it is the most common type of surgery to be followed by POI[6,18] and so the one likely to benefit the most from future pharmacological treatment.

MATERIAL AND METHODS

Study participants

The study included 56 non-consecutive patients undergoing elective laparoscopic cholecystectomy or segmental colectomy at Mataró Hospital (Barcelona, Spain), from October 2010 to February 2012 (Table 1). Exclusion criteria were: 1) previous major abdominal surgery; 2) intra-abdominal infection or inflammation; 3) the use of non-steroidal anti-inflammatory drugs, corticosteroids or mast cell stabilizers within 7 days prior to surgery; 4) treatment with prokinetics within 14 days prior to surgery; 5) colostomies/ileostomies; and 6) intra-abdominal radiotherapy within 30 days prior to surgery. Physical status was assessed before surgery according to the American Society of Anaesthesiologists Physical-Health Status (ASA-PS).

Study design

This is a prospective and non-randomized study. Patients were divided into three groups for each surgical procedure: 1) laparoscopic cholecystectomy (N=14), 2) laparoscopic segmental colectomy (N=32), and 3) open segmental colectomy (N=10). Laparoscopic or open colectomy procedures were assigned according to standard medical indications at our institution[19]: Patients with 1) anaesthetic contraindication, 2) severe cardiorespiratory insufficiency or 3) chronic liver disease underwent open colectomy. Cholecystectomy patients were included as it is a laparoscopic procedure that avoids manipulation of the GI tract and could therefore serve as control group to obtain basal/normal values of proteases without intestinal manipulation. Regarding clinical output, cholecystectomy patients were a reference for normal clinical recovery

as patients in this group seldom develop POI. All the procedures were elective and none of the patients presented any inflammatory condition at time of surgery. The study was approved by the Institutional Review Board of the Hospital de Mataró and informed consent was obtained from all participants prior to the study.

Table 1 Demographics and clinical characteristics of the patients.

Patients at enrolment	**Cholecystectomy (n=14)**	**Colectomy (n=42)**		**p-value**
		Laparoscopic (n=32)	**Open (n=10)**	
Age (years)*	44 (39-63)	73 (63-79)	76.50 (67-83)	0.0001[a+]
Sex (men)	9 (64.28)	18 (56.25)	7 (70)	0.703[b]
Body Mass Index (kg/m^2)*	25.90 (23.61-31.02)	26.37 (25.30-30.11)	24.26 (20.23-28.75)	0.226[a]
ASA-PS (grade I or II)	13 (92.8)	21 (67.41)	7 (70)	0.188[b]
Charlson index*	0 (0-1.25)	2 (2-3)	2.50(2-4)	0.0001[a+]
Duration of surgery (min)*	63.50 (53.75-96.25)	215 (177.5-252.5)	221.50 (162.50-246.30)	0.0001[a+]
Type of segmental colectomy (%)				0.412[b]
Right colectomy		8 (25.00)	1 (10.00)	
Left/sigmoid colectomy		17 (53.12)	5 (50.00)	
Rectal resection		7 (21.87)	4 (40.00)	
Type of disease (%)				
Cholelithiasis	14 (100.0)	-	-	-
Right colonic Neopl.	-	5 (15.65)	3 (25.00)	0.227[b]
Left colonic /sigmoid Neopl.	-	19 (56.37)	3 (25.00)	
Rectal Neopl.	-	6 (18.75)	4 (33.33)	
Cecal Neoplasia	-	2 (6.25)	2 (16.66)	

Data are expressed as number of cases (percentage), except* Median (IQR)
[a]Kruskal Wallis test
[b]Chi square test
+cholecystectomy group compared to laparoscopic and open colectomy
ASA-PS, American Society of Anaesthesiologists Physical-Health Status

Perioperative care

Surgical interventions were performed by four expert colorectal surgeons exclusively dedicated to colorectal surgery. They have completed the learning curve meaning they have performed at least 100 procedures. The four surgeons were assigned randomly and performed both laparoscopic and open colectomies. A multidisciplinary case discussion took place for each clinical case (except for cholecystectomy patients). All patients were informed about the procedure, were scheduled for operation and underwent a pre-operative risk assessment. Only colectomy patients were put on a low-fibre diet during preoperative days. Patients were fasted for 6h and premedicated with 5mg of diazepam (po) the evening before surgery. Nasogastric tube insertion was not routinely performed. Early feeding and deambulation were started 4-6h after surgery for cholecystectomy patients and 24h for colectomy patients. Perioperative management is detailed in Table 2.

Assessment of mast cell activation during surgery

Peritoneal lavage samples were collected from each patient at three consecutive time points: 1) immediately after opening the peritoneal cavity, 2) after initial inspection and the first handling of the bowel (15 min after the first sample), and 3) at the end of the surgical procedure. Collection was performed according to a modified method by The et al[15]. Briefly, the peritoneal cavity was gently flushed with 40ml of warm, sterile salt water, 0.9% NaCl. After 30s exposure, 15-20 ml of the lavage fluid was collected, centrifuged at 240 RCF for 10 min at 4ºC and the resulting supernatants were stored at -80ºC. A cocktail of protease inhibitors (P8340, Sigma-Aldrich, St Louis, MO, US) was added to samples (1:100) immediately after collection to prevent protein degradation by endogenous proteases.

Tryptase and chymase release assay

Tryptase and chymase content of peritoneal lavage and was measured by ELISA (E91070 and E96515, Uscn, Life Science Inc, Wuhan, China) following manufacturer's instructions.

Table 2. Perioperative care and management

	Cholecystectomy	Laparoscopic colectomy	Open colectomy
Colonic preparation	No	Enema (left-sided and rectum resection)	Enema (left-sided and rectum resection)
Anesthesia			
Induction	Propofol (2mg/kg), iv	Midazolam (1mg, iv) Propofol (2mg/kg, iv)	Midazolam (1mg, iv) Propofol (2mg/kg, iv)
Maintenance	Sevoflurane (1-2%)	Sevoflurane (1-2%)	Sevoflurane (1-2%)
Analgesia			
Intraoperative	Fentanyl (150ug, iv)	Bupivacaine (1.25mg/ml at 4ml/h rate). Non-thoracic epidural	Bupivacaine (1.25mg/ml at 4ml/h rate). Non-thoracic epidural
Postoperative (until POD2)	Dexketoprofen(50mg/8h) and/or paracetamol (1g/8h)	Bupivacaine (1.25mg/ml at 4ml/h rate). Epidural	Bupivacaine (1.25mg/ml at 4ml/h rate). Epidural
Postoperative (until discharge)	Dexketoprofen (50mg/8h) and/or paracetamol (1g/8h)	Dexketoprofen(50mg/8h) and/or paracetamol (1g/8h)	Dexketoprofen(50mg/8h) and/or paracetamol (1g/8h)
Postoperative fluid regimen	1000ml glucosaline 5% for 12h	500ml NaCl 0.9%/24h or 1500 ml 5% Glucosaline/24h *	500ml NaCl 0.9%/24h or 1500 ml 5% Glucosaline/24h *

*Until intake of liquid

Mast cell counts in colonic specimens

Full thickness colonic tissue samples were collected from patients undergoing open and laparoscopic colectomy. Tissue was fixed in 4% formaldehyde solution for 72h and embedded in paraffin. Transversal sections (5μm) were cut on a microtome.

Immunohistochemistry. Endogenous peroxidase was quenched by incubation in H_2O_2 in distilled water. Nonspecific binding of antibody was blocked by 1-hour incubation with normal swine serum (P12731C, Sigma-Aldrich, St Louis, MO, US). Endogenous biotin and avidin were blocked using avidin/biotin blocking kit (SP-2001, Vector Laboratories, Burlingame, CA, US). Sections were incubated overnight at 4ºC with primary antibody for human MC tryptase (1:500) (sc-32889,

Santa Cruz Biotechnology, CA, US). Biotinylated polyclonal swine anti-rabbit IgG (1:200, 1h at RT, E 0353; (Dakocytomation, Glostrup, Denmark)) was used as a secondary antibody followed by incubation with Avidin/peroxidase kit (Vectastatin ABC kit, Vector Laboratories, Burlingame, CA, US). Specific staining was developed with 3,3'-diaminobenzidine (peroxidase substrate kit, SK-4100, Vector Laboratories Inc, Burlingame, CA, US) and sections were counterstained with haematoxylin. Specificity of the staining was confirmed by omission of the primary antibody.

Toluidine blue staining. Transversal sections of the colon were also stained with a 0.5% toluidine blue solution (Scharlau, CI 52040, Sentmenat, Spain) for 5 minutes.

Quantification of tissue MC. Tryptase-positive cells and metachromatic toluidine blue stained cells were quantified under a light microscope at 40x magnification (Olympus CH 30, Tokyo, Japan). The number of positive cells was expressed as the mean of 15 non-overlapping high-power fields. For evaluation of percentage of degranulation, MC were categorized as 'intact mast cells' (those with intact membrane outline, with no granules outside of the cell and intense staining) or 'degranulated mast cells' (disrupted cell membrane and extrusion of granules)[20,21]. The number of degranulated mast cells was expressed as mean of percentage of degranulation of 10 non-overlapping high-power fields (60x).

Clinical assessment of POI and gastrointestinal recovery

Clinical assessment of gastrointestinal function. Recovery of GI function was monitored daily until discharge by medical staff using the following endpoints: Gastrointestinal index recovery 2 and 3 (GI-2 and GI-3) composite endpoint, time to tolerance of solid and liquid intake, time to first flatulence and time to be ready for discharge. GI-2 was defined as the maximum time between first tolerance of solid food and first defecation and was the primary endpoint for recovery and to define POI[22]. GI-3 was defined as the maximum time to first tolerance of solid

food and minimum time between first flatus or first bowel movement, the most recent event being the score counted [22]. Patients were considered to be ready for discharge when they met the following criteria: tolerance to solid and liquid diet, satisfactory pain control with oral analgesics and competent mobilization and physical autonomy[8].

Colectomy patients were classified as either POI-developers or non-POI developers according to GI-2 because this parameter has been correlated with colonic transit [23]. As mentioned above, GI-2 is defined by the later of these two events to occur: tolerance of solid food in absence of nausea or vomiting or first defecation monitored by expert nursing staff. Patients with GI-2 >3 days were considered to develop POI according to a previously validated definition of POI [2,24]

Gastrointestinal transit time. The oro-caecal transit time (OCTT) was determined 24 hours after surgery by *lactulose hydrogen breath test* (LHBT) as described elsewhere[25]. Patients fasted for 12 hours and then consumed 10g of lactulose (Duphalac, Solvaypharma S.A, Parets del Vallès, Spain) dissolved in 100ml of a liquid breakfast preparation of 200 Kcal (20.2 g of proteins, 15.6 g of fats and 43.4 g of carbohydrates, T-diet 20/2 from Vegenat, Badajoz, Spain). Breath samples were taken every 15 min for 4h. Breath H_2 concentration was recorded with Gastrolyzer (Bedfont Scientific Ltd. Rochester, Kent, England). A baseline breath H_2 concentration was measured for each patient before the ingestion of lactulose. OCTT was defined as the time between lactulose ingestion and a rise to 20 ppm above baseline in breath H_2. The 20ppm cut-off threshold was used to exclude false positives[25,26] and patients with high baseline values above 16 ppm (7 out of 39 patients) or with no detectable exhaled H2 (6 out of 39 patients) were excluded from the analysis[25]. Additionally, 14 healthy volunteers were included in the study to explore normal OCTT using this methodology. A group of healthy volunteers was included, to have a reference value of intestinal transit time in non-operated healthy subjects.

Statistical analysis

Categorical parameters (sex, ASA and type of surgery) were expressed as percentages of cases and compared using Chi-Square test. Quantitative variables were considered non-parametrically distributed and expressed as median and interquartile range (IQR). Protease concentration in lavages was analysed by Friedman test for repeated measures followed by Dunn's post-test. MC counts were compared by Mann-Whitney U test for independent samples. OCTT and clinical parameters were analysed by Kruskall-Wallis test and Dunn's post-test for independent samples. Colectomy patients were also divided regarding diagnosis of clinic POI and data re-analysed: Protease concentration in lavages, number of mast cells, percentage of degranulation and duration of surgery were analysed by Mann-Whitney U test (independent samples). The relationship between MC and clinical parameters was assessed by Pearson (bivariate) correlation test and partial correlation test.

All statistical analyses were performed using GraphPad Prism 6.0 except for correlation test for which IBM SPPS v22 software was used. A p value <0.05 was considered significant.

RESULTS

Demographics and clinical characteristics of the sample and surgical procedures

Table 1 summarizes the demographic characteristics of the patients undergoing surgical procedures. Sex and ASA-PS distribution and body mass index did not differ between cholecystectomy and colectomy patients. As expected, colectomy patients were older than cholecystectomy patients and Charlson Comorbidity Index was also higher. Duration of surgery was shorter for cholecystectomies than for both types of colectomy procedures without any significant difference between laparoscopic or laparotomic approaches. One laparoscopic colectomy and one cholecystectomy had to be converted to open procedures for technical/surgical reasons. Five patients from the colectomy group (with wound dehiscence, sepsis or pneumonia)

and 1 patient from cholecystectomy group (with intra-abdominal haemorrhage and wound infection) were excluded from the analysis of gastrointestinal transit time and GI clinical recovery.

Protease content in peritoneal lavage

Cholecystectomy did not trigger any detectable release of tryptase at any of the three time points. In contrast, chymase was released at the end of cholecystectomy (41.64 ng mL^{-1} (IQR 11.17-90.93)), P<0.010) (Figure 1). Laparoscopic colectomy did not trigger any detectable release of tryptase at the beginning (0.12 ng mL^{-1} (IQR 0.03-0.24)) or middle of surgery but did trigger a significant release of tryptase at the end (2.02 ng mL^{-1} (IQR 0.48-31.54)), P<0.0001). Chymase concentration was significantly higher at the middle (39.10 ng mL^{-1} (IQR 12.25-106.40)), P<0.050) and further increased at the end of laparoscopic colectomy (118.60 ng mL^{-1} (IQR 53.60-240.30)), P<0.0001). Open colectomy was associated with a release of both proteases at the end of surgery (tryptase: 4.99 ng mL^{-1} (IQR 0.417-13.60), P<0.05; chymase: 323 ng mL^{-1} (IQR 53.05-381.40), P<0.010)). Protease release did not differ significantly between laparoscopic and open colectomy at any point in the surgery although the latter tended towards an increased release at the end of the surgical procedure (Figure 1).

Mast cell count and distribution in colonic wall

Toluidine blue and tryptase immunohistochemistry stained cells displayed MC-specific features (Figure 2E, 2F and 2G). No metachromatic MCs were found in the mucosa, indicating that toluidine selectively stains connective tissue MC (MC_{CT}). Number of MC significantly decreased from the luminal to the serosal side of the colonic wall (Figure 2A,B) and the density and distribution of MC in colonic wall did not differ between laparoscopic and open surgery groups (Figure 2A,B). MC were frequently associated with blood vessels in the serosa and submucosa and only rarely associated with myenteric ganglia (Figure 2F).

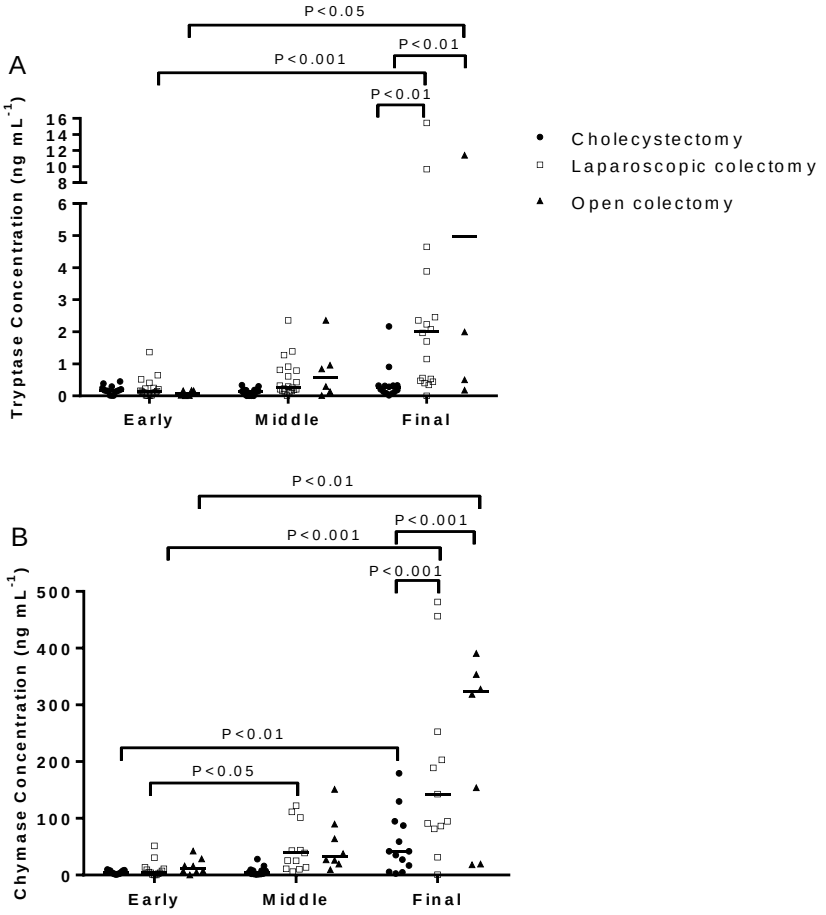

Figure 1. Mast cell tryptase (A) and chymase (B) concentration in peritoneal lavages collected at three different time points during cholecystectomy, laparoscopic and open colectomy. Dots represent individual values and horizontal lines represent median values expressed as ng mL^{-1}.

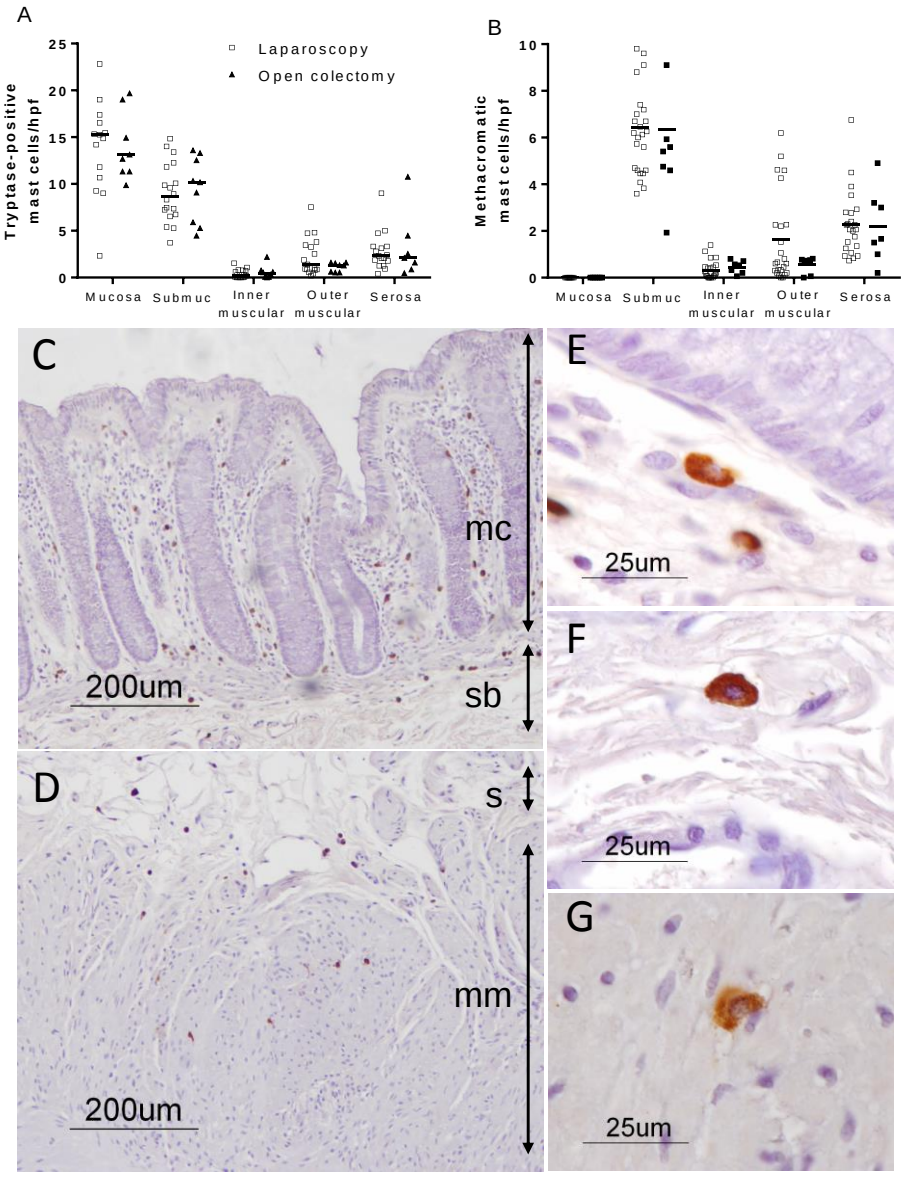

Figure 2. Quantification of mast cells in colonic tissue. Number of tryptase-positive (A) and metachromatic (connective tissue) mast cells (B) in colonic specimens collected at the end of laparoscopic and open colectomy. Dots represent individual values and horizontal lines represent median values (expressed as number of mast cells per high power field). Representative photomicrographs of tryptase immunostaining in human colonic samples (C,D,E,F,G): (C) Presence of MC in mucosa (mc) and submucosa (sb) at 10x;(D) MC in the outer muscular layer (mm) and serosa (s) at 10x. Detail of MC in mucosa (E), submucosa (F) and outer muscular layer (G) at 100x. Note intracitoplasmatic granules (E, F and G) and MC in close proximity to blood vessel in submucosa (F).

Clinical recovery of GI function

As we expected, GI-2 and GI-3 was longer for laparoscopic and open colectomy groups compared to the cholecystectomy group (Figure 3), indicating a slower recovery of GI function following colectomy. Colectomy patients also required more postoperative time before discharge and more time to tolerate oral intake. Time to first flatus was longer in laparoscopic colectomy compared to patients undergoing cholecystectomy and open colectomy. In contrast, clinical recovery did not differ between open and laparoscopic colectomy ($P>0.05$).

Oro-caecal transit time

Sex ratio of healthy volunteers (N=14, 6 men (42.85%) and 8 women (57.15%), did not differ from cholecystectomy and colectomy patients ($P>0.05$). The median age of the control group was 38 years (IQR 34-46) and the Charlson Index, 0 which did not differ from the cholecystectomy group ($P>0.05$). OCTT in healthy control subjects was 172.50 min (IQR 138.20-198.80) and did not differ from cholecystectomy patients who had a median postoperative OCTT of 172.5 min (IQR 116.30-198.80). The median OCTT of the whole group of colectomy patients was 240 min (IQR 165-250) without any statistical difference vs healthy subjects or cholecystectomy ($P>0.05$).

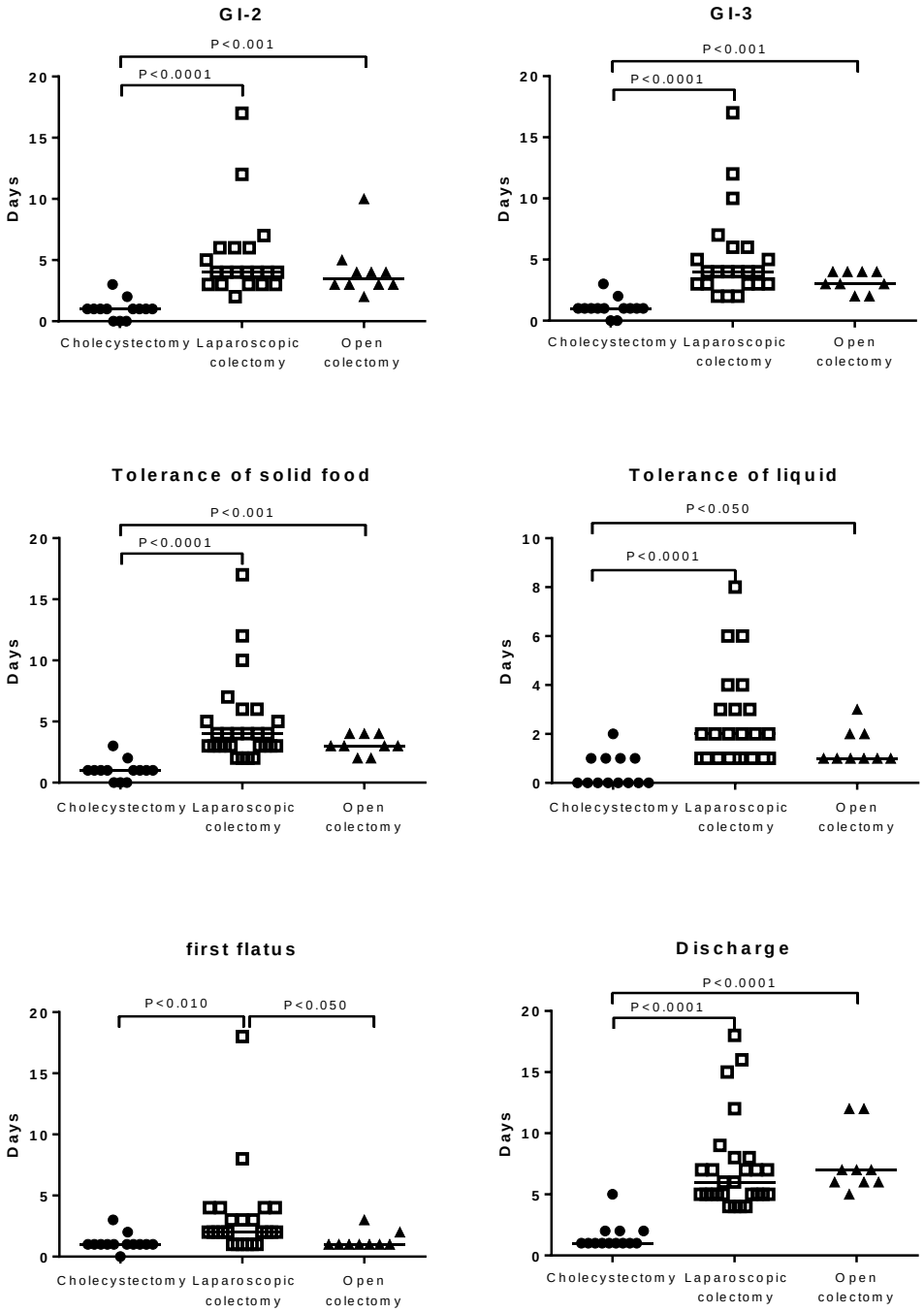

Figure 3. Clinical recovery of the gastrointestinal function after surgery. Dots represent individual values. Horizontal lines represent median values.

Evaluation of mast cells according to diagnosis of POI in colectomy patients

Colectomy patients were divided according to clinic diagnosis of POI and data were re-analysed in order to see how mast cell-related parameters were distributed in relation to POI incidence (Figure 4).

Non-POI patients and POI patients did not differ in age, sex, body mass index, Charlson index, duration of surgery and percentage of type of colectomy and disease (data not shown). In contrast, a higher proportion of ASA>3 were observed in patients developing POI (47.36% vs 6.6% in non-POI patients).

According to our definition of POI, 57% (15/27) of laparoscopic colectomies and 44% (4/9) of open colectomies developed prolonged POI compared to only 15% (2/13) of cholecystectomies. Moreover, colectomy patients diagnosed with POI had increased peritoneal tryptase (3170 ng mL^{-1} [IQR 1764-10971]) vs. non-POI patients (515.3ng mL^{-1} [IQR 365.9-1842.0]) P=0.002 and increased chymase (318.4ng mL^{-1} [IQR 94.59-456.3]) vs. non-POI patients (31.65ng mL^{-1} [IQR 18.6-142.6]) P=0.03.

In contrast, number and distribution of MCs and percentage of degranulation (non-POI patients: mucosa 16.23% [5.91-39.37]; submucosa 4.87% [0.0-7.08]; inner muscular 0.0% [0.0-11.67]; outer muscular 3.03% [0.0-24.04] and serosa 9.52% [3.75-17.36] vs. POI patients: mucosa 5.19% [1.33-21.72]; submucosa 7.14% [0.0-10.24]; inner muscular layer 0.0% [0.0-6.25]; outer muscular layer 0.0% [0.0-23.96] and serosa 0.0% [0.0-1.493]) did not differ among groups (P>0.05, Figure 4).

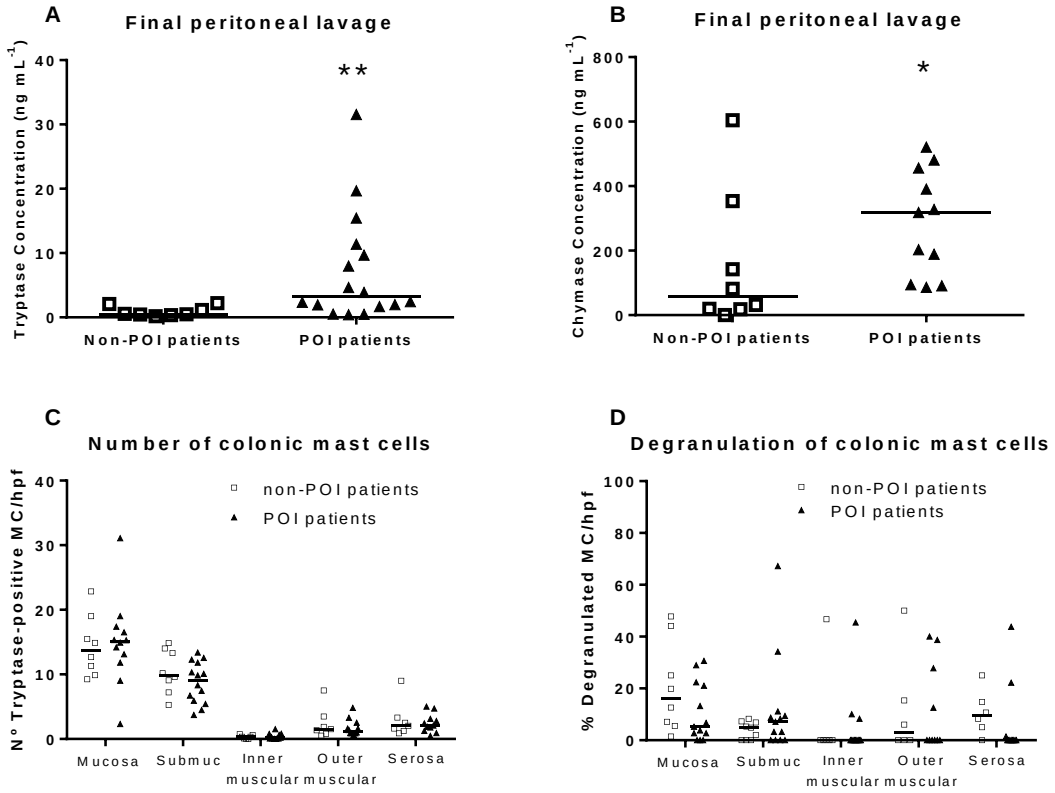

Figure 4. Colectomy patients divided according to diagnosis of postoperative ileus: Mast cell tryptase (A) and chymase (B) concentration in peritoneal lavages and number of tryptase-positive mast cells (C) and percentage of degranulation of mast cells (D) in colonic specimens collected at end of surgical procedures. Dots represent individual values and horizontal lines represent median values. Protease concentration is expressed as ng mL-^{1}. Quantification of mast cells is expressed as number of mast cells per high power field. Degranulation of mast cells is expressed as %. ** P<0.010,* P<0.05 vs non-POI diagnosed group.

Correlation between mast cell activation and time to discharge and GI-2.

Colectomy patients were analysed as a single group. Only discharge and GI-2 were included in the analysis because these parameters were found to be the most indicative factors of recovery. Age of patients correlated with GI-2 (r=0.409, P=0.029) while duration of surgery correlated with time to discharge (r=0.384, P=0.021). Of mast cell proteases, tryptase correlated with time to discharge (r = 0.422, P=0.032). However, duration of surgery and age of patients accounted for

this correlation as confirmed by partial correlation test. In fact, when we included these two parameters in the analysis –by means of partial correlation test- correlation for tryptase-discharge turned into r=0.34, P=0.092 after correction for the variable 'duration' and r = 0.392, P=0.052 after correction for the variable 'age of patients'.

DISCUSSION

Our exploratory study shows for the first time that colorectal surgery is associated with MC activation and the release of chymase and tryptase into the peritoneal cavity, irrespective of whether the colonic resection is performed by laparotomy or laparoscopy. Furthermore, protease release was greater in patients with POI. However, we could not establish a cause-effect relationship between MC activation and postoperative ileus because other factors such as duration of the surgery or age of the patients might have also influenced POI.

Our results agree with a previous study[15] that has shown that intestinal manipulation during abdominal surgery cause MC activation. In our study, however, MC activation was evaluated for three different surgical protocols all requiring intraperitoneal approach. Our findings show that colorectal surgery caused higher release of MC chymase and tryptase into the peritoneal cavity compared to cholecystectomy where only a moderate release of MC proteases was observed. This difference in protease release could also be influenced by the duration of the surgery. However, we believe this is not the case because protease concentration at 15 minutes (middle point for all groups) showed that cholecystectomy chymase (but not tryptase) release remained unaltered during cholecystectomies while in colectomies it was already elevated. Furthermore, when analysing non-POI colectomy patients (median duration of surgery 210 min), we found similar values of proteases as in cholecystectomy patients (median duration of 63 min). Consequently, our results strongly suggest that intestinal manipulation is the key factor in MC activation. Moreover, an unexpected finding was that both laparoscopic and open colectomies induced a similar release of MC proteases. Colectomies, independently of the surgical approach,

require intensive manipulation of the colon. Therefore, our results suggest that degranulation of MC does not differ much between laparoscopic and open approaches when part of the colon is removed. This is the first study to compare MC activation in intestinal surgery and the divergence of our results with other studies in non-intestinal surgeries [15] indicates, that in colorectal surgery, the handling and the resection of the colon are the main triggers of mast cell degranulation rather than the surgical approach (laparotomy vs. laparoscopy). This agrees with The's results that found mast cell activation in laparotomic hysterectomy but not in the laparoscopic and transvaginal approach[15].

Our results indicate a local response of MCs but we also aimed to evaluate whether systemic activation occurs upon intestinal manipulation. We observed that MC tryptase was not increased in plasma after colorectal surgery (data not shown). However the reduced number of patients in that part of the study limited the strength of our findings and further research is needed to confirm whether MC activation is only confined to the peritoneum. Moreover, in our study, open colectomy did not prolong clinical recovery compared to laparoscopic colectomy, in agreement with other studies [27–29]. Nonetheless, laparoscopic colectomy has been shown to decrease surgical trauma[30], reduce POI and improve recovery following abdominal surgery[3,8,9,31]. Perioperative care management also influences patient recovery. Vlug MS [8] and Van Bree SHW [9] clearly showed that open colectomy patients also benefited from fast-track, multimodal programs and have similar values for clinical recovery as laparoscopic patients receiving the same program. In our study, all colectomy patients received a standardized, similar and multimodal treatment. The lack of differences in our study could therefore result from similar perioperative care applied to all patients from which laparotomy patients benefited. Our results indicate that under a multimodal care program, the intensity of the manipulation of the colon is a key factor for postoperative motility recovery rather than the surgical approach itself. This is supported by other randomized studies that found no postoperative differences between both colectomy techniques [27,28]. Furthermore, our findings agree with a previous study in patients undergoing hysterectomy (where the colon is not removed) in which the surgical approach (laparotomy vs. transvaginal)

determined clinical recovery [15]. Criteria selection for open procedures in the colectomy group does not seem to entail a bias that could explain our finding. In any case, one would expect a bias towards a more delayed recovery in open colectomy patients according to their basal features. Likewise, duration of surgery and age of patients correlated with some clinical parameters but as we compared two similar surgery protocols that did not differ in duration or age of patients, and where perioperative protocol were similar, we can confirm these factors did not determine postoperative recovery in our patients.

Other studies have reported that the colon is the last part of GI tract to recover after surgery[32,33] which matches our findings. Our study demonstrated that the speed of clinical recovery was not associated to major changes in small intestine motility when assessed by oro-caecal transit time. It should be noted that lactulose by itself can slightly accelerate small intestinal motility [34]. It is therefore possible that such effect would mask the effect of POI in our measurement. Furthermore, the number of colectomy patients included in the analysis was limited due to inclusion criteria.

Our study also showed that peritoneal MC activation was related to occurrence of POI. Patients that were diagnosed with clinical POI according to a previously validated definition [2,24], had a significantly higher release of chymase and tryptase compared to patients that recovered earlier. MC activation is associated to increased visceral sensitivity[35,36], intestinal permeability [37] and altered motor functions [38] that could fit into the multifactorial scenario leading to POI. Nonetheless, we cannot conclude that MC degranulation alone is responsible for POI in our colectomy patients, as there are other variables that potentially affect clinical recovery. In the correlation analysis, duration of surgery and age of patients also correlated with time to discharge and GI-2 and so should be considered as possible influences on GI clinical recovery after colectomy. However, independently of other factors correlating with POI and the fact that our correlation analysis did not reveal a clear correlation between protease and GI-2/time to discharge, we report for the first time that POI patients after intestinal surgery presented a higher MC protease release than those who did not suffer POI. A therapeutic study using MC blockers

such as ketotifen or sodium cromoglycate should be conducted in order to find out whether preventing protease release could prevent POI.

A recent study using a new MC-deficient mouse model showed that there was not a role for MC in POI, casting doubt on the putative role of MC in inducing POI [17]. Despite this controversy, former models of murine POI and patients undergoing abdominal surgery [12–15] show degranulation of peritoneal MC and this is now further supported by the present study in a population of patients especially affected by POI. This study also describes MC distribution along the human colon wall, something scarcely reported in the literature[20,21]. However, we found no evidence that surgical handling of the colon was followed by recruitment of MC in the manipulated tissue, as reported in animal models of POI[39]. Furthermore, POI patients did not present changes in number of colonic MCs or morphological traits of degranulation that could suggest activation of intestinal-wall-resident MCs. However, MC have been shown to have piecemeal activation rather than full degranulation particularly in the gastrointestinal tract which could explain these results[37]. A more in-depth study using electron microscopy would be useful to address this question. In addition, as MCs in the serosa are peritoneal, and peritoneal MCs are difficult to stain or immunolabel (own observation and data not published), we think that this could have masked changes in the colonic wall, at least in the serosa.

To sum up, our study shows, for the first time, that colorectal surgery causes significant MC degranulation. In addition, colonic manipulation associated to resection seems to be the predominant factor determining peritoneal MC degranulation rather than colectomy surgical approach. Finally, higher levels of MC proteases in peritoneal lavage were found in patients with delayed GI recovery and POI. We suggest, therefore, that MC may play an important role in POI and efforts should be made to block this response regardless of the type of colectomy technique. Further studies are needed to verify MC involvement in POI and to explore whether MC stabilizers play a role in the prevention of POI following colorectal surgery.

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S.B designed and performed the studies related to mast cell activation, analysed data and wrote the paper. E.B, O.E and E.M designed and performed the study, analysed clinical and functional data and also contributed to writing the paper. J.R wrote the paper and contributed to analysing data. P.C and P.V designed the study and wrote the paper.

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CHAPTER 2

EFFECTS OF NERVE GROWTH FACTOR ANTAGONIST K252a ON PERITONEAL MAST CELL DEGRANULATION: IMPLICATIONS FOR RAT POSTOPERATIVE ILEUS.

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Key words: mast cells, NGF receptor, postoperative ileus

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ABSTRACT

Background: Stabilization of mast cell (MC) degranulation has been proposed to prevent postoperative ileus (POI). Nerve growth factor (NGF) mediates MC degranulation. The aim of the study was to evaluate whether NGF receptor antagonist K252a acts as a MC stabilizer in vitro and in vivo model of POI.

Methods: Peritoneal mast cells (PMCs) were obtained from Sprague-Dawley rats and were incubated with K252a and exposed to NGF or Compound 48/80 (C48/80). MC degranulation was assessed by β-hexosaminidase assay. POI was induced in rats by intestinal manipulation (IM). Rats were pre-treated with K252a (100µg/kg, sc) 20 min prior POI induction. 20 min after IM, rat mast cell protease 6 (RMCP-6) release was evaluated in peritoneal lavage. At 24h, intestinal transit (IT) and gastric emptying (GE) were evaluated. Ileal inflammation was assessed by myeloperoxidase (MPO) activity, expression of IL-6, NGF, TrkA, RMCP-2 and 6 and MC density within the full thickness ileum.

Key Results: C48/80 and NGF evoked degranulation of PMCs in a dose dependent manner. K252a prevented NGF-evoked, but not C48/80-evoked MC degranulation. IM evoked the release of peritoneal RMCP-6 and subsequently delayed IT and GE. IM increased MPO activity and expression of IL-6. In IM rats, K252a prevented up-regulation of IL-6 expression and reduced TrkA and RMCP-2 expression. IT, GE and inflammation were not affected by K252a.

Conclusions & Inferences: K252a inhibited NGF-evoked degranulation of PMCs *in vitro*. *In vivo*, K252a decreased IL-6 and RMCP-2 expression and PMC degranulation. This may be of relevance for the development of new therapeutic targets for POI.

ABBREVIATIONS

C48/80	Compound 48/80
CTMC	Connective tissue mast cells
GI	Gastrointestinal
GC	Geometric centre
IL 6	Interleukin 6
MMC	Mucosal mast cells
MC	Mast cell
MPO	Myeloperoxidase
NGF	Nerve growth factor
PMC	Peritoneal mast cells
POI	Postoperative ileus
RMCP	Rat mast cell protease
TrkA	Tropomyosin receptor kinase A

KEY MESSAGES

- Intestinal manipulation increased release of mast cell mediators into the peritoneal cavity in a model of rat postoperative ileus.

-NGF antagonist K252a inhibited degranulation of rat peritoneal mast cells *in vitro.*

-*In vivo*, K252a attenuated release of mast cell proteases (RMCP-6) from rat peritoneal mats cells and diminished expression of RMCP-2 within the intestinal wall in a model of postoperative ileus.

INTRODUCTION

Postoperative ileus (POI) is a motility disorder characterized by general hypomotility of the gastrointestinal (GI) tract evoked by intestinal manipulation during major abdominal surgery. POI leads to postoperative discomfort and prolonged hospitalization. Although many advances have been made in the perioperative management of patients, such as minimal invasive surgery or epidural local anaesthetics, there is a lack of etiologic treatments and therapeutic targets [1].

A major event evoked by intestinal manipulation during abdominal surgery is the activation of peritoneal mast cells (PMCs), observed in mice and humans [2–6], suggesting a role of mast cells (MCs) in the pathogenesis of POI. In addition, mast-cell deficient KO-mice suffer from a less severe POI, and MC stabilizing drugs such as ketotifen and doxantrazole prevent POI in mice [7]. Moreover, ketotifen improves delayed gastric emptying in patients undergoing abdominal surgery, although it does not successfully ameliorate POI [8]. Based on these studies, MC activation appears to play an important role in the pathophysiology of POI. Therefore, new strategies to block MC activity might become useful to treat or prevent POI.

Nerve growth factor (NGF) is a member of neurotrophin family exerting its actions mainly through the high-affinity NGF receptor, tropomyosin receptor kinase A (TrkA). TrkA activation by NGF leads to MC migration, activation or degranulation which can be antagonized by the specific TrkA receptor antagonist K252a [9–12]. Furthermore, inhibition of the NGF-TrkA pathways has been shown to prevent alterations of motility in models of IBS [13,14]. Thus, the TrkA receptor presents a potential target to modulate MC mediated inflammation and to normalize intestinal motility under inflammatory conditions.

We therefore investigated the effect of K252a as a potential antagonist of MC degranulation both *in vitro* and in an *in vivo* rat model of POI. The effect of treatment with K252a was evaluated by the following criteria: (a) intestinal transit and gastric emptying; (b) myeloperoxidase activity and interleukin-6 expression to determine intestinal inflammation in the manipulated intestine; (c) release of rat mast cell protease-6 (RMCP-6) in peritoneal lavage; (d)

number and protease expression of intestinal, wall-resident MCs, and (d) gene expression of NGF and TrkA.

MATERIAL AND METHODS

Animals

7-8 week-old (285-350g) male Sprague-Dawley rats (UAB Animal House facility) were kept under conventional conditions in an environmentally controlled room (temperature: 20-22ºC; photoperiod: 12h/12h light/dark cycle). Animals had free access to tap water and standard commercial pellet diet (2014 Harlan Maintenance). Animals were caged in groups of 2 to 3 upon arrival and allowed to acclimatize to the new environment for 5 days. All experimental procedures were approved by the Ethical Committee of the Universitat Autònoma de Barcelona (code 2478) and the Generalitat de Catalunya (code 7974).

Collection of peritoneal mast cells and *in vitro* activation with NGF

To obtain PMCs, animals were sacrificed by CO_2 inhalation, and 15 ml of Phosphate-buffered saline (PBS) was injected into the peritoneal cavity through a small incision in the abdominal wall. After 2 min of gentle abdominal massage, 10ml of the peritoneal lavage was extracted with a syringe. The peritoneal lavage was centrifuged at 240 RCF for 10 min at 4ºC. The pellet was resuspended in 1ml of Hank's Balanced Salt Solution (H9394, Sigma-Aldrich, St Louis, MO, US) supplemented with 11,25 µl of heparin (654754.0, Hospira, Madrid, Spain), pipetted on top of a 1-ml histopaque layer (11191, Sigma Aldrich) and centrifuged at 240RCF for 10min. The resulting pellet was resuspended in 2ml DMEM F-12 medium (12634-010, Invitrogen, Madrid, Spain) to obtain an enriched suspension of PMCs. To measure degranulation, MC suspensions were additionally washed twice with DMEM F-12 medium. Approx, $3\text{-}4\times10^4$ cells/well were then aliquoted in a 96-well plate in duplicate in a total volume of 100 µl DMEM F-12/well and set to rest for 45 minutes. Non-adherent cells were removed by replacing the medium twice using a

pipette. Cells were then incubated for 90min with NGF (1ng to 10µg/ml, N=3) or Compound 48/80 (C48/80; 0.1 to 100µg/ml, N=3) dissolved in DMEM F-12. To determine whether K252a affects the degranulatory response of MCs, cells were pre-incubated during 40 min with K252a (1.0nM to 200nM) or carrier (DMSO, final concentration 0.01%) added as 10 µl to each well. Cells were subsequently incubated with NGF (N=4, 10µg/ml) or C48/80 (N=4, 100µg/ml) for 90 min. Total cellular β-hexosaminidase content was released by lysing cells in designated control wells with 1% Triton-X 100 (Sigma-Aldrich, St Louis, MO, US). All incubations were performed in a humidified, 10% CO_2 incubator at 37ºC. Following incubation, plates were centrifuged (150g, 4°C, 5 min) and 60 µl of supernatant was collected from each well and stored at -80°C. NGF (N2513) and C48/80 (C2313) were purchased from Sigma-Aldrich. K252a was purchased from Tocris bioscience (1683, Ellisville, MO, US).

Assessment of mast cell degranulation *in-vitro* by β-Hexosaminidase assay

For determination of β-hexosaminidase, 60 µl of supernatant was added in duplicate to a fresh 96-well plate and mixed with an equal volume of 7.5 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide (N9376, Sigma-Aldrich) diluted in 0.2 M citrate buffer (pH 4.5). The plate was then incubated for 60 min at 37°C and the reaction was stopped by addition of 120 µl of 0.2 M glycine (pH 10.7) to each well. Absorbance at 360 nm and 460 nm was determined using a Victor-2 plate reader (PerkinElmer; Waltham MA, USA). The percentage of β-hexosaminidase was calculated as: {(a−b)/ (t−b)} ×100, where 'a' is the amount of release from stimulated cells, b is that released from non-stimulated cells and t is the total cellular content.

Prophylactic treatment of rats with k252a

Rats received an injection of K252a (100µg/kg, 1ml, sc) or vehicle (8% Ethanol, 1ml, sc) 20 min prior induction of the POI model: SHAM-vehicle (N=5), IM-vehicle (N=5), SHAM-k252a (N=5), IM-k252a (N=5). Peritoneal lavage was collected 20 min after intestinal manipulation while GI motility test, euthanasia and sampling were performed 24h after induction of the model.

Induction of postoperative ileus

Rats were anaesthetized by inhalation of isoflurane (4% and 2% for induction and maintenance respectively, IsoFlo, veterinaria ESTEVE, Barcelona, Spain). Body temperature was maintained at 37º C by placing the rat on a heating pad. Animals were prepared for sterile surgery. A 2cm-midline laparotomy was performed in all groups to expose the peritoneal cavity. Small bowel and cecum were carefully externalized onto sterile gauzes soaked and irrigated with sterile 0.9% NaCl solution to avoid tissue dryness. Distal jejunum, ileum, cecum and proximal colon were manipulated for 10 min with sterile cotton swabs. Abdominal wall and skin were closed performing a reverdin and subcuticular pattern respectively (Safil 3/0, BBraun Vetcare, Rubí, Spain). A single dose of buprenorphine (0.05mg/kg, s.c, Buprex, Schering-plough, Berkshire, UK) after surgery was administered for analgesia. Rats were returned to cages and allowed to recover from anaesthesia. No mortality or sings of clinical infection were observed during the postoperative period.

Collection of peritoneal lavage

Peritoneal lavage was collected 20 min after intestinal manipulation as following: during surgery, a syringe was inserted through the laparotomy and 7ml of sterile 0.9% NaCl solution was injected into the peritoneal cavity. After 20 min of exposure, 3.5ml of the solution were extracted from the peritoneal cavity. A cocktail of protease inhibitors (P8340, Sigma-Aldrich) was added to the sample (1:100) to prevent protein degradation by endogenous proteases. Samples were then centrifuged at 240RCF for 10 min at 4ºC. Supernatants were stored at -80ºC till further analysis.

Assessment of mast cell mediator release in peritoneal lavage

Mediator release of PMCs was measured by detecting extracellular RMCP- 6 in surgical lavage. For this purpose, a colorimetric assay was developed using a specific anti-RMCP-6 antibody. Appropriate controls omitting samples, primary and/or secondary antibodies were also performed to validate the assay. Peritoneal supernatant samples were diluted in carbonate coating buffer (1:2, Na_2CO_3: $NaHCO_3$, pH 9.6) and incubated onto microplates (Nunc Maxisorp flat bottom 96-well

plates, 300µl max volume, 44-2404-21) overnight at 4ºC. Non-specific binding of antibody was blocked by incubation with a solution of bovine serum albumin (A8531, Sigma-Aldrich) and 5% of normal donkey serum (D9663, Sigma-Aldrich) for 1hour at room temperature. Wells were incubated with a 1:1000 dilution of primary polyclonal antibody for rat RMCP-6 (32473, Santa cruz biotech, CA, US) for 2h at 37ºC. Donkey anti-goat IgG conjugated to horseradish peroxidase (1:200, 1h at 37ºC, 2020, Santa Cruz biotech) was used as a secondary antibody. Colorimetric reaction was developed by incubating the wells with a TMB substrate solution (3,3',5,5'-Tetramethylbenzidine, T0565, Sigma-Aldrich) for 10 min, at room temperature. The reaction was stopped by addition of 90µl 0.5M H_2SO_4. Absorbance was read immediately at 450nm using a microplate reader (Infinite F200, Tecan). For all washing steps, plates were rinsed four times with the washing buffer (Phosphate buffer salt, 0.05% Tween-20).

Functional studies: Gastrointestinal motility

Food was restricted to 11g the night before motility was assessed (restriction of 55%). The following day, 1,5ml of semiliquid and non-caloric phenol red solution (dissolved in 0.5% carboximethylcellulose/distilled water) was given by oral gavage (with round-shaped ending metallic feeding tube). Rats were killed by CO_2 inhalation 1 hour later. Stomach and small intestine were removed from the abdominal cavity. Stomach (Sto) and small intestine (SI) were separated and the later was divided into 9 equal, longitudinal segments (SI1-SI9). The phenol red concentration was determined in the stomach and individual intestinal segments as previously described [15]. The absorbance of phenol red was read at 560nm wavelength in a plate reader (ultrovision 2000, Pharmacia Biotech).

Gastric emptying (GE) was expressed as percentage of phenol red found in intestinal segments 1 to 9 referred to the total amount of phenol red found in the whole GI tract (including stomach). The formula used was the following: %GE = ([phenol red SI1 to SI9]/ [phenol red Sto to SI9]) x 100.

Intestinal transit was expressed as the geometric centre (GC) indicating the intestinal segment where phenol red was mainly located. The following formula was applied:

GC = ∑(%phenol red in each segment * segment number)/100 [4].

Sampling

Immediately after euthanasia (see previous section), full-thickness samples of distal ileum were removed and fixed in 4% paraformaldehyde in phosphate buffer overnight and paraffin embedded or snapped frozen in liquid nitrogen and stored at -80ºC until analysis.

Assessment of mast cell density in the ileum

5-µm transversal tissue sections of distal ileum were cut on a microtome from paraffin blocks and routinely processed for staining.

Toluidine Blue was used for staining of connective tissue mast cells (CTMCs). Tissue sections were stained with 1% toluidine blue (pH=7) for 20 minutes. Samples were then dehydrated and mounted in DPX medium.

Immunohistochemistry for RMCP-2 was used for identification of mucosal mast cells (MMCs) described as follows. Quenching of endogenous peroxidase was performed by 40-minute incubation of paraffin sections with 5% H_20_2 in distilled water. Non-specific binding of antibody was blocked by incubation with normal horse serum for 1 hour. Incubation of tissue sections with avidin and biotin (Avidin/biotin blocking kit, SP-2001, Vector laboratories) was performed to block endogenous biotin and avidin. Sections were incubated with primary monoclonal antibody for rat RMCP-2 (1:500, overnight at 4ºC, Moredun Animal Health, Edingburgh, UK). Biotinylated horse anti-mouse IgG (1:200, 1h at room temperature, BA-200; Vector Laboratories Inc, Burlingame) was used as a secondary antibody followed by incubation with Avidin/peroxidase kit (Vectastatin ABC kit, Vector laboratories) for 1h. Specific staining was performed with 3,3'-diaminobenzidine (Peroxidase substrate kit, SK-4100, vector laboratories) and counterstained with haematoxylin. Specificity of the staining was confirmed by omission of the primary antibody.

RMCP-2 positive and toluidine blue-stained cells were quantified in a blinded fashion under light microscope at 40x magnification (Olympus CH 30, Tokyo, Japan). The number of positive cells was expressed as the mean of counted cells on 15 non-overlapping high power fields.

Myeloperoxidase activity (MPO) assay

Ileum samples were weighted and homogenized in hexadecyltrimethylammonium bromide buffer (1ml/50mg tissue, 5g HTAB in 1L phosphate buffer 50mM, pH= 6.0) using GentleMACS (130-093-235, Miltenyi biotec). Samples were centrifuged at 1200rpm for 10 min at 4ºC and supernatants aliquoted until analysis. A standard was prepared with serial dilutions of purified MPO standard (M6908, Sigma) in HTAB buffer. 7µl of the samples at the appropriate dilution, standards and blank (HTAB buffer) were added to microplates (NUNC 96-well microplate, 12565210). O-dianisidine substrate solution was prepared by adding 16.8mg of O-dianisidine to 90ml of distilled water and 10 ml of phosphated buffered saline pH6.0. Before starting the reaction, 50µl of 1 % H_2O_2 were added to the substrate solution. To start the reaction, 200µl of mixture substrate solution were added to samples and absorbance of the reaction were read at 3min intervals during 15 minutes at 450 nm in a plate reader (Infinite F200, Tecan). Samples, blank and standard were assayed in triplicates. MPO activity was expressed as units of activity per mg of tissue (U mg^{-1}).

Reverse transcription–quantitative real-time PCR (RT-qPCR)

For extraction of total RNA, full-thickness ileum samples were homogenized in TRI reagent (Ambion, AM9738) using a homogenizer (IKA T10 basic ultra-turrax). Sample homogenates were mixed with chloroform for RNA separation and then with isopropyl alcohol for RNA precipitation. After washing steps, RNA was quantified by nanodrop (Nanodrop Technologies, Rockland, DE, USA) and converted into 20µl reaction volume of cDNA using a High Capacity cDNA Reverse Transcription Kit according to manufacturer's instructions (Applied Biosystems, US). RT-qPCR was performed using validated TaqMan gene expressions assays (Applied

Biosystems, US) with hydrolysis probes: Interlekin-6 (IL-6) (Rn01410330_m1), RMCP-2 (Rn01478347_m1), RMCP-6 (Rn00569857_g1), Nerve growth factor (NGF) (Rn01533872_m1) and TrkA (Rn00572130_m1). Gene expression was normalized to the reference expression of the housekeeping gene Actin beta (Rn00667869_m1). PCR reaction mixture was incubated on the ABI 7900 HT Sequence Detection System (Applied Biosystems). Samples were assayed in duplicates. Data were analysed using the Bio-Rad CFX Manager 2.1 software. Results were quantified using the $2^{-\Delta\Delta CT}$ method with respect to the expression of control groups and were expressed as fold-change relative to the house-keeping gene expression.

Statistical analysis

All data are expressed as Mean ± SEM. A robust analysis (one itineration) was used to obtain mean ± SEM for RT-qPCR data. Statistical analysis was performed using GraphPad Prism 6.0 software. One-way ANOVA with Sidak's multiple comparison test was used for all groups. A t-test for independent samples was performed for in vitro data when K252a was used. A p-value less than 0.05 was considered significant.

RESULTS

Effects of NGF and K252a on activation of peritoneal mast cell *in vitro*

A 90-min incubation of PMCs with 10µg/ml of NGF induced release of β-hexosaminidase ($P<0.05$, Figure 1 A). C48/80 also induced a significant release of β-hexosaminidase at 10 and 100µg/ml respectively ($P<0.05$, Figure 1 C). β-hexosaminidase release evoked by 10µg/ml of NGF was completely inhibited following pre-incubation with 200nM K252a ($P<0.05$, figure 1B). In contrast, K252a did not inhibit β-hexosaminidase release evoked by 100µg/ml of C48/80 (Figure 1 D). K252a by itself did not induce any release of β-hexosaminidase (not shown).

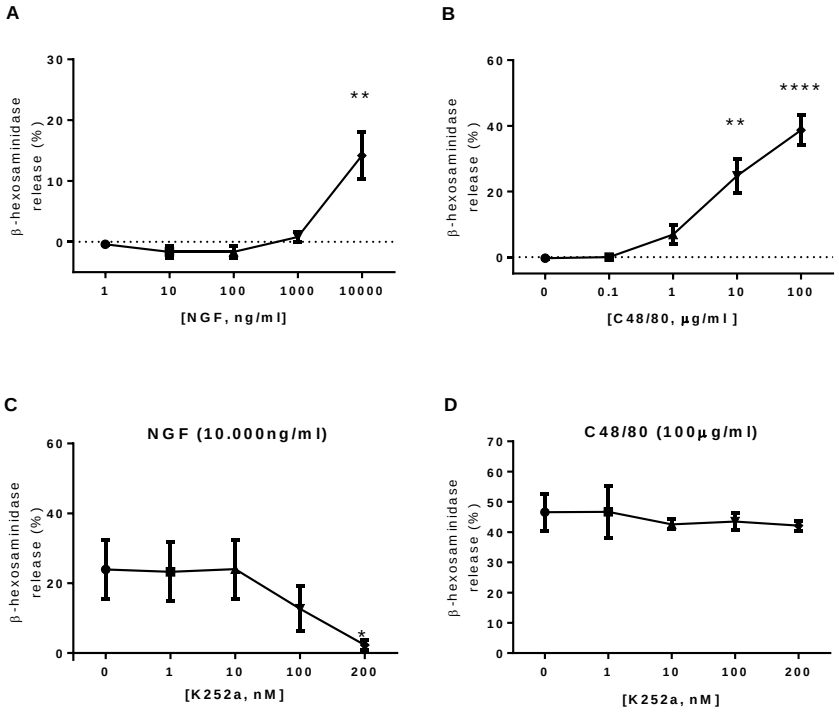

Figure 1. β-hexosaminidase release from peritoneal mast cells incubated with NGF (1-10000ng/ml) (A) and C48/80 (0-100µg/ml) (B) for 90min. Pre-incubation with K252a (0-20nM) for 40 min following incubation with 10000ng/ml NGF (C) or 100µg/ml C48/80 (D). Data are expressed as percentage of total cellular content. Dots represent mean values of N=3 (A, C) or N=4 (B, D). Vertical lines represent SEM. NGF, Nerve growth factor. C48/80, Compound 48/80. *, ** and **** P<0.05, P<0.01 and P<0.0001 respectively vs. basal group.

Effect of intestinal manipulation and K252a on peritoneal mast cell activation *in vivo*

Rats undergoing intestinal manipulation presented higher peritoneal release of RMCP-6 compared to sham groups (P<0.05, figure 2). Thus, indicating that intestinal manipulation induces mast cell degranulation in peritoneal lavage. Pre-treatment with K252a reduced slightly but not significantly RMCP-6 release in IM animals compared to vehicle (P=0.0502, figure 2).

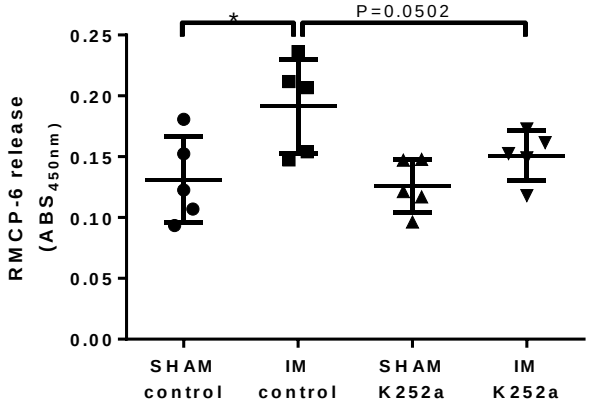

Figure 2. Release of RMCP-6 in peritoneal lavages collected 20 min after intestinal manipulation following pretreatment with K252a or vehicle. Data are expressed as Mean ± SEM of N=5. Dots represent individual values and vertical lines SEM. RMCP-6, Rat mast cell protease-6. * P<0.05.

Gastrointestinal transit following intestinal manipulation

Postoperative ileus-like symptoms following intestinal manipulation were observed in our rat model as characterized by delayed intestinal transit (P<0.05, figure 3 A) and delayed gastric emptying (P<0.05, figure 3 B) vs. SHAM-vehicle. K252a did not alter either basal intestinal transit or gastric emptying in SHAM animals and did not prevent the IM induced delay of GI transit and gastric emptying (Figure 3 A and B).

Intestinal wall inflammation following intestinal manipulation and pretreatment with K252a

MPO activity was increased in the ileal wall after intestinal manipulation (P<0.05 vs. SHAM-vehicle) but this effect was not prevented by K252a pretreatment (Figure 4 A).

Intestinal manipulation also increased the expression of IL-6 gene in the ileal wall compared to SHAM group (P<0.05). Treatment with K252a significantly reduced IL-6 up-regulation in IM rats (P<0.05 vs. IM-vehicle Figure 4 B).

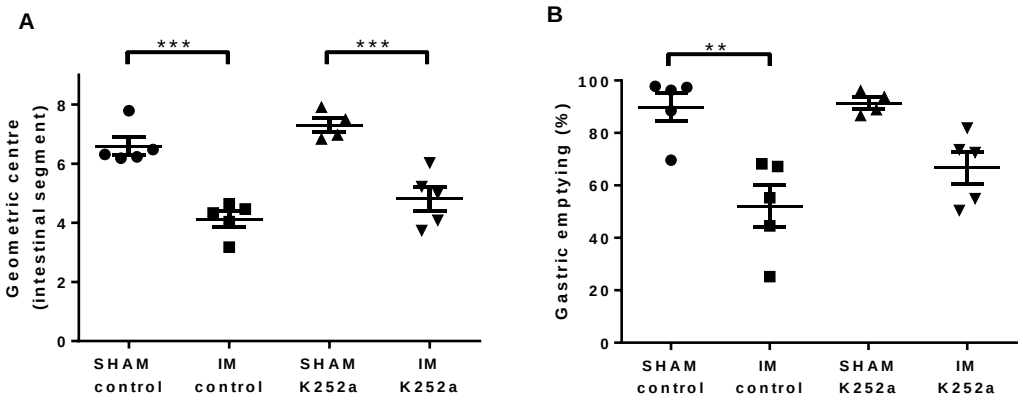

Figure 3. Intestinal transit (A) and Gastric emptying (B) 24h after intestinal manipulation following pre-treatment with K252a or vehicle. Data are expressed as Mean ± SEM of N=4-5. Dots represent individual values and vertical lines SEM. ** P<0.01, *** P<0.001.

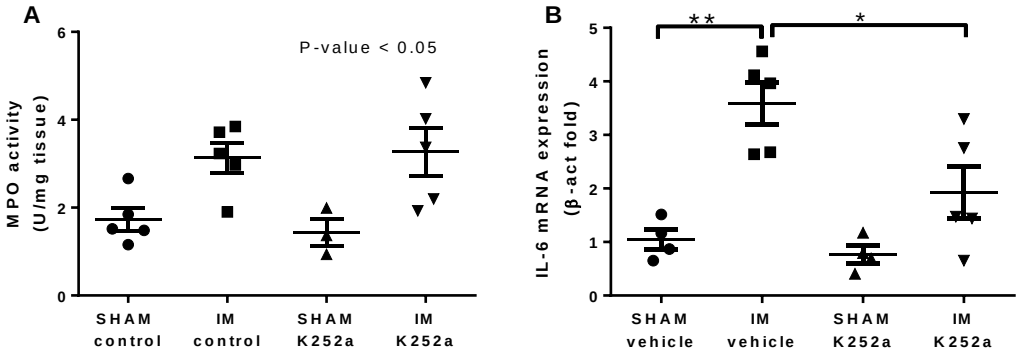

Figure 4. Evaluation of inflammation in ileum specimens collected 24h after induction of the model and following pre-treatment with K252a or vehicle. MPO cellular infiltrate assessed as MPO activity (A) and gene expression of pro-inflammatory IL-6 (B). Data are expressed as Mean ± SEM of N=3-5. Dots represent individual values and vertical lines SEM. A) P<0.05 is the overall P-value of ANOVA statistical test. B) *P<0.05, **P<0.01. MPO, myeloperoxidase; IL-6, Interleukine-6.

Mast cell distribution and density in intestinal wall following intestinal manipulation and K252a pretreatment

Only 1 to 2 CTMCs mainly located in the serosa were observed per slide. Furthermore, CTMC density was not altered by intestinal manipulation or K252a treatment. MMC density was also unaltered after intestinal manipulation (SHAM-vehicle 7.84 ± 0.78 vs. IM-vehicle 6.6 ±0.76 MMCs per 40x field, P>0.05) or K252a pretreatment (SHAM-K252a 7.06 ± 1.06 and IM-K252a 8.50 ± 1.44 P>0.05).

RMCP-6 gene expression in the ileal wall remained unchanged across the experimental groups while RCMP-2 expression was only decreased in IM-K252a compared to all other groups (P<0.05, figure 5A).

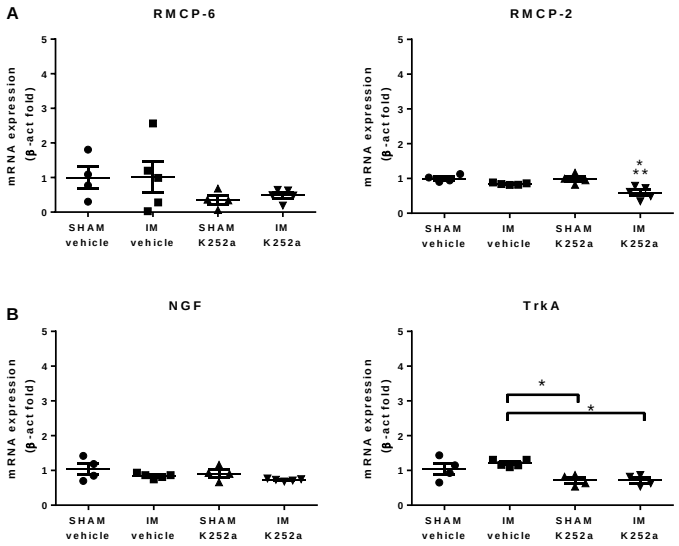

Figure 5. Gene expression of rat mast cell proteases RMCP-6 (A, left panel) and RMCP-2 (A, right panel) and Nerve growth factor (NGF, B, left panel) and its receptor TrkA (B, right panel) in ileum 24h after induction of the model and following pre-treatment with K252a or vehicle. Data are expressed as Mean ± SEM of N=4-5. Dots represent individual values and vertical lines SEM. RMCP-2 panel * P<0.05 vs IM-vehicle and ** P<0.01 vs. SHAM-vehicle and SHAM-K252a. TrkA panel *P<0.05.

NGF and TrkA gene expression

Intestinal manipulation or K252a treatment did not affect the expression of NGF within the wall of the ileum. However, K252a treatment reduced the expression of TrkA in both SHAM- and IM-treated animals compared to IM-vehicle treated rats ($P<0.05$, figure 5B).

DISCUSSION

In this study we demonstrate the ability of the tropomyosin-related kinase receptor antagonist K252a to inhibit NGF-evoked degranulation of PMCs *in vitro*. In addition, we demonstrate that K252a is able to block some of the functional consequences of intestinal manipulation, such as inflammation and mast cell degranulation, but it does not affect the motor inhibition observed in the POI model.

Previous studies demonstrated that NGF stimulates degranulation of PMCs [11,16] possibly via the NGF high-affinity receptor TrkA [13,17,18,9,19]. Our study confirms this by showing that stimulation of isolated PMCs with NGF evokes a degranulatory response. In addition, K252a prevented the NGF-evoked degranulation, without affecting the degranulation evoked by the MC degranulator C48/80 [11]. This suggests a specificity of K252a for the TrkA receptor in MCs.

We next evaluated the MC stabilizing properties of K252a *in vivo*, in the rat model of POI on the intestinal inflammation and transit. In line with previous studies, intestinal manipulation in our model resulted in a rapid degranulation of PMCs, as judged by the peritoneal increase of the CTMC specific product RMCP-6 [7,2,4,5]. Intestinal manipulation also resulted in a delay of the GI transit and produced inflammation of manipulated intestinal segments as previously reported [20,21].

K252a pretreatment attenuated the release of RMCP-6 *in vivo* following intestinal manipulation and expression of RMCP-2 within the intestinal wall, thus, further demonstrating that K252a has MC stabilizing properties. Jardí et al. demonstrated that MC express TrkA

receptor [13]. Taken together, these observations suggest that TrkA modulates MC activity and furthermore, that K252a could have a role for MC-mediated disorders.

Similarly, we also observed that K252a decreases IL-6 expression after intestinal manipulation. However, K252a did not prevent neutrophil activation after intestinal manipulation, as judged by tissue MPO activity. These findings suggest that, in our model, K252a is involved specifically in the inactivation of IL-6 secreted from intestinal resident cells including MCs or macrophages [22,23], cell types with TrKA receptors, rather than newly recruited neutrophils. Therefore, TrKA receptors seem not to interfere with other mechanisms leading to migration of circulating neutrophils as observed in other models [24,25]. In fact, only mononuclear cells have been identified as the most prominent cellular source for IL-6 in the intestine after intestinal manipulation [26].

Our study also demonstrates that K252a effectively blocks NGF receptor as TrkA mRNA levels were decreased in treated rats. NGF-TrkA interactions promote overexpression of TrkA on cell surface and this may explain our finding in rats receiving K252a [27]. This finding further corroborates that K252a inhibits MC activation as MCs express TrkA [13]. In fact, we reported attenuation of RMCP-6 release into the peritoneal cavity and the reduction of gene expression of RMCP-2 in the ileum after K252a pre-treatment.

However, GI motility was not improved by K252a. This is in contrast to a previous study in a rat model of food allergy reporting modulation of colonic motility in animals treated with K252a on a daily basis for 4 weeks [13]. We only observed a moderate effect of intestinal manipulation on delaying gastric emptying on K252a-treated animals compared to vehicle animals. So, we cannot confirm or discard an effect of K252a. We suggest that the lack of effect of K252a on motility in our study could be related to the fact that K252a was given only 20 min prior to intestinal manipulation, which is possibly not enough time to completely prevent intestinal manipulation-induced hypomotility.

In a similar way we cannot discard that K252a also acts on other cells expressing the TrKA receptor[28]. However, the fact that K252a specifically stabilizes mast cells allows us to

conclude that blocking of TrKA receptors can be a target for MC stabilization and therefore be useful in those diseases where MCs are involved.

In conclusion, K252a attenuated PMC degranulation, mucosal MC protease expression and up-regulation of IL-6 induced after intestinal manipulation in a POI model in the rat. Thus, showing 1) a potential use for treatment of MC-mediated disorders and 2) an anti-inflammatory effect. Therefore, we suggest that K252a and TrkA receptor should be further explored in intestinal inflammatory disorders with concomitant activation of MCs.

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S.B and J.R designed and performed the study, analysed data and wrote the paper. P.V designed the study and wrote the paper.

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No competing interests declared.

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CHAPTER 3

INTESTINAL MANIPULATION INCREASES GENE EXPRESSION OF VISCERAL-AFFERENT MEDIATORS IN DORSAL ROOT GANGLIA: ROLE FOR PERITONEAL MAST CELLS

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Key words: Afferent neural pathways, gastrointestinal motility, mast cells, postoperative ileus

Submitted to Neurogastroenterology and motility.

ABSTRACT

Background: Surgical handling of the bowel evokes degranulation of peritoneal mast cells (PMC) which has been demonstrated to play a role in postoperative ileus (POI). We aimed to investigate if MCs contribute to POI pathophysiology by activating intestinal afferent neural pathways.

Methods: POI was induced by intestinal manipulation in Sprague-Dawley rats. Additionally, compound 48/80 (C48/80) and ketotifen were used to modulate MC activity. Rat mast cell protease 6 (RMCP-6, ELISA) release was determined in peritoneal lavage 20 min after intestinal manipulation. At 24h, gastrointestinal transit was determined. Activation of dorsal root ganglia (DRG) was determined (PCR) for calcitonin gene-related peptide (CGRP), Protease-activated receptor-2 (PAR-2), Nerve growth factor (NGF) and TrkA receptor. Ileal wall inflammation was assessed by myeloperoxidase (MPO) activity, number of MCs, and PCR of IL-6, RMCP-2 and 6.

Key Results: Intestinal manipulation and exposure to C48/80 induced degranulation of PMCs, delayed gastrointestinal transit and up-regulated IL-6 and MPO activity. Intestinal manipulation, but not C48/80 up-regulated CGRP, PAR-2 and NGF/TrkA in DRGs. Ketotifen only improved gastric emptying and faecal output. Up-regulation of CGRP and TrkA expression in DRG was not prevented by ketotifen.

Conclusions and inferences: POI is accompanied by activation of DRGs thus confirming activation of splanchnic afferents. MCs are involved in POI dysmotility but, under our pharmacological approach, we were not able to prove that MCs are involved in DRG activation. Our results suggest that MCs should be targeted for treatment of dysmotility and that additional treatments should be employed to prevent DRG-mediated symptoms to ameliorate POI.

ABBREVIATIONS

CGRP	Calcitonin gene-related peptide
C48/80	Compound 48/80
CTMC	Connective tissue mast cell
GI	Gastrointestinal
IL 6	Interleukin 6
MMC	Mucosal mast cell
MC	Mast cell
MPO	Myeloperoxidase
NGF	Nerve growth factor
PAR-2	Protease-activated receptor 2
PMC	Peritoneal mast cell
POI	Postoperative ileus
RMCP	Rat mast cell protease
TrkA	Tropomyosin receptor kinase A

KEY MESSAGES

- Intestinal manipulation up-regulates gene expression of calcitonin gene-related peptide, nerve growth factor/TrkA and protease-activated receptor 2 in thoraco-lumbar dorsal root ganglia
- Intestinal manipulation induces activation of rat peritoneal mast cells and RMCP-6 release into the peritoneal cavity.
- Degranulation of peritoneal mast cells contributes to delayed gastrointestinal transit and inflammation during postoperative ileus.
- Modulation of mast cells with ketotifen and C48/80 does not modify gene expression of dorsal root ganglia.

INTRODUCTION

Postoperative ileus (POI) is a functional disorder following abdominal surgery characterised by hypomotility of the gastrointestinal (GI) tract. Although not life-threatening, POI prolongs hospitalization of patients and thus increases health care expenses [1].

In the rat GI tract, we find two different mast cell (MC) populations: mucosal mast cells (MMC) residing in the mucosa and expressing rat mast cell protease-2 (RMCP-2)[2,3] and connective tissue mast cells (CTMC) residing in the submucosa, muscular layer, serosa and peritoneal cavity and expressing RMCP-6 [2,4,5]. Degranulation of peritoneal mast cells (PMCs) following intestinal manipulation has been demonstrated, and has been proposed to play an important role in the pathogenesis of POI [6–8].

Extrinsic nerve endings supplying the GI tract are in close proximity to MCs [9,10] and a functional interaction between both has been described [11,12]. Activation of afferent systems evoked by intestinal manipulation has been documented during POI [13–15] and constitutes a first step to hypomotility [16–18]. Since MCs degranulate upon intestinal manipulation it is likely that a crosstalk exists between MCs and afferent nerves following intestinal manipulation.

This work aimed to elucidate interactions between MCs and afferent fibers of splanchnic nerves in an experimental model of POI in the rat. For this purpose we evaluated a) gene expression of selected genes in thoraco-lumbar dorsal root ganglia (DRG) and b) the effect of MC degranulator, Compound 48/80, and MC stabilizer, ketotifen on DRG gene expression.

MATERIAL AND METHODS

Animals

8 week-old (275-325g) male Sprague-Dawley rats (UAB Animal House facility colony stock) were used. Rats were kept under conventional conditions in an environmentally controlled room (temperature: 20-22ºC; photoperiod; 12h/12h light/dark cycle). Animals had free access to tap

water and standard commercial pellet diet (2014 Harlan Maintenance). Animals were caged in groups of 2 to 3 upon arrival and were allowed to acclimatize to the new environment for 5 days. All experimental procedures were approved by the Ethical Committee of the Universitat Autònoma de Barcelona (code 1398 and 2478) and the Generalitat de Catalunya (code 6309 and 7974).

Induction of postoperative ileus

Rats underwent only a laparotomy (SHAM group, N=7-8) or a laparotomy with intestinal manipulation (IM group, N=11-12) to induce POI. 20 min after induction, peritoneal lavage samples were collected. 24h post-surgery, animals were euthanized and intestinal transit and gastric emptying was measured followed by sampling of ileum and DRGs. POI was inducted by IM as follows. Rats were anaesthetized by inhalation of isoflurane (4% and 2% for induction and maintenance respectively, IsoFlo, veterinaria ESTEVE, Barcelona, Spain). Body temperature was maintained at 37º C by placing the rat on a heating pad. Animals were prepared for sterile surgery. A 2cm-midline laparotomy was performed in all groups to expose the peritoneal cavity. Small bowel and cecum were carefully externalized onto sterile soaked gauzes and irrigated with sterile 0.9% NaCl solution to avoid tissue dryness. Distal jejunum, ileum, cecum and proximal colon were manipulated for 10 min with sterile cotton swabs. Abdominal wall and skin were closed performing a reverdin and subcuticular pattern respectively (Safil 3/0, BBraunVetcare, Rubí, Spain). A single buprenorphine dose (0.05mg kg^{-1}, s.c, Buprex, Schering-plough, Berkshire, UK) was administered after surgery for analgesia. Rats were returned to cages and allowed to recover from anaesthesia. No mortality or sings of clinical infection were observed during the postoperative period

Pharmacological activation and stabilization of peritoneal mast cells

Pharmacological activation of MCs: rats were anaesthetized and prepared for sterile surgery as described above. After 2cm-midline laparotomy, a 8cm-segment of ileum was carefully externalized and place into an sterile Petri dish containing 6 ml of a 0.3mg mL^{-1} solution of

C48/80 (N=12) or sterile saline (N=12) for 1 min. After incubation, ileum was placed back into the peritoneal cavity. The surgical wound was closed as described above and animals were let to recover for 24h. No mortality or sings of clinical infection/anaphylaxis were observed during the postoperative period.

MC stabilization: Rats received an injection of ketotifen (10mg kg^{-1} in 2ml, ip) or vehicle (2ml of NaCl 0.9%, ip) 60 min prior induction of the POI model: SHAM vehicle (N=5), IM-vehicle (N=5) , SHAM-ketotifen (N=5), IM-ketotifen (N=5).

For both experimental protocols, 20 min after exposure/administration of drugs, peritoneal lavages samples were collected. 24h post-surgery, animals were euthanized and intestinal transit, and gastric emptying was determined following by sampling of ileum and DRGs.

Ketotifen fumarate salt (K2628) and Compound 48/80 (C2313) were purchased from Sigma-Aldrich, St Louis, MO, US.

Collection of peritoneal lavage

Peritoneal lavage was collected 20 min after intestinal manipulation or exposure to C48/80 as following: during surgery, a syringe was inserted through the laparotomy and 7ml of sterile 0.9% NaCl solution was injected. After 20 min of exposure, 3.5ml of the solution were extracted and a cocktail of protease inhibitors (P8340, Sigma-Aldrich, St Louis, MO, US) was added to samples (1:100) to prevent protein degradation by endogenous proteases. Samples were centrifuged at 240RCF for 10 min at 4ºC. The pellet was resuspended in 1ml of calcium- and magnesium-free Hanks Balanced Salt Solution (H9394, Sigma-Aldrich) containing 10% of fetal calf serum and 1000UI/ml heparin. The resulting solution was pipeted on top of a 1-ml histopaque layer (histopaque-1119, 11191, Sigma Aldrich) and centrifuged at 240RCF for 10min at 4ºC. The pellet was resuspended in 1ml of Hanks solution to obtain an enriched suspension of PMCs for morphological analysis (see below). Supernatants were kept frozen at -80ºC for further analysis.

For the ketotifen experimental protocol, PMC suspensions were further processed as described below.

Assessment of mast cell mediator release in peritoneal lavage

Mediator release of PMC was measured by detecting extracellular RMCP- 6 in surgical lavage. For this purpose, a colorimetric assay was developed using a specific anti-RMCP-6 antibody. Appropriate controls omitting samples, primary and/or secondary antibodies were also performed to validate the assay. Peritoneal supernatants samples were diluted in carbonate coating buffer (Na_2CO_3/$NaHCO_3$) and incubated onto microplates (NuncMaxisorp flat bottom 96-well plates, 300µl max volume, 44-2404-21) overnight at 4ºC. Non-specific binding of antibody was blocked by incubation with a solution of bovine serum albumin (A8531, Sigma) and 5% of normal donkey serum (D9663, Sigma) for 1hour at room temperature. Wells were incubated with a 1:1000 dilution of primary polyclonal antibody for rat RMCP-6 (32473, Santa cruz biotech, CA, US) for 2h at 37ºC. Donkey anti-goat IgG conjugated to horseradish peroxidase (1;200, 1h at 37ºC, 2020, Santa Cruz biotech, CA, US) was used as a secondary antibody. A colorimetric reaction was developed by incubating the wells with a TMB substrate solution (3,3',5,5'-Tetramethylbenzidine, T0565, Sigma) for 10 min at room temperature. The reaction was stopped by addition of 90µl 0.5M H_2SO_4. Absorbance was read immediately at 450nm using a microplate reader (Infinite F200, Tecan). For all washing steps, plates were rinsed four times with the washing buffer (Phosphate buffer salt, 0.05% Tween-20).

Assessment of mast cell morphology in peritoneal lavage

Cellular suspensions obtained from the surgical lavage were cytospined onto a slide (shandon 4 cytospin, 600rpm, 6 min, RT) and air-dried for 15 min, then fixed in Carnoy's fixative solution (60% pure ethanol, 30% Chloroform, 10% Acetic acid) for 15 minutes and stained with a 0.5% toluidine blue solution (Scharlau, CI 52040) at pH=7, for 5 min. Samples were dehydrated and mounted in DPX medium. MC counts and morphological assessment of degranulation was performed under light microscope at 40x magnification (Olympus CH 30, Tokyo, Japan) by an

observer blinded to the experimental groups. MCs were classified as either 'intact mast cells' (those with intact membrane outline, with no granules outside of the cell and intense staining) or 'degranulated mast cells' (disrupted cell membrane, extrusion of granules, poor staining) [19]. Degranulated MCs were expressed as percentage with respect total mast cells observed.

Postoperative fecal output

Fecal pellets were collected during the dark period (from 18.00 PM to 9.00 AM) before and after the surgery. For this purpose each animal was caged individually and put onto a metallic bottom grid. Fecal output was expressed as the percentage of fecal pellets produced by each animal in respect to its pre-surgical fecal pellet count.

Postsurgical assessment of gastric emptying and intestinal transit.

Food was restricted to 11g the night before motility was assessed (restriction of 55%). 1,5ml of semi-liquid and non-caloric phenol red solution (dissolved in 0.5% carboximethylcellulose/distilled water) was given by oral gavage (with round-shaped ending metallic feeding tube). Rats were euthanized by CO_2 inhalation 1 hour later. Stomach and small intestine were removed from the abdominal cavity. Stomach (Sto) and small intestine (SI) were separated and the later was divided into 9 longitudinal segments of equal length (SI1-SI9). Phenol red concentration was determined in the stomach and individual intestinal segments as previously described [20]. Absorbance for phenol red was read at 560nm wavelength in a plate reader (ultrovision 2000, Pharmacia Biotech).

Gastric emptying (GE) was expressed as percentage of phenol red found in intestinal segments 1 to 9 relative to the total amount of phenol red found in the whole GI tract (including stomach). The following formula was used for this calculation: %GE = ([phenol red SI1-SI9]/ [phenol red Sto to SI9]) x 100.

Intestinal transit was expressed as the geometric centre (GC) indicating the intestinal segment where phenol red is mainly located. The following formula was applied:

GC = ∑(%phenol red in each segment * segment number)/100 [21].

Sampling of ileum and DRG

Animals were euthanized 24h after intestinal manipulation. Full-thickness samples of distal ileum were removed and fixed in 4% paraformaldehyde in phosphate buffer overnight and subsequently embedded in paraffin. Thoraco-lumbar (T10-L2) DRGs and ileum were removed, snap-frozen in liquid nitrogen, and stored at -80º until analysis.

Assessment of mast cell density in the ileum

5-µm transversal tissue sections of distal ileum were obtained from paraffin blocks and routinely processed for staining.

Toluidine Blue was used for staining of CTMCs. For this purpose, tissue sections were stained with 1% toluidine blue (pH=7) for 20 minutes. Samples were dehydrated and mounted in DPX medium.

Immunohistochemistry for RMCP-2 was used for identification of MMCs as following. Quenching of endogenous peroxidase was performed by a 40-minute incubation of tissue sections with 5% H_20_2 in distilled water. Non-specific binding of antibody was blocked by incubation with normal horse serum for 1 hour. Incubation of tissue sections with avidin and biotin (Avidin/biotin blocking kit, SP-2001, Vector laboratories) was performed to block endogenous biotin and avidin. Sections were incubated with primary monoclonal antibody for rat RMCP-2 (1:500, overnight at 4ºC, Moredun Animal Health, Edingburgh, UK). Biotinylated horse anti-mouse IgG (1:200, 1h at room temperature, BA-200; Vector Laboratories Inc, Burlingame) was used as a secondary antibody followed by incubation with Avidin/peroxidase kit (Vectastatin ABC kit, Vector laboratories) for 1h. Specific staining was performed with 3,3'-diaminobenzidine (Peroxidase

substrate kit, SK-4100, vector laboratories) and counterstained with haematoxylin. Specificity of the staining was confirmed by omission of the primary antibody.

RMCP-2 positive and toluidine blue-stained cells were quantified in a blinded fashion under a light microscope at 40x magnification (Olympus CH 30, Tokyo, Japan). The number of RMCP-2 positive cells or toluidine blue-stained cells was expressed as the mean of counted cells on 15 non-overlapping high power fields.

Myeloperoxidase activity (MPO) assay

Ileum samples were weighted and homogenized in hexadecyltrimethylammonium bromide buffer (1ml/50mg tissue, 5g HTAB in 1L phosphate buffer 50mM, pH= 6.0) using GentleMACS (130-093-235, Miltenyibiotec). Samples were centrifuged at 1200rpm for 10 min at 4ºC and supernatants were obtained. A standard was prepared with serial dilutions of purified MPO standard (M6908, Sigma) in HTAB buffer. 7µl of the samples at the appropriate dilution, standards and blank (HTAB buffer) were added to microplates (NUNC 96-well microplate, 12565210). An O-dianisidine substrate solution was prepared adding 16.8mg of O-dianisidine to 90ml of distilled water and 10 ml of phosphate buffer pH6.0. Before starting the reaction, 50µl of 1 % H_2O_2 were added to the substrate solution. To start the reaction, 200µl of mixture substrate solution were added to samples and kinetics of the reaction were read at 3 min intervals during 15 minutes on a plate reader (450 nm, Infinite F200, Tecan). Samples, blank and standard were assayed in triplicates. MPO activity was expressed as units of activity per mg of tissue (U mg^{-1}).

Reverse transcription–quantitative real-time (RT-qPCR)

Total RNA was extracted from full-thickness ileal samples and DRG neurons (6 DRGs per animal,T10 to L2). Samples were homogenized (IKA T10 basic ultra-turrax) in TRI reagent (Ambion, AM9738). Homogenates were mixed with chloroform for RNA separation and then with isopropyl alcohol for RNA precipitation. After washing steps, RNA was quantified by Nanodrop (Nanodrop Technologies, Rockland, DE, USA) and converted into 20µl reaction

volume of cDNA using a High Capacity cDNA Reverse Transcription Kit according to manufacturer's instructions (Applied Biosystems). RT-qPCR was performed using validated TaqMan gene expressions assays with hydrolysis probes: Interlekin-6 (IL-6) (Rn01410330_m1), RMCP-2 (Rn01478347_m1), RMCP-6 (Rn00569857_g1), Calcitonin gene-related peptide (CGRP, Rn01511354_m1), CGRP receptor (Rn00688728_m1), Nerve growth factor (NGF, Rn01533872_m1), NGF-receptor TrkA (Rn00572130_m1) and protease-activated receptor 2 (PAR-2, Rn00588089_m1). Gene's expression was normalized to the reference expression of housekeeping genes Actine B (ileum, Rn00667869_m1) and Ribosomal protein S18 (Rps18) (DRG, Rn01428913_gH). The PCR reaction was performed on an ABI 7900 HT Sequence Detection System (Applied Biosystems). Samples were assayed in duplicates. Data were analysed using the Bio-Rad CFX Manager 2.1 software. Results were quantified using the $2^{-\Delta\Delta CT}$ method with respect to the expression of control groups and expressed as fold-change relative to house-keeping genes expression.

Statistical analysis

All data were expressed as Mean ± SEM. Statistical analysis was performed using GraphPad Prism 6.0 software (Graph Prism Software, Inc). For induction of POI and C48/80 experiments, all the parameters were compared using a t-test for independent samples. For ketotifen experiment, One-way ANOVA test with Sidak's multiple comparison test was used for all the parameters, except for % of phenol red distribution along the GI tract, for which a two-way ANOVA (factors being analysed were the intestinal segment and experimental treatment) with Turkey's multiple comparison test were run. A p-value less than 0.05 was considered significant.

RESULTS

Activation of dorsal root ganglia after intestinal manipulation

At 24h, intestinal manipulation increased mRNA expression of mediators related to viscero-sensorial function: CGRP, CGRP receptor, PAR-2 and NGF in thoraco-lumbar DRGs ($P<0.05$,

Figure 1 A). A trend toward increased expression for TrkA was also observed (P=0.054, Figure 1 A). These data suggest that intestinal manipulation activates extrinsic sensory fibers within the splanchnic nerves.

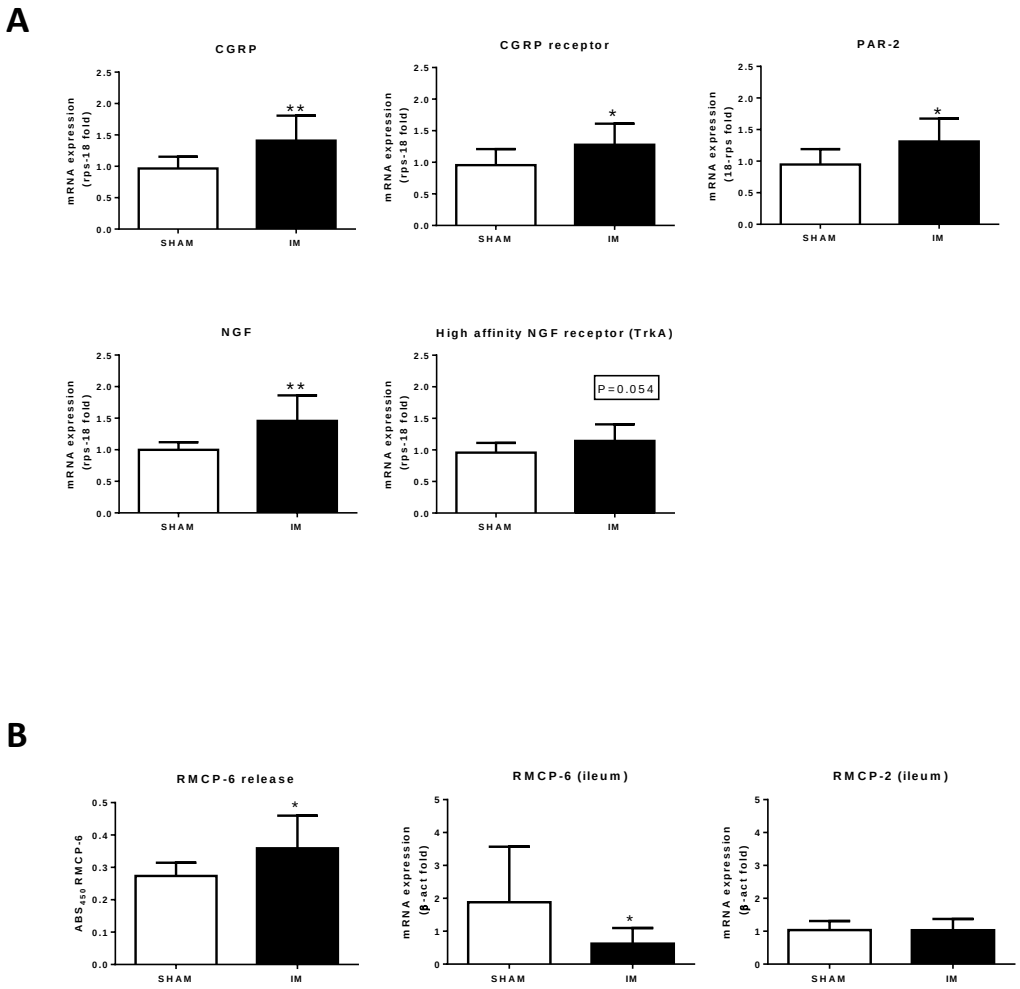

Figure 1. **PANEL A**. Intestinal manipulation up-regulates sensory-visceral markers in thoraco-lumbar dorsal root ganglia (DRG). Gene expression of calcitonin gene-related peptide (CGRP) and CGRP receptor, protease-activated receptor 2 (PAR-2), Nerve growth factor (NGF) and TrkA receptor. **PANEL B**. Characterization of mast cell response during postoperative ileus in peritoneal lavage and intestinal wall. (Left) Release of rat mast cell protease-6 (RMCP-6) into the peritoneal cavity 20 min after intestinal manipulation. Gene expression of (center) RMCP-6 and (right) RMCP-2 in ileal samples collected 24 hours after intestinal manipulation. * P<0.05, ** P<0.01, vs. SHAM. Data are expressed as mean ± SEM. N=7-12.

Postoperative ileus induction and mast cell activation after intestinal manipulation

24h after intestinal manipulation intestinal transit was delayed (3.97 ± 0.28 vs. 6.20 ± 0.28 in SHAM group, $P<0.05$). In addition, a trend towards delayed gastric emptying was observed (65.30 ± 8.05% vs. 84.07 ± 8.20% in SHAM group, $P=0.07$) accompanied by up-regulated ileal IL-6 mRNA expression (3.56 ± 1.02 fold change vs. SHAM, $P<0.05$).

20 min after intestinal manipulation, MC degranulation was observed in the peritoneal cavity: RMCP-6 release was increased by 23.74% in manipulated animals with respect to sham groups ($P<0.05$, Figure 1 B). At 24h after intestinal manipulation, RNA expression of RMCP-6 in manipulated rats was decreased compared to SHAM group ($P<0.05$, Figure 1 B). In contrast, RMCP-2 expression was not affected by intestinal manipulation (vs. SHAM group, Figure 1 B). Intestinal manipulation did not change neither number of MMCs (8.00 ± 0.21 in manipulated vs. 8.27 ± 0.53 in SHAM group, $P>0.05$) nor the number of CTMCs (0.04 ± 0.01 in manipulated vs. 0.03 ± 0.01 in SHAM group, $P>0.05$).

Effect of compound 48/80 on motility, inflammation and mast cell activation

Exposure to C48/80 mimicked the effects of intestinal manipulation as it also delayed intestinal transit ($P<0.05$), gastric emptying ($P<0.01$), evoked an up-regulation of the IL-6 expression and MPO activity compared to saline exposure ($P<0.05$, Figure 2 upper panel).

Exposure to C48/80 during 1 min induced a 30% decrease in the release for RMCP-6 in peritoneal lavage compared to exposure to saline ($P<0.05$) but did not induced changes in the expression of RCMP-6 andRMCP-2 in the ileal wall ($P>0.05$, Figure 2, lower panel).

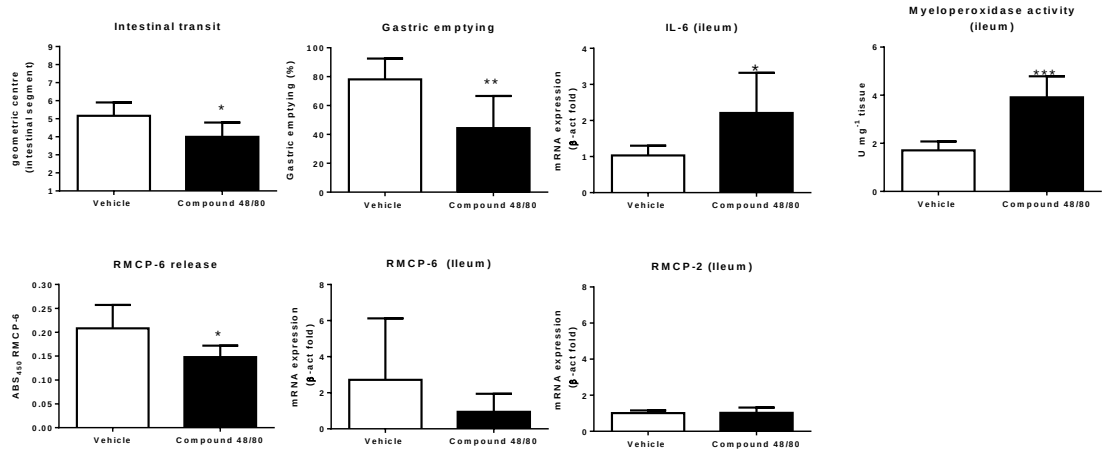

Figure 2. Effect of Compound 48/80 (C48/80) on intestinal transit, gastric emptying, gene expression of interleukin-6 (IL-6) and myeloperoxidase activity 24 hours after exposure to C48/80 (Upper panel). Rat mast cell protease-6 (RMCP-6) release into the peritoneal cavity 20 min after exposure to C48/80 and RCMP-6 and 2 expression in ileal wall 24h after exposure to C48/80 (lower panel). * P<0.05, *** P<0.001 vs. vehicle (saline). Data are expressed as mean ± SEM. N=6.

Activation of DGRs following exposure to C48/80

Exposure of the ileum to C48/80 did not affect the expression of mRNA for CGRP, CGRP receptor, PAR-2, NGF or TrkA when compared to vehicle group (P>0.05, Figure 3 A).

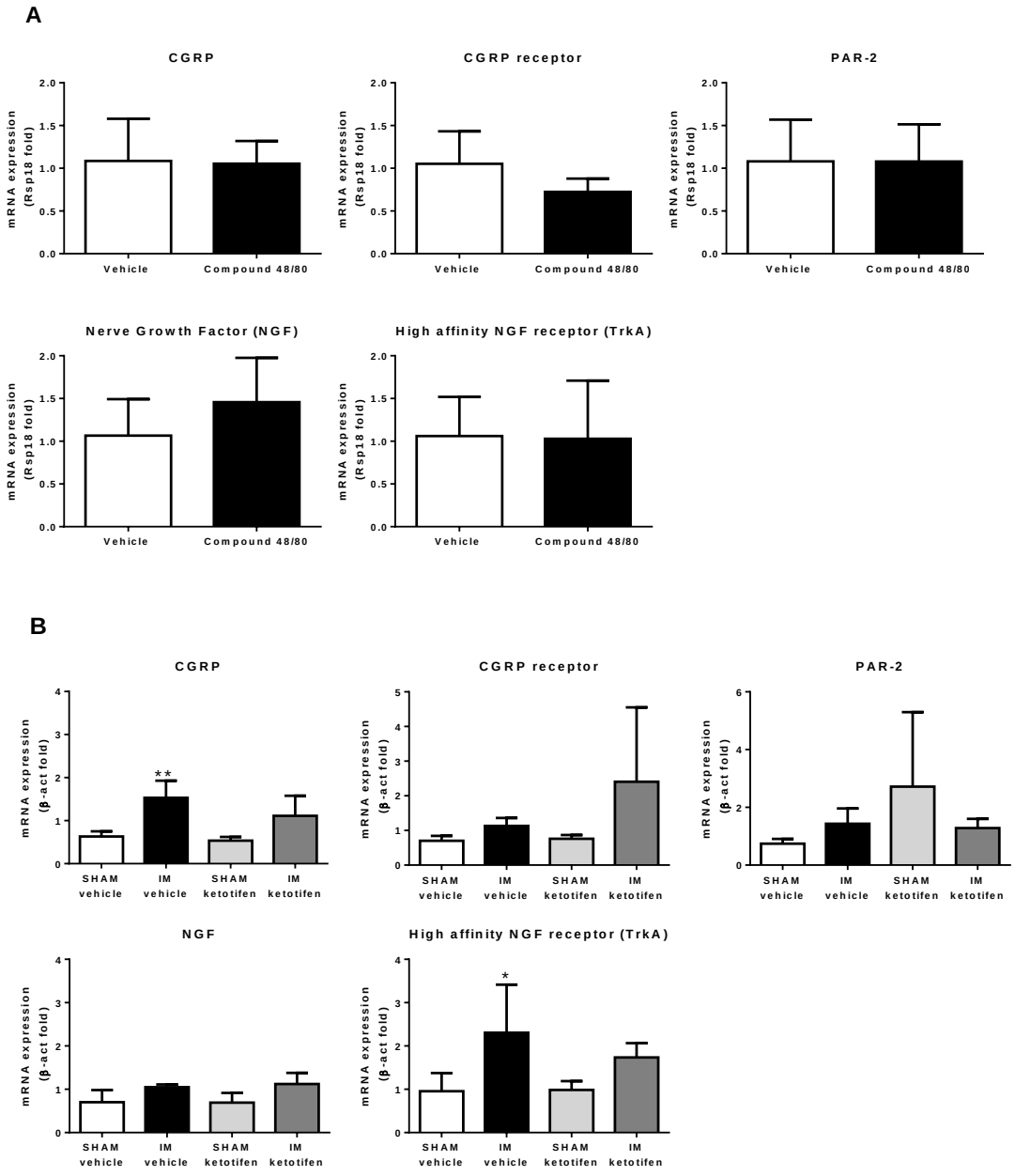

Figure 3. Expression of visceral sensitivity-related mediators in thoraco-lumbar dorsal root ganglia (T10-L2) after Compound 48/80 or ketotifen treatment. mRNA expression of Calcitonin gene-related peptide (CGRP), CGRP receptor, Protease-activated receptor 2 (PAR-2), Nerve growth factor (NGF) and TrkA receptor. Panel A: Effect of exposure to Compound 48/80, N=6. Panel B: Effect of ketotifen treatment, * P<0.05, **P<0.01 vs Sham-Vehicle, N=3-5. Data are expressed as mean ± SEM

Effect of ketotifen pretreatment on POI model

Intestinal manipulation delayed the intestinal transit and gastric emptying and reduced postoperative fecal output (IM-vehicle vs. SHAM-vehicle, $P<0.05$, Figure 4 A,B,C). In sham groups, ketotifen did not alter GI transit (SHAM-vehicle vs. SHAM-ketotifen, $P>0.05$, Figure 4 A,B,C). Nonetheless, in IM-ketotifen rats, CG was a 20% higher to that in IM-vehicle group ($P>0.05$). Delayed gastric emptying in IM-vehicle group (23.77 ± 11.53%) was improved after treatment with ketotifen but did not reach statistical significance (56.3 ±15.05%, $P>0.05$, Figure 4 B). Nonetheless, when plotting the amount of phenol red against each GI segment in the gastrointestinal histogram, phenol red in gastric content decreased after treatment with ketotifen (vs. IM-vehicle, $P<0.05$) indicating a partial effect of ketotifen in the prevention of POI by intestinal manipulation (Figure 4 D). In addition, ketotifen improved post-surgical fecal output suggesting improvement of large intestine propulsion (vs. IM-vehicle, $P<0.01$, Figure 4 C).

Pretreatment with ketotifen did not prevent the increase in mRNA expression of IL-6 induced by intestinal manipulation ($P>0.05$, Figure 4 E). Intestinal manipulation increased activity of MPO within the intestinal wall (IM-vehicle vs. SHAM-vehicle, $P<0.05$, Figure 4 F). Interestingly, ketotifen did not prevent the up-regulation of MPO activity but further increased MPO activity after intestinal manipulation (IM-ketotifen vs. IM-vehicle, Figure 4 F, $P<0.05$).

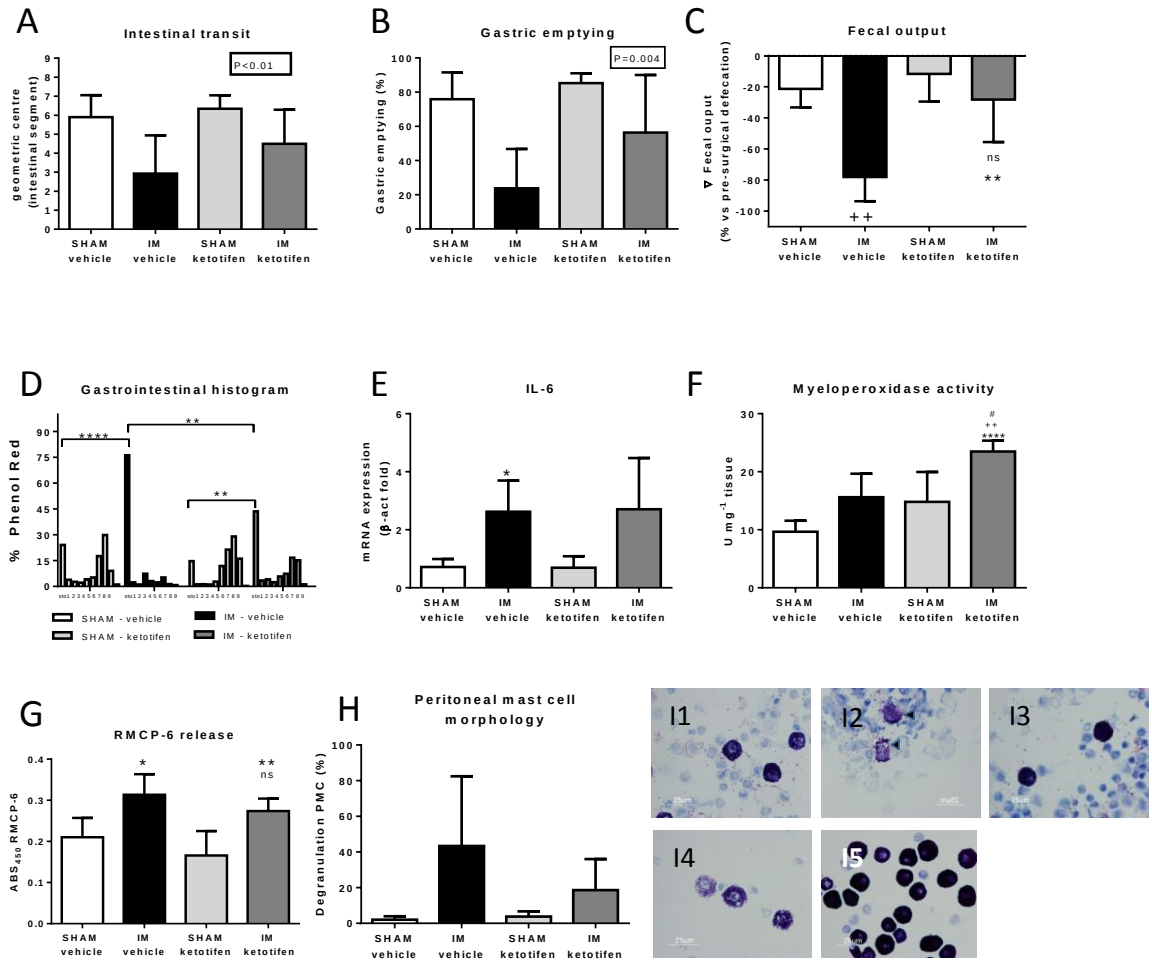

Figure 4. Effect of pre-treatment with ketotifen on the postoperative ileus model. (A) Intestinal transit, (B) Gastric emptying, (C) Percentage of inhibition of postoperative fecal output and (D) Distribution of phenol red marker along the gastrointestinal tract. **** P<0.01 vs Sham-vehicle, ** P<0.01 vs IM-vehicle and Sham-ketotifen, ++ P<0.05 vs. SHAM-vehicle, ns vs. SHAM-ketotifen. P-values on boxes represent overall P-value of ANOVA test. N=4-5. Sto, stomach.1-9, intestinal segment from the first to the ninth. (E) Interleukin-6 (IL-6) gene expression, * P<0.05 vs. SHAM-vehicle, N=4-5 and (F) Myeloperoxidase activity in ileum after treatment with ketotifen, # P<0.05 vs IM-vehicle, ++P<0.01 Sham-ketotifen, **** P<0.0001 vs Sham-vehicle, N=4-5. Data are expressed as mean ± SEM. (G) Peritoneal rat mast cell protease-6 (RMCP-6) release and (H) Percentage of degranulated peritoneal mast cells after treatment with ketotifen. *P<0.05 vs. SHAM-vehicle, **P<0.01 vs. SHAM-ketotifen, ns vs. IM-vehicle. N=3-5. (I1-I5) Representative photomicrographs of peritoneal mast cell cytospins stained with toluidine blue at 60x in (I1) SHAM-vehicle, (I2) IM-vehicle, (I3) SHAM-ketotifen and (I4) IM-ketotifen experimental groups. A more prominent disruption of the cell membrane and extrusion of granules can be noted after intestinal manipulation in picture B2 (black arrows) compared to other pictures. (I5) Cytospin of a non-operated rat as a reference of normal peritoneal mast cell morphology in a rat not subjected to laparotomy or/and intestinal manipulation.

Effects of ketotifen on mast cell activation after intestinal manipulation

Administration of ketotifen 60 min prior to surgery did not prevent the increase in peritoneal RMCP-6 release induced by intestinal manipulation (P>0.05, Figure 4 G). Analysis of PMC morphology by light microscopy revealed that intestinal manipulated-vehicle group presented MCs with granules with lower content along with poor staining and extrusion of granules outside of the cell outline, thus being compatible with features of MC degranulation (Figure 4 I2). Intestinal manipulation induced degranulation of 43.40 ± 22.56% which was only partially prevented by pre-treatment with ketotifen (18.60 ± 8.72%, Figure 4 H). On the other hand, MCs from the SHAM groups were characterized by highly-defined intracytoplasmatic granules and intact cell membrane (Figure 4 I1, I3). After treatment with ketotifen, PMCs in manipulated rats showed a staining pattern more similar to that in SHAM-groups than that in the IM-vehicle group (Figure 4 I4).

The number of MMCs in SHAM-vehicle (8.36 ± 0.45 MCs per high power field), IM-vehicle (7.60 ± 0.30 MCs), SHAM-ketotifen (8.57 ± 0.50 MCs) and IM-ketotifen (8.50 ± 0.25 MCs) were similar between the groups (P>0.05). Likewise, intestinal manipulation or ketotifen did not change the number of CTMCs in the SHAM-vehicle (0.02 ± 0.01 MCs), IM-vehicle (0.0 ± 0.0 MCs), SHAM-ketotifen (0.0 ± 0.0 MCs) and IM-ketotifen group (0.10 ±0.06 MCs), P>0.05. Expression of ileal RMCP-2 and -6 did not change by either intestinal manipulation or treatment with ketotifen (P>0.05).

Effects of ketotifen on activation of dorsal root ganglia following intestinal manipulation.

Ketotifen treatment did not prevent the increased mRNA expression of CGRP and TrkA receptor observed after intestinal manipulation (P>0.05, Figure 3 B).

DISCUSSION

In this study we report increased gene expression of specific sensory-related mediators in DRGs during POI in an experimental model. Specific MC degranulation or stabilization proved that dysmotility and inflammation observed in POI are mediated by degranulation of PMCs. However, we were unable to prove an involvement of MC in the increase of gene expression of sensory-related mediators induced by intestinal manipulation. We suggest that nociceptive alteration in POI is independent of MC activation.

This is, to our knowledge, the first report demonstrating that intestinal manipulation specifically evokes activation of gene expression of NGF, TrkA, CGRP and PAR-2 – all mediators directed related to visceral hypersensitivity. NGF, acting through TrKA receptors sensitises nerve terminals [22], induces visceral hypersensitivity [23–25] and changes colon motility [26]. Our study demonstrates that manipulation of the intestine induces an overexpression of NGF and of its receptor which would increase sensitivity and contribute to symptoms of POI.

CGRP induces neurogenic inflammation, mediates abdominal pain [27] and can also inhibit smooth muscle contractibility [28–30]. All of these actions could contribute to symptoms of POI. Furthermore, CGRP up-regulation observed in the present study is in line with other studies in which CGRP has been reported to be responsible for POI-like symptoms induced by activation of splanchnic nerves[16,18,31].

In addition, both NGF and CGRP have been related to MC activation [32,33] and this, together with the increase of their expression observed in our study, suggests a possible interaction between MCs and sensory mediators expressed in DGRs. Therefore, our hypothesis was that MC activation could contribute to the activation of DRGs by affecting the expression of TrkA and PAR-2 on nerve terminals following release of tryptase (and other possible mediators) from MCs.

Consequently we further aimed to investigate if MCs were involved in DRG activation. Intestinal manipulation elicited release of RMCP-6 from PMCs. This is the first study reporting such response in a rat model of POI and our results are in line with other studies performed on

mice and humans in which PMC degranulated upon intestinal manipulation [6–8,21,34]. However, in our model, MC response seems to be limited to PMCs since we found no differences in MC density or protease gene expression in the intestinal wall. This finding is in contrast to previous studies reporting increased MC infiltration after intestinal manipulation [35,36]. While the divergence of results may come from the methodology used for MC detection or from species differences, we suggest that MC hyperplasia requires more than 24h to develop and thus to be detected by changes in tissue MC density [4,37].

Despite the attention to the role of MC in POI, there is still some controversy regarding the involvement of MC in the pathophysiology of this disorder. While some studies point that MCs play a clear role [6,21,38] others suggest that POI development is independent of MC activation [34]. In our study, we addressed the involvement of MCs in symptoms of POI by a double pharmacological way: 1) by inducing specific MC degranulation with compound 48/80, largely proven as a specific mast cell degranulator, particularly of connective mast cells [39], and 2) by stabilization of MC with ketotifen, largely used in both experimental and clinical studies, including treatments of POI [6,37,40].

Compound 48/80 very efficiently reproduced all the motility changes induced by intestinal manipulation as well as the increase in the inflammatory parameters. In parallel, ketotifen in rats exposed to intestinal manipulation stabilised PMCs, which was verified by reduced morphological traits of degranulation on peritoneal cytospins, and improved gastric emptying and fecal output. Altogether, these results prove the role of PMCs in the induction of the gastrointestinal motor alterations as well as the inflammation observed in POI.

In contrast, pharmacological modulation of MC activation by either C48/80 or ketotifen did not alter expression of genes related to DRG activation. One could argue that intestinal manipulation may be a more intense stimulus that C48/80 and that, therefore, a much large activation of MCs could be induced by intestinal manipulation. However, the fact that C48/80 so effectively reproduced both inflammatory and motor changes observed in POI, let us to conclude that MC degranulation drives dysmotility and inflammation in POI but not DGRs stimulation. Furthermore, the fact that C48/80 on its own resembled all parameters of motor alteration and

inflammation indicates that DGRs would be mainly responsible for other symptoms present in POI, i.e. abdominal pain, but not motility disorders. In fact, a previous study demonstrated that treatment with ketotifen prevented delayed gastric emptying but did not ameliorate pain score in patients undergoing abdominal surgery [40].

In summary, our results indicate that clinical handling of POI should contemplate a double approach: 1) restoration of GI motor activity, where preventive treatments with ketotifen or other mast stabilizers could be very effective; and 2) prevention of hypersensitivity and hyperalgesia.

In summary, the present study describes the up-regulation in DRGs of NGF, TrkA, PAR-2 and CGRP, mediators that are related to hypersensitivity and visceral function, suggesting a potential role for POI pathogenesis. It also supports the role of MCs in POI and suggests these cells as a target for the development of new strategies to prevent POI in patients undergoing abdominal surgery. In addition, other strategies should also be provided to prevent neural activation, altogether leading to a better approach to treat the multi-mechanistic pathogenesis of POI.

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S.B and J.R designed and performed the study, analysed data and wrote the paper. P.V designed the study and wrote the paper.

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No competing interests declared.

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DISCUSSION

Over the last decade, using different experimental approaches, researchers have demonstrated that MCs contribute to the pathophysiology of POI [164,163,52,166,187]. As MCs communicate with nerve endings within the GI tract[131], we hypothesized that MC-nerve interactions are involved in the pathophysiology of POI. NGF is one of the mediators involved in MC-nerve interactions[189,190] and can induce MC degranulation[144]. Therefore, we proposed a role for NGF in mediating the activation of MCs during the development of POI. Thus, the main aim of this work was to gain knowledge on the mechanisms involved in the degranulation of MC upon intestinal manipulation, especially those involving nerve interactions and to further evaluate MCs as a target to prevent the development of POI.

Intestinal manipulation induces degranulation of peritoneal mast cells during colorectal surgery in human patients and in an experimental model of postoperative ileus in rats.

To evaluate if MCs degranulate during the development of POI we firstly focused on patients undergoing colorectal surgery. We observed increased release of MC proteases into the peritoneal cavity at the end of the surgical procedure. This finding suggests that resection of the colon induces degranulation of PMCs. These results are in line with those observed previously in abdominal surgeries that do not involve intestinal surgery[166,187]. However, our study was the first performed in patients undergoing intestinal handling, which is the main cause of POI. Therefore our findings are of relevance to the development of this condition. Furthermore, our findings were observed in patients developing POI. Thus, further supporting a potential role for MCs in POI as suggested by previous reports[166,187].

Another observation we report is that degranulation of PMCs is not dependent on the technique used for the resection of the colon. In fact, concentrations of released tryptase and chymase were similar between open and laparoscopic colectomies. This is in contrast to gynaecological surgery in which tryptase release was only detected following open hysterectomy [166].

Therefore, manipulation of the gut irrespective of the technique used appears to be sufficient to evoke degranulation of MCs. Pharmacological strategies, i.e. MC blocking agents,

should therefore be taken into consideration to prevent release of MC mediators during surgery and thereby prevent or diminish the severity of POI . Similar to the observations in patients undergoing colectomy, we demonstrated an increased release of mast cell protease in peritoneal lavage in our model of POI. This was confirmed in our two studies in the rat. Therefore, we conclude that intestinal manipulation elicits a similar PMC response in both human and rat. Our studies are the first reporting MC activation following intestinal manipulation in patients undergoing colectomy and in rats, and therefore provide new data that complement previous studies focusing on mice and on patients from non-gut surgeries [52,166,188,191].

In the present study we focused on RMCP-6 which is a specific protease of the major MC type residing in the peritoneal cavity. This allows us to conclude that CTMC activation constitutes a major response during the development of POI and efforts should be made to specifically block this type of MCs.

Summarised, these results indicate that PMCs are activated and release mediators that potentially can alter gut homeostasis leading to intestinal disease such as POI as previously reported[84]. To further corroborate if MC degranulation causes POI, we investigated the effect of drugs that modulate MC activity in the POI model.

Mast cells participate in experimental postoperative ileus in rat

The POI model we presented was characterized by delayed gastric emptying and intestinal transit, up-regulation of IL-6 gene expression and increased MPO activity in the intestinal wall. This is in line with previous animal studies reporting muscular layer inflammation and hypomotility in rat after intestinal manipulation[164,171].

The use of MC degranulator C48/80 induced a pattern of GI hypomotility and inflammation similar to that observed after intestinal manipulation. These findings suggest a detrimental role of MCs in POI which is supported by a previous study in which C48/80 also delayed intestinal motility and evoke an inflammatory response in the GI tract[52]. We verified that C48/80 induces β-hexosaminidase release from rat PMCs *in vitro* and in addition induces changes in the release of RMCP-6 from PMCs *in vivo*. We subsequently tested the effects of the MC

stabilizer ketotifen in the rat POI model. In our study, ketotifen did prevent delayed gastric emptying and reduced postoperative fecal output induced by intestinal manipulation. These results complement other studies that reported POI amelioration in mice and humans[52,187].

In summary, these results suggest that MCs are implicated in the development of inflammation and dysmotility occurring in the rat POI. This indicates MCs as a potential target for the development of new strategies to shorten POI in the clinical setting.

However, it should be taken into consideration that the actions of ketotifen are not specific to MCs, but can also act on other systems [192]. Complementary studies using MC deficient animal models would be needed to confirm our results. A recent study using MC deficient mice ($Cpa3^{Cre/+}$) indicated that absence of MCs does not result in the prevention of POI[191]. As immune responses may differ between mice and rat, a similar study using MC deficient rats would be required to confirm our observations.

Intestinal manipulation induces mast cell-independent up-regulation of gene expression of visceral sensory-related mediators in dorsal root ganglia.

In the next step of the present work, we investigated if MC-nerve interactions represent a mechanism by which MCs are involved in the pathogenesis of POI. We observed an up-regulation of CGRP, NGF, TrkA and PAR-2 gene expression in thoraco-lumbar dorsal root ganglia in the rat model of POI 24h after intestinal manipulation. This indicates a role of these mediators in the development of POI.

Since CGRP, NGF, TrkA and PAR-2 participate in MC-nerve communication[193,194], our results suggest a bi-directional interaction between MCs and nerve endings during the initiation of POI. The up-regulation of PAR-2 on nerve endings and RCMP-6 release after intestinal manipulation observed in our study could indicate that MCs do communicate with nerve endings during POI and therefore could result in alteration of motility and inflammation as reported in other studies [195–197]. Stimulated MCs have been also shown to release NGF[198] which can act on up-regulated TrkA receptors expressed in nerve terminations after intestinal manipulation. CGRP is a neuropeptide involved in activation of MCs [97]. Therefore, one can speculate that CGRP is

involved in the activation of MCs during POI, and in turn CGRP-induced MC degranulation can activate PAR-2 and TrkA. Furthermore, our results indicate DRGs as the source of CGRP that has been proved to lead to dysmotility in other POI models [199–202].

Nonetheless, the use of the mast cell degranulator C48/80 did not increase gene expression of the GCRP, NGF, TrkA or PAR-2 in the DRGs. Similarly, ketotifen did not prevent the up-regulation of the investigated genes induced by intestinal manipulation. Based on this result we conclude, that MCs are possibly responsible for motility disturbance and inflammation during POI but do not drive activation of DRGs. It could be argued that C48/80 was a less intense stimulus compared to the intestinal manipulation and therefore not sufficient to alter DRG gene expression, but the present study suggests that modulation of motility and inflammation by C48/80 and ketotifen was independent of DRG activation. This indicates that nerve endings do not represent a target for MC activation in the context of POI. This observation is in line with the suggestion that symptoms derived from activation of DRGs, such as abdominal pain or hypomotility, in non-manipulated segments[40] of GI tract should be targeted with strategies other than MC blockers[187].

NGF-antagonist K252a stabilized mast cells and prevented IL-6 expression in postoperative ileus rat model.

NGF is a well-known neurotrophin that induces degranulation of MCs[203,204] and participates in many functional motility disorders[205,137]. On this basis, we evaluated if NGF could serve as a pharmacological target in MC-mediated POI. Our study demonstrated that the alkaloid K252a, an antagonist of the NGF receptor TrkA, inhibits degranulation of PMCs evoked by NGF *in vitro,* which is in line with previous reports[143,206]. In addition, we also report, for the first time, inhibition of *in vivo* RMCP-6 release from PMCs and reduction of basal gene expression of RMCP-2 in experimental POI. All these data suggest that K252a has a therapeutic potential as a mast cell stabilizing agent. In addition we also described that K252a act on both MMCs and CTMCs suggesting that K252a could be efficient in MC-mediated disorders irrespective of the type of MC involved.

In the POI model, K252a also reduced IL-6 up-regulation induced by intestinal manipulation which is in agreement with other studies reporting amelioration of inflammation in experimental psoriasis, rhinitis and pulmonary infection following treatment with this agent[207–209]. Since MCs express TrkA receptors [137] and C48/80 induces over-expression of IL-6 as previously discussed in this work, MCs can be the mechanism responsible for IL-6 down-regulation after K252a treatment.

As an alternative, K252a could be acting on overexpressed TrkA on splanchnic nerve endings as we previously described. In that case, non-mast cell derived NGF could sensitize nerve endings and enhance CGRP release[210–212], or PAR-2 expression[213]. Therefore, overexpression of CGRP or PAR-2 induced by NGF/TrkA could be responsible for POI symptoms derived from DRG activation. This would be in agreement with our previous results in which DRG activation does not depend on MC degranulation.

In contrast to other parameters, K252a did not improve POI hypomotiliy. A previous study described intestinal motility modulation by K252a [137] but dosage or route of administration of K252a, used in this work, could underlie the lack of effect of this compound on motility in our study, and explain the divergence of our results from that of others.

In summary, in our study we observed that intestinal manipulation induced degranulation of PMCs in both human and rat. Manipulation of the intestine was also associated with an increase of gene expression of CGRP, NGF-TrkA and PAR-2 in thoraco-lumbar DRG, thus indicating the activation of splanchnic afferent fibers in the experimental model of POI. While MCs played a role in the inflammation and POI related dysmotility, C48/80 and ketotifen did not demonstrate a MC-dependent modulation of activation of DRGs. Therefore, under our experimental approach, activation of DRG appears to be independent of MC activity. Interaction between NGF and its receptor TrkA participate in degranulation of PMCs and inflammatory POI and therefore could be one of the mechanisms mediating MC involvement in POI. Furthermore, K252a could be used as MC blocking agent not only in POI but also in other disorders where MCs are involved.

CONCLUSIONS

1. In humans, colorectal surgery induced a local response of MCs characterized by release of proteases into the peritoneal cavity. This release was irrespective of the technique employed for resection of the colon, suggesting that the handling of the intestine to be removed, rather than the surgical technique, determined the degranulation of PMCs. The degranulation of PMCs following abdominal surgery was significantly correlated with the development of POI in surgical patients, thus indicating an association between MCs and POI.
2. In the rat model, intestinal manipulation lead to POI which was characterized by delayed gastrointestinal motility and intestinal inflammation. Intestinal manipulation also induced degranulation of MCs. This response was restricted to MCs residing in the peritoneal cavity and therefore belonging to the connective tissue MC type.
3. The mast cell degranulator Compound 48/80 induced a delay of gastric emptying and intestinal transit as well as an inflammatory response within the intestinal wall, thus, reproducing the pattern of POI symptoms observed after intestinal manipulation. Ketotifen reversed the delay of gastric emptying and restored the colonic propulsion induced by intestinal manipulation. All together, these results indicate involvement of MCs in the pathogenesis of POI.
4. Intestinal manipulation in the rat model increased the gene-expression of calcitonin gene-related peptide (CGRP), nerve growth factor (NGF), TrkA and protease activated receptor-2 (PAR-2) in thoraco-lumbar DRG. Therefore, intestinal manipulation seems to be associated to plasticity of extrinsic primary sensory neurons during the development of POI.
5. Exposure of the intestine to the mast cell degranulator compound 48/80 did not induced up-regulation of CGRP, NGF, TrkA and PAR-2 in DRG. The mast cell stabilizer ketotifen did not prevent up-regulation of these mediators following intestinal manipulation. Therefore, under this pharmacological approach, activation of DRG is independent of activation of MCs during the induction of POI. This suggests that

strategies other than MC stabilization should be employed in order to prevent DRG-derived symptoms after intestinal manipulation.

6. The NGF receptor antagonist, K252a, inhibited the degranulation of rat PMCs *in vitro* and *in vivo* in the experimental model of POI. In addition, K252a also decreased the basal RMCP-2 expression in the intestinal wall. Thus, we confirm that K252a could be employed as a MC stabilizing agent. In the POI model, K252a also diminished the up-regulation of IL-6 gene expression. Therefore, K252a partially resolves inflammation and modulates the immune response to intestinal manipulation in POI.

Intestinal manipulation evokes a response that involves hypomotility and inflammation of the GI tract, degranulation of peritoneal mast cells and activation of splanchnic afferent fibers leading to postoperative ileus. Of the components mediating this response is the interaction between Nerve growth factor and TrkA. Indeed, mast cell degranulation participates in the pathogenesis of postoperative ileus.

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PUBLICATIONS AND PARTICIPATIONS IN SCIENTIFIC MEETINGS DERIVED FROM THIS THESIS

Publications

Berdún S, Bombuy E, Estrada O, Mans E, Rychter J, Clavé P, Vergara P.

Peritoneal mast cell degranulation and gastrointestinal recovery in patients undergoing colorectal surgery. Neurogastroenterol Motil. 2015 Feb 9. doi: 10.1111/nmo.12525. [Epub ahead of print].

Submitted articles

Berdún S, Rychter J, Vergara P. Effects of nerve growth factor antagonist K252a on peritoneal mast cell degranulation: implications for rat postoperative ileus. Submitted to Journal of pharmacology and experimental therapeutics.

Berdún S, Rychter J, Vergara P. Intestinal manipulation increases gene expression of visceral-afferent mediators in dorsal root ganglia: role for peritoneal mast cells. Submitted to Neurogastroenterology and motility.

Scientific meetings

S Berdun, E Bombuy, O Estrada, E Mans, J Rychter, P Clavé, P Vergara. Peritoneal mast cell degranulation correlates with gastrointestinal recovery in patients undergoing colorectal surgery. Poster session. 20th United European Gastroenterology Week (UEGW). Amsterdam (The Netherlands) 2012.

S Berdun, E Bombuy, O Estrada, E Mans, J Rychter, P Clavé, P Vergara. A pilot study: human peritoneal supernatant presenting mast cell activation induced by intestinal surgery decreases gastric emptying in rat. Poster session. International mast cell and basophil meeting 2012. Berlín (Germany) 2012.

S Berdún, P Vergara. A refinement measure in a model of postoperative ileus in rat: comparative effects of buprenorphine and meloxicam. Poster session. 12th FELASA and 12th SECAL congress. Barcelona (Spain) 2013.

S Berdún, P Vergara. Intestinal manipulation activates peritoneal mast cells while it does not affect expression of mast cell proteases within the ileal wall in the rat. **Oral communication**. International mast cell and basophil meeting 2013. Udine (Italy) 2013.

S Berdun, E Bombuy, O Estrada, E Mans, J Rychter, P Clavé, P Vergara.
Correlació de la degranulació dels mastòcits peritoneals amb la recuperació gastrointestinal en pacients sotmesos a cirurgia colorectal. **Oral communication.** Congreso de la sociedad catalana de digestología. Lleida (Spain) 2014.

S Berdún, P Vergara. Intestinal manipulation induces degranulation of peritoneal mast cells and activation of dorsal root ganglia in postoperative ileus in rat. Poster session. Digestive disease week 2014. Chicago (US) 2014.