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# STUDY OF POST-INFLAMMATORY VISCERAL HYPERSENSITIVITY MECHANISMS AND INNOVATIVE TREATMENTS: EVIDENCE OF ADENOSINE $A_3$ RECEPTOR AGONISTS EFFICACY AND MICROBIOTA RELEVANCE IN ABDOMINAL PAIN PERSISTENCE

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# ABSTRACT

Visceral pain management is a major clinical problem since the lack of effective and safe drugs. The development of chronic visceral hypersensitivity frequently occurs in patients with a history of intestinal damage. Indeed, 20-50% of patients affected by Inflammatory Bowel Diseases (IBDs) manifest a chronic abdominal pain also in the remission phase of colitis. Despite the large number of sufferers, the mechanisms that drive the development of chronic abdominal pain remain largely unresolved and this presents a major barrier to progress in the development of new therapies. The present work aimed to: a) setup an animal model of chronic visceral pain closely related to the clinical manifestations for studying the pathophysiological mechanisms involved and looking for novel possible treatments; b) evaluate the efficacy of adenosine $A_3$ receptor ($A_3$AR) agonists in reducing post-inflammatory visceral hypersensitivity in rats; c) assess gut microbiota involvement in the persistence of visceral pain after the resolution of inflammation.

For these purposes we induced chronic visceral hypersensitivity in rats by the intra-rectal injection of dinitrobenzene sulfonic acid (DNBS, 30 mg in 0.25 mL EtOH 50%). Visceral pain was assessed by measuring the Visceral Motor Reflex (VMR) and the Abdominal Withdrawal Response (AWR) to Colo-Rectal Distension (CRD). Animals anxiety and depressive-like behaviour were investigated after DNBS injection. Histological and molecular characterization of bowel endothelium, enteric and central nervous system was carried out. As a consequence of the intestinal damage induced by DNBS, animals developed a visceral hypersensitivity which persisted also after colitis remission on days 14 and 21 up to 3 months. Visceral hypersensitivity was accompanied by depressive, but not anxiety behaviour in the animals. On day 14 a pharmacological characterization of pain by commonly used pain-relieving drugs (intraperitoneally *vs* intrathecally injected) revealed a mixed nociceptive, inflammatory and neuropathic pain originated by both the peripheral and central nervous system. At this time point, gut histological analysis highlighted a partial restitution of the *tunica mucosa*, transmural collagen deposition, infiltration of mast cells (MCs) and eosinophils, and upregulation of SP-nerve fibres which were surrounded with eosinophils and MHC-II-positive macrophages. At spinal cord level, a significant morphological activation of both microglia and astrocytes (Iba1- and GFAP-marked respectively), as well as an increased

number of astrocytes, was observed in the dorsal horn. The same cellular framework was found in the ventral horn. With the aim to individuate possible novel pharmacological approach, the acute effects of $A_3$AR agonists (MRS5980 and Cl-IB-MECA) were evaluated over time after DNBS injection and compared to the clinically-used drug linaclotide. $A_3$AR agonists significantly reduced DNBS-evoked visceral pain both in the post-inflammatory (14 and 21 days after DNBS injection) and persistence (28 and 35 days after DNBS) phases. Efficacy was comparable to that of linaclotide. Patch-clamp recordings showed that $A_3$AR agonists inhibited $Ca_v2.2$ in DRG neurons isolated from either control or DNBS-treated rats, this pharmacodynamic effect was confirmed by testing the selective $Ca_v2.2$ blocker PD173212 that fully reduced abdominal hypersensitivity to control values.

Among the factors involved in the complex physiology of the gut-brain axis, the microbiota plays a role of primary importance. Recent findings have linked microbiota alterations to gastrointestinal disorders characterized by abdominal pain. To investigate the microbiota capacity of modulating visceral sensitivity the effect of Faecal Microbiota Transplantation (FMT) from DNBS donors (in the post-inflammatory phase) to naïve rats has been evaluated. To prepare the animals to the FMT, they were pre-treated with antibiotics. The effect of FMT on visceral sensitivity, intestinal histology, permeability, immune response and neurotransmitters levels was studied. The faecal content was processed for assessing monoamines and the plasma for tryptophan metabolites. As expected, the antibiotic treatment induced an increase in visceral sensitivity in the animals. This effect was completely reverted by the re-colonization of intestine with a healthy microbiota, as well as by administering the vehicle. By contrast, the FMT from DNBS donors led to a further increase in visceral sensitivity in the animals. Three weeks after the interruption of the treatments this hypersensitivity progressively decrease again. This behavioural trend correlated with modifications in the gut microbiota. Studying possible mechanisms of this phenomena we highlighted that the effect induced by FMT was not related to an inflammatory response, neither to changes in gut permeability or monoamines levels in the gut. A significant reduction of tryptophan was observed in all the animals underwent the antibiotic treatment and the FMT (irrespective to the donor), as well as the DNBS donors (wherein it correlates with an increase in the production of 5-HT). Finally, to evaluate the therapeutic effect of FMT, 7 days after DNBS injection, the animals have been subjected to FMT from healthy controls. The transplant of a healthy microbiota in DNBS-treated animals counteracted the persistence of visceral pain after the colitis resolution, confirming an active involvement of microbiota in post-inflammatory visceral pain.

In conclusion, this work highlighted the complex nature of post-inflammatory visceral pain whose study required a multidisciplinary approach. Within this scenario, $A_3$AR agonists appear to be a promising resource for visceral pain management. The results obtained with the FMT strengthen the hypothesis of a direct involvement of microbiota in post-inflammatory visceral pain and so encourage to further study the therapeutic effect of microbiota transplantation.

# INTRODUCTION

## *1. Visceral Pain definition and features*

### *1.1. Pain*

Pain is the most common question for people to seek health care. Traditionally, pain has been viewed as a symptom for which a cause is diagnosed, and pain is eliminated by control or cure of the cause (McCaffery and Beebe, 1989). As its best, pain is the body's natural alarm system, warning us of injury. It prompts us to stop a harmful behaviour or look for medical attention. When pain persists, it is often a sign that the body's alarm system has broken down which means the pain signals are still active (The American Pain Foundation, 2010a). Untreated pain can have serious physiological, psychological and social consequences. It can limit the ability to sleep, perform everyday tasks and exercise. It also reduces mobility and appetite, aggravating other health problems. From the psychological point of view, it can lead to depression and anxiety which often reinforce the sensation of pain and place added strain on relationships (The American Pain Foundation, 2010b).

Besides, if we look at the definition of pain given by International Association for the Study of pain (IASP): "*Pain is an unpleasant sensory and emotional experience associated with potential or actual tissue damage or described in terms of such damage*", we realize that pain is always subjective and it means that psychosocial and spiritual concerns can modify the sensation of it (Woodruff, 2004). A person's response to pain is shaped by age, emotional state, sociocultural factors, past experiences with pain, the patient's knowledge of pain and the meaning of pain itself (Burke et al, 2011). The term 'total pain' refers to the etiological components of pain in addition to the noxious physical stimulus, that affect the patient's experience of pain: fear, social isolation, spiritual crisis, dependency, helplessness, anger and frustration (Radwany and Von Grueningen, 2012).

Several classification systems are available in the pain literature according to several variables, including its duration (acute, convalescent, chronic) and its pathophysiologic mechanisms (nociceptive, inflammatory, neuropathic) (Cherny et al., 1994). Moreover, on the base of the site of origin, pain can be further subcategorised as somatic or visceral pain.

### *1.2. Visceral pain*

Visceral pain is by definition pain sensed as arising from the internal organs of the body. It results from the activation of nociceptors of the thoracic, pelvic, or abdominal viscera (Kennedy and Abd-Elsayed, 2019). There are multiple etiologies for visceral pain, including:

- Inflammation (acute and chronic),
- Mechanical irritants (e.g., kidney stones);
- Infection;
- Disruption of normal mechanical processes (e.g., gastrointestinal dysmotility);
- Neoplasms (benign or malignant);
- Alterations in nerves carrying sensations from the viscera;
- Ischemia.

Visceral structures are highly sensitive to distension (stretch), ischemia and inflammation, but relatively insensitive to other stimuli that normally evoke pain such as cutting or burning (Aziz, 2019). Although there are many commonalities in the processing of somatic and visceral pain, there are also several important clinical distinctions (Kennedy and Abd-Elsayed, 2019). Firstly, pain cannot be evoked from all viscera. Besides injury to the viscera does not necessarily cause pain. Visceral pain is perceived more diffusely than noxious cutaneous stimulation with respect to location and timing, owing to relatively few visceral afferents with extensive receptive fields (Procacci et al., 1986). Pain from different visceral organs can have differing areas of presentation, e.g. bladder to perineal area, heart to left arm and neck, left ureter to left lower quadrant and loin. This diffuse nature and difficulty in locating visceral pain is due to a low density of visceral sensory innervation and extensive divergence of visceral input within the Central Nervous System (CNS). Subsequent development of symptoms may entail referred pain to parietal somatic structures within the same metameric field as the affected organ. Spatial discrimination of visceral pain is thus typically referred to superficial structures to produce secondary hyperalgesia of superficial or deep body wall tissues due to viscera-somatic convergence into shared spinal levels (discussed later) (Machmahon and Koltezenburg, 2006; Sikandar and Dickenson, 2012). This “referred pain” is better localized and therefore difficult to differentiate from pain of somatic origin. Furthermore, visceral pain is often associated with marked autonomic phenomena, including pallor, profuse sweating, nausea, muscle tension, gastrointestinal (GI) disturbances and changes in body temperature, blood pressure and heart rate (Procacci et al., 1986). The table below lists the general characteristics of visceral pain in humans, underlying the difference with somatic pain (Ness, 1995):

| Somatic | Visceral |
| --- | --- |
| • Can be superficial (skin, muscle) or deep (joints, tendons, bones)<br>• Nociceptors are involved<br>• It is well localized<br>• Usually described as throbbing or aching | • Involves hollow organs and smooth muscle nociceptors that are sensitive to stretching or hypoxia and inflammation<br>• Pain is usually referred, poorly localized, vague and diffuse<br>• May be associated with autonomic symptoms (e.g. pallor, sweating, nausea, blood pressure and heart rate changes) |

*Machmahon S.B., Koltezenburg M. Wall and Melzack's Textbook of Pain, 2006.*

### *1.3. Chronic visceral pain*

It has been estimated that ~40% of population experience a chronic or persistent pain from internal organs. Traditionally, chronic visceral pain has been categorized as either "organic," caused by a pathological lesion that is detectable by standard diagnostic measures, or "functional," where the etiology remains obscure and may be due to yet undefined changes in visceral sensitivity at either the peripheral or central level (Kennedy and Abd-Elsayed, 2019). Abdominal pain is the most common visceral pain and results in more than 15 million office visits per year. Chronic abdominal pain affects at least 10%–15% of the general population and is a characteristic feature of functional bowel disorders, such as irritable bowel syndrome (IBS), the inflammatory bowel diseases (IBDs), and is a growing problem associated with chronic opioid use (Longstreth et al., 2006; Mansfield et al., 2016; Regueiro et al., 2017)

### *1.4. Visceral pain in IBS*

The IBS is the most commonly diagnosed chronic gastrointestinal disease. Prevalence rates of IBS vary between 1.1% and 45%, based on population studies from countries worldwide (Longstreth et al., 2006). According to the Rome III criteria, IBS is defined based on the presence of chronic/recurrent abdominal pain or discomfort at least three days per month in the past three months associated with two or more of the following: (1) improvement with

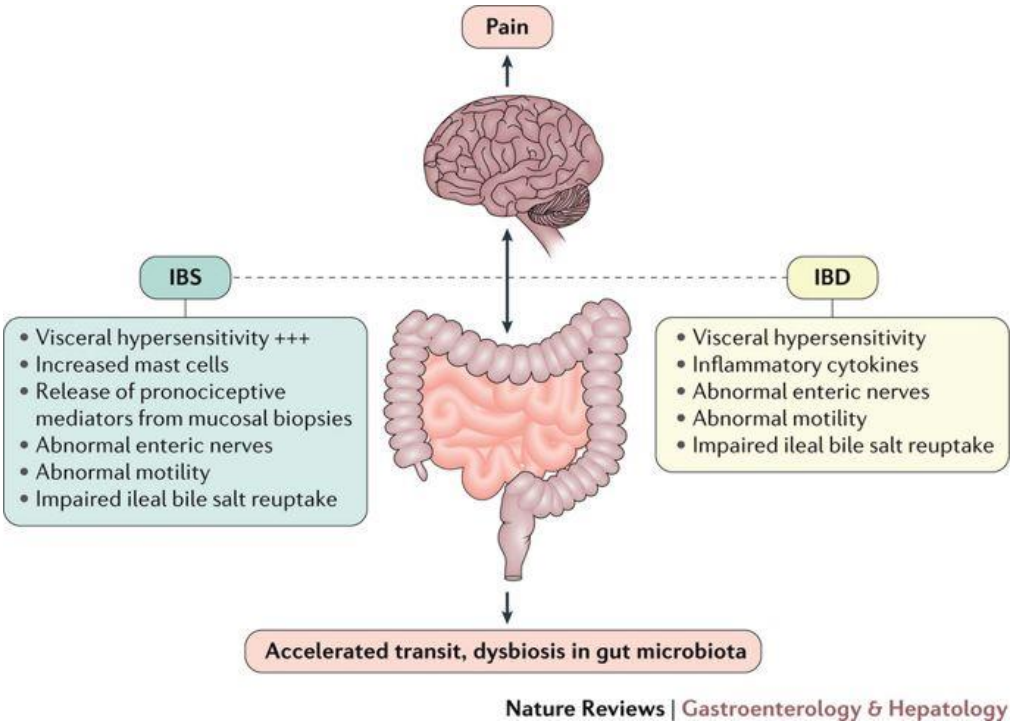
defecation; (2) onset associated with a change in frequency of stool; and (3) onset associated with a change in form (appearance) of stool. These criteria should be fulfilled for the previous three months with symptom onset at least six months before diagnosis (Longstreth et al., 2006). Rome III criteria subtype IBS according to the predominant bowel habit as IBS with constipation (IBS-C), IBS with diarrhea (IBS-D), mixed type, and unclassified (Longstreth et al., 2006). To this end, the definition of bowel-habit type is based on the patient's description of the stool form by referring to the Bristol Stool Scale (Lewis et al., 1997). Nevertheless, IBS symptoms cannot be explained by structural abnormalities, and specific laboratory tests or biomarkers are not available for IBS. Therefore, IBS is classified as a "functional" disorder whose diagnosis depends on the history of manifested symptoms (as above described) (Cervero et al., 2004). IBS patients can be further divided into two categories: sporadic (nonspecific) and postinfectious or inflammatory bowel disease-associated (PI-IBS). Meta-analyses suggest that gastroenteritis increases the risk of developing IBS sixfold, making it one of the strongest risk factors for IBS. PI-IBS shows clearly that acute inflammation followed by mucosal healing can cause the development of chronic IBS symptoms (El-Salhy et al., 2012; El-Salhy et al. 2014; Cairns et al., 2014). Early studies suggested adverse psychological features were less common in PI-IBS, but other studies suggest that hypochondriasis, anxiety, depression and somatization are important risk factors for developing PI-IBS, as they are for all IBS subtypes (Coyle et al., 1998; Sweitzer et al., 1999; Stuesse et al., 2001; Bandell et al., 2004; Larauche et al., 2008; Cairns et al., 2014). The prognosis for PI-IBS seems to be similar to other IBS subtypes (Tanga et al., 2004). Nevertheless, PI-IBS is a useful model for understanding mechanisms in IBS because it has a defined mode of onset making it easy to separate cause from effect. Regarding the pathophysiological mechanisms of IBS, though routine histologic examinations do not show significant colonic mucosal abnormalities in the majority of IBS patients, however, recent quantitative histologic, immunohistochemical, and ultrastructural analyses have indicated subtle organic alterations in these patients, like an increase in histamine levels in the gut (Morales-Soto and Gulbransen, 2018).

### *1.5. Visceral pain in IBD*

IBDs which are comprised of two main types, ulcerative colitis (UC) and Crohn's disease (CD), affects approximately 3.6 million people in the United States and Europe (Longstreth et al., 2006). Although considerable progress has been made in recent years, a major gap in knowledge of the pathogenesis of chronic visceral hypersensitivity in IBD patient remains and

consequently effective treatments are still lacking. IBD is characterized by chronic or relapsing immune activation and inflammation within the gastrointestinal (GI) tract that markedly alters GI function (Schirbel et al., 2010). When the gut is inflamed, there is breakdown of intestinal barrier function, abnormal secretion, changes in the patterns of motility, and visceral sensation, which contributes to symptom generation (diarrhoea, cramping, and pain) (Srinath et al., 2012).

Although abdominal pain has been traditionally associated with inflammatory flare-ups in IBD, a large subset of patients that suffer from IBD eventually develop chronic abdominal pain even after achieving clinical remission. This condition, called comorbid IBS (loosely termed "IBS-IBD"), is present in up to 46%–59% of patients with Crohn's disease and 36%–38% of patients with ulcerative colitis in remission (Keohane et al., 2010; Minderhoud et al., 2004; Halpin et al., 2012). Abdominal pain linked to these disorders is common in adults and pediatric patients and is a major contributing factor to their low quality of life and high morbidity. As a result, the Food and Drug Administration (FDA) now requires the use of abdominal pain as a patient-reported outcome to assess the efficacy of new therapies for Crohn's disease (Regueiro et al., 2017). The mechanisms that drive the development of chronic abdominal pain remain largely unresolved and this presents a major barrier to progress in the development of new therapies. Current therapies for IBD primarily focus on controlling inflammation with amino salicylates, corticosteroids, immunomodulators, and biologic agents. Although these agents clearly benefit the treatment of active inflammation, they do not address abdominal pain (Colombel et al., 2017) Opioids are the current frontline therapy for chronic abdominal pain, but chronic opioid use has serious complications and produces a condition called narcotic bowel syndrome that is characterized by abdominal pain (Camilleri et al., 2017). This is a serious and growing issue that requires a more sophisticated understanding of the causal mechanisms to permit the development of more effective therapies.




***Symptoms in IBD and IBS***

*Spiller and Major, Nature Reviews Gastroenterology & Hepatology, 2016*

### *1.6. Visceral pain comorbidities*

Visceral pain is reported to be frequently associated with both somatic and psychiatric disorders. Among the somatic comorbidities, the association between IBS and fibromyalgia is the most studied, with the IBS-symptoms occurrence in an estimated 48% (range, 32%–77%) of patients with fibromyalgia. Six studies examining the presence of IBS in patients with chronic fatigue syndrome reported a high degree of overlap, ranging from 35% to 92% (median, 51%) found that 38% of IBS patients self-reported back pain, 18% reported premenstrual syndrome, and 10% reported dysmenorrhea (Whitehead et al., 2001). All of these incidence rates are significantly higher than those in other gastrointestinal patients. Anxiety and depression are the most commonly reported psychiatric comorbidities in chronic visceral pain syndromes (Garakani et al., 2003; Frissora and Koch, 2005; Whitehead et al., 2007; Spetalen et al., 2009). This complex link between visceral sensation and psychological perceptions are mediated via the brain–gut axis. Moreover, stress-related changes in bowel habit can attest to the fact that the brain can influence gut function and sensation (Park et al., 2008). Several clinical studies have suggested that psychosocial comorbidity is a major contributor to the severity and impact on quality of life of visceral pain disorders such as IBS and somatic pain disorders such as fibromyalgia (Gupta and Silman, 2004; Boudewijn van Houdenhove and Egle, 2005; Resendiz-Figueroa et al., 2008; Mikocka-Walus et al., 2008). These findings are reinforced by a considerable volume of experimental research that links stress, anxiety, and depression to altered GI sensory and motor function as well as altered pain processing. Indeed, successful

management of patients with visceral pain disorders requires careful attention to these psychosocial factors, often in consultation with mental health professionals (Moloney et al., 2015). Depression and anxiety are also common in patients affected by IBD. A recent systematic review of 86 studies found that adults with IBD are more likely to develop anxiety and depression before IBD onset, and rates of anxiety and depression are higher in patients with IBD than in the general population, and higher in those with active IBD compared with inactive IBD (66.4% versus 28.2% respectively for anxiety, and 34.7% versus 19.9% for depression). Antidepressants are often used to treat the anxiety and depression that is commonly experienced by patients with IBD and IBS, wherein they have also been shown to be effective in treating associated gastrointestinal symptoms (Macer et al., 2017).

## *2. Visceral pain assessment in patients*

### *2.1. Pain rating scales*

"*…but when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind*". —Lord Kelvin Lecture "Electrical Units of Measurement",1893

A multitude of different instruments is currently used to assess chronic abdominal pain in IBS patients. Intensity is not the only factor important in the experience of pain, since pain occurs within a context. Pain intensity is influenced by the meaning of the pain to the patient and its expected duration (Turk and Melzack 1992). Pain is rarely caused by psychological factors but is associated with psychological and emotional effects such as fear, anxiety and depression. Moreover, in many cases, like GI diseases, pathology does not correlate well with pain (Wiesel et al., 1984; Boden et al., 1990; Turk and Melzack 1992). For these reasons, valid and reliable assessment of pain is essential for both clinical trials and effective pain management. To date, four commonly pain rating scales are available and used (Williamson and Hoggart, 2005; Mujagic et al., 2015).

- Visual Analogue Scale (VAS)
- Numerical Rating Scale (NRS)
- Verbal Rating Scale (VRS)
- Faces Rating Scale

The VAS is presented as a 10-cm line, anchored by verbal descriptors, usually 'no pain' and 'worst imaginable pain'. The patient is asked to mark a 100 mm line to indicate pain intensity. The score is measured from the zero anchor to the patient's mark. Using a millimetre scale to measure the patient's score will provide 101 levels of pain intensity. The NRS is a 11, 21- or

101-point scale where the end points are the extremes of no pain and pain as bad as it could be, or worst pain. There is no published information about the distribution or error of data obtained using the NRS. However, the scale is interval level and can provide data for parametric analysis. The VRS comprises a list of adjectives used to denote increasing pain intensities. The most common words used to be: no pain; mild pain; moderate pain; and severe or intense pain. For ease of recording these adjectives are assigned numbers. These rank numbers can lead to the misapprehension that intervals between each descriptor are equal, but this is not the case (Jensen and Karoly, 1992) and could be a source of error. Facial expression drawings (‘‘faces scales’’) are a popular method of pain severity assessment in pediatric populations. Faces scales use a series of facial expressions to illustrate a spectrum of pain intensity. Numerous face-based rating scales are available (Chambers et al., 1999). A multitude of different instruments is currently used to assess chronic abdominal pain in IBS patients. Of these questionnaires, the validated IBS-Symptom Severity Scale includes the broadest measurement of pain-related aspects. Anyway, the multitude of different instruments to measure chronic abdominal pain in IBS makes it difficult to compare endpoints of published studies (Mujagic et al., 2015).

The complexity of visceral nociceptive processes, as illustrated above, has imposed a challenge in assessing visceral perception in IBS. Traditionally, visceral perception has been tested by delivering a certain controlled sensory stimulus and measuring the nociceptive response evoked by it. The GI tract contains afferents that encode a wide range of intensities and modalities of stimulation including electrical, mechanical, thermal, and chemical (including nutrient) stimuli (Keszthelyi et al., 2012). Choosing the correct stimulation method or interpreting differential responses to multiple stimuli may be of superior relevance in delineating pathological mechanisms. For instance, electrical stimulation is often thought of as non-physiological and therefore of limited use in assessing GI sensation. Furthermore, manipulation of the environment in which a sensory stimulus is given, before and after nutrient infusion or heterotopic stimulation, for instance, can yield important physiological and pathophysiological information above and beyond that gained purely from quantitative sensory testing alone (Mayer et al., 2009). With respect to the assessment of visceral perception, perhaps the most important aspect is the objectivity and robustness of the measuring the magnitude of the pain response, used as primary endpoint for the experiment. Measurement of the pain response using verbal scores and scales to assess perceptive intensity and thresholds of nociception is largely influenced by subjective components, making it prone to reporting bias. Brain imaging and neurophysiological measurements, on the other hand,

theoretically offer a more objective assessment of nociceptive responses. However, these outcome measures are also greatly affected by cognitive-affective mechanisms, posing a challenge to the search for a surrogate marker of altered visceral perception in IBS (Keszthelyi et al., 2012).

### *2.2. Perceptional response to mechanical stimuli (rectal distension)*

Until recently, employing gut distensions has most widely been used to test visceral perception in IBS. Lowered rectal pain threshold is a hallmark of IBS patients (Bouin et al., 2002). It has been reported that 35–60% IBS patients exhibit increased visceral sensitivity to rectal balloon distension (Delvaux, 2002; Ludidi et al., 2012; Piche et al., 2010).

Both rectal and sigmoid distensions have been employed, although the overwhelming majority of studies have applied rectal distensions. In fact, the more accessibility and the grater content of sensitive afferents make the rectum an ideal target in assessing visceral nociception (Kyloh et al., 2011). Rectal visceral sensitivity can be tested by using polyethylene bags in conjunction with a barostat. The barostat is a device that maintains a constant pressure within an air-filled polyethylene bag and can measure variations in rectal tone by recording changes in the intra-rectal pressure and volume (van der Schaar et al., 1999). Rectal distension can be done according to a certain protocol, during which different pressures are applied to the rectal wall. During this procedure, the intensity and quality of perception can be measured by means of rating scales. Several different distention protocols have been applied to assess visceral perception in IBS (Keszthelyi et al., 2012). The lowered sensory thresholds seen in IBS have been shown to be elicited by rapid rates of rectal distension, whereas in the majority of patients the perception threshold during slow rectal distension pressure is not different from that in normal control subjects. It has also been postulated earlier that rapid phasic distensions preferentially stimulate serosal afferents (Lembo et al., 1994). These afferents are particularly chemosensitive to inflammatory and enteroendocrine mediators such as serotonin (5-HT), which suggests a role for nociception in conditions in which sensitization of visceral afferents is involved (Hugon, 1973). Furthermore, complex distension protocols with non-random presentation of stimuli such as double random staircase distension reduce psychological response bias by making the stimulus unpredictable to the subject (Mayer et al., 2008), which is desirable considering that IBS patients may be particularly prone to such bias due to anticipatory anxiety. However, these complex protocols usually require more distension levels and increasing the number of

interrogations about sensations may introduce bias or errors in a fatigued and stressed or disinterested participant (Keszthelyi et al., 2012).

During rectal distension, several parameters of visceral sensitivity can be collected to assess perceptional status. These include perceived intensity of the stimulus, extent of the viscerosomatic referral area and the threshold at which different sensations (pain, discomfort, urge) appear during the distension protocol (Posserud et al., 2007). These components of visceral perception are not uniformly used by various groups, making comparison of study results troublesome (Keszthelyi et al., 2012). As for the intensity of perception, different scales including numeric rating scales, verbal rating scales, verbal descriptor scales, magnitude estimation, and visual analog scales are employed. Visual analog scales have emerged as having psychometric properties that are superior to the other pain scaling methods because they fulfill multiple criteria for ideal pain measurement and assessment (Price et al., 2007). These criteria include ratio scale properties, high test-retest reliability and repeatability, the capacity to detect small differences, internally consistent measures of clinical and experimental pain, sensitivity to variables that increase or decrease pain, detection of individual differences in pain sensitivity, and ease of use (Price et al., 1994). The majority of the research groups investigating visceral perception using the barostat techniques have focused on identifying the thresholds for perception in IBS patients because these have been shown to be lowered compared with healthy volunteers. However, threshold measurements of pain perception are limited since they fail to assess alterations in pain intensity that may occur over a wide range of noxious intensities. On the other hand, investigation of the perceived intensity of the given stimulus as well as recording the viscerosomatic referral area can give extra information on processing of visceral stimuli (Mertz et al., 1995; Posserud et al., 2007). In addition to lowered thresholds to visceral stimuli, marked alterations in somatic referral patterns in response to balloon distension of the colon have been described in patients with IBS. This is a strong indication for altered spinal processing of visceral sensory information and is a manifestation of central sensitization (Kingham and Dawson, 1985; Keszthelyi et al., 2012). Currently, distension paradigms differ in duration and type of inflation (tonic vs. phasic), level of bias (ascending vs. randomized distension), and measurement of response magnitude (perceptual threshold or rating). Despite the fact that not all patients exhibit hypersensitivity to rectal distensions, the barostat procedure appears to be a reliable and valid approach to assess visceral nociception. The barostat has also shown robust changes following administration of a narcotic analgesic even in small crossover samples, further supporting the measure's validity for assessing pain perception (Delvaux et al., 1999; Lembo

et al., 2000). It is also important to note that these perceptual alterations were independent of changes in compliance or tone (Bradette et al., 1994). Evidence from crossover studies points to considerable reliability and validity for rectal distensions to testing visceral perception, reviewed extensively by Mayer et al. (2008). Furthermore, the barostat method in conjunction with verbal reporting has shown to have high sensitivity and specificity for detecting visceral hypersensitivity in IBS patients (95.5 and 71.8%, respectively) (Bouin et al., 2002). Noteworthy, visceral sensitivity studies are generally performed in the fasted state. However, bowel symptoms in IBS patients often exacerbate after food consumption (Simren et al., 2001). There is ample evidence that GI nutrient content may either be the source of or contribute to abnormal, painful, or uncomfortable visceral perception in IBS (Ragnarsson and Bodemar, 1998). Rectal and colonic perception thresholds in IBS patients have been shown to be lowered following intraduodenal infusion of fat. Furthermore, a liquid meal was shown to enhance rectal sensitivity in IBS, with a fatty meal producing more pronounced effect compared with a carbohydrate meal (Simrenet al., 2007; Keszthelyi et al., 2012). It has been postulated that enteroendocrine cells in the intestinal mucosa respond to changes in luminal contents by releasing mediators such as cholecystokinin and 5-HT, which in turn activate specific receptors on primary afferent nerve terminals. Cholecystokinin, a peptide released in response to intake of fat, has been postulated to sensitize mechanoreceptors because it has been demonstrated to increase rectal nociception in healthy volunteers (Sabate et al., 2002). 5-HT, on the other hand, could also be a mediator of meal-induced nociception as higher postprandial levels of 5-HT have been reported in IBS patients (Bearcroft et al., 1998). Therefore, testing of visceral sensitivity in IBS patients in a postprandial state can also have an added value in assessing visceral perceptional status (Keszthelyi et al., 2012).

### *2.3. Perceptional response to non-mechanical stimuli*

Besides mechanical stimulation, a number of other stimulation modalities have been used to assess visceral sensitivity. Transmucosal electric nerve stimulation, for instance, induces perception by nonspecific stimulation of afferent pathways without the involvement of a specific receptive unit. However, whereas IBS patients had lower sensory thresholds to mechanical stimulation of the small intestine compared with controls, thresholds to electrical intestinal stimulation were similar in IBS patients and controls (Accarino et al., 1992). A disadvantage of using electrical stimulation is the nonspecific activation of nerve endings, resulting in neural discharge from nerves, which might not otherwise be activated during nociceptive processes.

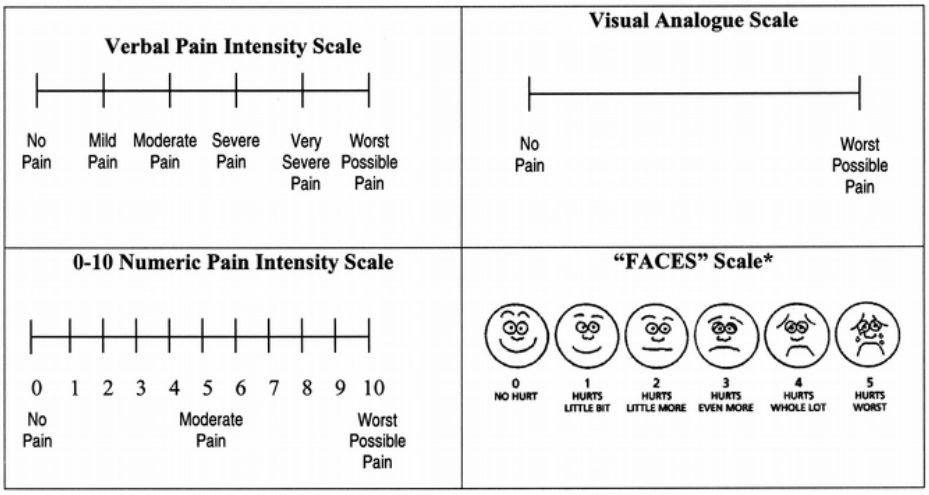
Thermal stimulation of the gut can be provided via intraluminal bags by recirculating water at adjusted temperatures. Thermal stimuli are also potentially applicable in conjunction with mechanical and electrical stimuli for the evaluation of visceronociceptive responses. It has been demonstrated that, similarly to mechanical stimuli, IBS patients also demonstrate lower thresholds to thermal stimulation of the rectum (Li et al., 2004). Chemical stimuli have been used widely to study pain mechanisms. Capsaicin is commonly applied as a sensitizing stimulus in studies of somatic pain. Hammer et al. (1998) demonstrated that intestinal capsaicin infusion resulted in a sensation of warmth and burning, in addition to the generation of cramps. This cramping sensation was independent of changes in bowel motility or biomechanics and the authors concluded that capsaicin was acting directly on mechanosensitive afferents. In addition to capsaicin producing these unique sensory characteristics, it was also noted that the time to first sensation of a second capsaicin infusion was significantly reduced, suggesting the induction of peripheral sensitization of capsaicin-sensitive afferents (Hammer et al., 1998). Capsaicin has therefore also been successfully used to evaluate chemical hypersensitivity in functional dyspepsia (Fuhrer et al., 2011). On the other hand, drawbacks of chemical stimuli include relative long latency time to onset of effects, compared with other stimulation modalities, and the fact that these effects are often not reproducible (Ness and Gebhart, 1990). Therefore, validation of results using chemical stimulation in IBS is still warranted.

### *2.4. Brain Imaging and fMRI*

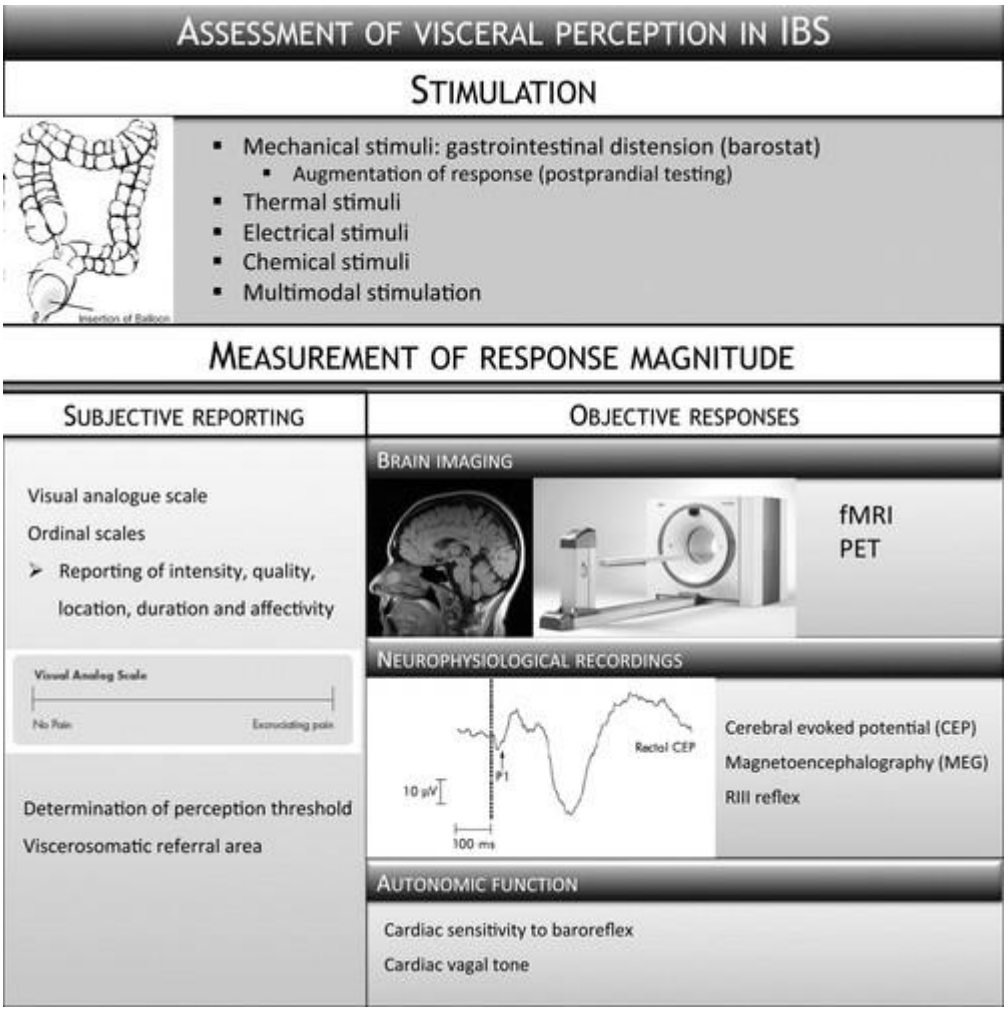
In the past decade, accumulating evidence on visceral nociceptive processes has emerged from results of brain imaging studies. The most commonly used brain imaging entity, functional magnetic resonance imaging (fMRI), is based on the difference in the magnetic properties of oxygenated and deoxygenated blood, specifically the difference in decay time of the magnetic resonance signal from oxyhemoglobin and deoxyhemoglobin, referred to as the blood oxygenation level-dependent technique. This difference in signal characteristics allows for the localized detection of regional cerebral blood flow (rCBF). These regional changes in blood flow, volume, and oxygenation of hemoglobin derive from changes in neuronal activity and, thus, regions of activation may be identified by subtracting rCBF during a control condition from blood flow during a stimulus condition or by correlating regional blood flow with the intensity or time course of a stimulus or its perception (Turner, 1992). A major advantage of fMRI is that it is non-invasive and non-cumulative, allowing subjects to be studied repetitively. fMRI has an excellent spatial resolution (2–5 mm), especially in the more

superficial layers. Limitations are seen in the deeper structures, such as the brain stem and thalamus, owing to pulsation artifacts. The temporal resolution is poor (1–3 s), and therefore fMRI is not a specific tool for investigating the neuronal activity directly related to the painful stimuli. Since the exogenous brain activity takes place within the first 150 ms poststimulus, the response may miss the fast-occurring activity and model, instead, the endogenous activity rather than brain responses due to pain. Anyway, comparison of images recorded during resting and active periods following administration of a nociceptive stimulus, for instance rectal distension, reveals regions of increased cortical activity, and these measures have been used to increase understanding of the functional properties of the brain (Keszthelyi et al., 2012).

Another technique, Positron emission tomography (PET) scanning, utilizes biological molecules synthesized with radiolabeled isotopes, such as $^{15}O$-labeled water to monitor regional changes in cerebral blood flow. The most commonly used in visceral pain research is $H_2{}^{15}O$. In the normal brain, rCBF is closely coupled to regional cellular metabolism; therefore $^{15}O$-labeled water serves as a reliable monitor of cerebral interneuronal activity (Heiss and Herholz, 2006). Alterations in rCBF can then be mapped to specific intracerebral structures and foci, using either MRI or a computerized stereotactical method. PET has excellent spatial resolution (2–5 mm) and allows the operator to tag important biological molecules that bind to targeted receptor groups or glucose metabolism in active neuronal tissue. PET is superior in imaging radiopharmaceuticals and/or other ligands because it offers the ability to study receptor distribution and explore the site of action. However, the temporal resolution of PET is poor (minutes), and since the subject receives a considerable dose of radiation, group analyses are needed for meaningful results, interpreting endogenous brain activity following pain rather than exogenous brain activity following painful stimulation. Another major disadvantage is the expense and limited availability of a PET scanner (Keszthelyi et al., 2012).




***Representative images of VRS, VAS, NRS and faces scale***




***Schematic overview of assessment of visceral perception in irritable bowel syndrome (IBS)***

*Keszthelyi et al., American Journal of Physiology-Gastrointestinal and Liver Physiolog, 2012*

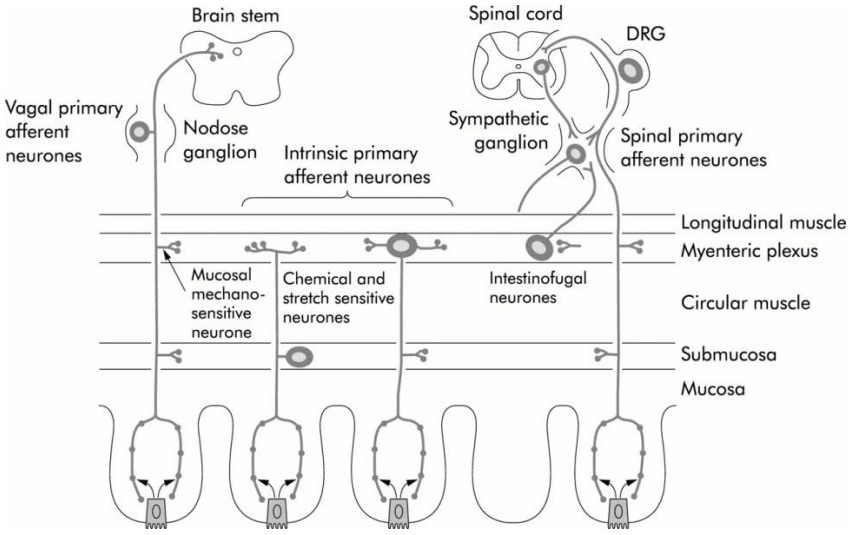
## *3. Visceral pain pathways*

The perception of pain involves the integration of neurobiological pathways within the enteric, peripheral, and central nervous systems (Schirbel et al., 2010). The gastrointestinal tract differs from all other peripheral organs. An extensive intrinsic nervous system, termed enteric nervous system (ENS) controls digestive and defensive functions of the intestine even when it is completely separated from the central nervous system (CNS). The ENS, however, is not autonomous. Indeed, neuronal control of gastrointestinal function is an integrated system involving interactions between local enteric reflexes, reflexes that pass through sympathetic ganglia and reflexes that pass from the gut and back through the CNS (Furness, 2012).

### *3.1. Enteric nervous system*

The rich sensory innervation of the gastrointestinal tract comprises intrinsic sensory neurones contained entirely within the gastrointestinal wall, intestine-fugal fibres that project to prevertebral ganglia, and vagal and spinal afferents that project into the CNS (Grundy, 2002). The ENS regulates and coordinates almost all aspects of intestinal function including gut motility, transport of fluid and electrolytes, secretion of mucins, production of cytokines, and the regulation of epithelial barrier function (Furness, 2012). Each of the aspects of physiology are compromised in IBDs and IBS patients, whereby it is not surprising that there is an increasing amount of research interest in elucidating the role of the ENS in the pathogenesis of these GI disorders (Lakhan and Kirchgessner, 2010). Enteric ganglia are organized into two major ganglionated plexuses, the myenteric (Auerbach's) and submucosal (Meissner's) plexus. They contain a variety of functionally distinct neurons, including primary afferent neurons, interneurons, and motor neurons, synaptically linked to each other in microcircuits (Furness, 2012). The cell bodies of vagal and spinal visceral afferents are contained within the nodose and dorsal root ganglia. Central projections of these neurones enter the brain stem and spinal cord, respectively, and make synaptic connection with second order neurones that distribute visceral information throughout central neuronal structures (Joshi and Gebhart, 2000) Projections from the nucleus tractus solitarus are mainly to hypothalamic and limbic structures associated with behavioural and emotional aspects of sensory processing. The peripheral terminals of vagal and spinal afferents locate in mucosal layers, muscle, and in the serosal and mesenteric attachments (Berthoud and Neuhuber, 2000). Nerve terminals in the serosa and in muscle convey mechanosensory information relevant to distension and contraction of the bowel wall. However, the afferent information conveyed by vagal and

spinal mechanosensitive afferents is very different, as revealed by direct electrophysiological recordings of afferent traffic en route to the CNS (Sengupta et al., 1990). Vagal afferents have low thresholds of activation and reach maximal responses within physiological levels of distension. In contrast, spinal afferents, although many have corresponding thresholds for activation, are able to respond beyond the physiological range and encode both physiological and noxious levels of stimulation. This different stimulus response profile is consistent with the hypothesis that vagal afferents are involved in physiological regulation while spinal afferents are responsible for mediating pain. However, recent evidence implicates vagal afferents both in the mediation of sensation and in the modulation of sensory experience. The morphological appearance of afferent terminals in the gastrointestinal wall, visualised by fluorescent microscopy, suggests that these endings may also subserve an "efferent" sensorimotor function. Gastrointestinal afferents are thought to have collateral branches that supply blood vessels and innervate the enteric ganglia where they have the potential to modulate blood flow and enteric reflex pathways as a consequence of release of transmitters from their varicose nerve terminals (Sternini et al., 1992). Besides, viscerofugal neurons have cell bodies within the myenteric plexus, but have projections out of the gut wall, via extrinsic nerve trucks, to prevertebral ganglia. These viscerofugal neurons sense and receive information regarding mechanical distension of the intestine and transmit this information to postganglionic sympathetic visceromotor neurons in the prevertebral ganglia (Furness et al., 2012). In addition, enteric neurons are supported by enteric glial cells (EGCs), that can modulate enteric neuron function (Furness et al., 2012; Morales-Soto and Gulbransen, 2018). Enteric glia forms a cellular and molecular bridge between enteric nerves, entero-endocrine cells, immune cells, and epithelial cells, depending on their location (Sharkey et al., 2015). EGCs function can be profoundly changed by many factors, such as pro-inflammatory cytokines, bacteria, and neurotransmitters. Thus, EGCs may undergo dynamic processing under pathological conditions and serve overlapping functions such as modulating intestinal barrier function, mucosal immunity and enteric neurotransmission via releasing various substances (Wang et al., 2016). The role of enteric glia will be further discussed below.




***Arrangement of the primary afferent neurones within the intestine***

*Grundy, Gut, 2002*

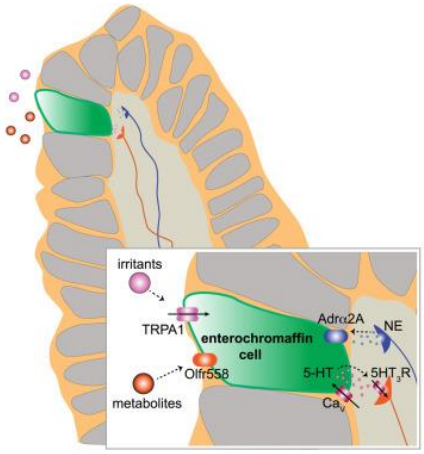
### *3.2. Peripheral visceral neurotransmission*

Afferent fibres innervating viscera project to the CNS through autonomic sympathetic and parasympathetic nerves - a dual sensory innervation (McSwiney, 1944; Cervero, 1994). Some spinal afferents travel along hypogastric, lumbar colonic and splanchnic nerves to terminate in thoracolumbar regions as part of sympathetic innervation, traversing both prevertebral and paravertebral ganglia en route to the spinal cord (Sengupta and Gebhart, 1995). Vagal and pelvic afferents respectively terminate in the brainstem and lumbosacral cord and contribute to parasympathetic innervation. Visceral fibres can serve 'sensory' and 'afferent' functions: the former can evoke conscious sensations and the latter regulate autonomic flow (Langley, 1903). Accordingly, sensory afferents innervating the gastrointestinal and urinary tracts serve regulatory functions of the gut (e.g. absorption, secretion, propulsion) and contribute to consciously evoked sensations such as pain and fullness (Janig et al., 1996). Visceral sensory afferents are almost exclusively thinly myelinated Aδ-fibres and unmyelinated C-fibres. However, the distinction between nociceptive afferents and non-nociceptive afferents is not clear in visceral neurotransmission compared to somatic nociception, given the functional division of mechano-sensitive visceral receptors into two physiological classes (Sengupta and Gebhart, 1994; Janig et al., 1996). 'High-threshold receptors' in organs such as the heart, oesophagus, colon, ureter and uterus respond only to noxious mechanical stimuli. 'Low-threshold receptors' are intensity-encoding and thus respond to a range of innocuous to noxious stimuli. An important contrast with somatic nociception is the role of low-threshold

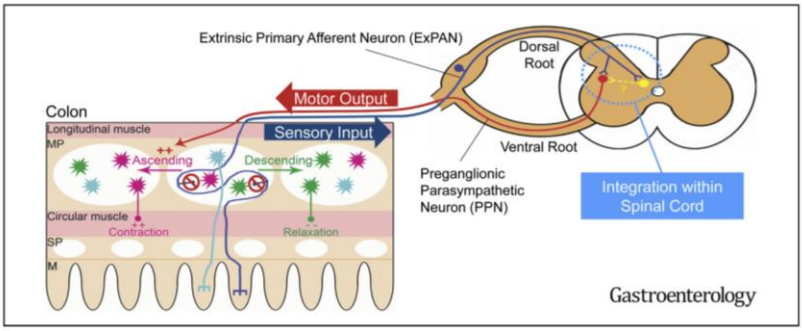
Aβ-fibres, which *only* convey innocuous mechanical sensations in normal conditions (Sikandar and Dickenson, 2012).

Viscera are also innervated by so-called 'silent' nociceptors, more accurately designated as mechanically insensitive afferents (MIAs). These can acquire mechanosensitivity following inflammation and have been thoroughly characterised in significant proportions in rodent pelvic and splanchnic innervations of colorectum and in human microneurographic studies of cutaneous C-afferents (McMahon and Koltzenburg, 2019).

In addition to the enteric nervous system, gut epithelium is another site for detecting dietary, microbial, and inflammatory stimulus. Among the epithelial cells, serotonergic entero-chromaffin (EC) cells are proposed to fulfil this role by acting as chemosensors. Studies conducted on cultured intestinal organoids showed that EC cells express specific chemosensory receptors, are electrically excitable, and modulate serotonin-sensitive primary afferent nerve fibers via synaptic connections, enabling them to detect and transduce environmental, metabolic, and homeostatic information from the gut directly to the nervous system (Bellono et al., 2017). Recently, a research group of Pittsburgh demonstrated that extrinsic primary afferent neurons (ExPANs) link visceral pain to colon motility through a spinal reflex in mice. Indeed, proper colon function requires signals from ExPANs located in spinal ganglia. Besides, ExPAN stimulation initiates myenteric neuron activity and subsequent colon contractions, but only in the presence of an intact spinal cord, confirming the presence of a sensory-parasympathetic spinal circuit regulating colonic motility. The described circuit demonstrated how visceral pain is linked to colon motility and explain why pain and dysmotility co-occur in GI disorders (Smith-Edwards et al., 2019). Anyway, these results complicate further the network at the base of visceral sensitivity and might preclude the possibility of achieving a selective modulation of pain.



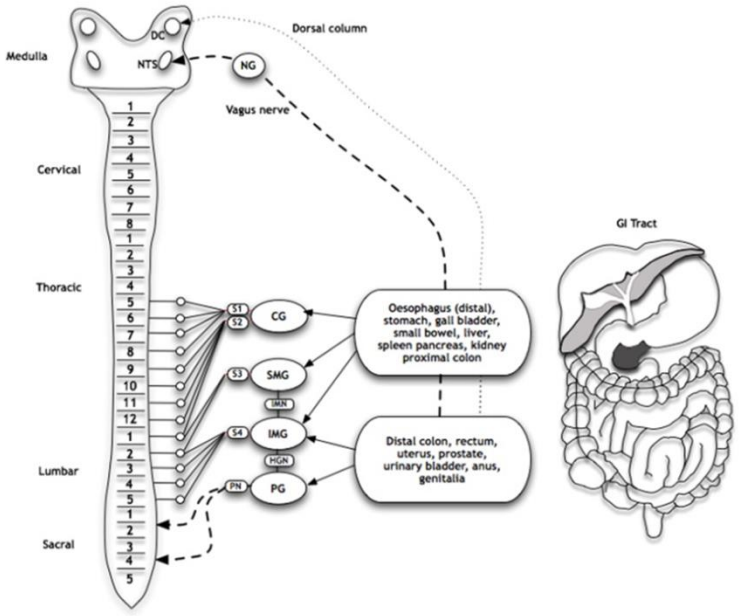

*Bellono et al., Cell, 2017*




*Smith-Edwards et al., Gastroenterology, 2019*

### *3.3. Viscerosomatic convergence*

The neurophysiological convergence of visceral and somatic afferent inputs to the CNS is thought to underlie referred visceral pain, where noxious stimulation of viscera triggers pain referred to somatic sites (Cervero, 1983; Mertz et al., 1995). Viscerosomatic convergence may occur as a result of the scarcity of visceral afferent fibres with spinal cord terminations. In fact, the relative contribution of visceral afferent fibres to the total spinal cord afferent input is less than 10%. Visceral afferent terminals also show extensive divergence and intraspinal distribution compared to cutaneous afferents (Gebhart, 2000). Because of viscerosomatic convergence, somatic injury and visceral inflammation can respectively alter central processing of visceral and somatic inputs (Cameron et al., 2008). Axons can send peripheral terminals to anatomically distinct segments to produce pain sensations distant to the primary site (Coderre et al., 1993). Viscerosomatic convergence also accounts for altered central nociceptive processing through sensitization of primary afferent pathways, ultimately modifying neuronal input at sites of convergence in the spinal cord or higher centres (Ness and Gebhart, 1991). This convergence of visceral and somatic messages may be one reason for visceral pains often accompanying somatic pain conditions or vice versa. In addition there can be viscero-visceral convergence whereby pain from one organ is referred to another (Sikandar and Dickenson, 2012).




***Innervation of the rat GI tract***

*Sikandar and Dickenson, Curr Opin Support Palliat Care, 2012*

### *3.4.Central modulation of visceral pain*

The ascending fibers of the second order neurons are organized into the spinothalamic, the spinoparabrachial, and the spinoreticular tracts, depending on where the cell body of the third-order neuron is located (Almeida et al., 2004). The final primary synapse occurs at cell bodies within the brain. For the spinothalamic tract, the 3rd order neuron is within the thalamus, which acts as the primary hub for the central pain matrix (Morton et al., 2016). The thalamus is somatotopically organized such that noxious signals from the spinal cord are sent to specific regions of the primary somatosensory cortex for the localization of the signal. In contrast, the cortical localization for visceral pain is typically less precise since the ascending signal often innervates the spinal cord at multiple levels and pain signals from visceral and somatic sources may be transmitted by the same 2nd order spinal neuron (viscerosomatic convergence). Within the central pain matrix, the thalamus signals to brain regions that process the emotional component of the pain signal, such as the amygdala, insula, anterior cingulate cortex, hippocampus, and nucleus accumbens (Wilder-Smith, 2011; Bushnell et al., 2013). In healthy individuals, activation of the central pain matrix provides the appropriate behavioral responses (unpleasant emotion, guarding, and/or immobilization of the affected site) to promote recovery and to learn avoidance to prevent future injury (Navratilova and Porreca, 2014). Descending antinociceptive brainstem pathways are also activated by the

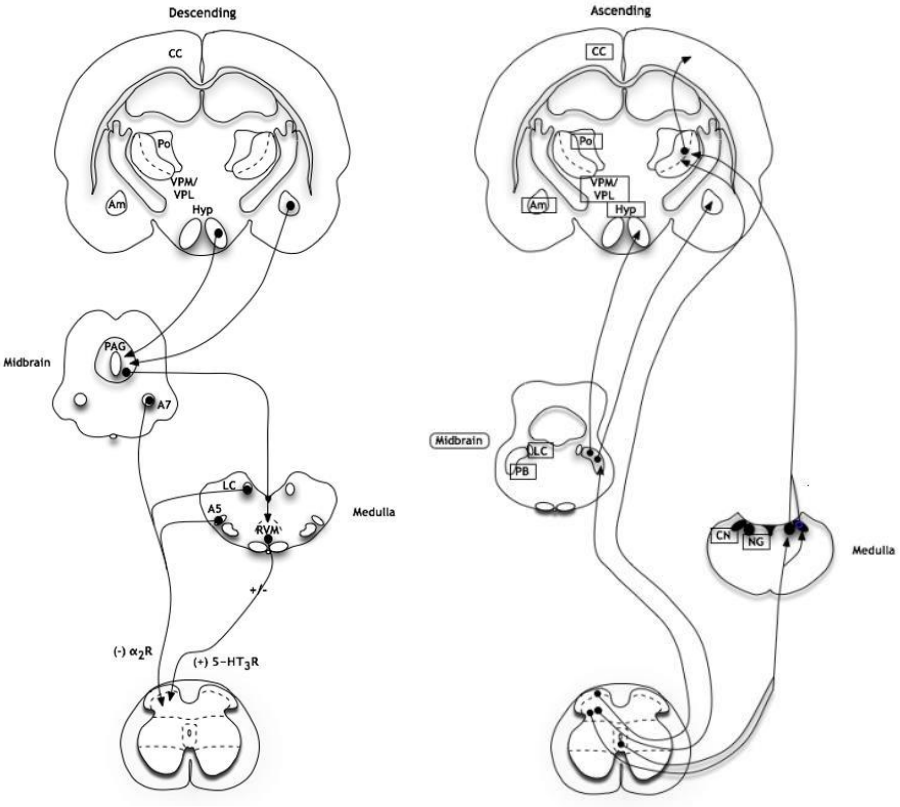
central pain matrix to decrease noxious signalling at the dorsal horn of the spinal cord by changing the excitability of the 2nd order neuron within the spinal cord (Heinricher et al., 2009; Denk et al., 2014). In fact, pain modulation of the central nervous system occurs via descending pathways, with multiple areas involved, e.g. the amygdala and hypothalamus. The periaqueductal gray region (PAG) plays an important role within this pathway through its projections to the medulla, which relay descending inhibitory signals to ascending pain pathways. Another proposed mechanism arises from the nucleus raphe magnus and nucleus reticularis, which are collections of cells derived from the rostral ventromedial medulla (RVM). This system projects to the spinal and medullary dorsal horns to modify nociceptive signals. Profound anti-nociceptive effects occur when opiates are injected into these brain regions in animal models (Giamberardino, 2009). The RVM receives input from the PAG and is thought to be a joint final relay center in a network of descending inhibitory pathways of nociception from central sites. The RVM forms connections with primary pain afferents in the second and third order neurons that transmit nociceptive signals to higher brain centers (Cervero and Laird, 2009). The PAG also projects to the amygdala and other cortical sites that regulate emotion. Emotional responses, stress, and anxiety are believed to play a significant role in the integration and processing of pain. Synaptic plasticity in the amygdala was found in a rat model of visceral pain (Neugebauer et al., 2004). Moreover, it has been demonstrated that altered central processing of visceral stimuli in IBS is at least in part mediated by symptoms of anxiety and depression, which may modulate the affective–motivational aspects of the pain response. Indeed, patients with IBS experienced significantly more pain and discomfort upon rectal distensions in the scanner, despite unaltered rectal sensory thresholds. Anxiety and depression scores were associated with these subjective stimulus ratings, but not with rectal sensory thresholds. Anxiety symptoms in IBS were significantly associated with pain-induced activation of the anterior midcingulate cortex and pregenual anterior cingulate cortex. Depression scores correlated with activation of the prefrontal cortex (PFC) and cerebellar areas within IBS. Group comparisons revealed significant activation in the IBS versus controls in the anterior insular cortex and PFC (Elsenbruch et al., 2010).

### *3.5.Gut-Brain axis*

The “gut-brain axis” is a theoretical model depicting bidirectional neural pathways linking cognitive, emotional and autonomic centres in the brain to neuroendocrine centres, the enteric nervous system and the immune system. Bodily visceral functions (e.g. digestion, nutrient resorption, gaseous exchange, excretion) require complex regulation in which the CNS is

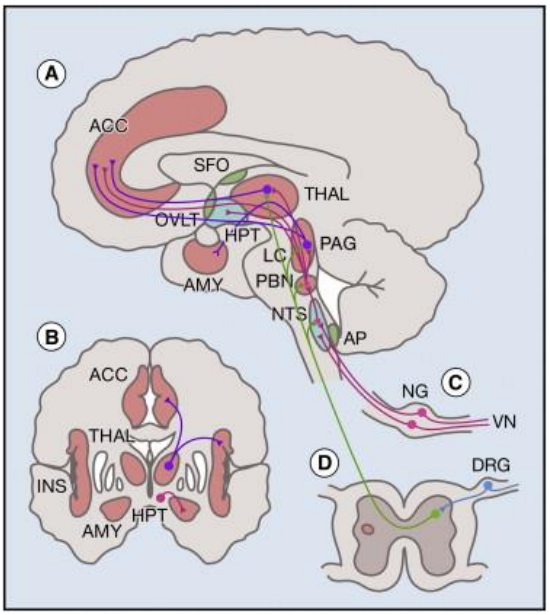
highly integrated with the peripheral and enteric nervous systems and hormonal controls. Accordingly, altered brain-gut interactions can contribute to autonomic dysregulation of the gut and associated pain and perceptual changes in visceral disorders like IBS (Mayer, 1999).

Vagal afferents project to the nucleus tractus solitarius (NTS) in the brainstem with cell bodies in nodose ganglion. Spinally-converging visceral afferents terminate in the dorsal horn with second order neurones projecting to higher centres through the dorsal column pathway (DC), parabrachial pathway and spinothalamic tract, as previously described (Hunt and Mantyh, 2001). Studies involving DC lesions have shown suppressed inhibition of exploratory behaviour induced by noxious visceral stimulation and inhibition of potentiated visceromotor reflexes evoked by colorectal distension during inflammation (Palecek and Willis, 2003). Superficial dorsal horn projections mostly form the spinoparabrachial pathway, associated with autonomic and affective responses to painful stimuli (Bester et al., 1995). Along with NTS projections from vagal afferents, spinoparabrachial projections are transmitted to limbic and cognitive higher centres including parts of the brain involved in affect, such as the amygdala, hypothalamus and periaqueductal grey (PAG) (Bester et al., 1997; Hunt and Mantyh, 2001).




***Ascending and Descending pathways in visceral nociception (in rodents).***

*Hunt and Mantyh, Nature Review in Neuroscience, 2001*




***Viscerosensory Paths and Centers in the Human Brain***

*Critchley and Harrison, Neuron, 2013*

## *4. Chronic visceral pain mechanisms*

Distinct from the well-established protective and adaptive functions of acute pain, pain persisting beyond tissue healing is maladaptive and serves no known physiological function. In contrast to acute pain, the mechanisms involved in the development and maintenance of persistent pain are not fully understood and visceral pain complexity make this research further difficult. The study of events immediately after acute gastrointestinal diseases, like infections or inflammations, showed a number of abnormalities that can persist even after overt remission of inflammation and can be involved in the chronicization of symptoms like pain. These abnormalities include increased gut permeability, immune response, increased mucosal serotonin availability, sensitized enteric nerves and altered gut microbiota (Spiller and Major, 2016). Anyway, a single factor is not likely to be responsible for the several presentations of chronic visceral pain (Sinagra et al., 2012).

### *4.1. Increased gut permeability*

Acute gastroenteritis produces an increase in gut permeability in all infected individuals, both adults and children. This change lasts at least 12 weeks and up to 4 years in some individuals with PI-IBS. The increased permeability persisted even in patients whose bowel habit had returned to normal, which might indicate that increased permeability increases susceptibility

to IBS symptoms but is not sufficient to cause symptoms without other factors (Johansen et al., 1989; Spiller et al., 2000). On the other hand, elevated small and large intestinal permeability in both PI-IBS and IBS cases was detected without an obvious infectious precipitant (Dunlop et al., 2006). Such evidence does suggest that increased permeability might represent a common pathway for other factors to influence the gut. Furthermore, the real-life stress of public thesis defence, which more closely models the stressors patients associate with exacerbation of their IBS symptoms, has been shown to increase small bowel permeability, an effect that seemed to be blocked by the mast cell stabilizer cromoglycate (Vanuytsel et al., 2014). Rat studies, which enable improved control of other variables, clearly show that permeability is increased by psychological stress (induced by water avoidance or crowding), acting via mucosal mast cells and vasoactive intestinal peptide (Keita et al., 2013). Most of these changes can be mimicked by corticotropin-releasing factor (CRF) injections and blocked by CRF antagonists in these rat models (Larauche et al., 2009). If permeability tests represent barrier function, then such effects imply increased exposure of the immune system to bacterial and food antigens. This process could lead to immune activation that further deranges barrier function. Such abnormalities have also been indicated in IBD: genome-wide association studies on the aetiology of IBD have implicated a range of genetic loci related to pathways of epithelial barrier function (Khor et al., 2011). Increased gut permeability is reported in both disease-free relatives and patients with Crohn's disease or ulcerative colitis, supporting the hypothesis of its role as a predisposing factor with many causes (Buning et al., 2012). In patients with IBD-IBS mucosal permeability is markedly elevated compared with both healthy individuals and patients with IBD without IBS-like symptoms (Vivinus-Nebot et al., 2014). Given the similar symptoms, it is perhaps not surprising that being diagnosed as IBS increases the risk of subsequently being diagnosed with IBD (Larsson et al., 2012). Whether incident cases of IBD in people with prevalent IBS represent delayed diagnosis or evolution of the same condition cannot be determined. Plainly, other risk factors, either genetic or environmental, are needed in addition to increased permeability before IBS or IBD manifests (Spiller and Major, 2016).

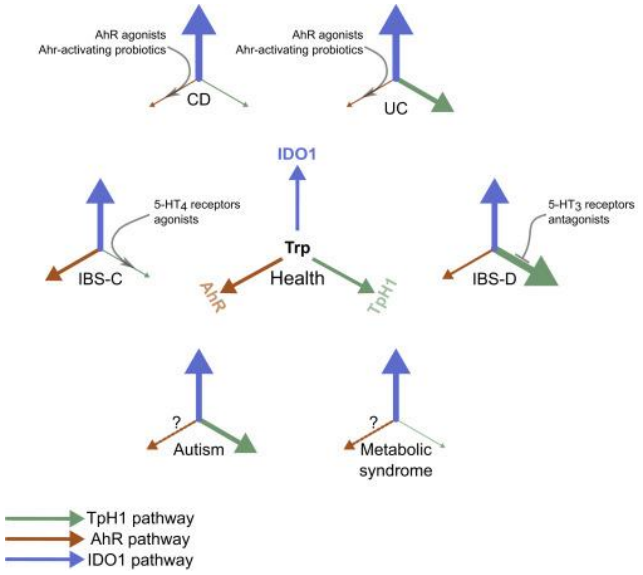
### *4.2. Abnormal serotonin availability*

Serotonin, or 5-hydroxytryptamine (5-HT), is contained in enterochromaffin cells found in the intestinal crypts. 5-HT stimulates motility and secretion of water into the lumen (Mawe et al., 2013). These effects are terminated by transport into enterocytes and neurons by the serotonin transporter (SERT), whose impairment in inflammatory conditions can lead to excess mucosal

serotonin availability. Increased rectal enterochromaffin cell numbers have been reported in PI-IBS after C. jejuni infection and in IBS-D, associated with a tenfold increase in 5-HT release in biopsy samples, which correlated with pain severity (Dunlop et al., 2003; Cremon et al., 2011). The striking clinical benefit of inhibiting 5-HT synthesis with a THP-1 inhibitor or blocking 5-HT3 receptors with 5-HT3 receptor antagonists in IBS-D, does confirm that increased 5-HT availability has a role in symptom generation (Andresen et al., 2008; Garsed et al., 2014). Postprandial plasma levels of 5-HT has been found to be elevated in PI-IBS and IBS-D. The increased levels might reflect either increased release or impaired reuptake by SERT, which is decreased in the platelets of patients with IBS-D (Dunlop et al., 2005; Atkinson et al., 2006). Human studies of the role of 5-HT in IBD are more limited. Increased enteroendocrine cell numbers staining for 5-HT and peptide YY have been seen in lymphocytic colitis. However, both an increase and decrease in numbers of these cells have been reported in Crohn's disease and ulcerative colitis. When frank ulceration occurs, the enterochromaffin cells are destroyed, their numbers decrease and 5-HT levels fall, but increases in enterochromaffin cells can occur during intestinal regeneration, hence the confusion in the literature (Magro et al., 2002; El-Salhy et al., 2012; Massironi et al., 2016). In IBD-IBS, enterochromaffin cell numbers are normal but such patients show increased TPH-1, the rate-limiting enzyme in serotonin synthesis, possibly implying greater 5-HT turnover than those without symptoms (Minderhou et al., 2007). Moreover, a role for serotonin in inflammation-induced neurogenesis has been shown in experimental models, being enhanced by 5-HT4 receptor agonists and inhibited by 5-HT4 receptor antagonists (Belkind-Gerson et al., 2015).

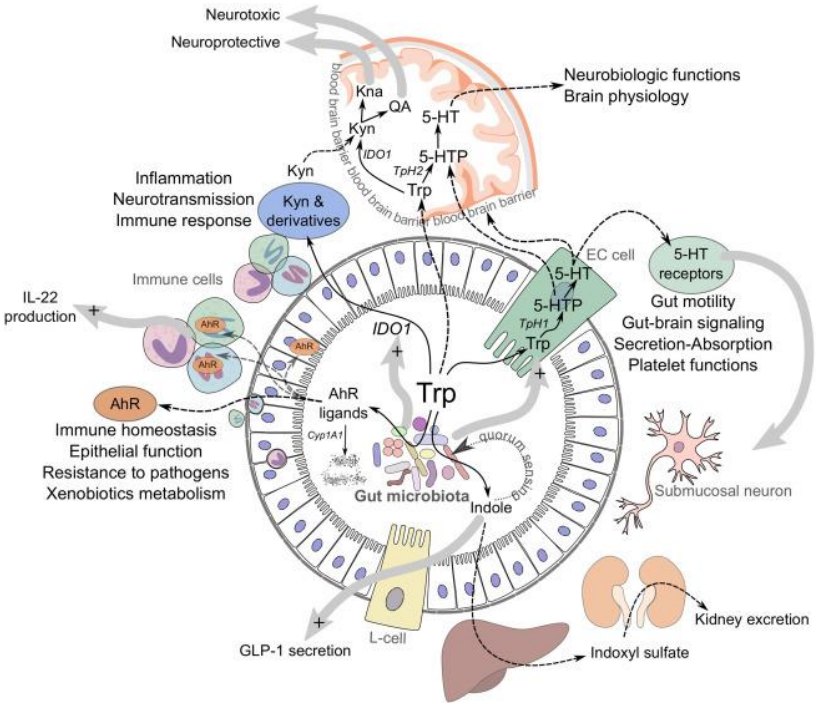
Abnormalities in the metabolism of tryptophan were found in different GI disease, including $IBD_S$ and different types of IBS, resulting in a dysregulation of serotonin synthesis. There are neural processes in the gastrointestinal tract which can be influenced by local alterations in serotonin concentrations with subsequent relay of signals along the scaffolding of the brain-gut axis to influence neurotransmission at central level (Agus et al., 2018). In the gut, the three major Trp metabolism pathways leading to serotonin, kynurenine, and indole derivatives are under the direct or indirect control of the microbiota. Interesting is the ability of microbiota to control host tryptophan metabolism along the kynurenine pathway, thereby simultaneously reducing the fraction available for serotonin synthesis and increasing the production of other neuroactive metabolites (O'Mahony et al., 2015; Kennedy et al., 2017). Since gut microbiota is clearly affected in both IBS and IBDs, a long-term dysregulation in serotonin metabolism due to gut dysbiosis was proposed as possible mechanism for visceral

symptoms persistence in these patients, though there are poor evidence about this correlation (Spiller and Major, 2016).




***Perturbations to Trp Metabolism in Diseases***

*Agus et al., Cell Host & Microbes, 2018*




***Integrated Trp Metabolism under the Control of the Gut Microbiota in Host Physiology***

*Agus et al., Cell Host & Microbes, 2018*

### *4.3. Dysbiosis in gut microbiota*

A growing body of preclinical and clinical evidence supports a relationship between the complexity and diversity of the microorganisms that inhabit our gut and health status. Under normal homeostatic conditions this microbial population helps maintain intestinal peristalsis, mucosal integrity, pH balance, immune priming and protection against invading pathogens. Furthermore, these microbes can influence centrally regulated emotional behaviour through mechanisms including microbially derived bioactive molecules (amino acid metabolites, short-chain fatty acids, neuropeptides and neurotransmitters), mucosal immune and enteroendocrine cell activation, as well as vagal nerve stimulation. Furthermore, the gut microbiota regulates several host biochemicals with know neuromodulatory properties, including endocannabinoides, neuropeptides and biogenic animes (Sjogren et al., 2012).

Post-infectious IBS (PI-IBS), which bears close resemblance to IBS-D, is a surprisingly common result of acute gastroenteritis (primarily of bacterial aetiology) with a reported incidence of between 5–32% (Thabane et al., 2009). Small intestinal bacterial overgrowth (SIBO), defined by an excessive amount of bacteria in the small intestine, may be a cause of IBS. SIBO arises when the homeostatic mechanisms that regulate enteric bacterial populations are compromised. Small intestinal dysmotility and reduced gastric acid secretion are the most common predisposing processes. Studies in IBS patients have revealed delayed transit, intestinal dysmotility, and abnormalities in the migrating motor complex—all of which could account for a predisposition to SIBO (Dukowicz et al., 2007; Aziz et al., 2017). The reduction in microbial richness shown with rifaximin therapy may be responsible for its modest clinical benefit in diarrhoea-predominant IBS patients, but SIBO was not tested in these patients and the underlying mechanism of action remains to be clearly defined. Antibiotics significantly alter gut microbial ecology (namely a collapse in diversity) and disrupt interactions with host metabolism (Perez-Cobas, 2014). A consequence of this is the frequent disturbance in bowel habit, particularly with broad-spectrum antibiotics (5–62%), and numerous animal and human studies provide evidence of the role antibiotics play in IBS pathogenesis (Goldenberg et al., 2015).

Preclinical studies have shown that mice raised in a sterile environment from birth, and as such, without gastrointestinal bacteria (germ-free), exhibit an exaggerated stress response (Clarke et al. 2013; Sudo 2012; Sudo et al. 2004), and a blunted response to inflammatory pain (Amaral et al. 2008). Similarly, antibiotic-induced alteration of gastrointestinal microbiota decreased visceral pain-related response elicited by intraperitoneal acetic acid injection or intracolonic capsaicin infusion in mice (Aguilera et al. 2015), and also decreased

visceral sensitivity in naïve rats (Hoban et al. 2016). Further studies demonstrated that early life exposure to antibiotic alters microbial composition and leads to enduring effects on visceral pain in rodents (O'Mahony et al., 2009). Evidence that this also occurs in adulthood was shown in a prospective study in which consecutive patients prescribed antibiotics for non-GI complaints were more than three times as likely to report chronic bowel symptoms as controls (Maxwell et al., 2002). Recently, faecal matter from IBS patients characterised by hypersensitivity to colorectal distension was transplanted to germ-free rats, and the response to colorectal distension was enhanced in these animals (Crouzet et al. 2013). While preclinical evidence for the efficacy of prebiotics in manipulating visceral hypersensitivity is limited, there is evidence for a role of the microbiota in regulating response to visceral pain through probiotic administration. In contrast to the provocative preclinical evidence for a role for gut microbiota in visceral pain, clinical studies remain inconclusive with a large 'non-responder' population in many probiotic trials (Larauche et al. 2012c; Kannampalli et al. 2014). On the other hand, cohorts are not always well-characterized when microbiome is studied in a disease context. Yet, the use of drugs such as proton pump inhibitors or laxatives, which are more often used by patients with IBD or IBS, has a large impact on the gut microbiota composition. Considering these effects, correction for these medications is essential for identifying disease-associated microbial features and avoiding false positive associations due to changes in GI acidity or bowel mobility. Nevertheless, numerous studies have shown alterations in the gut microbiota in both IBS and IBD. Moreover, although the gut microbiota composition has been described as stable across individuals in different population cohorts even in the presence of high inter individual taxonomic variation, a large number of microbial pathways were shown to be disrupted in patients with IBD or IBS (Vich Vila et al., 2018).

This change might be secondary to changes in a range of factors that are now understood to influence the gut microbiota, such as diet, medical treatment, mucosal immune response, gut transit and redox potential within the gut lumen, but dysbiosis might also contribute to symptoms (Rajilic-Stojanovic et al., 2015). The loose stool induced by both IBS-D and IBD exerts selective pressure on gut microorganisms. Thus, enterotypes associated with loose stool include taxa with the capacity for rapid growth or adherence to the mucosa, features that promote avoidance of washout (Vandeputte et al., 2016). A meta-analysis of studies of patients with Crohn's disease shows that, although there are many disparities between often underpowered studies, there is a consensus that Bacteroidetes and Enterobacteriaceae are increased in abundance whereas Firmicutes are decreased (Wright et al., 2015). An increase

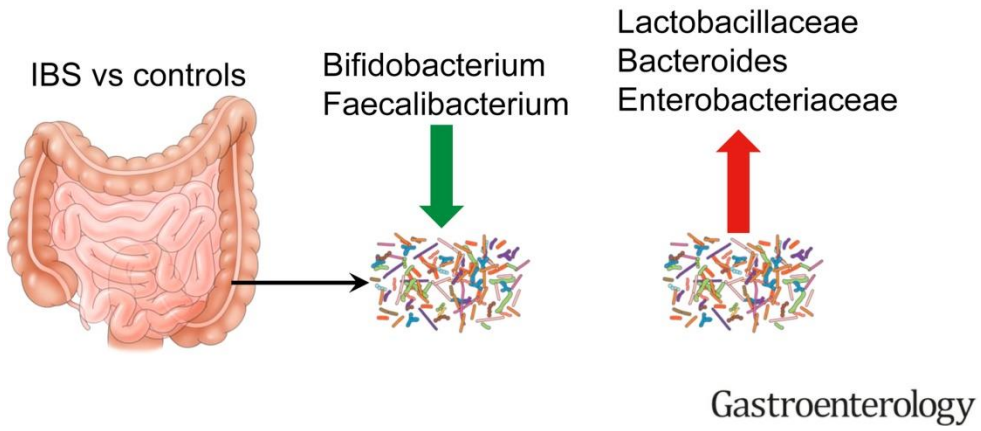
in Bacteroidetes has also been observed in PI-IBS. However, differences in the gut microbiota between IBD and IBS were found (Jalanka-Tuovinen et al., 2014).

Evidence suggests that dysbiosis in IBD might, in large part, reflect the response of a complex microbial community to the environmental stress of intestinal inflammation. In the healthy gastrointestinal tract, a radial oxygen gradient exists due to the diffusion of oxygen from the host mucosa into the gut lumen (Marshall et al., 2004; Dunlop et al., 2006; Vicario et al., 2010). Accordingly, bacteria adherent to the colonic mucosa have higher oxygen tolerance and catalase expression relative to faeces-associated species (Vicario et al., 2010). As inflammation is an oxidative state, it might be expected to promote the outgrowth of aerotolerant taxa such as Proteobacteria and Actinobacteria. Indeed, the mouse pathogen Citrobacter rodentium has been shown to gain a fitness advantage by promoting epithelial aerobic respiration and increasing oxygenation of the mucosal surface (Vanuytsel et al., 2014). Alternatively, several lines of evidence have shown that intestinal inflammation induces the production of small molecules that serve as terminal electron acceptors for facultative anaerobes such as Enterobacteriaceae (Soderholm et al., 2002). Thus, metabolic alterations associated with inflammation and/or pathobiont colonization might act as microbial stressors and promote the outgrowth of dysbiotic species. The heterogeneity of IBS inevitably means that reports of disturbed gut microbiota have been conflicting. In one study, patients with IBS with a similar gut microbiota to healthy individuals had higher scores for depression than patients whose gut microbiota differed (Jeffery et al., 2012). This finding raises the possibility of grouping patients with IBS into those whose symptoms predominantly relate to central processing and psychological factors, and those with a gut-predominant aetiology who have abnormal gut microbiota. The role of diet in determining the gut microbiota should not be forgotten. Patients with both IBS and IBD symptoms modulate their diet in important ways, often avoiding foods high in fermentable oligosaccharides, monosaccharides, disaccharides and polyols (FODMAPs) (Anderson et al., 2015).

However, it is yet unclear how, or to what extent, microbiota confined to the gastrointestinal tract can influence visceral pain behaviour (Moloney et al. 2016). A number of different receptor types are involved in the process of peripheral sensitisation including the TRPV family, proteinase-activated receptors, cholecystokinin receptors, serotonin receptors, cannabinoid receptors as well as an array of ion channels including ATP-gated ion channels, voltage-gated sodium and calcium channels and acid-sensing ion channels (Akbar et al. 2009). The gastrointestinal microbiota can activate these receptors directly or indirectly through immune responses at the mucosal surface during infection, inflammation and

autoimmunity (Cassel et al. 2008; El Aidy et al. 2014; Kamada et al. 2013; Mazmanian et al. 2005; Round and Mazmanian 2009). Also, a number of different molecules, including formyl peptides and protease, polyunsaturated fatty acid (PUFA), SCFA, as well as hormones and neurotransmitter were found involve in the effects of microbiota (Husebye 1997; Cummings and Macfarlane 1997; Cenac et al. 2008; Vergnolle 2009; Lyte 2013, 2014; Cani et al. 2013; Cenac et al. 2015). However, the extent to which these mechanisms either individually or collectively have a role in the aetiology of visceral pain remains unaddressed. Gastrointestinal microbiota can also stimulate the release of the body's natural pain-suppressing biomolecules including opioids from innate neutrophils and monocytes, endocannabinoids from colonic tissue as well as other pain modulators including monoamines (Muccioli et al. 2010; Boue et al. 2014; Oleskin and Shenderov 2016).

Recently the role of spinal microglia in the mediation of visceral pain has received some attention (Saab et al. 2006; Bradesi 2010). Microglia are critically involved in neuronal events at various stages in development and adulthood, including synaptic remodelling to improve neuronal network signalling (Schafer et al. 2012; Schafer and Stevens 2015). In contrast, the absence of a complex host microbiota led to increased microglial populations, defects in microglia maturation, activation state and differentiation, alterations to microglia morphology (with longer processes and increased branching, terminal points and clubbing at synaptic boutons) and a compromised immune response to bacterial or viral infection (Erny et al. 2015). The observed alterations in microglial phenotype were reversed with recolonisation of gut microbiota, following 6-week cohabitation of germ-free mice with control mice. The findings from this study have redefined the ideology that the relationship between our microbiota, immune system and neurodevelopment is moot in adulthood, as a healthy and diverse gastrointestinal microbiota is essential for the continuous preservation of healthy microglia and proper brain function throughout our lifespans (Cryan and Dinan 2015). It is plausible that such events also occur to spinal microglia. However, to date, no studies have investigated the effects of gastrointestinal microbiota modulation on spinal microglia activation in visceral pain.




*Pittayanon et al., Gastroenterology, 2019*

### *4.4. Altered gut immune response*

The GI tract has a complex innate and adaptive mucosal immune system that is capable of monitoring the luminal content for a diverse array of innocuous antigens including commensal microbiota and food antigens (oral tolerance) versus invasion of the host by potentially toxic pathogens. The immune cells that reside in the intestine mucosa, mesenteric lymph nodes, and Peyer's patches make up the gut-associated lymphoid tissue (GALT). Cells of the GALT, dendritic cell, macrophages and B-cells make up the antigen-presenting cells and shape the responses of a heterogeneous population of T cells. Such response can be tolerogenic against commensal bacterial antigens or immunogenic against invading pathogens. Together, the cells of the GALT play a role in both innate and adaptive immunity and are pivotal for maintaining immune homeostasis in the gut. The maintenance of a delicate balance between tolerance and immune system activation is key for overall gut health with abnormalities in this equilibrium leading to pathologies of the gut including IBD, CD, and food intolerances (Mann and Li; 2014; Reboldi and Cyster, 2016; Vitale et al., 2016).

Significant increases in the number of T lymphocytes, macrophages and mast cells, enteroendocrine cells, and IL-1β mRNA expression were observed in submucosal biopsies of PI-IBS patients compared with healthy volunteers (Spiller et al., 2000; Gwee et al., 2003; Wang et al., 2004).

Immune cells contribute significantly to both neuropathic and inflammatory pain: on one hand, distinct immune cell types release mediators that act on the terminals of nociceptors to drive peripheral sensitization; on the other hand, in the CNS, microglia, astrocytes and T cells modulate neurotransmission and spinal cord pain circuitry to drive central sensitization.

In acute inflammation, pain often parallels the immune response and diminishes with resolution of inflammation. In chronic diseases such as rheumatoid arthritis and colitis, persistent immune triggers such as cytokines mediate long-lasting pain. In this regard, multiple studies have shown that cytokine and chemokine signalling pathways impact pain-like behaviours in animal models of inflammatory and neuropathic pain (White et al., 2005; Cook et al., 2018).

TNF is one of the most important cytokines mediating pain sensitization in the periphery. In the CNS, microglia and astrocytes also release TNF, which acts on CNS neural circuitry to modulate pain signalling (Ferreira et al., 1988; Jin et al., 2006; Ji et al., 2014). IL-1β is a cytokine produced downstream of the inflammasome and is involved in driving pain sensitivity in a number of diseases including gout and rheumatoid arthritis. IL-1β was one of the first cytokines demonstrated to cause hyperalgesia upon both local challenge and systemic administration in mice (Ferreira et al., 1988; Fukuoka et al., 1994). Both tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) levels were found increased in the spinal cord of animals after the induction of colitis by TNBS. In these animals the colonic inflammation is strictly associated with the development of persistent post-inflammatory visceral hypersensitivity (Lu et al., 2019). IL-6 and the chemochines are also mediators of neuroimmune communication in pain and are involved in both peripheral and central pain sensitization (Xu et al., 1997; Jiang et al., 2016; Cook et al., 2018). IL-17A is one of the main cytokines driving inflammation and pathology in several autoimmune and inflammatory diseases (Pinto et al., 2010; McNamee et al., 2011). Neutralizing endogenous IL-17A prevented pain-like behaviours in mice, with this effect being mediated via the expression of TNF, IL-1β and CXC-chemokine ligand 1 (CXCL1) and neutrophil recruitment34. IL-17A was also observed to directly induce rapid phosphorylation of protein kinase B and ERK in DRG cell cultures, which contain nociceptors (Richter et al., 2010).

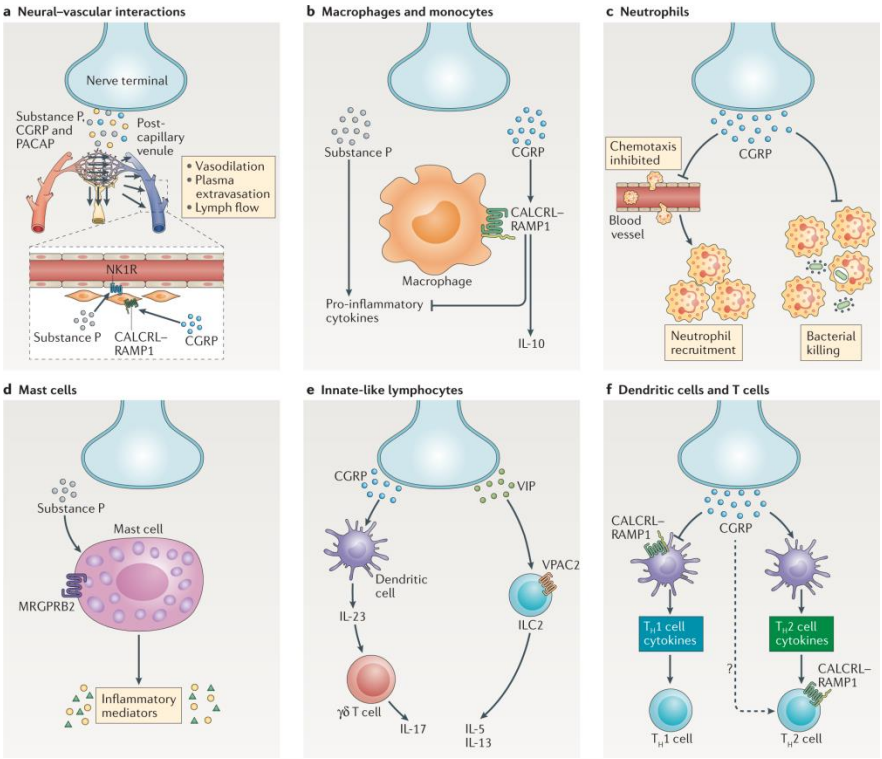
Besides, IL-10, a key anti-inflammatory cytokine, possesses anti-nociceptive properties (Milligan et al., 2005; Krukowski et al., 2016). IL-10-mediated suppression of pain may involve both its inhibitory effect on the expression of pro-algesic cytokines such as TNF, IL-6 and IL-1β and a direct effect on neurons (Shen et al., 2013). Despite this, the serum levels of either IL-5, IL-6, IL-10, and TNF-α were found significantly higher in patients with IBS compared to healthy controls (Liebregts et al., 2007; Vara et al., 2018). In rats, a similar colon pattern of cytokine mRNA levels was observed in the post-inflammatory phase of colitis: higher IL-6 and IL-10 and no modifications of TNF- production (Salameh et al., 2019).

Gram-negative bacterial infections are also frequently painful. LPS, which is a major component of Gram-negative bacterial cell walls, induces pain when injected into mice. Recent work suggests that LPS can directly act on nociceptors, as nociceptors express Toll-like receptor 4 (TLR4), which mediates LPS detection (Diogenes et al., 2011; Meseguer et al., 2014). In a mouse model of urinary tract infection with uropathogenic Escherichia coli, pelvic pain has been found driven by LPS in a TLR4- dependent fashion independent of bacterial colonization or inflammation (Rudick et al., 2010). LPS-induced systemic immune activation leads to visceral hyperalgesia in rats (Nozu et al., 2017). In accordance, TLR4 blockage counteracted visceral hyperalgesia associated to a high-fat diet in mice (Tramullas et al., 2016).

It has been also demonstrated that neuronal signalling via the secreted neuropeptides CGRP and substance P could generate immediate vasodilation, increasing blood flow and oedema independently of the immune response in a process called neurogenic inflammation. Innate and adaptive immune cells also express receptors for CGRP, substance P and other neurotransmitters, indicating a role for complex neural–immune communication through these neuropeptides to potently regulate innate and adaptive immunity. Substance P has a robust effect on monocytes and macrophages and induces their release of proinflammatory cytokines, including IL-1, TNF and IL-6, via ERK–p38 MAPK-mediated NF-κB activation (Lotz et al., 1988; Sun et al., 2008; Lim et al., 2017). CGRP has a predominantly anti-inflammatory effect on myeloid cells, inducing the downregulation of cytokine production, oxidative burst synthesis and antigen presentation and the upregulation of the antiinflammatory cytokine IL-10 in macrophages and dendritic cells (DCs) (Nong et al., 1989; Yaraee et al., 2005; Baliu-Pique et al., 2014; Russell et al., 2014). One intriguing area that remains to be characterized is the impact that the mechanisms described above have on pain. Nociceptor interactions with neutrophils have been less well characterized, but recent studies show a key role for nociceptors in regulating neutrophil recruitment and activation (Strausbaugh et al., 1999; Baral et l., 2018). Yet, a tight interplay between nociceptors and mast cells has long been postulated owing to their anatomic co-localization. Mast cells are found in close apposition to nerve fibres in barrier tissues, particularly CGRP– and substance P+ neurons (Stead et al., 1987; Arizono et al., 1990; Alving et al., 1991). Increases in the number of nerves–mast cell contacts have been reported in models of inflammatory and allergic diseases, as well as in parasitic infection of the colon. Both Substance P and CGRP alone are sufficient to cause mast cell activation, degranulation and release of inflammatory mediators including histamine, leukotrienes, tryptases, PGD2 and TNF (Arizono et al., 1990;

Jarvikallio et al., 2003; Yosipovitch et al., 2016). Like mast cells, DCs have been found to be in close contact with nociceptors in peripheral tissues. Indeed, nociceptor regulation of DCs has an important role in modulating immunity (Riol-Blanco et al., 2014; Kashem et al., 2015). The increased numbers of T and mast cells in mucosal biopsies of IBS patients significantly correlated with abdominal bloating frequency and symptoms of dysmotility-like dyspepsia (Chadwick et al., 2002; Cremon et al., 2009). In addition, mast cells were found to be located in closer proximity to nerve fibers in IBS patients vs controls while the number of mast cells in close proximity to nerves significantly correlated with the severity and frequency of abdominal pain and discomfort (Barbara et al., 2004).

Analysis of the supernatants obtained after incubation of mucosal biopsies revealed that the supernatants from IBS patients contained increased amounts of pro-inflammatory mediators, such as histamine, serotonin, polyunsaturated fatty acid metabolites and proteases (Cenac et al., 2007; Buhner et al., 2009; Cenac et al., 2015). Each of these mediators can contribute to aberrant visceral pain perception as *in vitro* studies revealed that the marked increase in intracellular calcium in rat DRG neurons was at least partially inhibited by the application of a 5-HT3 antagonist, histamine 1 or 2 receptor antagonists, and a protease antagonist (Nasser et al., 2014). *In vivo,* supernatants from colonic biopsies of IBS patients, but not controls, caused somatic and visceral hyperalgesia and allodynia in mice, when administered into the paw or colon, respectively (Cenac et al., 2007). Although the evidence for aberrant immune activation seems overwhelming in PI-IBS, care must be taken when interpreting these results as the evidence supporting persistent immune cell infiltration or low-grade inflammation in IBS is conflicting. Indeed, various research groups found no differences in immune cell numbers or cytokine mRNA expression in mucosal biopsies of PI-IBS and IBS patients compared with healthy volunteers (Mearin et al., 2009; Braak et al., 2012; Wouters et al., 2016a; Bennett et al., 2016). Braak et al. even reported decreased numbers of mast cells, macrophages and T cells in the colonic mucosa of 66 IBS patients compared with 20 healthy volontaries (Braak et al., 2012). Also, at mRNA level, decreased levels of genes linked to chemokine function or IL-10 were detected among IBS patients (Macsharry et al., 2008; Chang et al., 2012). More recently, Bennet et al. found no differences in cytokine mRNA expression levels in sigmoid colon biopsies when analyzing 109 IBS patients vs healthy volunteers (Bennett et al., 2016). These reports which fail to demonstrate immune infiltration in the colorectal mucosa of PI-IBS patients, also fail to demonstrate a correlation between immune infiltration and visceral pain perception (Braak et al., 2012).




***Neuro-immune interactions***

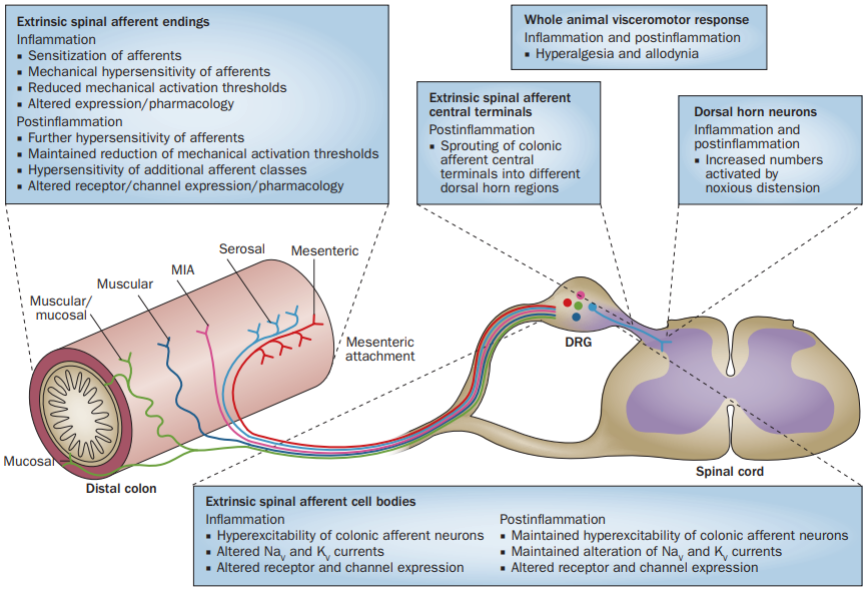
*Baral et al., Nature Reviews Immunology, 2019*

### *4.5. Altered enteric nervous signalling*

Acute inflammation damages enteric nerves and disconnects them from their gut targets. As nerves regrow, they sprout and subsequently undergo complex remodelling in the process of reconnecting with their targets. This process has been clearly described in experimental model of colitis (Byers et al., 2003; Simpson et al., 2008; Liebregts et al., 2009).

In the enteric nervous system, inflammation causes a rapid loss of enteric neurons and viscera-fugal neurons. Of the remaining enteric neurons, specific subpopulations located in the myenteric and submucosal ganglia become hyperexcitable and synaptic transmission between them is facilitated. These changes in neuronal function result in decreased secretion and disrupted motility (Mawe et al., 2009). In the prevertebral ganglia, although synaptic input from viscera-fugal neurons is reduced, sympathetic viscera-motor neurons are actually hyperexcitable. Many of the neuronal changes evident during inflammation are still present after resolution of inflammation, with hyperexcitable enteric neurons and facilitated synaptic transmission still evident in the post-inflammatory state. This hyperexcitability results in increased neuronal activation in the nucleus of the solitary tract or the dorsal horn of the spinal cord, respectively (Mawe et al., 2009; Spiller and Major, 2016). In whole-animal studies, this process translates to enhanced pain responses to either gastric or colorectal

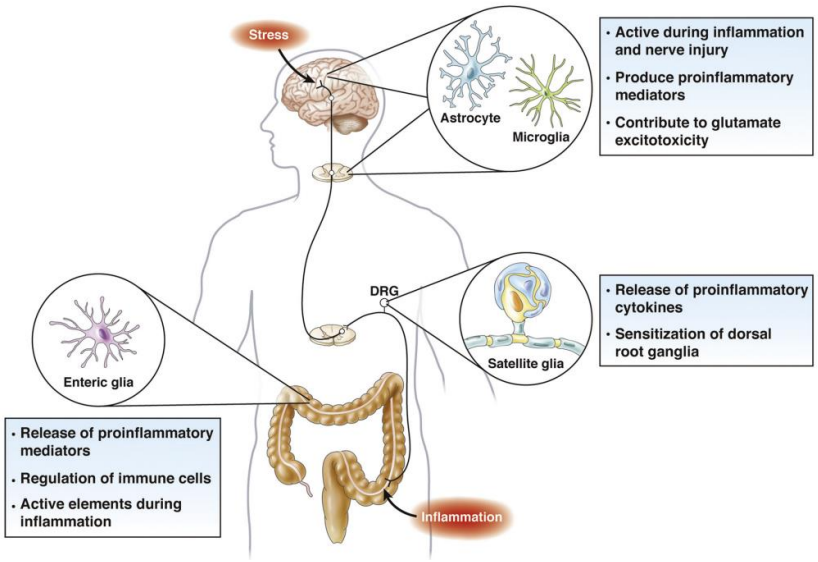
distension. In this context, an increased density of colonic afferent central terminals was also observed, as there is a sprouting of these terminals into different regions of the dorsal horn of the spinal cord. This plasticity results in increased numbers of dorsal horn neurons in the spinal cord being activated in response to noxious colorectal distension. Many of these changes are still present or are even enhanced following resolution of inflammation (Brierley and Linden, 2014). Histochemical studies of nerves in diseased terminal ileum resected from patients with Crohn's disease show anatomical distortion with abnormal varicosities and a chaotic increase in staining for a wide range of neuropeptides (Belai et al., 1997). Tachykinergic innervation of the submucosal blood vessels increases markedly in ulcerative colitis (de Fontgalland et al., 2014). Increases were also seen in nerves co-expressing substance P and transient receptor potential cation channel subfamily V member 1 (TRPV1), with a decrease in somatostatin-expressing nerves. An earlier study in patients with IBD-IBS showed increased TRPV1-positive nerves with a strong correlation between number of TRPV1-positive fibres and pain. The same group also investigated patients with IBS and found an increase in total nerve fibres, including those staining for substance P, mast cells and lymphocytes. Multivariate regression analysis showed that TRPV1-immunoreactive fibres and mast cells were related to the abdominal pain score, suggesting that these nerve fibres might contribute to visceral hypersensitivity (Akbar et al., 2008; Akbar et al., 2010). The occurrence of such changes in both IBS and IBD suggests a common explanation for pain occurrence, though the mechanisms by which these signalling alterations perpetuate over time are not elucidated (Spiller and Major, 2016).




***Neuroplasticity of afferent pathways during and after resolution of gut inflammation***

*Brierley and Linden, Nature Reviews Gastroenterology & Hepatology, 2014*

### *4.6. Peripheral and central Glia involvement in visceral pain*




***Major populations of glia that contribute to chronic pain.***

*Morales-Soto and Gulbransen, Cellular and Molecular Gastroenterology and Pathology, 2019*

Glia (from Greek γλοία meaning 'glue') pertains to non-neuronal cells in the central (CNS) and peripheral nervous system (PNS) that nourish neurons and maintain homeostasis. In addition, glia are now increasingly appreciated as active regulators of numerous physiological processes initially considered exclusively under neuronal regulation (Grubišić and Gulbransen, 2016).

Multiple distinct types of glial cells expressing are encompassed by the term ‘enteric glia’. These diverse populations of cells reside within myenteric and submucosal ganglia (intraganglionic), within inter-ganglionic nerve fibre tracts, below the mucosal epithelial cells (subepithelial), and are associated with nerve fibres interspersed between smooth muscle cells (intramuscular) (Gulbransen and Sharkey, 2012). Current theories suggest that neuronal plasticity involving the sensitization of visceral afferent sensory nerve fibers and broad alterations to the brain-gut axis contribute to the development of chronic abdominal pain. Much of this theory is based on evidence demonstrating changes in neuronal sensitivity, firing patterns, and network activity in the periphery, brain, and spinal cord. Although little is still known regarding the mechanisms that drive these alterations to neurons and their networks, it is increasingly clear that these properties are regulated by glia (Morales-Soto and Gulbransen, 2019). Enteric glia regulates enteric neurons activity and interacts with various non-neuronal cell types in the gut wall such as enterocytes, enteroendocrine and immune cells and is

therefore emerging as important local regulators of diverse gut functions. The intricate molecular mechanisms that govern glia-mediated regulation are beginning to be discovered, but much remains unknown about the functions of enteric glia in health and disease. Reactive enteric glia, as with reactive astrocytes, display a marked hypertrophy, increased proliferation and vary expression of both cytoplasmic and surface proteins under certain conditions (Grubišić and Gulbransen, 2016). Notably, under pathological conditions, enteric glia can transform into antigen-presenting cells that promote inflammation by attracting immune cells. Moreover, enteric glia can modulate enteric neural circuits in a number of ways, including terminating the actions of neurotransmitters from synapses, supplying neurons with neurotransmitter precursors and by generating neuroactive substances (Gulbransen and Sharkey, 2012; Sharkey et al., 2015).

Recently, a research group in Chicago demonstrated that the intestinal inflammation induced by DNBS in rats alters the ENS functionality by affecting the interaction between enteric neurons, nociceptors and glia. In that context the activation of enteric glia resulted to be actively involved in visceral hypersensitivity (Delvalle et al., 2018). Interestingly glia activation, triggered by the intestinal damage, persists even in the remission phase of colitis (Ippolito et al., 2015), spreading vertically to the dorsal root ganglion where it was found an increased coupling between satellite cells and neuron that was positively correlated with visceral pain (Huang et al., 2010; Hanani et al., 2010). This upward activation of glia along the sensitive pathways would suggest an involvement of central glia in the persistence of pain after the peripheral damage resolution.

Indeed, it is now acknowledged that the development of central sensitization engages not only neuronal, but also glial processes (Dodds et al., 2016). Within the CNS, glia is the non-neuronal, immune-like cell population that constitute the vast majority of cells. Glia comprises satellite glial cells in the ganglia, and microglia, astrocytes and oligodendrocytes within the spinal cord and brain. The anatomical co-localization of astrocytes and microglia in the spinal cord, combined with pre- and postsynaptic neurons, forms a key site of interaction termed the ‘tetrapartite synapse’ (Deleo et al. 2006; Ren and Dubner, 2015). In this context, the reactivity state and control of astrocytes and microglia is critical in maintaining healthy CNS activity. Many of the proinflammatory responses of glia are important in protecting against challenges that disrupt the homeostatic balance of the CNS (Maier and Watkins, 1998). However, under certain conditions, glial reactivity is not advantageous and can instead be detrimental to neuronal function, such as during the manifestation of persistent pain. In response to strong or persistent receptor stimulation, microglia switch from a surveillance

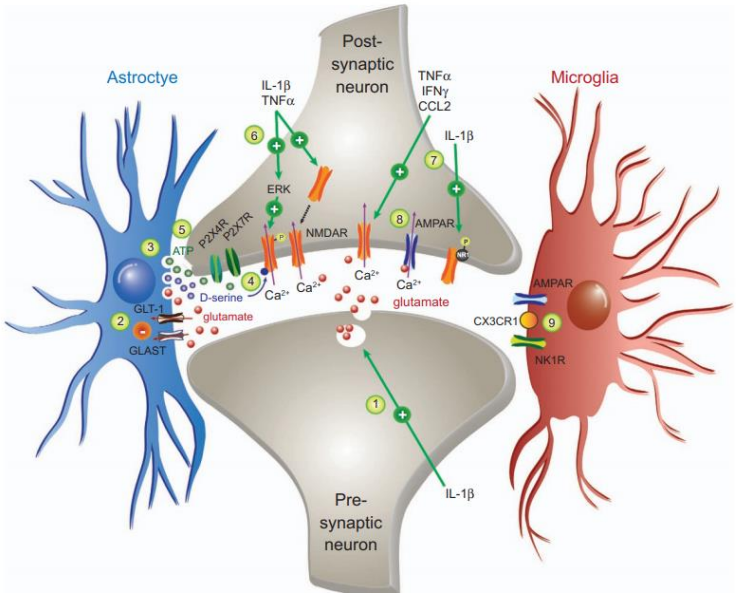
state to an active response state, and astrocytes transition from a regulatory to reactive state (Ji et al., 2013). Under these circumstances, the release of proinflammatory mediators by glia can contribute to ongoing nociception, by inducing long-lasting plastic changes of synaptic connectivity that enhances the transmission of ascending nociceptive information (Milligan and Watkins, 2009; Grace et al., 2014). Glial proliferation, morphological changes and increases in protein expression can persist for months after initial injury, even beyond tissue healing (Beggs et al., 2012; Schwaller et al., 2015). Moreover, proinflammatory mediators and glial-derived neurotransmitters can reciprocally stimulate glia in an autocrine and paracrine manner, thereby amplifying a positive feedback loop of unfavorable activity (Anderson et al., 2004; Shiga et al., 2011; Zhang et al., 2014).

Many studies are also investigating the impact of early-life stressors, such as maternal separation or injury, on long-lasting glial alterations in the adult. Such events can be the 'first hit' that primes glia to over-respond and be detrimental in restoring 'second hit' immune challenges later in life. The hypothesis is that adverse life events provoking long-term heightened glial reactivity may lead to greater sensitivity to future harmful stimuli. Priming of spinal glia may also provide an explanation for why some people are predisposed to developing IBS as result of gastrointestinal inflammatory or infections. In fact, the initial scenario of gastroenteritis preceding IBS could represent the 'first hit' of irritation that sensitizes the neuroimmune system, later contributing to disease progression. The relevance of inflammation in glia priming could also explain why IBS symptoms were reported in ~40% of patients with IBDs in apparent remission (Halpin and Ford, 2012).

The studies on glia and IBD employed rodent models of di- or trinitrobenzene sulfonic acid-induced colitis and chronic pancreatitis. In these preclinical models, marked increases in reactivity were described for microglia in the spinal cord and hippocampus, and activated satellite glia in the dorsal root ganglia. This is associated with an upregulation of TNFα levels, and closer apposition between satellite glial cells and primary afferent neurons in the dorsal root ganglia via enhanced neuron–glia gap junction coupling. Microglia activaction was associated with an increased visceromotor reflex activity and abdominal withdrawal reflex to graded colonic distension. Intracerebroventricular, intrathecal or systemic minocycline (a microglia inhibitor) or intrathecal administration of an anti-TNFα antibody attenuated pain behaviors in these animals (Riazi et al., 2008; Huang et al., 2010; Kannampalli et al., 2014; Song et al., 2014; Liu et al., 2012). In support of the direct involvement of microglia in visceral pain, the injection of fractalkine (a microglia activator) reproduced the visceral nociception in naïve rats (Milligan et al., 2005). Unlike microglia, the role of spinal astrocytes

in visceral pain has been poorly investigated, though both these cell types were found activated in chronic pain conditions (Li et al., 2019; Ji et al., 2019).

In summary, heightened spinal glial reactivity and proinflammatory signaling may contribute to ongoing peripheral inflammation, as well as enhancing pain by central sensitization. This raises another interesting question as to whether centrally derived neurogenic inflammation, generated in part by neuroimmune signaling, contributes to the perpetuation of other inflammatory diseases. Indeed, neurogenic inflammatory processes have been implicated in the exacerbation of IBD, cystitis and endometriosis. In this setting, enhanced afferent signalling in response to the tissue insult may facilitate lesion development by a positive feedback loop (Dodds et al., 2016). Although the link between glia activation and visceral pain persistence has to be further clarified, these preliminary results support the pro-nociceptive role of glia in mediating visceral hyperalgesia and make it a promising target for controlling chronic visceral pain.




***Glia and Visceral Pain.***

*Dodds et al., Translational Psychiatry, 2016.*

### *4.7. Stress*

While the previous description was presented as a “bottom-up” model of sensitization leading to chronic visceral pain, direct sensitization of the central pain matrix can drive a “top-down” mechanism wherein stress and negative emotions can promote enhanced perception of nociception in the absence of overt peripheral injury (Scarinci et al., 1994; Lampe et al., 2003; Maizels et al., 2012; Racine et al., 2012). The body's response to stress is composed of

two parallel systems: the quick “flight or fight” of the sympatho-medullary axis and the slower hypothalamic-pituitary-adrenal (HPA) axis. The sympathetic response to acute stress mobilizes epinephrine and norepinephrine to change blood-flow away from the skin and GI tract toward the muscles along with providing a burst of energy and a dampening of pain perception to allow the individual to run or fight for survival. The neuroendocrine response mediated by the HPA axis causes release of cortisol in humans or corticosterone in rodents (CORT) to mobilize glucose reserves to restore homeostasis after an acute stressor, or to cause long-term changes in metabolic function and neuronal sensitivity following chronic stressors. Typically, the sympathetic response will habituate to repeated stressors, whereas the HPA response may or may not habituate depending on the type, duration, and variability of the stressor (Greenwood-Van Meerveld and Johnson, 2017). As implied by the name, the HPA axis is initiated when paraventricular nucleus of the hypothalamus secretes corticotropin-releasing hormone (CRH) into the hypophyseal portal circulation in response to a stressor. After binding to corticotrophs in the anterior pituitary, CRH causes the release of adrenocorticotropic hormone into the systemic circulation after being cleaved from its acid precursor protein, proopiomelanocortin. After binding in the adrenal cortex, adrenocorticotropic hormone induces de novo synthesis of CORT from a cholesterol-derived steroid precursor, which then enters systemic circulation bound to a carrier protein (cortisol binding globulin). In addition to its metabolic functions, CORT binding to its high affinity mineralocorticoid receptor (MR) and low affinity glucocorticoid receptor (GR) within brain regions such as the hippocampus, the paraventricular nucleus of the hypothalamus, and some cortical regions induces negative feedback to terminate the response of the HPA axis, while binding at the amygdala opposes the feedback inhibition by increasing CRH expression and facilitation of the stress axis (Sapolsky et al., 1983; Reul and de Kloet, 1985; Herman and Cullinan, 1997; Schulkin et al., 1998; Shepard et al., 2000). In particular, the central nucleus of the amygdala (CeA) integrates viscerosensory signaling with neuroendocrine and autonomic responses to stressors and is primed to influence both stress and pain signaling. (Myers and Greenwood-Van Meerveld, 2010; Johnson and Greenwood-Van Meerveld, 2015; Johnson et al., 2015). Exposure to chronic stress causes neuronal remodelling in specific regions and the net effect of this neuronal remodelling is the exacerbation of pain perception and the promotion of chronic pain symptomatology due to the loss of anti-nociceptive and anti-stress signalling within the central pain matrix combined with facilitation of nociceptive and stress-responsive signalling. These remodelled pain circuits also impinge

on the function of key brainstem regions that modulate descending pain inhibition (Greenwood-Van Meerveld and Johnson, 2017).

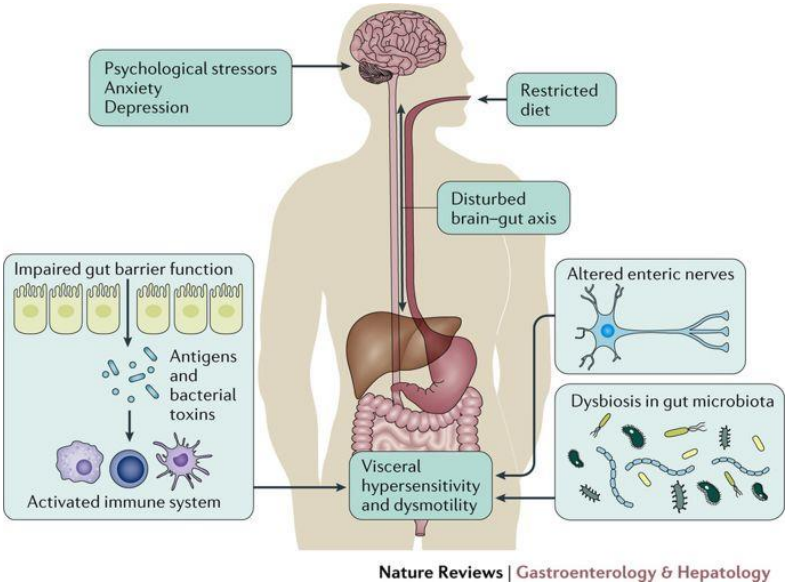
Clinically, the effect of chronic stress on visceral pain is best illustrated by the high co-morbidity of anxiety, depression, and other psychiatric disorders with functional pain disorders, such as IBS (Drossman et al., 2011; Hooten, 2016). Because there are multifactorial mechanisms that can induce chronic visceral pain, further research is necessary to identify specific mechanisms underlying the development of chronic stress-induced visceral pain. Neuroimaging studies in IBS patients have shown that there is altered brain activation in response to a nociceptive stimulus suggesting central sensitization (Weaver et al., 2016). In particular, the amygdala has been found to consistently demonstrate altered activation to visceral stimulation in IBS patients (Greenwood-Van Meerveld and Johnson, 2017).

To understand the central pathways and cellular mechanisms underlying changes observed in the human brain and to design novel therapeutics for stress-induced visceral pain, there are multiple rodent models of stress. The Wistar Kyoto rat is a spontaneous or genetically-induced model in which a high anxiety trait is associated with elevated colonic sensitivity as demonstrated by an increased visceromotor response (VMR) induced by low levels of CRD (Greenwood-Van Meerveld et al., 2005; Gibney et al., 2010; Bravo et al., 2011; Johnson et al., 2012). In other models, adult animals are exposed to stressors such as restraint stress, water avoidance stress, and a chronic variable stress (Gué et al., 1997; Bradesi et al., 2005; Winston et al., 2010) In these models, there is an increase in plasma CORT resembling that seen in IBS patients and an increase in visceral sensitivity (Leserman and Drossman, 2017). On the other hand, animal models of early-life stress were used for longitudinal studies aimed at understanding the life-long effects of early life adversity on visceral pain perception. Preclinical studies in these rodent models have provided important experimental evidence to suggest that brain circuits are primed by exposure to stress or pain during early life, predisposing individuals to chronic pain disorders as adults (Greenwood-Van Meerveld and Johnson, 2017).

### *4.8. Genetic predisposition*

The number of documented IBD susceptibility loci continues to increase, with more identified as regional datasets are combined enabling comparison of geographically distant populations (Liu et al., 2016). These loci include NOD2, IRGM, ATG16L1 and IL23R genes dealing with the detection and response to gut bacteria. By comparison, the understanding of IBS genetics lags far behind, although there are a number of initiatives such as the international network

GENIEUR (Genes in Irritable Bowel Syndrome Europe; www.GENIEUR.eu) aiming to improve the phenotyping and power of future studies. Most studies have been investigations of candidate genes and have been underpowered (Czogalla et al., 2015). However, this meta-analysis did support the link between IBS and polymorphisms in the TNFSF15 gene, a member of the TNF superfamily that influences interferon production (Zucchelli et al., 2011; Swan et al., 2013; Czogalla et al., 2015). This gene was found to be a risk factor for Crohn's disease in an analysis of 86,640 Europeans and 9,846 Asians with a relative risk of 1.14 (Liu et al., 2015). This data supports the idea that an excessive response to immune stimulation could predispose to both IBD and IBS. However, the same IBS study that showed the link with TNFSF15 found no association with 30 other IBD genes, reinforcing the argument that IBD requires other less common factors than are required for IBS (Zucchelli et al., 2011). This aspect might explain why IBS (an incidence of 1 in 10) is 100 times more prevalent than IBD (an incidence of 1–3 per 100,000) (Burisch et al., 2015).




***Potential shared mechanisms in IBS and IBD in remission.***

*Spiller and Major, Nature Reviews Gastroenterology & Hepatology, 2016.*

### *5. Visceral pain therapy*

Although some visceral pain disorders are not life-threatening, they still contribute significantly to a large segment of healthcare resource consumption and have a considerable negative impact on lives with psychological distress, disturbance of work and sleep and sexual dysfunction (Hungin et al., 2003). For this reason, the treatment of visceral as well as

somatic pain is progressively becoming independent of the accompanying disease and pain itself is regarded as a syndrome, rather than a symptom or by-product of illness (Cervero and Laird, 1999).

Patients with visceral pain present unique challenges because the pain is often poorly localized and is associated with strong autonomic reactions and changes in visceral function. Pain management, in turn, may further alter visceral function, with opioid effects on the gastrointestinal tract providing a good example. These unintended treatment effects on visceral function can exacerbate the pain or lead to additional discomfort, thus showing that rational and effective pain management needs to be based on an understanding of the anatomic and physiologic basis of visceral function and pain (Gebhart and Bielefeldt, 2016).

Since IBS symptoms do not have an apparent structural or biochemical explanation, the therapy included psychotherapy- behavioural therapy, nutrition and drug treatment of the predominant symptoms (bulking agents, antidiarrheals, antispasmodics and tricyclic antidepressants). Currently, the most efficacious therapies against visceral hypersensitivity are mainly directed to treat bowel dysfunction, while drugs able to directly target the related-pain are still unsatisfactory (Camilleri and Boeckxstaens, 2017).

As previously described, there is clinical overlap between IBD and IBS, with IBS-like symptoms frequently reported in patients before the diagnosis of IBD, and a higher than expected percentage reports of IBS symptoms in patients in remission from established IBD. So, the therapeutic approach for IBD and IBS pain relieving is often the same (Schirbel et al., 2010; Furness, 2012).

### *5.1. Dietary interventions*

Food is often a trigger of abdominal symptoms in patients with IBS, and the mechanisms evoked by food ingestion have been reviewed extensively elsewhere (Bohn et al., 2013; Farré et al., 2013). In relation to colonic function, these triggers include stimulation of colonic motility through a vagally mediated reflex, inhibition of colonic water absorption and stimulation of colonic transit and high amplitude propagated contractions by carbohydrates that reach the colon and their metabolic products. On the other hand, deficiency of fibre in the diet is often considered as a factor predisposing to constipation, which may then cause abdominal pain (Burkitt et al., 1976; Suares et al., 2011).

A number of different diets are now promoted to treat IBS symptoms, and these include regimens that exclude carbohydrates, fermentable foods, gluten, and substances that might create food-related antibodies (Camilleri et al., 2008). The more commonly employed by

patients are diets low in Fermentable Oligo-, Di-, Mono-saccharides And Polyols (FODMAPs) and excluding gluten (Werlang et al., 2019). There are numerous evidences that the intake of FODMAPs is associated with development of symptoms of IBS, including pain. The proposed mechanisms include increasing water retention in the small intestine through the osmotic effects of the molecular entities and rapid fermentation by intestinal bacteria, leading to production of gas and short chain fatty acids with luminal distension resulting in sensations of pain and bloating and stimulation of abnormal motility (Gibson et al., 2015).

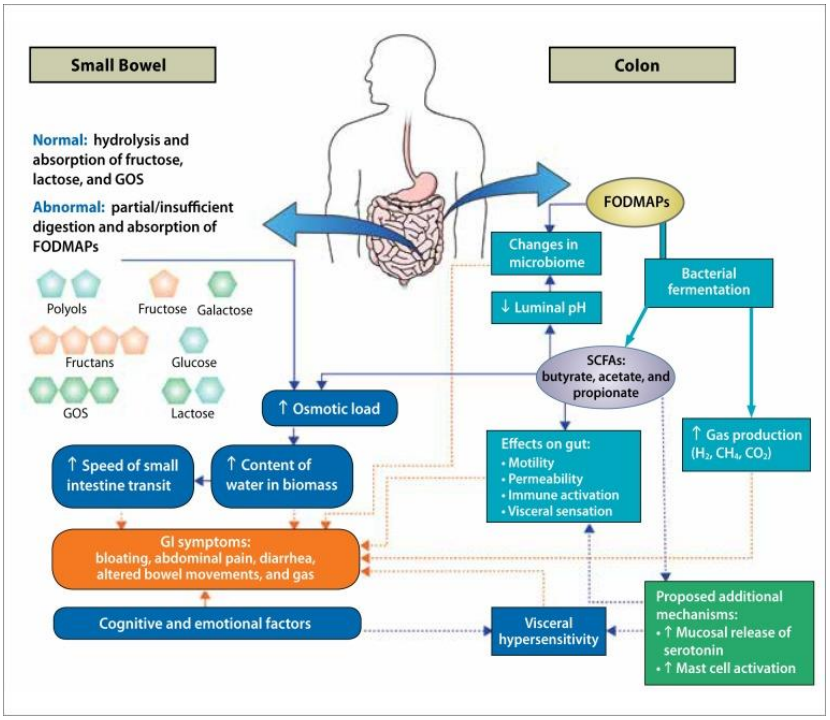
The low-FODMAP diet intervention for IBS patients consists of 3 distinct phases: the restriction or elimination phase, the re-introduction or re-challenge phase, and the maintenance or personalized phase (Staudacher and Whelan, 2016; McIntosh et al., 2016). During the initial phase, patients eliminate FODMAPs from their diets. Importantly, the low-FODMAP diet is meant to last only 4 to 6 weeks, and it is essentially a method to determine whether symptoms are related to specific foods. It is not designed for long-term use. During the second phase, after noting symptom improvement or resolution, foods containing FODMAPs are reintroduced gradually, with the goal of identifying tolerance to individual ingredients and specific symptom triggers among fermentable carbohydrates. This phase lasts several weeks, if not longer, as foods are slowly reintroduced. After reviewing and interpreting results from the food re-challenge phase, the goals of the third phase are to continue the intake of foods that were well-tolerated and to restrict foods that produced symptoms. As the tolerance to different FODMAPs can change over time, patients can attempt to reintroduce their trigger foods a few months after symptom control if they so desire (Böhn et al., 2015).

Systematic review and meta-analysis have documented the greater benefit of low FODMAPs diet over control treatment for overall symptom severity and severity of abdominal pain and bloating (Böhn et al., 2015; Marsh et al., 2016; Eswaran et al., 2016). The mechanism of benefit is assumed to relate to reduced colonic fermentation or greater microbial diversity and reduced total bacterial abundance (Halmos et al., 2015; Valeur et al., 2016). More specifically, a recent study of 20 patients with diarrhoea-predominant IBS (IBS-D) or IBS-M reported that low FODMAP diet decreased serum levels of proinflammatory interleukin (IL)-6 and IL-8, as well as levels of faecal bacteria, like Actinobacteria, *Bifidobacterium* and *Faecalibacterium prausnitzii*, as well as faecal total SCFAs and n-butyric acid compared with baseline (Hustoft et al., 2016). Lower levels of Bifidobacteria were confirmed with low FODMAP diet in other study (Staudacher and Whelan, 2016). Conversely, increased

Actinobacteria richness and diversity were demonstrated in another study on IBS patients (McIntosh et al., 2015).

In general, the low FODMAP diet remains controversial, and it still presents short-term and long-term limitations, including a high level of restriction that may be required in individual patients, the need for monitoring by an expert dietician, potential for developing nutritional deficiencies, potential for changes in gut microbiota, lack of predictors of response as well as relative efficacy compared with other dietary, psychological or pharmacological interventions for IBS (Molina-Infante et al., 2016).

Even the elimination of gluten from the diet of IBS patients has demonstrated efficacy beyond patients with celiac disease (Biesiekierski et al., 2011; Biesiekierski et al., 2013; Vazquez-Roque et al., 2013). The main limitations of the current literature on gluten-free diets for IBS lie in small study sample sizes and concern for contamination of the vehicle of gluten exposure. To this point, a large meta-analysis reviewing 1726 studies evaluating the efficacy of a gluten-free diet on the management of IBS recently found insufficient evidence to recommend this diet for IBS symptoms, as findings were not statistically significant (Dionne et al., 2018). Isolation of gluten from the diet without also removing other potential symptom-driving substances is both difficult to study and nebulous for IBS patients. It is possible that many IBS patients improve on a gluten-free diet, as it also reduces fructan intake, a significant component of modern wheat products (Skodje et al., 2018).




***Proposed mechanisms of FODMAP ingestion and symptoms in IBS***

*Werlang et al., Gastroenterol Hepatol (N Y), 2019*

### *5.2. Probiotics supplementation*

The World Health Organisation define probiotics as “live microorganisms, which when taken in adequate amounts, confer a health benefit on the host”. Probiotics, derived from the Latin “for life”, have been used in fermented foods for millennia. The ancient Egyptians are known to have consumed fermented milk products, Laban Rayeb and Laban Khad, as early as 9000 years ago, with fermentation growing in popularity due to its ability to preserve food and also possibly as a digestion aid (Gogineni et al., 2013).

Probiotics may exert their beneficial effects on the host through various mechanisms: (1) Pathogen suppression—competition for nutrients and mucosal space, and the production of bacteriocins (anti-bacterial proteinaceous toxins); (2) Improvement of barrier function—tight junction (spaces between adjacent epithelial cells) homeostasis; (3) Immunomodulation—pathogen-associated molecular patterns (PAMPs) are sensed by dendritic cells which influence B and T cell regulation; and (4) Neurotransmitter production—a number of lactic acid bacteria are capable of producing serotonin and gamma-aminobutyric acid (GABA) which may influence the communication between the gut and brain (gut–brain axis) (Sánchez et al., 2017).

The latest consensus definition of prebiotics by the International Scientific Association for Probiotics and Prebiotics defines them as “*a substrate that is selectively utilised by host microorganisms conferring a health benefit*” (Gibson et al., 2017). Prebiotics such as inulin and inositol are found in foods including leeks, asparagus, Jerusalem artichokes, garlic, and onions (Slavin, 2013). Some of the most common prebiotics are described below.

- Inulin: Belonging to the fructan family of dietary fibres, inulin is non-digestible. It is therefore able to pass through the small intestine intact and reach the colon. Here, inulin is fermented—particularly by *Bifidobacterium* species and other lactic acid-producing bacteria—boosting the numbers of these health beneficial bacteria (Slavin, 2013). Fermentation products of inulin offer colon cancer-preventing properties, and short chain fatty acids (SCFAs) are also produced during fermentation.
- β-glucan: This exopolysaccharide is found naturally in cereal grains, bacteria, and fungi (Wang et al., 2017). It has been shown to have prebiotic properties on the growth of *Bifidobacterium* and *Lactobacillus* species (Russo et al., 2012). Fermentation of β-glucan by *Bifidobacterium infantis*, in particular, was shown to increase the production of SCFAs (Jaskari et al., 1998).
- Fructooligosaccharides (FOSs): Present in wheat, honey, onion, garlic, and banana, FOSs are short-chain carbohydrates which resist digestion in the small intestine. In the

colon, they promote *Bifidobacterium* and are converted to SCFAs, and also contribute to faecal matter, improving bowel movement. Conversely, they inhibit the pathogen *Clostridium perfringens* in the colon (Belorkar et al., 2016).

Synbiotics are products containing both prebiotics and probiotics, whereby they act synergistically. In a synbiotic, the prebiotic component is designed to selectively stimulate either growth or metabolism by the probiotic bacteria and may therefore be considered for use where there may be survival challenges for the probiotic alone (Pandey et al., 2015). In addition, they may also stimulate certain commensal bacteria in the GI tract (Markowiak et al., 2017).

Scholarly reviews have appraised the potential effects of the intestinal microbiota on intestinal motility and sensation, autonomic nervous system, hypothalamic-pituitary-adrenal axis, enteric nervous system, mucosal barrier and neuroimmune signalling (Distrutti et al., 2016). The interactions between the gut microbiota, stress and the central nervous system have emerged suggesting that visceral pain-related disorders may be candidates for symptom relief through therapeutic alterations of the microbiome (Moloney et al., 2016). These observations suggest that therapeutic interventions that alter the microbiome may have beneficial effects in patients with IBS.

IBS patients were found to have an abnormal pro-inflammatory IL-10/IL-12 ratio, which was normalised—in conjunction with symptom alleviation—by the consumption of the probiotic *Bifidobacterium infantis* (O'Mahony et al., 2005). Neural mechanisms involved in visceral pain perception also seem to be influenced by certain bacterial species, such as the induction of μ-opioid and cannabinoid receptors by *Lactobacillus acidophilus* (Quigley, 2013). Earlier studies had shown that probiotics affect bowel function and bloating in patients with IBS-D treated with individual probiotics such as *Bifidobacterium infantis* or combination probiotics (Kim et al., 2003; Kim et al., 2005; Whorwell et al., 2006). Ford *et al*. reported benefit of probiotics over placebo for global symptoms in IBS (Ford et al., 2014a). However, the data do not provide sufficient information to specifically assess effect on abdominal pain. A 2015 systematic review and meta-analysis of probiotics in IBS included 15 trials, of which only 2 had sufficient data to assess effects on abdominal pain. These studies used combination *Escherichia coli* (DSM 17252) and *Enterococcus faecalis* (DSM 16440) or *E. coli* (DSM 17252) alone as the probiotics compared with placebo (Enck et al., 2008; Enck et al., 2009; Didari et al., 2015). However, recent randomised controlled trials demonstrated no significant benefit of probiotics preparations over placebo in the treatment of pain in adults with IBS in contrast to the benefit observed in some (but not all) trials in children with regard

to frequency and intensity of abdominal pain, for example, with a combination of three Bifidobacterial species or a single bacterial species (Weizman et al., 2016; Spiller et al., 2016; Giannetti et al., 2017). Treatment with *Lactobacillus paracasei* in mice (Eutamene et al. 2007) and the probiotic mix VSL#3 in rats (Distrutti et al. 2013) were both shown to be effective in ameliorating the maternal separation stress-induced hypersensitivity during colorectal distension. In a restraint stress-induced increase in visceral hypersensitivity to colorectal distension, *Bifidobacterium lactis* significantly attenuated the nociceptive response (Agostini et al. 2012), while *Bifidobacterium infantis* (McKernan et al. 2010), Lactobacillus paracasei NCC2461 (Verdu et al. 2006) and *Lactobacillus reuteri* (Kamiya et al. 2006) resulted effective in blunting nociceptive response during colorectal distension in naïve animals. VSL#3 was also shown to prevent visceral hypersensitivity induced by inflammation via intracolonic instillation of 4% acetic acid when given prophylactically (Dai et al., 2012), while *Bifidobacterium infantis* ameliorated visceral hypersensitivity to colorectal distension in the trinitrobenzenesulphonic acid-induced (TNBS) model of colitis in rats (Johnson et al. 2011). Moreover, *Lactobacillus rhamnosus* and *Bifidobacterium animalis ssp. lactis* were shown to exhibit protective effects on intestinal barrier function in the TNBS model of colitis in mice by restoring barrier integrity, reducing inflammatory cytokines and increasing the levels of intestinal tight junction proteins (Laval et al. 2015; Martin et al. 2016). In summary, effects of probiotics in the treatment of abdominal pain appear to be strain dependent and may be more significant in children. Further studies are required to address which bacterial strains and which patients are most likely to respond.

Besides, increasing research is revealing the interaction between psychiatric disorders including generalised anxiety disorder, major depressive disorder, and schizophrenia and IBS (Fadgyas-Stanculete et al., 2014). Both animal and human studies have demonstrated a link between altered gut microbiota and depression (Zhou and Foster, 2015). Worryingly, a UK study identified that IBS has the potential to cause fatal outcomes from suicide, with depression not accounting for all the variance in suicidal ideation (Miller et al., 2004). Probiotics capable of conferring mental health benefits through interactions with commensal gut bacteria have been coined “psychobiotics”. The psychophysiological effects of psychobiotics include psychological effects on emotional and cognitive processes, systemic effects on the hypothalamic–pituitary–adrenal axis, and neural effects via neurotransmitters and neurotrophic proteins (Sarkar et al., 2016). In a human study using functional magnetic resonance imaging (fMRI), 4 weeks of a multi-strain probiotic altered emotional processing compared to placebo (Tillisch et al., 2014). On the other hand, germ-free (GF) mice have an

exaggerated stress response with heightened levels of stress hormones—a finding that was reversed by treatment with *Bifidobacterium infantis* (Sudo et al., 2004).

### *5.3. Non-absorbed antibiotic, rifaximin*

Rifaximin produced significant improvements in core symptoms of IBS-D in patients treated with up to three 2-week courses of therapy. With second repeat treatment, the most significant benefit was the relief of urgency and bloating, with borderline benefit on abdominal pain and stool consistency (Chey et al., 2015; Chang et al., 2015). The mechanism of benefit of rifaximin is still unclear. In the past, it was assumed that the benefit reflected beneficial effects on small bowel bacterial overgrowth or direct anti-inflammatory actions that countered effects of bacterial products (Pimentel et al., 2016). However, there is evidence that breath hydrogen measurements after lactulose or glucose load, a no-invasive method to diagnose SIBO, may reflect oro-caecal transit rather than bacterial overgrowth (Simrén and Stotzer, 2006; Yu et al., 2011). A recent study appraised several potential mechanisms of action of rifaximin in the treatment of non-constipated patients with IBS, including permeability, expression of barrier proteins and faecal microbiome, but there were no significant effects relative to placebo (Acosta et al., 2016). In summary, the efficacy and the mechanism leading to relief with this non-absorbable antibiotic are unclear.

### *5.4. Antispasmodics and prokinetics*

In addition to visceral hypersensitivity, abnormal GI motility is recognised as an important pathophysiological mechanism. Increased or decreased GI transit is indeed reported in IBS-D and IBS-C, respectively (Manabe et al., 2010). Moreover, patients with IBS develop increased small bowel motility in response to meal ingestion and the stress hormone, corticotropin-releasing factor (CRF), associated with episodes of abdominal pain (Fukudo et al., 1998). As antispasmodics reduce GI contractility, these compounds may be beneficial in IBS and are indeed widely used in Europe. A European systematic review identified nine placebo-controlled studies of antispasmodics in IBS, but many did not use standardised diagnostic criteria, and all were of low to intermediate quality since they were performed before the development of the Rome criteria for study design (Narducci et al., 1986; Tack et al., 2006). Abdominal pain was improved in seven of the studies, bowel symptoms improved significantly versus placebo in two studies and four of the studies reported global symptom severity improvement. Reviewers concluded that there is level II evidence suggesting that antispasmodics may improve abdominal pain, but that there is lack of evidence to support

global symptom improvement. Afterwards, a Cochrane review concluded that there was weak evidence for the benefit of some antispasmodics for abdominal pain and global symptom relief, although it was unclear which individual classes were effective (Quartero et al., 2005). Among the antispasmodics, the strongest data were for otilonium bromide. This compound targets L-type and T-type calcium channels, and muscarinic type 2 and tachykinin neurokinin (NK)-2 receptors, possibly contributing to its increased efficacy. The efficacy of otilonium bromide in IBS has been confirmed in four studies, including significant improvement of abdominal pain and bloating severity with otilonium bromide versus placebo, or reduction in the number of pain episodes and severity of abdominal distension, improved well-being and global assessment, but not in bowel symptoms (Baldi et al., 1991; Battaglia et al., 1998; Glende et al., 2002; Clavé et al., 2011).

Antispasmodics are generally well tolerated, apart from anticholinergics which can cause atropine-like side effects, including constipation (Ford et al., 2008).

Peppermint oil and its active ingredient, l-menthol, are smooth muscle calcium channel antagonists that may cause muscle relaxation and, therefore, serve as an antispasmodic. Peppermint oil and menthol also have κ-opioid agonistic properties that may alter gut sensitivity, have been reported to possess anti-inflammatory effects and have serotonergic (5-$HT_3$) antagonistic properties (Hawthorn et al., 1988; Juergens et al., 1998; Galeotti et al., 2002; Walstab et al., 2014). Menthol, the active component in peppermint oil, is also widely used in medicinal preparations for the relief of acute and inflammatory somatic painful conditions. Recent evidence has indicated that this menthol-induced analgesia is mediated by activation of the temperature sensing ion channel, transient receptor potential ion channel melastatin subtype 8 (TRPM8) (Liu et al., 2013). TRPM8 has been identified on colonic primary afferent neurons, TRPM8 mRNA in colonic DRG neurons and TRPM8 protein on nerve fibres throughout the wall of the colon. It is expressed by nociceptive visceral afferents, where TRPM8 has antinociceptive properties (Harrington et al., 2011). Visceral hypersensitivity has been relieved in rat by a combination of essential oils from peppermint and caraway. In this effect seems involved mycobiome modulation (Botschuijver et al., 2018). In a systematic review and meta-analysis of five randomised controlled trials of an older formulation of peppermint oil that included 197 patients on the active treatment arm and 195 on placebo, the analysis favoured peppermint oil (RR 2.23 (95% CI 1.78 to 2.81)) over placebo. Peppermint oil was significantly superior to placebo for global improvement of IBS symptoms and improvement in abdominal pain (Khanna et al., 2014). However, multicentre trial for testing a new formulation of peppermint oil with sustained release into the small

bowel showed no superiority over placebo, although pain, bloating and urgency were significantly reduced (Cash et al., 2016).

### *5.5. Serotonin receptors agonist/antagonist*

Another neurotransmitter which seems to active visceral afferent fibers is serotonin (5-HT). 5-HT released from EC cells may affect several subtypes of enteric neurones (intrinsic and extrinsic sensory neurones as well as motor and secretomotor neurones) and final effector cells (smooth muscle cells and enterocytes). The role of 5-HT in GI motility and pain perception is rather complex (Gershon and Tack, 2007; Kendig and Grider, 2015), and intervention in the 5-HT signalling pathway may, therefore, impact several mechanisms involved in IBS symptoms, including pain. Several serotonin receptor subtypes have been characterized, of which 5HT3, 5HT4, and 5HT1b are the most important for GI function (De Ponti, 2004). 5-HT4 receptors are another key target for pharmacological intervention. Their stimulation with agonists potentiates peristalsis initiated by 5HT1 receptor stimulation, they are therefore useful in constipation predominant form of IBS and in chronic constipation. The partial 5-HT4 agonist, tegaserod, has been proposed to reduce visceral sensitivity by inhibiting substance P expression in the dorsal horn of spinal cord (Bradesi and Mayer, 2007). Tegaserod has been demonstrated in several randomized, placebo-controlled trials to relieve global IBS symptoms as well as individual symptoms of abdominal discomfort, number of bowel movements and stool consistency (Ford et al., 2009a). One important aspect of partial agonists is that they may surrogate the functions of 5-HT when its release is impaired but may work as antagonists by opposing the effect of endogenous 5-HT (which is a full agonist) in case of 5-HT overload. In addition, partial agonists may help to overcome, at least in part, the problem of 5-HT4 receptor desensitisation. Noteworthy, 5-HT4 receptors are present in human atrial cells and when stimulated may cause atrial arrhythmias (Fayyaz and Lackner, 2008). Indeed, tegaserod was pulled out of the market by because of the risk of heart attacks and strokes. Recently, FDA approved the reintroduction of Zelnorm (tegaserod) for the treatment of IBS-C, but with some restrictions (FDA, 2018).

On the other hand, 5HT3 antagonists (alosetron and cilansetron) prevent the activation of 5HT3 receptors on extrinsic afferent neurons and can decrease the visceral pain associated with IBS (Andresen et al., 2008). These agents also retard small intestinal and colonic transit and are therefore useful in diarrhea-predominant IBS. Several randomized, controlled trials have shown that alosetron relieves pain, improves bowel function, and provides global symptom improvement in women with diarrhea-predominant irritable bowel syndrome.

However, ischemic colitis and severe complications of constipation have been major concerns leading to voluntary withdrawal of alosetron from the market followed by remarketing with a comprehensive risk management program (Bielefeldt, 2016).

Considering the limited availability of the serotonin 5-HT3 antagonists, the global market of drugs for diarrhoeal disorders is still led by agents developed 40–60 years ago, including opioid receptor agonists (eg, loperamide), bile acid binders (eg, cholestyramine) and tricyclic antidepressants (eg, amitriptyline) (Barbara et al., 2014).

### *5.6. Antidepressants*

Visceral pain syndromes, including IBS, may be effectively treated by a variety of therapies that modulate the interactions between the central and enteric nervous systems. Clinical observations and preliminary data suggest that antidepressants may be efficacious for the treatment of these syndromes (Crowell et al., 2004). Tricyclic antidepressants (TCA) are the first-line pharmacologic treatment for symptom improvement in gut-brain axis disorders where pain is a prominent feature. Their mode of action is by 5-HT and NA reuptake inhibition in combination with additional receptor antagonistic properties (5-$HT_{2A}$ and $_{2C}$, muscarinic$_1$, histamine$_1$). There are slight variations comparing different TCAs from these aspects where the tertiary amines (amitriptyline, imipramine) are more prone to produce side effects from their greater antimuscarinic and antihistaminic actions compared to the secondary amines (desipramine, nortriptyline). These side effects, particularly sedation and constipation, can be used also to treat some aspects of IBS such as sleep disturbance and diarrhoea, if present. it was also shown that escitalopram, an SSRI, did not have the same positive effect on abdominal pain as TCAs, thus supporting the more limited use of SSRIs for treating pain due to their lack of NA effects. Anyway, it is important to consider that potential benefit with higher dosages of TCAs (particularly the tertiary amine agents) is compromised by their greater potential for side effects (Törnblom and Drossman, 2018).

A recent review indicates that antidepressants may have even a beneficial effect on IBD course, given that psychological factors play an important role in IBD activity and antidepressants have been reported to have anti-inflammatory properties (Macer et al., 2017). A systematic review of animal models of colitis has found that desipramine and fluoxetine reduce the risk of colitis and improve inflammatory markers, with little evidence of adverse effects.4 (Mikocka-Walus et al., 2009)

The efficacy of antidepressants on pain syndromes is best supported by reviewing meta-analyses (Ford et al., 2009b). Nevertheless, these analyses have a number of flaws or

inconsistencies in the design, analyses of efficacy, safety, as documented elsewhere, leading to significant questions regarding the generalizability for this class of pharmacological agents (Ford et al., 2014b; Camilleri, 2015). The recent literature is questioning whether antidepressants are safe drugs when used over the long term for non-psychiatric indications, but several confounding factors may influence this link (eg, severity of depression and other psychiatric comorbidities) and causality has not been proven. For example, selective serotonin reuptake inhibitors (SSRIs) affect bone health, and there is epidemiological evidence of dementia with long-term antidepressant treatment, based on a population-based, retrospective, case-control analysis using the Taiwan National Health Insurance Research Database of patients enrolled from 2005 to 2011 (Yadav et al., 2008; Lee et al.,2016).

### *5.7. Secretagogues*

Effects of the chloride channel activator, lubiprostone, on bowel function and pain scores in IBS-C have generally shown consistent efficacy for spontaneous bowel movement rate over 3 months and somewhat reduced efficacy in relief of reduction in IBS pain and discomfort in the third month of a 3-month clinical trial (Johanson et al., 2008).

One recent type of visceral analgesic relates to the functional effects of guanylate cyclase-C (GC-C) receptor stimulation in the gut (Waldman and Camilleri, 2018). The analgesic properties are mediated by a cascade of intracellular events starting with GC-C stimulation that gives rise to intracellular cyclic guanosine monophosphate (cGMP) production. cGMP transported to the extracellular space modulates the conduction properties of nociceptive neurons located in the submucosa. This concept is based on mechanistic studies in animal models and has been shown to be relevant in the treatment of the pain component of IBS (Johnston et al., 2010). The effect of GC-C stimulation on fluid excretion results in an accelerated gut transit that restricts its use to those patients with a bowel habit dominated by constipation. Linaclotide was the first substance available for clinical use after proving to be effective in IBS-C, followed by plecanatide that is available at some markets with the same indication (Chey et al., 2012; Rao et al., 2012; Miner at al., 2017). Linaclotide, appears to increase the proportion of adequate relief and global relief responders, as well as improving weekly frequency of bowel movements and reducing pain. It also relieved pain in patients with severe symptoms, as well as discomfort and bloating ratings during 12 weeks of treatment (Johnston et al., 2010; Rao et al., 2014; Castro et al., 2013). On the base of these evidence, US Food and Drug Administration's (FDA) recommend linaclotide for IBS-C (Videlock et al., 2013).

### *5.8. Peripheral Opioid Receptor Agonists/Antagonists*

Stimulation of the visceral μ-receptor has for long been the first-line treatment option in conditions involving chronic diarrhea, including IBS-D. From the historical use of opioids with both peripheral and central effects, the advent of loperamide, a μ-receptor agonist that does not penetrate the blood-brain barrier was a major break-through. One limiting factor has been that a substantial proportion of patients do not tolerate this treatment due to the development or aggravation of abdominal pain or constipation. Therefore, μ-opioid agonists are used during acute exacerbations of pain in patients with IBS (Hellström et al., 2011).

A new therapeutic option, eluxadoline, has agonistic properties on μ- and κ-receptors, and antagonistic properties on δ-receptors. Animal studies showed promise that eluxadoline could reduce visceral hypersensitivity, one of the key pathophysiologic mechanisms in IBS, and normalize transit in diarrhea models where the combined μ- and δ- receptor effects normalized transit over a wider dose range compared with loperamide (Wade et al., 2012; Lacy, 2016). Due to a limited bioavailability after oral administration, the central effects of opioid receptor stimulation are avoided and clinical trials with treatment given for up to 52 weeks have not shown signs of abuse potential or opioid withdrawal effects after treatment termination (Fujita et al., 2014; Fant et al., 2017). Eluxadoline, at 100 and 200 mg, resulted in greater improvements in bowel movement frequency and urgency, global symptoms, IBS symptom severity score, IBS quality of life and adequate relief (Dove et al., 2013). The adverse events of pancreatitis and sphincter of Oddi spasm led to specific exclusions of patients with a history of bile duct obstruction, pancreatitis, severe liver impairment or severe constipation, and in patients who drink more than three alcoholic beverages per day. Nevertheless, the FDA Adverse Event Reporting System received information on 99 cases of pancreatitis, 39 cases of sphincter of Oddi spasm and 220 cases of abdominal pain within 10 months of the availability of the medication to patients with IBS-D (FDA report, 2016).

### *5.9. Histamine receptor antagonist*

Mast cells and their mediators, in particular histamine, serotonin and proteases, are increasingly recognised as contributing to the pathogenesis of IBS (Camilleri and Boeckxstaens, 2016). Most insight in the role of inflammation in chronic abdominal pain comes from studies evaluating patients with postinfectious (PI)-IBS. Colonic biopsies of patients with PI-IBS reveal no signs of overt inflammation but show persistent minor

increases in epithelial T lymphocytes and mast cells, suggesting that long-term inflammatory changes may be responsible for colonic hypersensitivity. Yet, it has been found a significant correlation between mast cell density and pain perception in patients with IBD who were in remission (Wouters, 2016a).

Of interest, histamine is released by IBS colonic biopsies and induces visceral hypersensitivity to colorectal distension in murine models. Recently, evidence was reported that histamine sensitises TRPV1 on neurons from murine DRG and on human submucosal neurons in rectal biopsies via activation of H1 receptors (HRH1) (Wouters et al., 2016b). Moreover, supernatant of IBS biopsies sensitised murine DRG neurons, an effect also mediated via HRH1. H4 and H1 receptor antagonists dose-dependently reduce and even normalise post-inflammatory visceral hypersensitivity via different underlying mechanisms but with a synergistic effect (Deiteren et al., 2014).

The mast cell stabiliser, ketotifen, was tested in a prior single-centre trial, and it increased the threshold for discomfort on rectal balloon distension in patients with IBS with visceral hypersensitivity, reduced global IBS symptoms and severe abdominal pain, and improved health-related quality of life (Klooker et al., 2010). The potential unblinding resulting from sedating effects of ketotifen raised questions about its therapeutic potential. As ketotifen also possesses HRH1 antagonistic properties and HRH1 has been implicated in visceral hypersensitivity, a follow-up study evaluating the HRH1 antagonist, ebastine, indeed showed a significant improvement in global relief and abdominal pain (Wouters et al., 2016b). Ebastine is a second generation H1R antagonist free of any significant influence on the central nervous system as it does not penetrate the blood-brain barrier. Of interest, a recent study detected histamine levels in urine samples of patients with IBS that were modulated by a low FODMAP diet. Detection of histamine in urine or other samples may represent an interesting approach to select patients responding to HRH1 antagonists (McIntosh et al., 2015).

### *5.10. NK receptor antagonists*

Recently developed models have focused considerable attention on two sites of neural processing: primary afferents and the spinal dorsal horn (Wesselmann et al., 2009). Sensitization of the primary afferents has been shown to occur via changes in the intrinsic properties of sensory neurons that regulate excitability and in gene expression for nociceptive-specific membrane proteins (Latremoliere and Woolf, 2009). Peripheral sensitization resulting from prolonged periods of stimulation also releases at least two central neural mediators,

glutamate and substance P (SP) that increase the efficacy of synaptic transmission between primary afferents and spinal neurons (central sensitization) (Wesselmann et al., 2009). NK1 and NK2 receptors are abundantly expressed in the GI tract and mediate robust and long-lasting contractions of smooth muscle in the gut. Inflammation of viscera increases central and peripheral NK1 receptor expression, and visceral hyperalgesia is attenuated in NK1 receptor knockout mice. Receptor antagonists act peripherally and centrally to attenuate viscera motor responses induced by colorectal distention. Also, NK2 receptor activation is involved in stimulation of sensory nerves and activation of visceral reflexes. Based on these characteristics, NK2 receptor antagonists have been evaluated as treatments for IBS. In a phase II, dose-finding study, ibodutant, a highly selective NK2 antagonist with high oral bioavailability, revealed improvement in patients with IBS-D (Corsetti et al., 2015). A more recent multinational, double-blind, placebo-controlled study showed dose-dependent improvement of overall symptoms, abdominal pain and stool pattern in IBS-D in females, but not in males. The tolerability of the compound was reported to be excellent (Tack et al., 2016). Recently, also the role of NK3 receptors in inflammation-induced gut hyperalgesia was recognized.

Like SP, also glutammate has a clear role in nociceptive systems, though specific relevance of this neurotrasmitter in visceral pain systems are yet to be precisely defined, (Wesselmann et al., 2009).

### *5.11. Gabapentenoids*

Gabapentenoids, pregabalin and gabapentin, agents are α2δ ligands that generally bind potently to an auxiliary protein associated with voltage-gated calcium channels, reducing depolarisation-induced calcium influx at nerve terminals which reduces the release of several excitatory neurotransmitters including glutamate, noradrenaline, substance P and calcitonin gene-related peptide, which are involved in pain mechanisms (Camilleri et al., 2007). Prevention of neurotransmitter release by pregabalin and gabapentin occurs only in pathologic states when calcium channels are being up-regulated and activated. Pregabalin and gabapentin are predominantly central acting analgesic (Dooley et al., 2000; Patel et al., 2000; Field et al., 2006). Gabapentinoids have been shown to reduce visceral hypersensitivity in experimental animals as well as symptoms of irritable bowel syndrome in humans (Diop et al., 2002; Lee et al., 2005; Million et al., 2007; Houghton et al., 2007). Not only do gabapentinoids reduce nociceptive neurotransmission centrally but also improve bowel compliance to distention perhaps through blocking alpha-2 delta subunits in smooth muscle (Davis et al., 2012).

Pregabalin has been tested in pharmacodynamics studies in healthy controls and in patients with IBS, with inconsistent results on effects on colonic compliance and sensation thresholds (Houghton et al., 2007; Iturrino et al., 2014).

## *6. New therapeutic opportunities*

### *6.1. Emerging pharmacological targets in visceral pain management*

As argued in the previous paragraphs, the mechanisms underlying pathophysiological events that produce chronic visceral pain are still poorly understood, so therapeutic targets for treating visceral pain provide a very active and rapidly changing field of research. Several chemical and molecular factors in the intestine have a potentially significant role in IBS, particularly in IBS-D, including mast cell products such as histamine, proteases and tryptase, and mucosal messenger RNAs (mRNA), proteins and microRNAs (miRNA) (Camilleri et al., 2016b).

Between the channels in the enteric nervous system, ASICs and KCQN result particularly important. The activation of acid sensing ion channels (ASICs) which respond to decreases in pH can also contribute to mechanic-sensation from viscera. In the GI tract, ASIC1a has an inhibitory effect, ASIC2 has mixed effects and ASIC3 appears excitatory (Page et al., 2005).

Voltage-gated potassium channels are required for action potential firing and are also involved in spontaneous trains of action potentials after nervous system injury. Particularly KCNQ (Kv7) channels came into focus because of the anti-nociceptive effect of the specific KCNQ opener retigabine in animal models of neuropathic and visceral pain (Blackburn-Munro et al., 2005; Hirano et al., 2007).

The over-expressed colonic brain-derived neurotrophic factor (BDNF) has been reported to be associated with abdominal pain in patients with IBS. Functionally, TrkB (BDNF receptor), or BDNF knockdown significantly suppressed visceral hypersensitivity in mice (Wang et al., 2016). On the contrary the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) resulted able to support survival, regeneration and functioning of several neuronal populations: dopaminergic, enteric, sympathetic, parasympathetic, motor, cholinergic neurons and of sensory neurons (Boucher et al., 2000). So, modulation of these peptides could be study in order to restore a normal visceral sensitivity.

The role of Vascular Endothelial Growth Factor (VEGF) in pain is rapidly increasing. Its instillation into the mouse bladder promotes a significant increase in peripheral nerve density together with alterations in bladder function and visceral sensitivity. The VEGF pathway is

being proposed as a key modulator of neural plasticity in the pelvis and enhanced VEGF content may be associated with visceral hyperalgesia, abdominal discomfort, and/or pelvic pain (Malykhina et al., 2012).

In the GI tract, another class of receptors highly studied belong to the family of protease-activated receptors (PARs). PARs are expressed both on the apical and basolateral sides of intestinal epithelial cells, these receptors might be activated both by luminal proteases (including microbial proteases) and by tissue proteases. PAR activation causes visceral hypersensitivity, modifies intestinal motility and intestinal epithelial permeability. All these functions take an important part in IBS symptoms generation (Cenac et al., 2007). Trypsin and tryptase expression and release were increased in colonic biopsies from IBS patients compared with control subjects. Biopsies from IBS patients (but not controls) released mediators that sensitized murine sensory neurons in culture. Sensitization was prevented by a serine protease inhibitor and was absent in neurons lacking functional protease-activated receptor–2 (PAR2). Supernatants from colonic biopsies of IBS patients, but not controls, also caused somatic and visceral hyperalgesia and allodynia in mice, when administered into the colon (Cenac et al., 2007). According to this serpin superfamily, endogenous serine protease inhibitor, and leukocyte elastase turn out seems to have a novel and clinically relevant role in the crosstalk between neurons and T cells in the modulation of neuropathic pain (Vicuña et al., 2015). Recently, a study demonstrated that proteases, through the activation of PAR2, were able to induce the release of TRPV4 endogenous agonists, which were found up-regulated in tissues from patients with IBS (Vergnolle et al., 2016). According to these evidence thermosensive ion channels, TRPV1 and TRPV4, resulted implicated previously in visceral mechanosensation. In fact, deletion of TRPV1 or TRPV4 impairs afferent fibre transmission of mechanical stimuli in the colon (Jones et al., 2005). TRPV1 and TRPA1 showed a pivotal role in visceral hypersensitivity at the peripheral and spinal cord level during acute colitis and gastric disease. Further, histamine- or serotonin mediated visceral hypersensitivity depend on TRPV4 expression in sensory neurons (Cenac et al., 2010). TRPV4 appears as a common mechanism to several known mediators of visceral hypersensitivity (Kondo et al., 2009). Histamine can also sensitize the nociceptor transient reporter potential channel V1 (TRPV1) contributing to visceral hypersensitivity in animals (Cenac et al., 2010).

In this contest, it is interesting to note that, unlike the other protease, elastase effects seem instead to involve another type of receptor, the toll-like receptor-4, but the exact mechanism is still unknown (Vergnolle et al., 2016). Toll-like receptors (TLRs) are critical pattern

recognition molecules of the innate immune system. Interestingly, innate immune receptor expression resulted changed in the gastrointestinal tract of animals with stress induced IBS-like symptoms (McKernan et al., 2009).

Growing evidence suggests that, in IBS, the epithelial barrier, gut microbiota, food antigens and bile acids elicit abnormal responses in the key regulators of sensorimotor functions, involving the hypothalamus–pituitary–adrenal (HPA) axis. Corticotropin releasing factor (CRF) receptor mediate the stress response, activating the hypothalamic-pituitary-adrenal and sympathic-adrenal axes. In animals both acute stress, colitis or central CRF administration increases colorectal distension-evoked nociceptive responses and in all the cases visceral hypersensitivity can be reversed by CRF1 antagonists. Yet, in human administration of CRF increases colonic motility and hypersensitivity to distension, effects that are exacerbated in patients with IBS (Taché et al., 2009). Other neuropeptides that have similar potential for a role in visceral pain sensation include CGRP, somatostatin and cholecystokinin (Wesselmann et al., 2009).

Over recent years, increasing attention has been placed also toward the adenosine system, which represents a crucial link between the enteric neuromuscular layer and the immune components of the gut. Studies in rodents have shown a significant involvement of adenosine in the control of intestinal secretion, motility and sensation, via activation of A1, A2A, A2B or A3 purinergic receptors, as well as the participation of ATP in the regulation of enteric functions, through the recruitment of P2X and P2Y receptors (Antonioli et al., 2008). Great interest is focusing on the involvement of ATP and adenosine in the pathophysiology of intestinal disorders, with particular regard for inflammatory bowel diseases (IBDs), intestinal ischemia, post-operative ileus and related dysfunctions, such as gut dysmotility, diarrhoea and abdominal discomfort/pain. Current knowledge suggests that adenosine contributes to the modulation of enteric immune and inflammatory responses, leading to anti-inflammatory actions (Antonioli et al., 2008). Expression of nociceptive-specific P2X3 ATP receptors is up-regulated in colonic nerve fibres of individuals with IBS (Burnstock et al., 2009). It is interesting to observe that P2X3 receptors seems not involved in sensory signalling under physiological conditions whereas they modulate visceral hypersensitivity during acute and in the post-inflammatory phase, although via different mechanisms of sensitization (Deiteren et al., 2015). Spinal microglial P2X4 receptors are also activated after visceral injury. The consequent microglia activation evokes inflammatory cytokines release that contribute to central sensitization (Burnstock et al., 2009). Furthermore, the activation of neuronal P2X7 receptor-Pannexin-1 complex resulted as mediators of death of enteric neurons during colitis

(Gulbransen et al., 2012). Recent evidences suggest a P2X7-dependent-glutamate release in cerebro-cortical nerve terminals of neuropathic animals (Silverman et al., 2009; Mannelli et al., 2015). Opposite of ATP, the neurotransmitter adenosine seems mediate pain suppression. In fact, the spinally injection of two ectonucleotidases, able to generate adenosine in nociceptive neurons, showed long-lasting adenosine $A_1$ receptor-dependent antinociceptive effects in inflammatory and neuropathic pain models (Zylka, 2011).

### *6.2. Adenosine receptors*

The main focus for the development of adenosine targets as analgesics to date has been $A_1$Rs due to its antinociceptive profile in various preclinical pain models. The usefulness of systemic $A_1$R agonists may be limited by other effects (cardiovascular, motor), but enhanced selectivity for pain might occur with partial agonists, potent and highly selective agonists, or allosteric modulators. $A_{2A}$R agonists exhibit some peripheral pronociceptive effects, but also act on immune cells to suppress inflammation and on spinal glia to suppress pain signalling and, therefore may be useful for inflammatory and neuropathic pain. $A_{2B}$R agonists exhibit peripheral proinflammatory effects on immune cells, but also spinal antinociceptive effects similar to $A_{2A}$R agonists (Sawynok, 2016). $A_3$Rs are now demonstrated to produce antinociception in several preclinical neuropathic pain models, with mechanistic actions on glial cells, and may be useful for neuropathic pain. Endogenous adenosine levels can be augmented by inhibition of metabolism (via adenosine kinase) or increased generation (via nucleotidases), and these approaches have implications for pain. Endogenous adenosine contributes to antinociception by several pharmacological agents, herbal remedies, acupuncture, transcutaneous electrical nerve stimulation, exercise, joint mobilization, and water immersion via spinal and/or peripheral effects, such that this system appears to constitute a major pain regulatory system (Sawynok, 2016).

It has been demonstrated that increasing endogenous adenosine levels through selective adenosine kinase inhibition produces powerful analgesic effects in rodent models of experimental neuropathic pain through the $A_3$ adenosine receptor ($A_3$AR, now known as ADORA3) signalling pathway (Little et al., 2015). These effects were prevented by blockade of spinal and supraspinal $A_3$AR, lost in $A_3$AR knock-out mice, and independent of opioid and endocannabinoid mechanisms. $A_3$AR activation also relieved non-evoked spontaneous pain behaviours without promoting analgesic tolerance or inherent reward (Little et al., 2015). Also, $A_3$AR agonists resulted effective in several models of chronic neuropathic pain, including that caused by chemotherapeutic agents, was also proved (Bennett et al., 1988;

Chen et al., 2012). Of particular interest are the highly selective $A_3$AR agonists developed by Tosh et al. (2015) which lack the cardiovascular side effects known for other AR agonists, such as $A_1$AR agonists which also provide pain relief (Luongo et al., 2012). Critically, engaging the $A_3$AR mechanism did not alter nociceptive thresholds in non-neuropathy animals and therefore produced selective alleviation of persistent neuropathic pain states (Little et al., 2015).

Exploring the mechanistic basis for the protective effect of $A_3$AR agonists in chronic neuropathic pain, it has been found that pain relief is dependent on GABAergic transmission, but not cannabinoid or opioid receptors, in the spinal cord, and that both peripheral sensory neurons and brain are involved. Moreover, when peripherally administered $A_3$AR agonists can correct the imbalance in dorsal horn cytokines, oxidative pathways, and glutamatergic transmission associated with the pain state. $A_3$AR agonists are demonstrated to be also protective in other pain models, such bone cancer pain (Little et al., 2015; Varani et al., 2013; Yan et al., 2016). Thus, $A_3$AR agonists promise to be safe and effective in the treatment of chronic neuropathic pain of various etiologies.

Moreover, recently, we found that the selective stimulation of $A_3$AR inhibits N-type voltage-gated $Ca^{2+}$ channels ($Ca_v2.2$) opening and decreases the electrically-evoked excitation of isolated rat dorsal root ganglia (DRG) neurons. This mechanism could explain the anti-hyperalgesic effect of $A_3$AR agonists across different models (Coppi et al., 2019).

The possibility of targeting adenosinergic system also in visceral pain management is supported by preclinical evidence attesting the involvement of adenosine receptors in the analgesic effect of paeoniflorin in rats with neonatal maternal separation-induced visceral hyperalgesia (Zhang et al., 2009). Asano and Takenaga (2017) reported that the adenosine $A_{2B}$ receptor is involved in visceral hypersensitivity in animal models of IBS and blocking the $A_{2B}$ receptor suppresses both somatic and visceral pain. Additionally, the same research group reported that a specific inhibitor for each subtype of adenosine $A_{2B}$ receptor and phosphodiesterases mediated the inhibitory effect of aminophylline on maternal separation- and acetic acid administration-induced visceral hypersensitivity to colorectal distension (CRD) (Asano et al., 2017). Recently, Hou et al. (2019) demonstrated that the inhibitory effect of electroacupuncture on visceral pain is mediated by adenosine receptors activation in mice with inflammatory bowel disease. In this context, electroacupuncture also determine an increase in the expression of $A_1$R, $A_{2A}$R, and $A_3$R and a decrease in the expression of $A_{2B}$R in the colon tissue. $A_3$R selective activation has an anti-inflammatory activity through the

inhibition of pro-inflammatory cytokine expression associated with the inhibition of NF-κB signaling pathways in murine DSS colitis in vivo (Ren et al., 2015).

The efficacy showed by adenosine receptors modulation in visceral pain, joined with the recent evidence identifying adenosine $A_3$ receptor as an effective and safe target for achieving pain relief in different models of persistent pain, put these receptors first in the list of possible new treatments for chronic visceral pain.

On the other hand, contrasting data were reported about the involvement of $A_3R$ in the regulation of intestinal motility. Ren et al. (2015) reported that $A_3$ receptors expression localized on epithelium, excluding a direct action on enteric nerves functionality (Ren et al., 2015). Nevertheless, immunofluorescence showed a predominant distribution of $A_3$ receptors in normal myenteric ganglia and an increased density during colitis (Antonioli et al., 2010). Yet, functional disruption of $A_3R$ in $A_3^{-/-}R$ mice alters intestinal motility (Ren et al., 2011). The effect of $A_3R$ regulation on intestinal functionality is an important point to know in the development of therapies for visceral pain.

## *7. Faecal Microbiota Transplantation (FMT)*

### *7.1. Faecal Microbiota Transplantation in IBS and IBD*

The complex interplay between genetic, microbial, and environmental factors is likely responsible for the phenotypic heterogeneity in IBS, and the successful treatment of different subsets may therefore require specific microbial manipulations (as introduced in the previous paragraphs). Host specificity has been suggested to be a desirable property for probiotic bacteria and is therefore recommended as one of the selection criteria (Rinkinen et al., 2003). Of the studies that found a statistically significant improvement in “global symptoms” (14 out of 29 studies) and a clinically meaningful improvement in “abdominal pain” (only 8 of all 35 trials), the majority were multi-strain products (~2:1). Furthermore, only multi-strain trials found a clinically meaningful improvement in quality of life in IBS patients (Williams, 2008; Lorenzo-Zúñiga et al., 2014). The mechanisms underlying this difference in efficacy remain to be defined and are likely complex.

However, in an in vitro intestinal epithelial cell inflammation model, MacPherson et al. demonstrated that, in comparison to single-strains, a multi-strain challenge resulted in a greater reduction of inflammation-modulated genes—findings that indicated a synergistic effect of bacterial combinations in resolving inflammation and maintaining cellular

homeostasis, which the authors concluded reinforces the rationale for using multi-strain probiotic formulations (Macpherson et al., 2017).

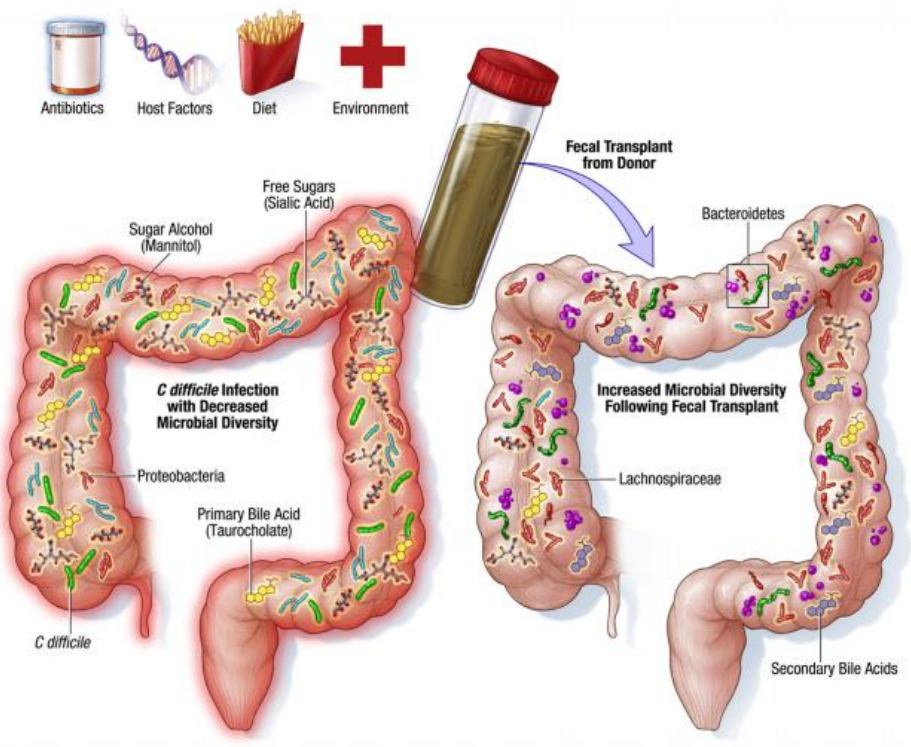
The more frequent success of multi-strain probiotics in IBS may be owed to a synergistic effect on disease-modulated genes. Considering the advantages of combining multiple strains in respect to the use of a single bacterial strain, it is natural to question whether the transfer of a whole healthy microbiota could bring even more benefits.

Faecal Microbiota Transplantation (FMT), also called faecal bacteriotherapy or stool/faecal transplantation, is the infusion or engraftment of liquid filtrate faeces from a healthy donor into the gut of a recipient to treat many diseases, including Clostridium difficile infection, IBD, obesity, and insulin resistance. Evidence for this range in therapeutic response is provided by studies using the somewhat controversial technique of transplantation of faecal matter from healthy donors to sufferers of functional gastrointestinal disorders (Choi et al. 2016). In one study involving IBS and IBD sufferers (Borody et al. 1989), FMT was effective in 36% of the patients, mildly improved discomfort in 16% and non-effective in the rest 47%. Other clinical studies and case studies have utilised FMT for alleviation of chronic constipation (Andrews and Borody 1993), refractory IBS (Pinn et al. 2015) and IBD (Angelberger et al. 2013; Bennet and Brinkman 1989) with varied success. Another recent Open-Labeled Study on Fecal Microbiota Transfer in IBS patients confirmed improvement in abdominal pain which resulted associated with the relative abundance of *Akkermansia muciniphila* (Cruz-Aguliar et al., 2019). While such studies provide evidence for a role of microbiota in alleviating certain symptoms associated with gastrointestinal discomfort or visceral sensitivity, the studies provide little empirical data relating to long-lasting changes in gastrointestinal microbiota or alterations to immune, endocrine, inflammatory or neurotransmitter systems. Moreover, given the heterogeneity of chronic pain and the complex and diverse nature of the microbiome, individual interventions may be developed.

Nevertheless, the principle of the gut microbiota as a therapeutic target has generated much enthusiasm. Prebiotic, Probiotic and FMT approaches have been tried in both IBD and IBS but understanding of cause–effect relationships continues to evolve. Several mechanisms underlying the therapeutic effects of FMT on chronic pain were proposed, including direct competition of pathogenic bacteria with commensal microbiota, protection of the intestinal barrier, restoration of secondary metabolism, and stimulation of the intestinal immune system (Guo et al., 2019). To date FMT is approved only for the therapy of Clostridium difficile infection, where the microbiota transplant is highly efficacious with the ability to restore healthy microbial ecology (Cammarota et al., 2016). The same efficacy has not been achieved

when FMT is employed for the treatment of other diseases. However, enteric microbiota alterations in other type of gastrointestinal disease, as colitis, might be more resistant to restoration than those in C difficile infection, possibly requiring more intense and prolonged therapy. Clostridium difficile infection is a pure ecological problem characterized by a defect in gut microbiota barrier properties (Leffler and Lamont, 2015; Gupta et sl., 2016). Besides, IBD is far more complex and involves a deregulation of the host-microbes cross talk. The goal of FMT in IBD is thus not only to correct the dysbiosis, but also to restore a normal dialog between the host immune system and the microbiota. Data are still scarce, but the results of the first studies suggest a complex effect of FMT in IBD (Pigneur and Sokol, 2016). A recent study investigating the Fecal Microbiota Transplantation for Chronic Active Ulcerative Colitis (FOCUS) showed the efficacy and safety of multidonor, intensive-dosing, faecal microbiota transplantation in patients (Paramsothy et al., 2017). The efficacy of serial FMT has been demonstrated also in patients affected by chronic intestinal pseudo-obstruction in which bloating, and pain resulted effectively alleviated 2 weeks after FMT (Gu et al., 2017). Increasing the dose and/or repeating FMT ameliorates the prognosis also in patients with IBS (El-Salhy et al., 2019).

Although serious adverse events including bacteraemia and perforations have been reported, the majority of adverse events of FMT appear to be mild, self-limiting and gastrointestinal in nature. In some cases, a credible association was not established due to the lack of controlled data. Plainly, there is a need for standardized, randomized controlled trials to qualify and quantify the risks associated with FMT (Baxter and Colville, 2016). However, even if FMT for IBS has still a long way to go, the preliminary evidences are promising. Ongoing and future research should be oriented in understanding the best FMT protocol of treatment to employ for chronic visceral pain relief, since the literature is heterogeneous and not clear on this point.




***Mechanisms underlying successful treatment of recurrent CDI with FMT***

*Kelly et al., Gastroenterology, 2015a*

### *7.2. Faecal Microbiota Transplantation procedure*

The FMT procedure involves identification of a donor, screening of donor stool for pathogens, stool preparation (including cryopreservation if necessary), preparation of the recipient (antibiotic treatment and enteric cleansing), infusion of the donor stool and post-procedural management of the patient, including the monitoring of adverse events. Hence, the treatment is somewhat unwieldy compared with antibiotic treatment alone.

To reduce and prevent the occurrence of adverse events, strict donor screening tests of FMT are recommended (Choi et al., 2016; Cammarota et al., 2017; Wang et al., 2019). The guidelines in the United States and European consensus conference both suggest to use a donor questionnaire to meet the exclusion and inclusion criteria (Bakken et al., 2011; Smits et al., 2013; Cammarota et al., 2017; Vindigni and Surawicz, 2017). To evaluate any recent potentially harmful behaviors, screened donors should undergo an additional interview on the same day of the donation (Routy et al., 2018). Furthermore, standard donor screening protocols should be set up to lower the risks of infection transmission from the donor to recipient, and a suitable donor ought to receive both blood and stool examinations within 4 weeks before donation (Cammarota et al., 2017).

The similarity of microbial species is expected between the recipient and his/her close relative; therefore, adaptive immunity in the mucosal immune system might present more tolerance towards the microbiota from the donor (Bakken et al., 2011). Nevertheless, additional clinical evidence has proven no association between donor and FMT outcomes (Weingarden et al., 2014; Shankar et al., 2014). Unrelated FMT volunteer donors may be more beneficial in cases where genetics play a contributing factor in the disease, like IBD (Kelly et al., 2015a).

The optimal preparation of fecal material to manage remains to be determined. It had been debated whether fresh or frozen fecal material was the better choice for FMT at the beginning of the FMT practice. Currently, several randomized clinical trials and meta-analysis demonstrated that frozen FMT has the same efficacy as fresh FMT in clinical improvement of recurrent or refractory CDI (Lee et al., 2016; Wise, 2016; Tang et al., 2017). Fresh fecal material should be processed within 6 h of donor production, and it can be stored at room temperature until further processing. Approximately 50 g (minimum amount of 30 g) of fecal material is mixed with approximately 150 mL of sterile normal sodium chloride by blender. The mixture is filtered with a filter or gauze to clear away large particulate matter, which may obstruct the endoscope channel. Finally, the filtrate is infused into 60-mL syringes (generally 4–5 tubes) and infused to the recipient's GI tract (Sokol et al., 2016; Cammarota et al., 2017; Vindigni and Surawicz, 2017).

In recent years, several stool banks have been established worldwide in succession. Collecting stool from a set of prescreened donors, preparing and dividing the donated fecal material, and freezing aliquots of screened fecal material are processed in a stool bank. Additionally, the final fecal material must be strictly managed by clearly labeling, tracking, and storing at −80 °C. On the day of FMT, the fecal suspension is thawed in a warm (37 °C) water bath, and the normal saline solution is mixed to obtain an expected suspension volume. It is worth noting that repetitive thawing and freezing ought to be avoided, and infusion should be performed within 6 h of thawing (Cammarota et al., 2017; Vindigni and Surawicz, 2017). Nevertheless, the appropriate volume of fecal infusion is difficult to define. Larger volumes of fecal material have shown better FMT outcomes in CDI patients, as demonstrated by a systemic review article, and up to a four-fold higher risk for failure was noted for infusion volumes less than 50 g compared to the larger volume (Gough et al., 2011).

Regardless of the source of fecal material or chosen route of administration, patients undergoing FMT need support and education prior to treatment (Blackburn et al., 2015). Recipient should not be administered with any form of antibiotics 12–48 h before

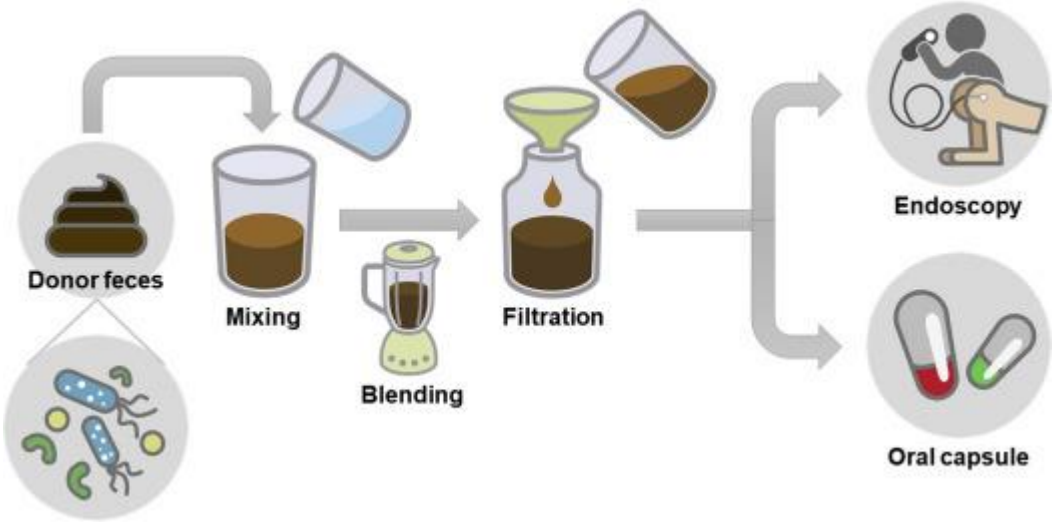
fecal infusion (Cammarota et al., 2017). Practical preparation for the FMT procedure is similar to that of any other endoscopic procedure, including a standard bowel preparation. The bowel should be virtually free of contaminated fecal material prior to the donor feces infusion to ensure a healthy graft (Blackburn et al., 2015). Some studies suggested loperamide use one hour before FMT in order to ensure that the transplanted feces stay at least 4 h long in the intestines (Brandt and Aroniadis, 2013; Evrensel and Ceylan, 2016; Goldenberg et al., 2018).

The current administration of fecal material by means include upper GI route (via esophagogastroduodenoscopy, nasogastric, nasojejunal, or nasoduodenal tube), lower GI route (via colonoscopy or retention enema), and oral capsule. In general, FMT via the upper GI route can be administered in patients with an inflamed colon; however, the discomfort sensation during tube placement, risks of aspiration, and inability to evaluate the colon mucosa or to collect mucosa tissue samples are weak points. FMT via colonoscopy has superiority in recolonizing the entire colon with favorable bacteria, and bowel cleaning can probably reduce the number of residual organisms and spores to visualize the entire colon, but it is a relatively risky, expensive, and invasive procedure. FMT via retention enema is more affordable and less invasive than colonoscopy, but the donor fecal material cannot be delivered to the entire colon and is limited to the distal colon. Oral capsule for FMT administration has the advantages of less invasion and high patient acceptability, but the expense and large capsule burden are its disadvantages (Cammarota et al., 2014; Vindigni and Surawicz, 2017).

If more was known about the elements in stool that make FMT work, it might be possible to develop a simpler FMT procedure. For instance, if only certain faecal bacteria are needed for successful FMT, the production of synthetic bacterial suspensions could be standardized, scaled up and potentially commercialized. Such a system would also reduce the risk of transferring potential pathogens such as enteric viruses and parasites that might be difficult to detect during routine microbiological screening of donor stool (Wang et al., 2019).

Most clinical trials and systemic reviews presented that some minor adverse events, such as abdominal discomfort, diarrhea, constipation, and low-grade fever, were transiently noted after FMT, and uncommon severe side effects were often associated with the possible complications of endoscopy and sedation (Wang et al., 2019). Moreover, some case reports and cohort studies showed that small populations of patients experienced IBD flares after FMT (De Leon et al., 2013; Quera et al., 2014; Fischer et al., 2016). The definite mechanism of IBD flare after FMT is still unclear, although Quera et al. suggested that

transient bacteremia may result in altered intestinal permeability, resulting in a flare (Quera et al., 2014).




***The schematic diagram of fecal microbiota transplantation process***

*Wang et al., Journal of the Formosan Medical Association, 2019*

### *8. Animal models of visceral pain*

Rats and mice are the most commonly used animal models used to assess colonic physiology, pathophysiology, and new treatment approaches for visceral pain. In general, there are multiple, well-established methodologies to universally quantify visceral nociception in rats and mice. The most commonly used technique involves recording devices such as electromyographic (EMG) electrodes or strain gauges implanted on the external oblique abdominal musculature, to quantify the number of reflex abdominal muscle contractions in response to graded colonic balloon distension. Measurement of isobaric distension pressures with a barostat offers numerous strengths, such as an objective assessment of pseudoaffective nociceptive reflexes, allowing for assessment of colonic compliance, as well as mimicking the approach used in clinical studies in which sensitivity is assessed in response to rectosigmoid distension. However, there are also weaknesses that must be considered in the data interpretation. For example, the animals may be exposed to stress during the procedure as a result of the novel laboratory environment or may be restrained to reduce movement artifact during EMG recording. An acclimatization period to the experimental environment has been shown to significantly reduce animal stress and allows for the assessment of visceral sensitivity in a freely moving animal. However, anesthesia will always be necessary to insert the colorectal balloon; it should be kept as brief as possible to avoid interference with the

nociceptive reflex. An additional approach to assess colonic sensitivity is by the visual assessment of the abdominal withdrawal reflex (AWR) in response to colorectal distension (CRD). A benefit of this technique is that the stereotyped nociceptive behavior is induced by a brief distension; however, the AWR is a subjective behavioral measure, requiring larger samples sizes to demonstrate significant differences between treatment groups. An alternative approach to the use of a colonic balloon distension paradigm is to directly stimulate visceral nociceptors through colonic infusion of algesic chemicals, such as capsaicin or mustard oil. Such substances produce spontaneous nocifensive behaviors (perianal licking, abdominal retraction or compression, and hindlimb stretching) and take advantage of spontaneous evoked nocifensive behaviors. A note of caution in the data interpretation is that the inflammatory component of the stimulus suggests that the model may more relevant to inflammatory pain rather than functional visceral pain (Greenwood-Van Meerveld et al., 2015).

### *8.1. Genetic/Spontaneous models of visceral hypersensitivity*

Knockout models provide the opportunity to investigate the role of a specific gene in the regulation of colonic sensitivity. For example, there is a significant decrease in colonic sensitivity to CRD in corticotropin-releasing factor (CRF)-1 receptor knockout mice, suggesting the importance of CRF in colonic sensitivity (Trimble et al., 2007). Further studies in knockout mice have demonstrated the importance of other signalling molecules and transporters in the regulation of colonic sensitivity, including brain-derived neurotrophic factor (BDNF), guanylate cyclase C (GC-C), serotonin (5HT), and interleukin 10 (IL-10), human excitatory amino acid transporter 2 (EAAT2), and the serotonin reuptake transporter (SERT) (Greenwood-Van Meerveld et al., 2015).

Multiple studies have confirmed that the Wistar-Kyoto (WKY) rat has a hypersensitive response to colonic distension (Gunter et al., 2000). No single mechanism has been demonstrated to be responsible for the hypersensitive response in WKY rats. Recent studies have shown that both central and peripheral CRF receptors are differentially expressed and that selective CRF antagonists can inhibit colonic hypersensitivity (O'Malley et al., 2010; Bravo et al., 2011; Johnson et al., 2012; Buckley et al., 2014). Interpretation of data in genetic models is complicated and represents a significant challenge. For example, gene deletion may affect the overall health of the animal along with endogenous compensatory and/or redundant mechanisms that mask the true effect of the loss of the gene. An issue with the WKY rats is that, the inbred nature of this strain may limit the translational relevance because they may

only model the pain experienced by a subgroup of patients with functional visceral pain (Greenwood-Van Meerveld et al., 2015).

### *8.2. Early Life Stress models of visceral pain*

Increasing evidence from clinical studies suggests that a history of Early Life Stress (ELS) serves as a risk factor for the development of adult pathologies including but not limited to GI disorders such as IBS, with affected patients being two to four times more likely to report an adverse experience during childhood (Brierley et al., 2009). Despite the strong correlation between ELS and decreased health-related quality of life in adults due to GI-related abnormalities, the mechanism by which ELS underlies these changes is still unknown. Although the complex nature of the human ELS experience cannot be completely simulated in animal models, animal models of ELS are important tools to develop our understanding of how adverse neonatal experiences alter brain-gut communication that may lead to the development of abnormal visceral perception. The most well studied model of ELS is maternal separation (MS), which involves removal of pups from mother and nest, most commonly for 3 h/day on postnatal (PN) day 2–14 (Plotsky and Meaney, 1993). The purpose of this paradigm is to mimic childhood neglect and abuse through separation and subsequent alterations in maternal care, including altered licking and grooming behaviors and arched-back nursing (Plotsky and Meaney, 1993). MS pups exhibit decreased weaning body weight at PND22 compared with controls that are left undisturbed in their home cage on PND2, potentially introducing the effects of malnutrition as a side effect of neglect in this ELS model. In adulthood, there are contradictory results on visceral sensitivity depending on the duration of separation. However, a 3 h/day separation has been shown to result in visceral hypersensitivity, as evidenced by an increased viscero-motor response (VMR) to CRD in male Long-Evans rats (Coutinho et al., 2002). This model is useful to investigate the relationship between ELS and subsequent development of visceral hypersensitivity, in conjunction with hyper-reactivity of the HPA axis, two commonly comorbid symptoms in disorders such as IBS (Dinan et al., 2006; Whitehead et al., 2010). The odor-attachment learning (OAL) model of ELS is a classical conditioning model that utilizes predictable or unpredictable odor-shock pairings to mirror an attachment to an abusive caregiver (Sullivan et al., 2000). Conditioning occurs from PND8-12 wherein rat pups are experiencing both a sensitive and a hyporesponsive period. These are evolutionary advantages that allow the pups to be more sensitive to maternal odors to find the dam in the cage for care and nursing (Sullivan and Wilson, 1994). Therefore, by utilizing an odor and modest shock, this model is

able to mimic patterned interactions between pup and dam. In adulthood, this is the first model that induces female-specific visceral hypersensitivity in adult Long-Evans rats. Following OAL, female rats with a history of unpredictable ELS exhibit an increased VMR to CRD, an effect that has been shown to be estrogen dependent (Chaloner et al., 2013). This model of ELS has far reaching relevance to translational research as it parallels the female predominance found in patients who experience visceral pain (Bradford et al., 2012). A third model of ELS, limited nesting, aims to mirror the neglect and abuse found in areas of poverty or lower socioeconomic standing (Gilles et al., 1996). From PND2 to PND9, all bedding material is removed, and the dam and pups are placed on a wire cage bottom with only a single paper towel for nesting material. This limitation of bedding material causes disruptions in normal maternal care similar to those exhibited by dams during the MS protocol, however, the limited nesting model does not require removal of pups from the dam at any time during the experimental paradigm. This advantage also eliminates differences in weaning weights seen in the MS model (Prusator et al., 2015). Another model of ELS utilizes neonatal colonic irritation using colonic infusion of mustard oil or repeated CRD in neonates (Al-Chaer et al., 2000). Colonic irritation results in altered neuronal excitability and permeability within the colon, as well as visceral hypersensitivity of adult animals (Al-Chaer et al., 2000; Lin et al., 2003; Chaloner et al., 2010). This model may be relevant in instances of repeated physical or sexual abuse, and potentially in patients who experience some type of colonic inflammation during childhood. Rodent models of ELS are pivotal tools in the investigation of the mechanisms that underlie visceral pain following early life trauma. However, ELS studies as a whole, as well as each paradigm, are not without challenges. Overall, the initial consideration of protocol, strain, and sex differences present major considerations (Greenwood-Van Meerveld et al., 2015).

### *8.3. Stress-Induced models of colonic hypersensitivity in adulthood*

As a model of stress targeting only the amygdala, implantation of corticosterone (CORT) micro-pellets on the dorsal surface of the central nucleus of the amygdala (CeA) were initially shown to increase colonic sensitivity to innocuous distension (Greenwood-Van Meerveld et al., 2001). An interesting feature of this model is its relevance to patients with IBS based on imaging studies showing heightened activation of the amygdala in IBS patients in response to colonic distension (Naliboff et al., 2003; Wilder-Smith et al., 2004). Careful stereotaxic targeting of the CeA is necessary, since placement of the CORT micro-pellet in adjacent nuclei does not reproduce the colonic hypersensitivity (Myers et al., 2007).

Another model of stress is that induced by animal restraints. The most typical protocol is a 1- or 2-h acute stress protocol in which the animal either has its forelimbs wrapped with tape to restrict movement and grooming behaviour or is placed in a restraint apparatus (cage or tube) that prevents turning and grooming. A strength of the model is the robust and reproducible nature of the colonic hypersensitivity induced by restraint stress. This acute restraint stress induces an increase in colonic sensitivity to distension as measured by EMG or AWR quantification. A stronger model of stress-induced colonic hypersensitivity is that represented by the Water Avoidance paradigm. In this experimental model of stress, the rat is placed on a dry platform surrounded by water in attempt to mimic a psychological stressor. However, this model may also engage the fear neurocircuitry, which could evoke freezing behaviors in the colonic distension paradigm and affect interpretation of data using this model. With the exception of two studies (Larauche et al. 2012a, 2012b), water avoidance stress (WAS) protocols in rats induce colonic hypersensitivity to distension. A single exposure to WAS has been demonstrated to induce colonic hypersensitivity either immediately, within 60-min of the acute stressor, or after recovery from the stressor, 24-h poststress (Myers et al., 2012).

To eliminate the possibility of rats habituating to a single repetitive stressor, several variable stress protocols have been developed that expose animals to a set of randomly presented stressors. The unpredictable nature of the stressors prevents acclimatization to the procedures. The duration of colonic hypersensitivity to distension, measured through EMG or AWR, was strain dependent with a duration of action of up to 48 h poststress. A common issue is that in rat models of stress-related visceral pain the nature of the stressors, including whether they are physical vs. psychological, acute vs. chronic, or predictable vs. unpredictable, can have a profound influence not only on the outcome of the investigation but also on the biological processes involved (Greenwood-Van Meerveld et al., 2015).

### *8.4. Colonic irritation models of hypersensitivity*

Acetic acid has been used to induce colonic hypersensitivity to distension through two different mechanisms. Low-concentration (~1.0%) acetic acid produces a transient sensitization of colonic afferents (Langlois et al., 1994; Plourde et al., 1997). Higher concentrations of acetic acid produce a mild damage to the colon, producing inflammation-associated hypersensitivity. In an attempt to produce a noninflammatory model of colonic hypersensitivity with potential translational relevance to IBS, Bourdu et al. (2005) characterized the effect of repeated butyrate enemas on both colonic hypersensitivity to distension and colonic histology. Six enemas of butyrate induced colonic hypersensitivity,

reversible with morphine and requiring C-fibers, without evidence of inflammation or histological damage (Bourdu et al., 2005). On the other hand, the colonic installation of a low volume of capsaicin or mustard oil induces pain through direct activation of receptors on afferents as well as establishing an acute inflammation through tissue damage and is typically used to evaluate the analgesic properties of novel therapeutics. Stereotypic pain behaviors, such as abdominal licking, stretching, retraction, or compressing on the cage floor, are counted for 20 –30 min after irritant administration (Greenwood-Van Meerveld et al., 2015).

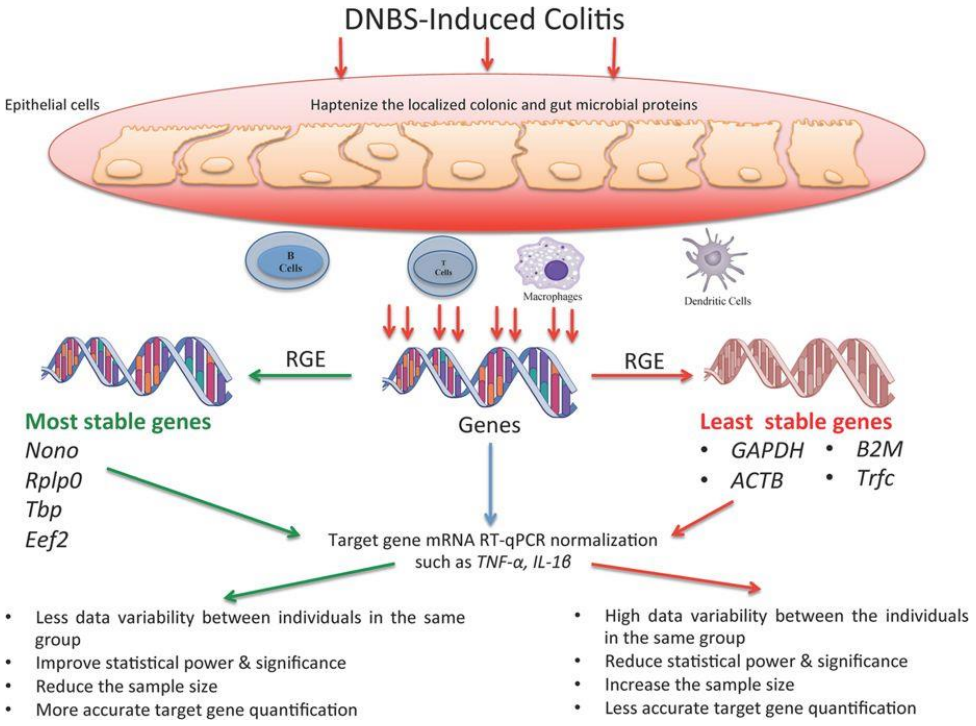
### *8.5.Models of post-infection/inflammatory visceral pain*

These models attempt to mirror the postinflammatory/infective hypersensitivity that develops in some IBS patients. In an attempt to model postinfective IBS, colonic sensitivity has been assessed in rat and mouse models following an acute bacterial gastroenteritis. In rats, *Citrobacter rodentium* induced a significant increase in AWR score, which was inhibited by treatment with a traditional herbal medicine or alosetron (Hu et al., 2013). In mice, *Campylobacter jejuni* infection caused long-term hyperexcitability of colonic DRG neurons (Ibeakanma et al., 2009). However, a clear increase in the EMG response to balloon distension was found only if the mouse was exposed to an additional stressor (Ibeakanma et al., 2011). Trichinella spiralis infection has also been used to produce a long-term colonic hypersensitivity to distension in rats and mice. At 8 wk postinfection, the EMG response to distension was significantly increased along with increases in glutamatergic receptor expression (Yang et al., 2009). Notheworty, additional precautions should be exercised when using the post-infective models to protect the experimenters from the pathogens (Greenwood-Van Meerveld et al., 2015).

Based on the current understanding of post-inflammatory- (PI-) visceral hypersensitivity (PI-IBS), in an ideal animal model, the animals should not only completely recover from the acute intestinal infection or inflammation but also show intestinal features such as visceral hypersensitivity, motility dysfunction, and changes in permeability or secretion (Qin et al., 2012). Owing to their low cost and rapid onset of disease, chemical models are considered invaluable to the study of various aspects of IBS (Hughes et al., 2009; Giamberardino, 2009). Among the irritants used to induced post-inflammatory IBS there are acetic acid, DSS, and TNBS. Following recovery from an acute colitis induced by these chemicals, determined by gross morphology, histology, and/or tissue immune activation markers (cytokines), these animals develop a hypersensitive response to colonic distension. The standard protocol for acetic acid-induced post-inflammatory colonic hypersensitivity is to administer and enema of

4% acetic acid, followed by a buffered saline enema, with colonic hypersensitivity lasting 7 days post-enema. Strong evidence for post-inflammatory colonic hypersensitivity following DSS colitis is lacking in recent literature. Although hypersensitivity to distension was demonstrated at 10 days post-DSS administration (Chen et al., 2013), colonic sensitivity was similar to control animals at ~40 days post-colitis (Eijkelkamp et al., 2009). Besides, in rats, colonic hypersensitivity has been observed 14-112 days post-TNBS enema. 2,4,6-trinitrobenzenesulfonic acid (TNBS) is a hapten that produces an acute colonic inflammation when administered as an enema in combination with 25–50% ethanol (EtOH). Ethanol is a prerequisite to break the colonic mucosal barrier to allow penetration of TNBS into the lamina propria where TNBS haptenize the localized colonic and gut microbial proteins to become immunogenic, thereby triggering the host innate and adaptive immune responses (Qin et al., 2011). Although inflammatory changes in the colonic tissue can be measured within hours of the enema, most protocols investigate colonic sensitivity to distension, with EMG or AWR quantification, 3–7 days post-enema, which will represent different amounts of inflammatory damage depending on the concentration of TNBS and EtOH in the enema (te Velde et al., 2006). Assessing colonic sensitivity in rats and mice with active colitis likely increases the risk for perforation by the balloon catheter due to the potential presence of ulcerated and/or necrotic tissue (te Velde et al., 2006). In general, TNBS model is associated with severe and sometimes bloody diarrhoea, weight loss and intestinal wall thickening however symptoms vary depending upon the type of rodent used, as well as the timing, dose and degree of exposure to TNBS used in the study (Qin et al. 2011). The features of IBS, such as visceral hypersensitivity, motility dysfunction, alteration in histopathology, and changes in permeability and secretion, have also been identified in the experimental animals after recovery from the initial inflammation induced TNBS (Qin et al. 2012). Moreover, both tegaserod and linaclotide have been shown to inhibit TNBS-induced post-inflammatory colonic hypersensitivity, providing evidence for the translational relevance of the model (Greenwood-Van Meerveld et al., 2006; Castro et al., 2013). All these evidences made TNBS the most commonly used model of pot-inflammatory IBS. TNBS is considered as a hazardous chemical due to its highly oxidative properties so 2,4-DiNitroBenzene Sulfonic acid (DNBS) is currently regarded as a preferred choice to induce IBS. Despite the numerous aspects similar to IBS patients found in the TNBS and DNBS models, the experimental protocols are variegated and poorly defined because of the great variability in the animal's responses to the damage (Giamberardino et al., 2009). Indeed, the recovery from the colitis does not guarantee the existence for colonic hypersensitivity to balloon distension (Zhou et al., 2009). Thus, the

acute effects of the initial inflammatory insult (loose stool/ diarrhoea, weight loss, occult or explicit bleeding) needs to be monitored during the recovery period to aid in predicting which animals may develop post-inflammatory colonic hypersensitivity. Optimally, colonic sensitivity to distension should be assessed before testing a therapeutic intervention with only those animals with verified colonic hypersensitivity being used for subsequent testing (Greenwood-Van Meerveld et al., 2015).




***Summary of DNBS effects on the reference genes expression (RGE) stability in the gastrointestinal mucosa***

*Eissa et al., Scientific Reports, 2017*

## AIMS OF THE STUDY

The aims of the study are the establishment and the characterization of an animal model of persistent visceral pain closely related to the clinical manifestations in order to study the pathophysiological mechanisms involved and to research novel possible treatments.

For these purposes we will set up a persistent visceral hypersensitivity in rats through the intra-rectal injection of 2,4-DiNitroBenzene Sulfonic acid (DNBS). Visceral pain persistence will be assessed by measuring the Abdominal Withdrawal Response (AWR) and the Visceral Motor Reflex (VMR) to Colo-Rectal Distension (CRD) weekly after DNBS injection. Anxiety- and depressive-like behaviour will be also assessed in these animals. The effect of systemic and/or intrathecal administration of spasmolytics, anti-inflammatory drugs, opioids, antiepileptics and antidepressants will be investigated in order to characterize the nature of the observed pain. Histological and molecular characterization of intestine will be carried out for evaluating colon damage, fibrosis, eosinophil infiltrate, mast cells concentration and substance P level. An immunofluorescence analysis will be performed to evaluate immune interaction with the peripheral afferents on colon and microglia (Iba-1 positive cells) and astrocytes (GFAP positive cells) number and morphology in the spinal cord.

In the second part of this work we will study the effect of modulating adenosine $A_3$ receptors on post-inflammatory visceral pain. We will evaluate the effect of acute intraperitoneal administration of a new highly selective $A_3$ adenosine receptor agonists, MRS5980, and the golden standard, Cl-IB-MECA in DNBS treated animals. The involvement of $A_3$ receptors activation and $Ca^{2+}$ modulation in the anti-hyperalgesic effect of MRS5980 and Cl-IB-MECA will be studied by both behavioural tests and electrophysiological analysis on DNBS isolated DRG neurons. The efficacy of these molecules will be compared with a reference drug for IBS therapy, linaclotide.

In the third part of the work the role of microbiota in post-inflammatory visceral pain will be studied. Firstly, the effect of Faecal Microbiota Transplantation (FMT) from DNBS-treated to naïve rats on visceral sensation will be investigated. To allow the transplant of the microbiota the animals will undergo an antibiotic treatment. To reduce the impact of antibiotics on visceral sensitivity we will test different protocols, and the least impacting will be used in the following experiments. Moreover, we will study the mechanisms underlying microbiota-mediate visceral sensitivity alterations by exploring the effect of FMT on gut permeability, cytokines profile and neurotransmitters (5-HT and DA) levels. We will also assess the levels of tryptophan and kynurenine in the plasma and monoamines in the faeces. In parallel, the

analysis of microbiota composition will be performed. Finally, we will investigate the effect of transplanting a healthy microbiota in DNBS animals on the development of post-inflammatory visceral pain.

## MATERIALS AND METHODS

### *1. Animals*

For all the experiments described below, Male Sprague-Dawley rats (Envigo, Varese, Italy) weighing approximately 220-250 g at the beginning of the experimental procedure, were used. Animals were housed in CeSAL (Centro Stabulazione Animali da Laboratorio, University of Florence) and used at least 1 week after their arrival. Four rats were housed per cage (size 26 × 41 cm); animals were fed a standard laboratory diet and tap water ad libitum, and kept at 23 ± 1 °C with a 12 h light/dark cycle, light at 7 a.m. All animal manipulations were carried out according to the Directive 2010/63/EU of the European parliament and of the European Union council (22 September 2010) on the protection of animals used for scientific purposes. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the described experiments was obtained from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines (McGrath and Lilley, 2015). All efforts were made to minimize animal suffering and to reduce the number of animals used.

### *2. Induction of colitis*

Colitis was induced in accordance with the method described previously by Fornai et al. (2006) with minor changes. In brief, during a short anesthesia with isoflurane (2%), 30 mg of 2,4-dinitrobenzenesulfonic acid (DNBS) in 0.25 ml of 50% ethanol was administered intrarectally via a polyethylene PE-60 catheter inserted 8 cm proximal to the anus. Control rats received 0.25 ml of saline solution.

### *3. Drug administrations*

Morphine hydrochloride (S.A.L.A.R.S., Como, Italy) was subcutaneously (10 mg $kg^{-1}$) and intrathecally (1 μg in 15μL) administered. Ibuprofen (100 mg $kg^{-1}$ Carbosynth, Compton, UK) and otilonium bromide (20 mg $kg^{-1}$ Carbosynth, Compton, UK) were orally administered. Pregabalin (Sigma-Aldrich, Milan, Italy) was intraperitoneally (30 mg $kg^{-1}$) and intrathecally (100 μg 15μL) administered. Amitryptiline hydrochloride (Sigma-Aldrich, Milan, Italy), was intraperitoneally (15 mg $kg^{-1}$) and intrathecally (60 μg 15μL) administered. Desamethasone (0.6 mg $kg^{-1}$ Carbosynth, Compton, UK) was dissolved in 1% DMSO saline solution and

intraperitoneally administered. The other compounds were dissolved in saline solution for subcutaneous and intraperitoneal administrations, in CMC 1 % for oral administrations and in PBS for intrathecal injections. All the substances were injected 15 min before the test (n=5).

### *4. Assessment of visceral sensitivity by Viscero Motor Response (VMR)*

The visceromotor response (VMR) to colorectal balloon distension were used as objective measure of visceral sensitivity in animals. Two EMG electrodes were sutured into the external oblique abdominal muscle under deep anaesthesia and exteriorised dorsally (Christianson and Gebhart, 2007). VMR assessment were carried out under light anaesthesia (Isoflurane 2%). A lubricated latex balloon (length: 4.5 cm), assembled to an embolectomy catheter and connected to a syringe filled with water was used to perform colo-rectal distension. The baloon was inserted into the colon and positionated 6.5 cm from the anus and and was filled with increasing volumes of water (0.5, 1, 2, 3 mL). A smaller balloon (length: 2 cm), was used to perform rectal distension by positioning it in the rectum and by filling it with 1.5 mL of water. The electrodes were relayed to a data acquisition system and the corresponding EMG signal consequent to colo-rectal stimulation were recorded, amplified and filtered (Animal Bio Amp, ADInstruments, USA), digitised (PowerLab 4/35, ADIinstruments, USA), analysed and quantified using LabChart 8 (ADInstruments, USA). To quantify the magnitude of the VMR at each distension volume, the area under the curve (AUC) immediately before the distension (30 s) was subtracted from the AUC during the balloon distension (30 s) and responses were expressed as percentage increase from the baseline. The time elapsed between two consecutive distension was 5 min. The measurements were carried out before (pretest) and 7, 14 and 21 days after DNBS administration (control group n=10, DNBS group n=10).

### *5. Assessment of visceral sensitivity by Abdominal Withdrawal Reflex (AWR)*

The behavioural responses to CRD were assessed in the animals by measuring the Abdominal Withdrawal Reflex (AWR), a semi-quantitative score described previously in conscious animals (Chen et al., 2014). Briefly, rats were anesthetized with isoflurane, and a lubricated latex balloon (length: 4.5 cm), attached to polyethylene tubing, assembled to an embolectomy catheter and connected to a syringe filled with water was inserted through the anus into the rectum and descending colon of adult rats. The tubing was taped to the tail to hold the balloon in place. Then rats were allowed to recover from the anaesthesia for 30 min. AWR measurement consisted of visual observation of animal responses to graded CRD (0.5, 1, 2, 3 mL) blinded observers who assigned AWR scores: No behavioral response to colorectal

distention (0); Immobile during colorectal distention and occasional head clinching at stimulus onset (1); Mild contraction of the abdominal muscles but absence of abdomen lifting from the platform (2); Observed strong contraction of the abdominal muscles and lifting of the abdomen off the platform (3); Arching of the body and lifting of the pelvic structures and scrotum (4). The measurements were carried out once week for 7 weeks after DNBS administration (control group n=10, DNBS group n=10).

### *6. Assessment of depression-related behaviour by Forced Swim Test (FST)*

The FST was carried out as previously described (Slattery and Cryan, 2012). All experimental animals were first habituated to the testing room 30 minutes prior to testing. A pre-swim (15 min) was conducted 24 hours prior to the test. On test day, all animals were introduced again to the Plexiglas cylinder (46 cm tall; 21 cm in diameter) filled with water (24 °C) to a depth of 30 cm. Test sessions (5 min) were recorded by video camera positioned directly above the cylinder. Animals were removed from their home-cage and placed into the tank. After 5 min, animals were removed from the tank, dried and replaced back into their home-cage. The tank was then emptied, and fresh water was replaced into the tank between animals. The parameters of interest were the time mobile and the distance travelled. Data acquisition and analysis were performed automatically using ANY-maze Software® (Stoelting Co., USA) (control group n=10, DNBS group n=10).

### *7. Assessment of anxiety-related behaviour by Open Field Test (OFT)*

The open field paradigm was performed as previously described by O' Mahony et al. (2011). The open field arena was brightly lit (1000 lux). This is considered to be stressful to rats as they are not given the opportunity to retreat into dark corners. The arena 90 cm in diameter was placed on the floor. A video tracking system was fixed above the experimental platform. All rats were habituated to the testing room for 30 min. Rats were placed gently into the centre of the arena: the total distance travelled and the time they spent in the centre of the field within 5 min was recorded. The arena was cleaned between each rat with 30% ethanol to avoid leaving an abnormal odour. Data acquisition and analysis were performed automatically using ANY-maze Software® (Stoelting Co., USA) (control group n=10, DNBS group n=10).

### *8. Assessment of anxiety-related behaviour by Elevated Plus Maze Test (EPMT)*

The elevated plus maze test was performed as previously reported by Bissiere et al. (2006). Briefly, the apparatus consisted of two open arms (50 cm x 10 cm), two closed arms (50 cm x

10cm x 30 cm) and a central platform (10 cm x 10 cm) on a stand that was 70 cm high off the floor. A video tracking system was fixed above the experimental platform. All animals were habituated to the testing room for 30 min. Then rats were placed in the central platform of the maze and allowed to explore the maze for 5 min. The percentage of time spent in the open arms, in the closed arms and in the centre were measured. The maze was cleaned between each rat with 30% ethanol to avoid leaving an abnormal odour. Data acquisition and analysis were performed automatically using ANY-maze Software® (Stoelting Co., USA) (control group n=10, DNBS group n=10).

### *9. Histological evaluation of colon damage*

The evaluation of colon damage was performed both macroscopically and histologically, in accordance with the criteria previously reported by Antonioli et al. (2007). The macroscopic criteria were: presence of adhesions between colon and other intra-abdominal organs (0-2); consistency of colonic faecal material (indirect marker of diarrhoea; 0-2); thickening of colonic wall (mm); presence and extension of hyperaemia and macroscopic mucosal damage (0-5). Microscopic evaluations were carried out on haematoxylin/eosin-stained sections of formalin-fixed full-thickness samples obtained from the distal colon. The microscopic damage was evaluated in accordance with the criteria reported previously by Antonioli et al. (2010): 1) mucosal architecture loss (0-3); 2) goblet cell depletion (0, absent; 1, present); 3) crypt abscess (0, absent; 1, present); 4) cellular infiltration (0-3); 5) *tunica muscularis* thickening (0-3). Data obtained were used to calculate mean values ± SEM for each experimental group, which were plotted in graphs. The significance of differences was evaluated by one-way ANOVA followed by Tukey's multiple comparison test. P values $\leq 0.05$ were considered significantly different compared to controls (n=4).

### *10. Evaluation of collagen deposition and inflammatory cells in the colon wall*

Collagen deposition was localized by the Sirius Red/Fast Green (SR/FG) staining, which allowed to distinguish collagen and non-collagen proteins as red and green deposits, respectively, as described in detail by Segnani and colleagues (2015). Mast cells (MCs) and eosinophils were detected by histochemical toluidine blue and haematoxylin/eosin staining, respectively, and quantified as previously reported (Pellegrini et al., 2016). Activated macrophages were revealed by immunofluorescence staining of the MHC-II antigen as previously reported by Mowat and Bain (2013) and described in the following paragraph (control group n=4, DNBS-Day 14 group n=4).

### *11. Immunodetection of SP-positive fibres and MHC-II-positive macrophages in the colon*

*Immunoenzymatic histochemistry.* Colonic sections were processed as previously described (Pellegrini et al., 2016). Briefly, after an overnight incubation at 4°C with rabbit anti-SP immunoglobulins, biotinylated immunoglobulins, peroxidase-labelled streptavidin complex, 3.3'-diaminobenzidine tetrahydrochloride and eosin-staining were employed.

*Confocal immunofluorescence histochemistry.* SP-positive fibres and MHC-II-positive macrophages were detected by means of double immunofluorescence protocols. Colonic sections were unmasked with heat and incubated overnight at 4°C with the combined primary antibodies anti-SP and anti-MHC class II. The sections were then exposed to appropriate fluorophore-conjugated secondary antibody or biotinylated secondary antibody followed by fluorophore-conjugated streptavidin and nuclear counterstaining with TO-PRO3 as previously described (Ippolito et al., 2015). Sections were examined under a Leica TCS SP8 confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany) (control group n=4, DNBS-Day 14 group n=4).

### *12. Image analysis for the histochemical and immunohistochemical staining of the colon*

The sections were examined by a Leica DMRB light microscope, equipped with a DFC480 digital camera (Leica Microsystems, Cambridge, UK), and quantitatively analysed using the Image Analysis System 'L.A.S. software version 4.5' (Leica Microsystems, Cambridge, UK), as previously described. Two blind investigators (C.I. and C.S.) independently evaluated the eosinophil and MC density (cell number / respective analysed areas [$mm^2$]) as well as the SR- and SP-positivity expression (positive pixels percentage [PPP]) as previously described (Segnani et al., 2015; Pellegrini et al., 2016). Data obtained were used to calculate mean values ± SEM for each experimental group, which were plotted in graphs. The significance of differences was evaluated by Student's t test for unpaired data. P values $\leq 0.05$ were considered significantly different compared to controls (control group n=4, DNBS-Day 14 group n=4).

### *13. Analysis of spinal cord Iba-1 and GFAP positive cells by immunofluorescence*

The lumbar segments of rat spinal cord were exposed from the vertebral column via laminectomy and formalin-fixed by standard protocols previously described (Di Cesare Mannelli et al., 2013). Cryostat sections (5 μm) were washed thrice with phosphate-buffered saline (PBS), permeabilized with 0.3% Triton X-100 in PBS (PBST) for 10 min and then were incubated, at room temperature, for 1 h in blocking solution (1% bovine serum albumin

in PBST). Slices were incubated overnight at 4°C in PBST containing rabbit primary antibodies. The primary antibody used was directed against Iba1 (ionized calcium binding adapter molecule 1; rabbit, 1:250; Wako, Richmond, VA, USA) for microglial staining or against GFAP (glial fibrillary acidic protein; rabbit, 1:500; DAKO, Carpinteria, CA, USA) for astrocyte staining. The following day, slides were washed thrice with PBS, and then incubated, for 1 hour, with blocking solution in goat anti-rabbit IgG secondary antibody labeled with Alexa Fluor 488 or 568 respectively (1:200; Life Technologies-ThermoFisher Scientific, Milano, Italy) and DAPI (4', 6-diamidin-2-fenilindolo; 1:2000; Life Technologies-ThermoFisher Scientific), a nuclei marker, in PBST for 5 min., at room temperature in the dark. After three washed in PBS and a final wash in distilled water, slices were mounted using ProLong Gold (Life Technologies-ThermoFisher scientific) as a mounting medium. Negative control sections (no exposure to the primary antibody) were processed concurrently with the other sections for all immunohistochemical studies, to exclude the presence of non-specific immunofluorescence staining or cross-immunostaining. Digitalized images were collected at 100×, 200× or 400× total magnification by a motorized Leica DM6000B microscope equipped with a DFC350FX. Quantitative analysis of GFAP- and Iba1-positive cells was performed by collecting independent fields in the dorsal and ventral horn of each mouse spinal cord. GFAP and Iba1-positive cells were counted using the "cell counter" plugin of ImageJ (NIH, Bethesda, Maryland, USA). Values were expressed as means ± S.E.M. and the statistical analysis was performed by *t*-test. Each value represents the mean of 8 field per spinal cord, both dorsal and ventral horn (control group n=4, DNBS-Day 14 group n=4).

### *14. Adenosine $A_3$ receptor agonists administration*

The selective $A_3$ receptor agonist, 2-Chloro-$N^6$-(3-iodobenzyl)-adenosine-5′-N-methyluronamide (Cl-IB-MECA; Tocris Bioscience, Bristol, UK), and the selective $A_3$ receptor antagonist, 3-Propyl-6-ethyl-5-[(ethylthio)carbonyl]-2 phenyl-4-propyl-3-pyridine carboxylate (MRS1523; Sigma-Aldrich, Milan, Italy), were dissolved in 5% DMSO and 5% TWEEN 20 saline solution for *in vivo* administration. The new, highly selective (10,000-fold vs each of the other 3 receptor subtypes), $A_3$ receptor agonist, MRS5980, (1S,2R,3S,4R,5S)-4-(2-((5-Chlorothiophen-2-yl)ethynyl)-6-(methylamino)-9H-purin-9-yl)-2,3-dihydroxy-N-methylbicyclo[3.1.0]hexane-1-carboxamide, recently synthetized by the research group of Prof. Kenneth Jacobson, NIDDK, NIH, Bethesda, US (Fang et al., 2015) was dissolved in 5% DMSO saline solution for *in vivo* administration. N-[[4-(1,1-dimethylethyl)phenyl]methyl]-N-methyl-L-leucyl-N-(1,1-dimethylethyl)-O-(phenylmethyl)-L-tyrosinamide (PD173232) was

purchased from Alomone labs (Jerusalem, Israel; https://www.alomone.com) and dissolved in 5% DMSO and 5% TWEEN 20 saline solution for *in vivo* administration. Linaclotide (Allergan, UK) was dissolved in water and orally administered 1 h before starting the behavioural tests (Eutamene et al., 2010). Cl-IB-MECA, MRS5980 and PD173232 were administered i.p. 15 min before the test. MRS1523 was administered i.p. 15 min before Cl-IB-MECA and MRS5980 (n=5).

### *15. Faecal Microbiota Transplantation (FMT)*

First experimental set: Faecal material was collected from controls and DNBS treated animals (between 14 and 21 days after the intra-rectal injection of the inflammatory agent) and placed into tubes containing freezing solution (sterile saline solution with 12.5% glycerol), tubes were left on ice for 60 min and later homogenized for two min on ice using a hand-held pellet pestle device with sterile, re-usable pestles. When fully homogenized, the suspended pellets were frozen and stored at -80°C until utilized for the FMT procedure. To this end, rats were randomized into the following treatment groups (n=5): control (no antibiotic treatment, no FMT); abx + FMT from CTR (antibiotic treatment followed by FMT from controls); abx + FMT from DNBS (antibiotic treatment followed by FMT from DNBS treated animals). Antibiotic mix was prepared in autoclaved drinking water and provided in 500 ml clear glass slippers or were suspended in 1% carboxymethylcellulose sodium salt (CMC; Sigma-Aldrich) and administered by oral gavage. The antibiotic/anti-fungal regime was as follows: day 1-3 rats received a daily oral gavage of amphotericin B (1 mg $kg^{-1}$), day 4-17 rats received a daily gavage of metronidazole 100 mg $kg^{-1}$ while the antibiotic mix (ampicillin 1 g $L^{-1}$, vancomycin 0.5 g $L^{-1}$ and neomicin 1 g $L^{-1}$ was added to drinking water), day 18-24 daily oral gavage with ampicillin 1 g $L^{-1}$, vancomycin 0.5 g $L^{-1}$, neomicin 1 g $L^{-1}$, metronidazole 100 mg $kg^{-1}$, amphotericin B 1 mg $kg^{-1}$. FMT was carried out via oral gavage with a faecal suspension (50 mg $ml^{-1}$) in a final volume of 3 ml. FMT was performed six times on days 24-28 and 35 from the beginning of antibiotic regime. Behavioural tests were performed 10, 17 and 24 days after the last FMT (n=5).

Second experimental set: Faecal material was collected from controls and DNBS treated animals (between 14 and 21 days after the intra-rectal injection of the inflammatory agent) and placed into tubes containing sterile saline solution, tubes were left on ice for 60 min and later homogenized for two min on ice using a hand-held pellet pestle device with sterile, re-usable pestles. When fully homogenized, the suspended pellets were directly utilized for the FMT procedure. To this end, rats were randomized into the following treatment groups (n=5):

control (no antibiotic treatment, no FMT); abx + vehicle (antibiotic treatment followed by vehicle administration); abx + FMT from CTR (antibiotic treatment followed by FMT from controls); abx + FMT from DNBS (antibiotic treatment followed by FMT from DNBS treated animals). Antibiotic mix was prepared in autoclaved drinking water and provided in 500 ml clear glass slippers or were suspended in 1% carboxymethylcellulose sodium salt (CMC; Sigma-Aldrich) and administered by oral gavage. The antibiotic/anti-fungal regime was as follows: day 0-6 rats received a daily oral gavage of amphotericin B (1 mg $kg^{-1}$) and metronidazole (100 mg $kg^{-1}$) while the antibiotic mix (ceftazidime 1 g $L^{-1}$, vancomycin 0.5 g $L^{-1}$ and neomicin 1 g $L^{-1}$ was added to drinking water). FMT was carried out via oral gavage with a faecal suspension (50 mg $ml^{-1}$) in a final volume of 3 ml. FMT was daily performed ten times on days 7-11 and 20-24 from the beginning of antibiotic regime. Behavioural tests were performed at the end of the antibiotic treatment, 24h and 7 days after each FMT set and once week after the last treatment.

Third experimental set: Faecal material was collected from controls animals and placed into tubes containing sterile saline solution, tubes were left on ice for 60 min and later homogenized for two min on ice using a hand-held pellet pestle device with sterile, re-usable pestles. When fully homogenized, the suspended pellets were directly utilized for the FMT procedure. To this end, rats were randomized into the following treatment groups (n=6): controls; DNBS + vehicle (intrarectal injection of DNBS 30 mg followed by vehicle administration); DNBS + FMT from CTR (intrarectal injection of DNBS 30 mg followed by FMT from controls). FMT was carried out via oral gavage with a faecal suspension (50 mg $ml^{-1}$) in a final volume of 3 ml. Four set of 5 consecutive FMT were performed on days 7-11, 14-18, 21-25 and 28-32 after DNBS injection. Behavioural tests were performed 7 day after DNBS injection, 3 days after each FMT set and once week after the last treatment (n=5).

### *16. Detection of DNBS in faecal samples by Liquid Chromatography – Mass Spectrometry (LC-MS)*

The LC-MS/MS analysis was carried out using a Varian 1200L triple quadrupole system (Palo Alto, CA, USA) equipped by two Prostar 210 pumps, a Prostar 410 autosampler and an Electrospray Source (ESI) operating in negative ions. Raw-data were collected and processed by Varian Workstation vers. 6.8 software. The column used was a Pursuit XRs C18 30 mm length, 2 mm internal diameter and 3 µm particle size, at constant flow of 0.25 mL $min^{-1}$, employing a binary mobile phases elution gradient. The solvents used were 10 mM formic acid solution (solvent A) and 10 mM formic acid in acetonitrile (solvent B) according to the

elution gradient as follows: initial at 90 % solvent A, which was then decreased to 10 % in 4.0 min, kept for 3.0 min, returned to initial conditions in 0.1 min and maintained for 3.0 min for reconditioning, to a total run time of 10.0 min. The column temperature was maintained at 30 °C and the injection volume was 5 μL. The analyses were acquired in Multiple Reaction Monitoring (MRM) using 150 ms of dwell time and the ion transitions reported:

**MRM parameters**

| **Compound** | **Precursor Ion (m/z)** | **Quantifier Ion (m/z) [CE (V)]** | **Qualifier Ion (m/z) [CE (V)]** |
|---|---|---|---|
| ISTD | 262 | 183 [25] | 122 [30] |
| DNBS | 247 | 109 [40] | 183 [20] |

Stock solutions of DNBS and ISTD were prepared in acetone at 1.0 mg $mL^{-1}$ and stored at 4 °C. Working solutions of analyte were freshly prepared by diluting stock solution up to a concentration of 1.0 μg $mL^{-1}$ and 0.1 μg $mL^{-1}$ (working solution 1 and 2 respectively) in mixture of mQ water:acetonitrile 50:50 (v/v). The ISTD working solution was prepared in acetonitrile at 10 μg $mL^{-1}$ (ISTD solution). A five levels calibration solutions were prepared in mQ water:acetonitrile 50:50 (v/v) by adding proper volumes of working solution (1 or 2) of each analyte and 10 μL of ISTD solution. Final concentrations of calibration levels were: 0, 5.0, 10.0, 25.0 and 50.0 ng $mL^{-1}$ of analyte in the sample. All calibration levels were analyzed six times by LC-MS/MS method described above. The calibration curve of analyte was obtained by plotting the peak area ratios (PAR), between analyte and ISTD quantitation ions, versus the nominal concentration of the calibration solution. A linear regression analysis was applied to obtain the best fitting function between the calibration points. In order to obtain reliable limit of detection (LOD) and limit of quantitation (LOQ) values, the standard deviation of response and slope approach was employed. The estimated standard deviation of response of analyte was obtained by the calculated standard deviation of y-intercept (SDY-I) of regression line. The calculated values of LOD and LOQ were 2.0 ng $mL^{-1}$ and 6.0 ng $mL^{-1}$ respectively. The sample was obtained by suspending the rat/mouse faeces (about 1.7 g) in 16 mL of phosphate buffer solution. An aliquot of 1 mL of sample solution was transferred in microcentrifuge tube, added of 10 μL of ISTD solution and then centrifuged (room temperature for 5 min at 800 g). The supernatants were transferred in autosampler vials and analysed by the LC-MS/MS method described above. In order to verify the evaluated limit of

quantitation in real sample, an aliquot of control sample was added of 5 ng $mL^{-1}$ of DNBS and analysed by the LC-MS/MS method.

### *17. Plasma Lipopolysaccharide Binding Protein (LBP) dosage by ELISA*

Plasma LBP concentrations were determined using the Enzyme Immunoassay Kit (Enzo®, Life Sciences). Rat blood samples were collected in heparinized tubes after decapitation. Samples were centrifuged immediately at 14000 rpm for 15 minutes and the resulting plasma was frozen at -80°C until analysis. LBP concentrations were determined using the Enzyme Immunoassay Kit for free mouse and rat LBP as per manufacturers' instruction (Enzo®, Life Sciences). Sensitivity: Range 1-50 ng/ml (n=4-6).

### *18. Monoamines analysis by High Performance Liquid Chromatography (HPLC)*

Rat colons or faecal pellets were placed into chilled mobile phase spiked with N-methyl-serotonin, an internal standard (N-methyl-5-HT; 4 ng/20 µl; Sigma-Aldrich, Ireland). Samples were weighed, individually sonicated in 500 µl buffer and centrifuged (14 000 rpm, 4°C, 20 min). Supernatant was collected and diluted 1/2 in HPLC mobile phase. The supernatants derived from faecal pellets were filtered by 0.22 µm filter before the anlysis. The mobile phase contained 0.1 M citric acid, 0.1 M sodium dihydrogen phosphate, 0.01 mM EDTA (Sigma-Aldrich, Ireland), 5.6 mM octane-1-sulphonic acid (Sigma) and 11% (v/v) methanol (Sigma-Aldrich, Ireland) and was adjusted to pH 2.8 using 1M sodium hydroxide (Sigma-Aldrich, Ireland). The monoamines and metabolites DA, homovanillic acid (HVA), dihydroxyphenylacetic acid (DOPAC), 5-HT, 5-hydroxyindoleacetic acid (5-HIAA) and noradrenaline (NA) were measured using HPLC with electrochemical detection. Twenty microlitres of supernatant was injected onto the HPLC system which consisted of a SCL 10-Avp system controller, LC-10AS pump, SIL-10A autoinjector (with sample cooler maintained at 4°C), CTO-10A oven, LECD 6A electrochemical detector (Shimadzu) and an online Gastorr Degasser (ISS, UK). A reverse-phase column (Kinetex 2.6u C18 100× 4.6 mm, Phenomenex) maintained at 30°C was employed in the separation (flow rate 0.9 ml/min), the glassy carbon working electrode combined with an Ag/AgCl reference electrode (Shimdazu) was operated at +0.8 V and the chromatograms generated were analysed using Class-VP 5 software (Shimadzu). Monoamines were identified by their characteristic retention times as determined by standard injections which were run at regular intervals during the sample analysis. Concentrations were calculated using analyte: internal standard peak height ratios and expressed as nanograms of neurotransmitter per gram of tissue weight (n=4-6).

### 19. *Kynurenine/Tryptophan analysis by High Performance Liquid Chromatography (HPLC)*

Plasma samples were spiked with internal standard (3-Nitro l-tyrosine) prior to being deproteinised by the addition of 20 µl of 4M perchloric acid to 200 µl of sample. Samples were centrifuged at 12000 rpm for 20 minutes at 4°C and 100 µl of supernatant transferred to a HPLC vial for analysis on the HPLC system (UV and FLD detection). All samples were injected onto a reversed phase Luna 3 µm C18 (2) 150 × 2 mm column (Phenomenex), which was protected by Krudkatcher disposable pre-column filters (Phenomenex) and SecurityGuard cartridges (Phenomenex). The mobile phase consisted of 50 mM acetic acid, 100 mM zinc acetate with 2.5% (v/v) acetonitrile and was filtered through sterile 0.45µm polyvinylidene fluoride (PVDF) Stericup™ Millipore filter unit and vacuum degassed prior to use. Compounds were eluted isocratically over a 30-minute runtime at a flow rate of 0.3 mls/min after a 20 µl injection. The column was maintained at a temperature of 30°C and samples/standards were kept at 8°C in the cooled autoinjector prior to injection. The fluorescent detector was set at an excitation wavelength of 254 nm and an emission wavelength of 404 nm. The UV detector was set to 330 nm. L-tryptophan and kynurenine were identified by their characteristic retention times as determined by standard injections which were run at regular intervals during the sample analysis. Analyte: Internal standard peak height rations were measured and compared with standard injections and results were expressed as ng/ml of plasma (n=4-6).

### *20. Gene expression analysis by quantitative RT-PCR (qRT-PCR)*

Total RNA was extracted using the mirVana$^{TM}$ miRNA Isolation kit (Ambion/life technologies) according to the manufacturer's recommendations. For each group, 4–6 animals were used. RNA concentration and quality were determined using a Nanodrop 1000 (Thermo Scientific). Quantitative PCR was carried out in a LightCycler480 System using PowerUp™ SYBR® Green Master Mix (Applied Biosystems) and specific probes designed by Applied Biosystems to rat occludin, ZO-1, TNF-α, IL6, IL10 and Tgf-β, while using β-Actin as an endogenous control. Experimental samples were run in triplicate with 4 µL cDNA per reaction. To check for amplicon contamination, each run contained no template controls in triplicate for each probe used. Cycle threshold (Ct) values were recorded. Data was normalised using β-Actin and transformed using the $2^{-\Delta\Delta Ct}$ method. No significant differences were observed in the mRNA expression levels of β-Actin between groups (n=4-6).

### *21. Statistical analysis*

Behavioural measurements were performed on 5-10 animals for each treatment carried out in 2 different experimental sets. All the experimental procedures were performed by a researcher blind to the treatment. Results were expressed as mean ± S.E.M. The analysis of variance of behavioural data was performed by one-way ANOVA with Bonferroni's significant difference procedure used for post-hoc comparisons. Statistics of electrophysiological data was performed by Student's paired or unpaired t-test or by one-way ANOVA followed by Bonferroni's post-test, as appropriate. P values of less than 0.05 were considered significant. Data were analyzed using the "Origin 9" software (OriginLab, Northampton, USA).

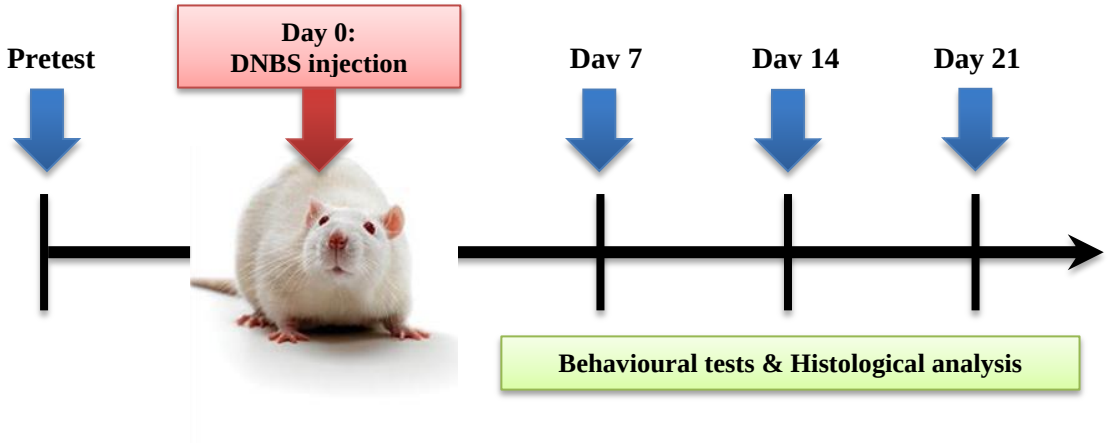
# RESULTS

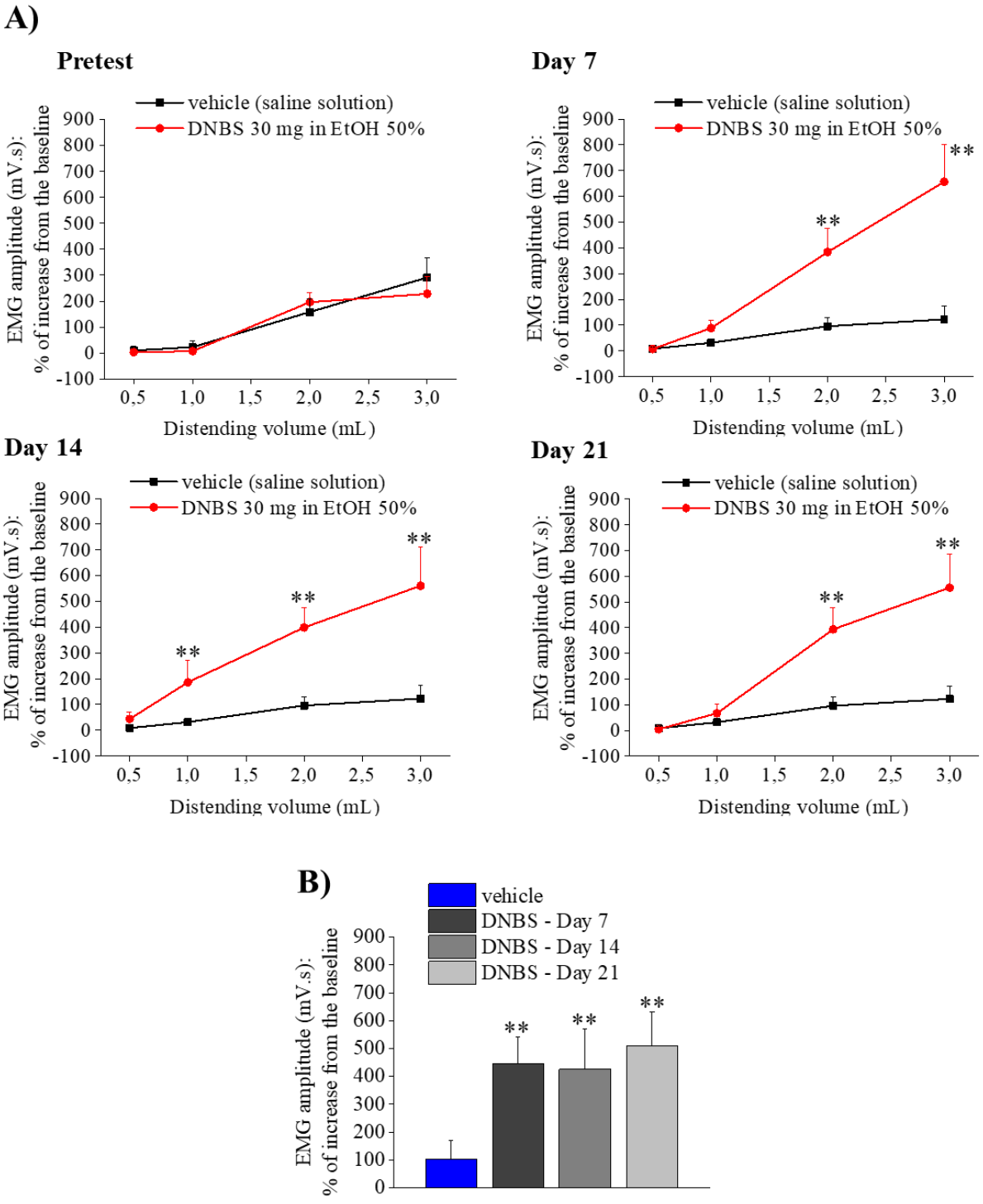
## 1. *Setting up and analysis of a rat model of persistent abdominal pain*

### *1.1 Assessment of visceral sensitivity after the intra-rectal injection of DNBS*

Colitis was induced in rats by the intra-rectal injection of DNBS (30 mg dissolved in 0.25 mL EtOH 50%). Control animals were administered with saline solution. Visceral sensitivity was assessed in the animals by measuring the viscero-motor response to colo-rectal distension before (Pretest) and 7, 14 and 21 days after DNBS injection (Fig 1A). No difference were found in the experimental groups before the treatments (Fig 1, *pretest*). The injection of DNBS caused a huge and persistent increase of visceral sensitivity in the animals. In fact, DNBS-treated animals showed a viscero-motor response to colo-rectal distension induced by 2 and 3 mL significantly higher than vehicle-treated animals up to 21 days after the injection (Fig 1A). On day 14, DNBS-treated animals were also responsive to 1 mL, showing a visceral sensitivity threshold further lowered (Fig 1A). The abdominal contraction in response to a rectal stimulus was measured in the same animals (Fig 1B). A smaller balloon (2 cm) was positionated at rectal level and filled with 1.5 mL of water to specifically induce rectal distension. The response of DNBS-treated animals resulted significantly augmented in comparison to that of controls at each time tested (Fig 1B). The nocifensive response of animals to colorectal distension was measured by assigning a score to their abdominal withdrawal response (AWR) to colo-rectal distension (Fig 1C). The behavioural response of vehicle-treated animals to colo-rectal distension was constant over time and proportional to the stimulus applied. DNBS-treated animals showed an abdominal response significantly higher than that of controls, resulting responsive also to low volume of distension (0.5-1 mL). This effect was observed up to 13 weeks after DNBS injection (Fig 1C).

***Experimental scheme:***




A)
Pretest
Day 7
Day 14
Day 21
vehicle (saline solution)
DNBS 30 mg in EtOH 50%
EMG amplitude (mV.s):
% of increase from the baseline
Distending volume (mL)
**
B)
vehicle
DNBS - Day 7
DNBS - Day 14
DNBS - Day 21

C)

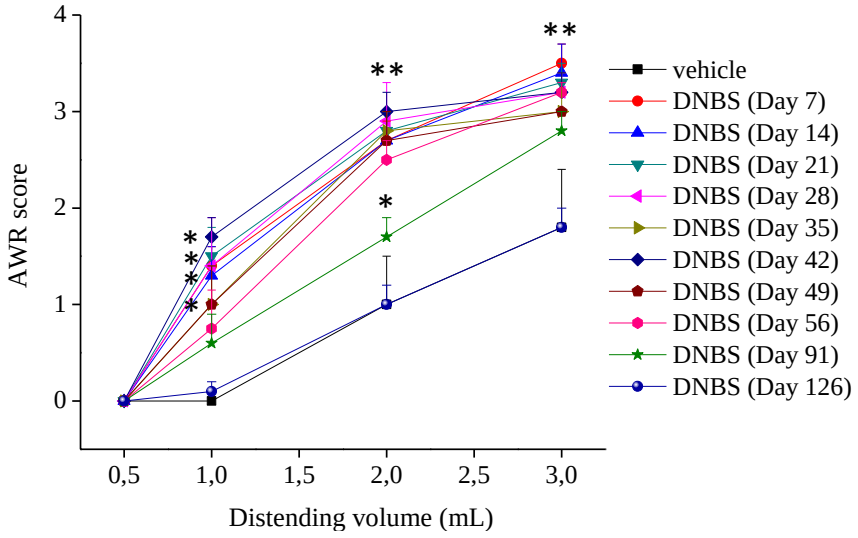



***Figure 1. Measurements of the visceral sensitivity in the animals after DNBS injection.*** *A) Viscero-motor response to colo-rectal distension; B) Viscero-motor response to rectal distension; C) Abdominal withdrawal reflex in response to colo-rectal distension. Each value represents the mean ± S.E.M. of 10 animals per group. *P < 0.05 and **P < 0.01 vs vehicle treated animals.*

### *1.2. Assessment of behavioural alteration in DNBS-treated animals*

The behavioural alterations frequently associated with the presence of spontaneous pain, as depression, anxiety and reduction of locomotor activity were investigated trhought the Forced Swim Test (FST), the Open Field Test (OFT) and the Elevated Plus Maze Test (EPMT). FST is used to highlight depressive-like behaviours in the animals. Control rats swim, struggle and climb the wall of the tank to stay afloat in the water. This behaviour was expressed both as the time the animals spent moving and as the distance travelled. DNBS-treated animals showed a decreased motility in comparison with controls on Day 7, 14 and 21, pointing out a depressive disorder in these animals (Fig 2A). OFT was performed to evaluate the locomotor activity and the explorative behaviour (expressed as distance travelled and wall rearings, respectively) of DNBS-treated animals in comparison to controls (Fig 2B). Both the distance travelled and the number of walls rearings performed by DNBS-treated animals were significantly lower than that of controls 7 days after damage induction. By contrast, no difference was relieved between the experimental groups on Day 14 and 21 (Fig 2B). EPMT was used to evaluate anxiety-like behaviour in the animals. DNBS-treated animals did not show significant

differences in the % of time spent in the open arms, in the closed one and in the centre with respect to the controls. In both the groups, the animals preferred to spend their time in the closed arms instead of the open arms (Fig 2C).

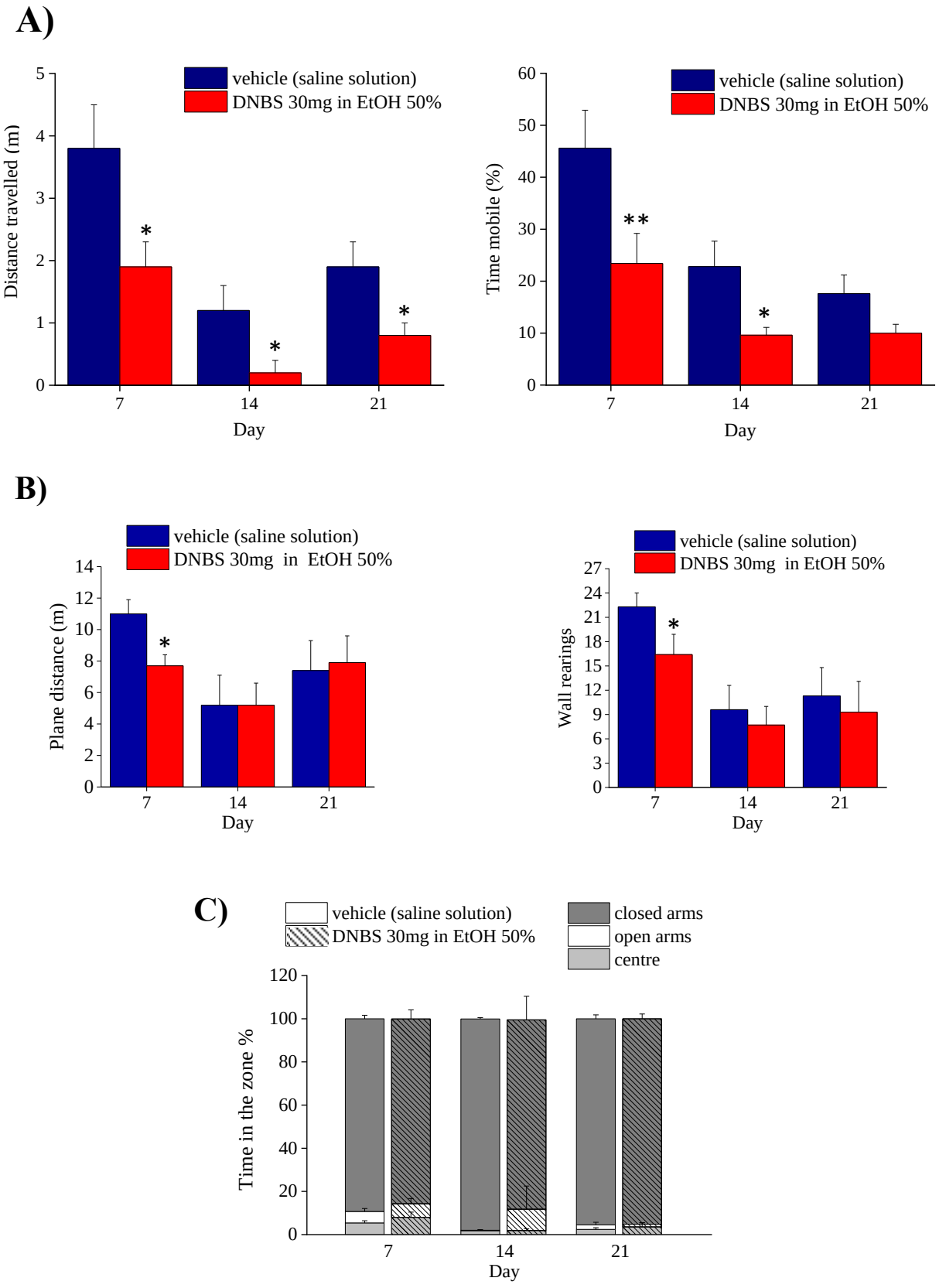


***Figure 2. Measurements of the depressive and anxiety related behaviours in DNBS-treated animals.*** *A) Forced Swim Test; B) Open Field Test; C) Elevated Plus Maze Test. Each value represents the mean ± S.E.M. of 10 animals per group. *P < 0.05 and **P < 0.01 vs vehicle treated animals.*

### *1.3. Histological assessment of colon damage*

The animals were sacrificed 3, 7, 14 and 21 days after DNBS injection and the colon was harvested and processed for both macroscopic and microscopic analysis (Figure 3A and 3B, respectively). The *Macroscopic Damage Score* was used to quantify the tissue damage degree (Fig. 3A). Macroscopically, the damage induced by DNBS showed a peak 3 days after the injection and progressively decreased between 7 and 21 days after DNBS injection. In fact, though the *Macroscopic Damage Score* of DNBS-treated animals on Day 21 resulted still significantly higher than that of controls, it appeared considerably lowered than that of Day 3 (Fig. 3A). The *Microscopic Damage Score* followed the trend of the *Macroscopic Damage Score* at the respective time points (3, 7, 14 and 21 days; Figure 3b and Figure 3C).

Colitis had a peak 3 days after DNBS injection, the colon appearing extensively inflamed, infiltrated and thickened with diffuse ulcerations and necrosis, and loss of its main layers. After 7 days from DNBS injection colonic samples still showed a considerable epithelial injury. The tissue largely recovered its primary structures, though there were many ulcers and areas with surface epithelium loss, transmural immune cell infiltration (predominantly neutrophils, lymphocytes, and MCs), crypt abscesses, altered goblet cells and oedema. At day 14 after DNBS treatment the colon appeared still significantly thick with inflammatory infiltration. The *tunica mucosa* resulted mostly restored apart from spot loss of epithelial surface, probably resulting from healing processes on previous deep ulcers. The crypts were elongated with irregular diameters and shapes. On day 21, although the thickening of the colonic wall persisted, the presence of inflammatory infiltrate was reduced and almost exclusively limited to the submucosa. No hyperplasia of the epithelial cells was detected, and the structure of the crypts was comparable to that of controls (Figure 3C).

On the base of these results, the optimal timing for studying persistent visceral hypersensitivity in the presence of significantly restored intestine morphology was established at day 14. The subsequent behavioural and histological evaluations on DNBS-treated animals were performed at this time point.

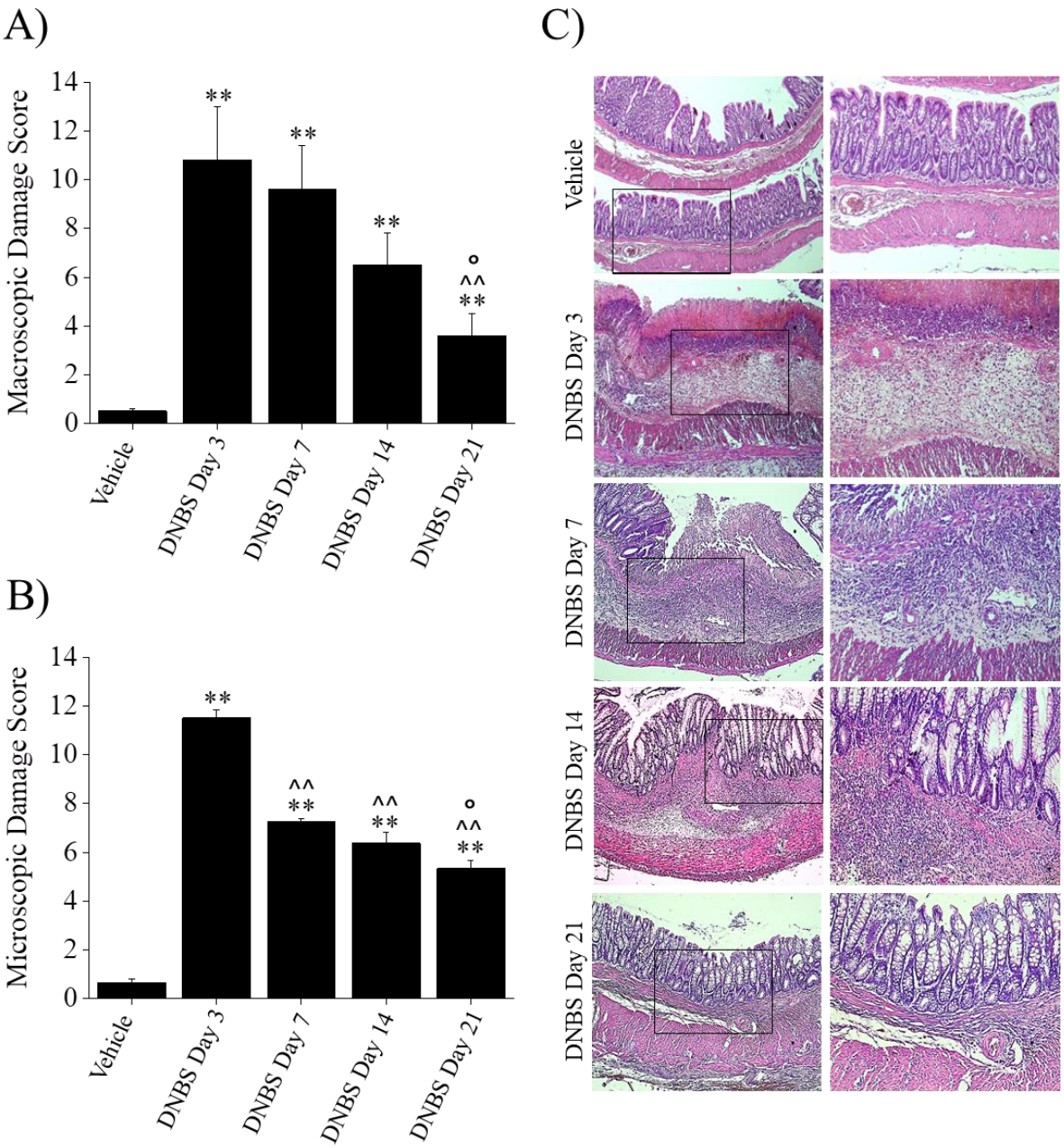
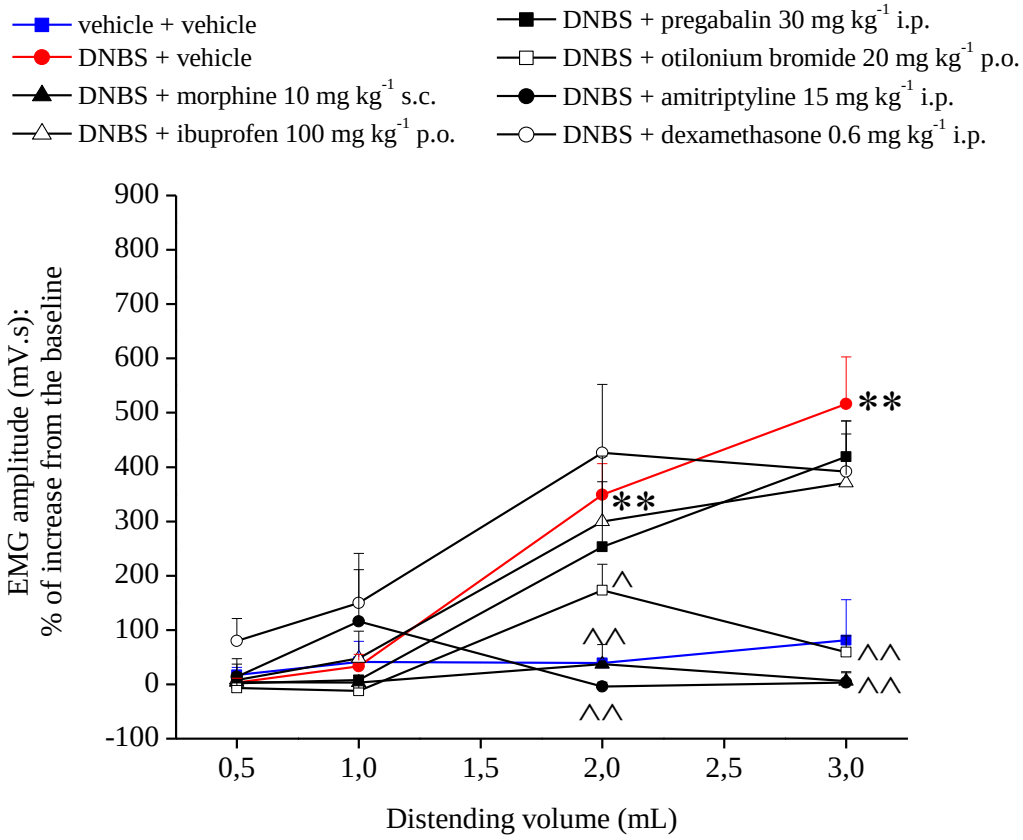



***Figure 3. Histological evaluation of colon damage after DNBS injection***. *A) Macroscopic damage score; B) Microscopic damage score. C) Representative pictures of haematoxylin/eosin-stained sections of full-thickness colon. Original magnification: 5x (left column), 10x (right column). Each value represents the mean ± S.E.M. of 4 animals per group. **P < 0.01 vs vehicle treated animals. ^^P<0.01 vs DNBS day 3 treated animals. °P<0.05 vs DNBS day 7 treated animals.*

### *1.4. Effect of the systemic administration of reference drugs*

To characterize the type of pain observed, we evaluated the effect of the acute systemic administration of different drugs on visceral hypersensitivity 7, 14 and 21 days after the injection of DNBS (Fig 4A, B and C respectively). Visceral sensitivity was assessed by measuring the viscero-motor response to the colorectal distension 15 min after compounds administration (Fig 4). The acute administration of morphine (5-10 mg $kg^{-1}$ s.c.) and amitriptyline (15 mg $kg^{-1}$ i.p.) completely suppressed the viscero-motor response to colorectal distension in DNBS-treated animals 7, 14 and 21 days after colitis induction (Fig 4A-C). Otilonium bromide (20 mg $kg^{-1}$ p.o.) significantly reduced visceral hypersensitivity induced by DNBS on day 7 (Fig 4A), while on day 14 and 21 the drug resulted less effective (Fig 4 B and C). On the contrary, pregabalin (30 mg $kg^{-1}$), which was ineffective in reducing visceral hypersensitivity on day 7 (Fig 4A), became progressively active starting from Day 14 (Fig 4 B and C). Ibuprofen (100 mg $kg^{-1}$ p.o.) was not able to significantly reduce visceral pain induced by DNBS (Fig 4A-C). In line with ibuprofen, also dexamethasone (0.6 mg $kg^{-1}$ i.p.) resulted ineffective (Fig 4A-C). The administration of vehicles did not elicit any effect in DNBS-treated animals (Fig 4A-C). Considering that there was no substantial difference between the effect of drugs on day 14 and 21, hereinafter we reported only the results gathered on Day 14, as representative of the post-inflammatory phase. Figure 5 shows the effect of the acute systemic administration of the same reference drugs on the abdominal withdrawal reflex in DNBS-treated animals. This measure allows to evaluate also the affective component of visceral pain, since it was performed in awake animals, unlike the EMG recording. Control animals showed a gradually response to the increasing stimuli and this response was significantly augmented in DNBS treated animals. The systemic administration of morphine was able to make DNBS-treated animals completely insensitive to the colorectal distension. In fact, the animals treated with morphine (10 mg $kg^{-1}$) showed a responsiveness even lower than that of controls. On the other hand, amitriptyline (15 mg $kg^{-1}$) and otilonium bromide (20 mg $kg^{-1}$) conserved their efficacy on visceral hypersensitivity without affecting the normal intestinal sensitivity. Dexamethasone (0.6 mg $kg^{-1}$ i.p.) resulted ineffective also in this case while ibuprophen (100 mg $kg^{-1}$) became partially effective. By contrast pregabalin (30 mg $kg^{-1}$) lost some of its effectiveness on visceral pain in conscious animals (Fig 5).

A)


vehicle + vehicle
DNBS + vehicle
DNBS + morphine 10 mg kg-1 s.c.
DNBS + ibuprofen 100 mg kg-1 p.o.
DNBS + pregabalin 30 mg kg-1 i.p.
DNBS + otilonium bromide 20 mg kg-1 p.o.
DNBS + amitriptyline 15 mg kg-1 i.p.
DNBS + dexamethasone 0.6 mg kg-1 i.p.
EMG amplitude (mV.s):
% of increase from the baseline
900
800
700
600
500
400
300
200
100
0
-100
0,5
1,0
1,5
2,0
2,5
3,0
Distending volume (mL)
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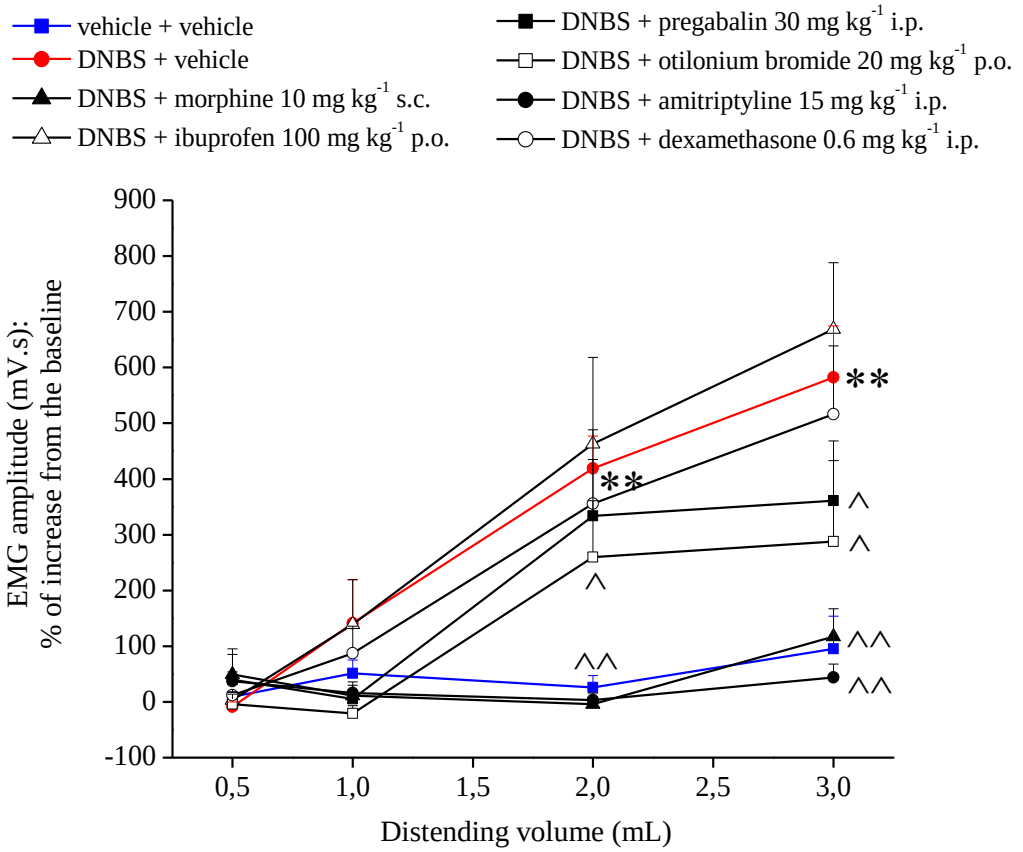
B)


vehicle + vehicle
DNBS + vehicle
DNBS + morphine 10 mg kg-1 s.c.
DNBS + ibuprofen 100 mg kg-1 p.o.
DNBS + pregabalin 30 mg kg-1 i.p.
DNBS + otilonium bromide 20 mg kg-1 p.o.
DNBS + amitriptyline 15 mg kg-1 i.p.
DNBS + dexamethasone 0.6 mg kg-1 i.p.
EMG amplitude (mV.s):
% of increase from the baseline
900
800
700
600
500
400
300
200
100
0
-100
0,5
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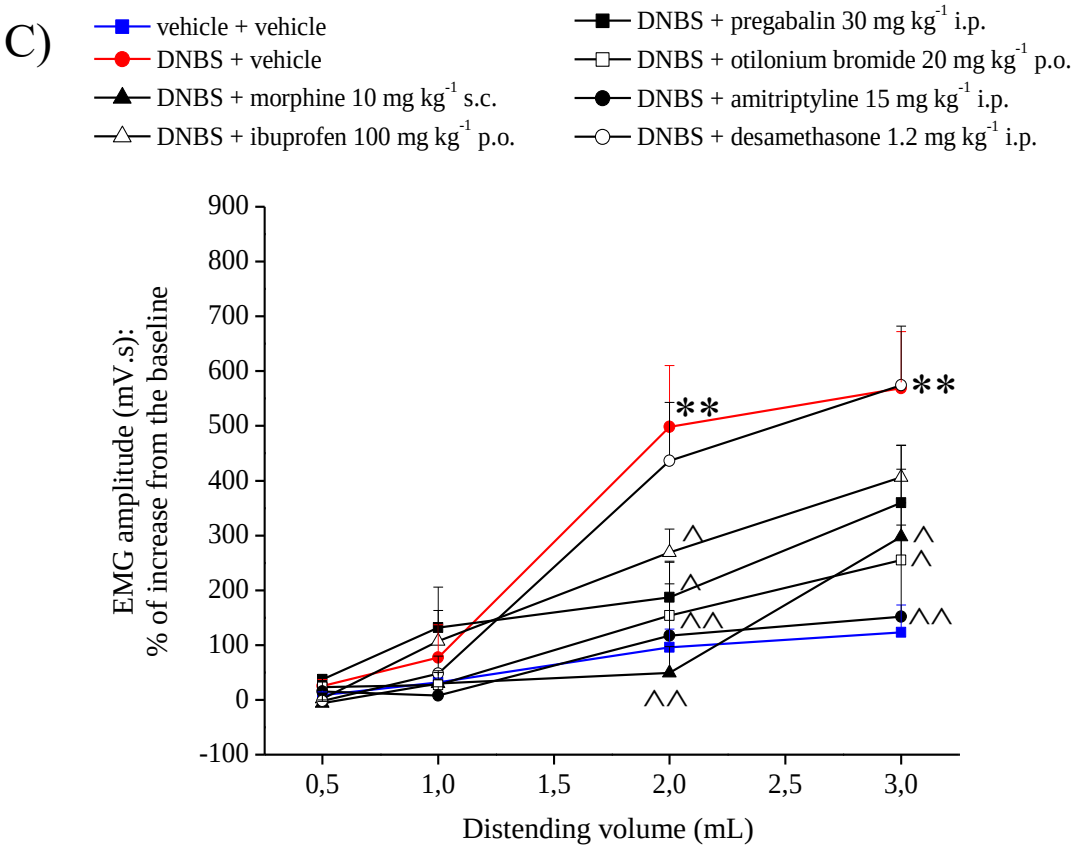



***Figure 4. Effect of the systemic drugs administration on viscero-motor response to colo-rectal distension.*** *The tests were performed 7(A), 14(B) and 21(C) days after DNBS injection. All the drugs were administered 15 min before starting the test. Each value represents the mean ± S.E.M. of 5 animals per group performed in 2 experimental sets. *P < 0.05 and **P < 0.01 vs vehicle + vehicle treated animals. ^P<0.05 and ^^P<0.01 vs DNBS + vehicle.*

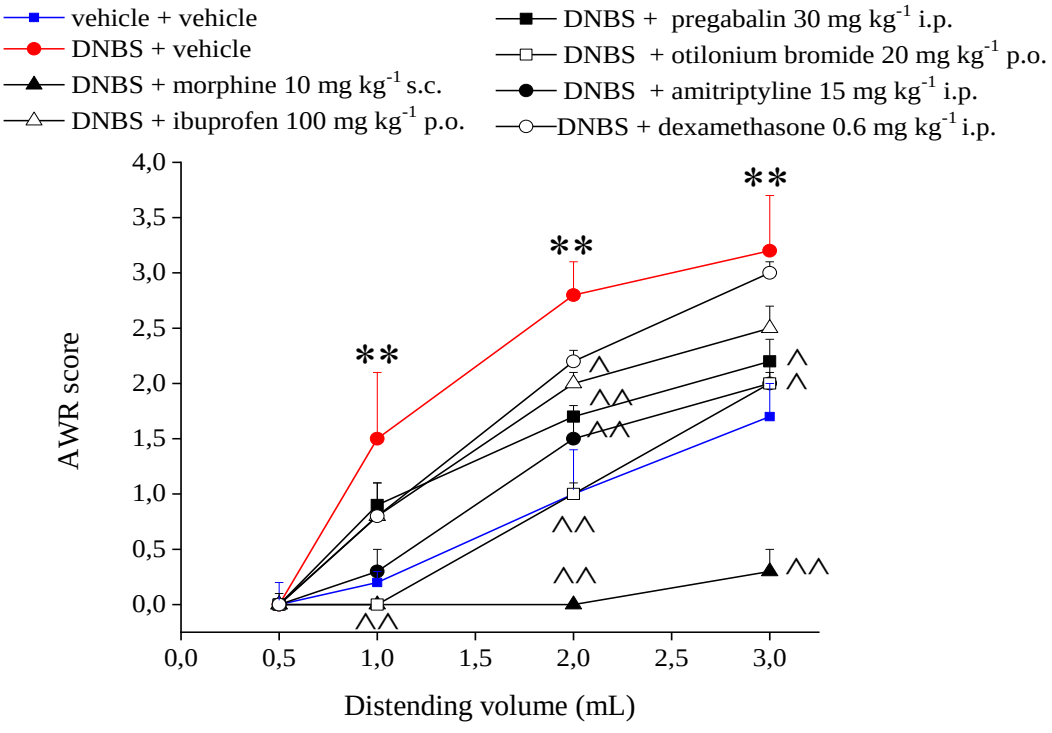


***Figure 5. Effect of the systemic drugs administration on abdominal withdrawal reflex in response to colo-rectal distension.*** *The tests were performed 14 days after DNBS injection. All the drugs were administered 15 min before starting the test. Each value represents the mean ± S.E.M. of 5 animals per group performed in 2 experimental sets. *P < 0.05 and **P < 0.01 vs vehicle + vehicle treated animals. ^P<0.05 and ^^P<0.01 vs DNBS + vehicle.*

### *1.5. Effect of the intrathecal administration of reference drugs*

In Figure 6 it is shown the effect of the intrathecal administration of morphine, amitriptyline and pregabalin on both the viscero-motor response (Fig 6A) and the abdominal withdrawal reflex (Fig 6B) to colorectal distension in DNBS-treated animals. The intrathecal administration of pregabalin (100 µg) strongly reduced the viscero-motor response to colorectal distension in the animals under anaesthesia (Figure 6A), while resulted ineffective in reducing visceral pain in awake animals (Fig 6B). Amitriptyline (60 µg), intrathecally administered, showed a partial effect on the abdominal withdrawal response of animals to colo-rectal distension (Fig 6B) and no significant effects on their viscero-motor response (Fig 6A). Likewise, morphine (1 µg i.t.) significantly reduced the abdominal withdrawal reflex in DNBS-treated animals, without showing any effect on the viscero motor response to colorectal distension in the same animals under anaesthesia.

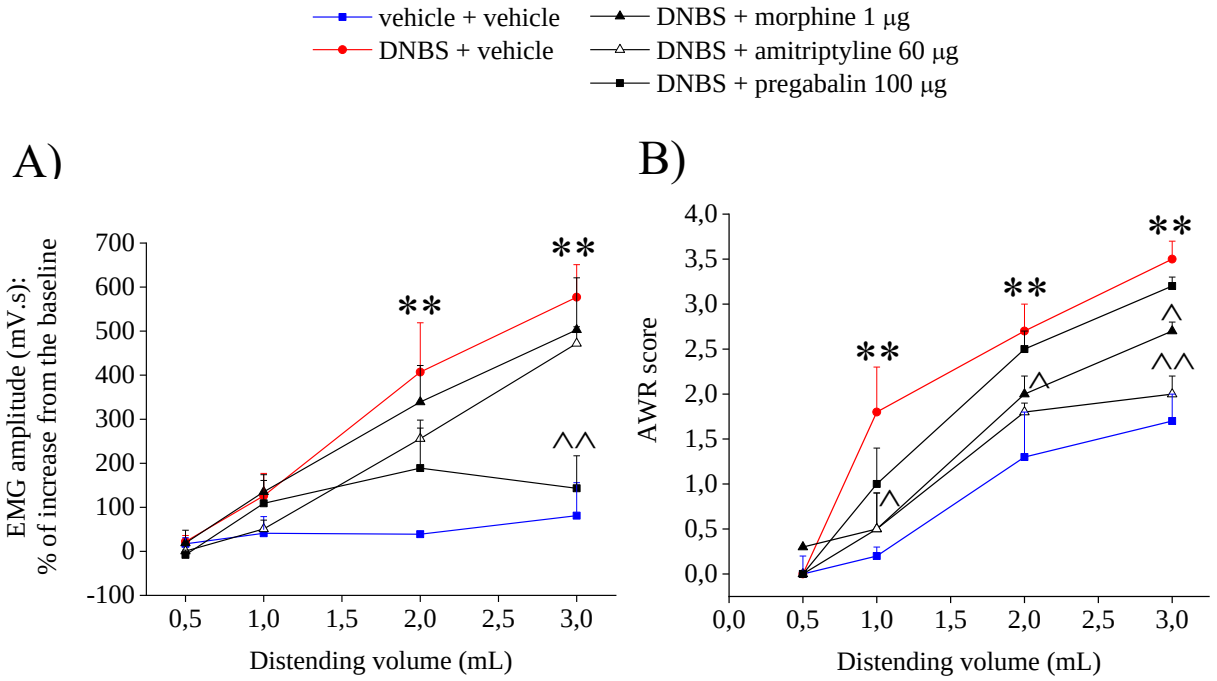



***Figure 6. Effect of the intrathecal drugs administration on visceral hypersensitive induced by DNBS in the animals.*** *The effect of the drugs was evaluated on both the viscero-motor response (A) and the abdominal withdrawal reflex (B) to colo-rectal distension. The tests were performed 14 days*

*after DNBS injection. All the drugs were administered 15 min before starting the test. Each value represents the mean ± S.E.M. of 5 animals per group performed in 2 experimental sets. *P < 0.05 and **P < 0.01 vs vehicle + vehicle treated animals. ^P<0.05 and ^^P<0.01 vs DNBS + vehicle.*

### *1.6. Histological evaluation of inflammatory cells, fibrosis and SP-immunostained fibres*

At day 14 from DNBS treatment the partial restitution of the tunica mucosa was associated with inflammatory cell infiltration and significant transmural deposition of SR-positive collagen fibres (Fig 7A). MCs, occasionally found in controls along the colonic submucosal vessels and rarely within the tunica muscularis, were significantly increased in density in the whole wall of colon from DNBS-treated rats (Fig 7B). Eosinophil density significantly rose throughout the inflamed colonic wall, as compared with the low amount of eosinophils registered at mucosal and submucosal levels in controls (Fig 8). Several eosinophils (Figure 8) and macrophages expressing MHC-II antigen (Fig 9) were found in close proximity with SP-immunostained fibres in the lamina propria and tunica muscularis, where the SP-fibres resulted upregulated and mainly localized in the myenteric ganglia and circular layer of DNBS colon, compared to controls (Fig 8).

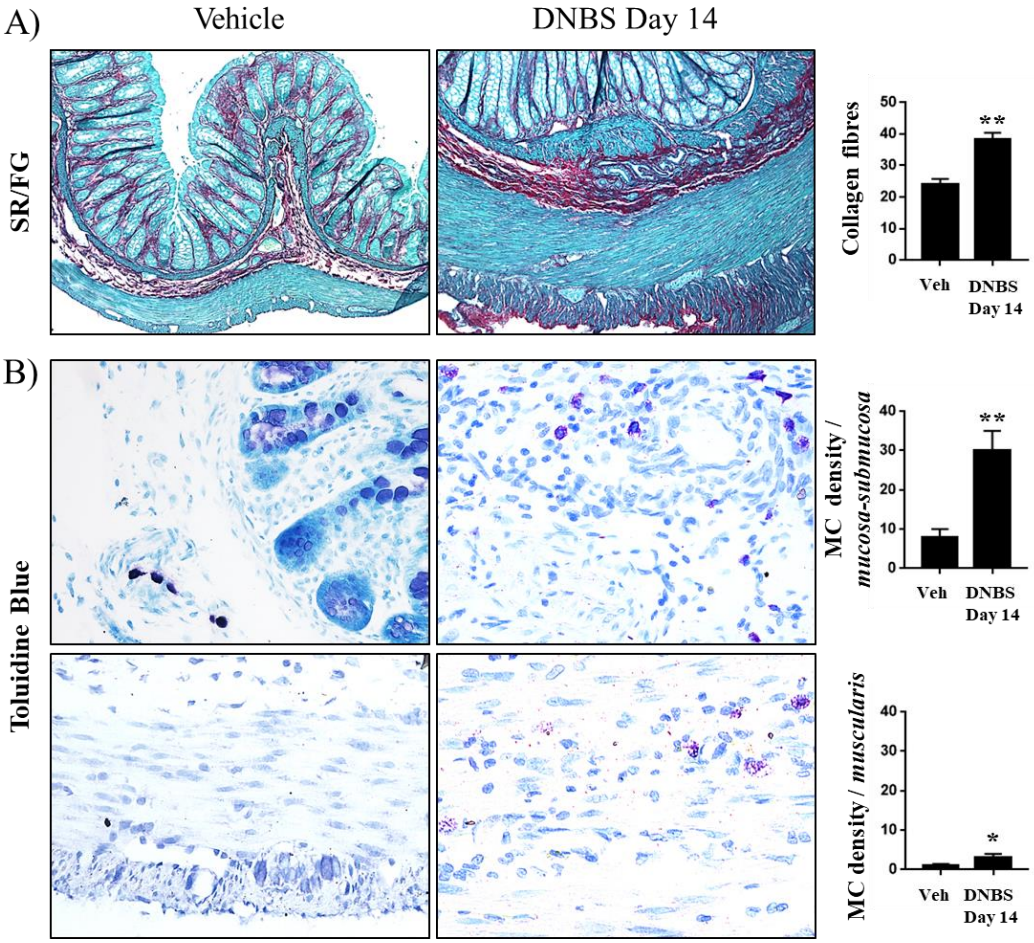


***Figure 7. Histologic evaluation of colonic fibrosis and mast cell (MC) infiltration***

*Representative pictures of (A) Sirius Red-Fast Green (SR/FG)-, and (B) toluidine blue-stained colonic sections obtained from controls or animals treated with DNBS at day 14. The column graphs display the mean values of the positive pixels percentage (PPP) of SR-stained fibres, and MC density per mm$^2$ of colonic areas (cells/mm$^2$) ± S.E.M. obtained from 4 animals for each group. *P≤0.05, **P≤0.01 versus vehicle-treated animals. Original magnification: 20x (a), 100x (b).*

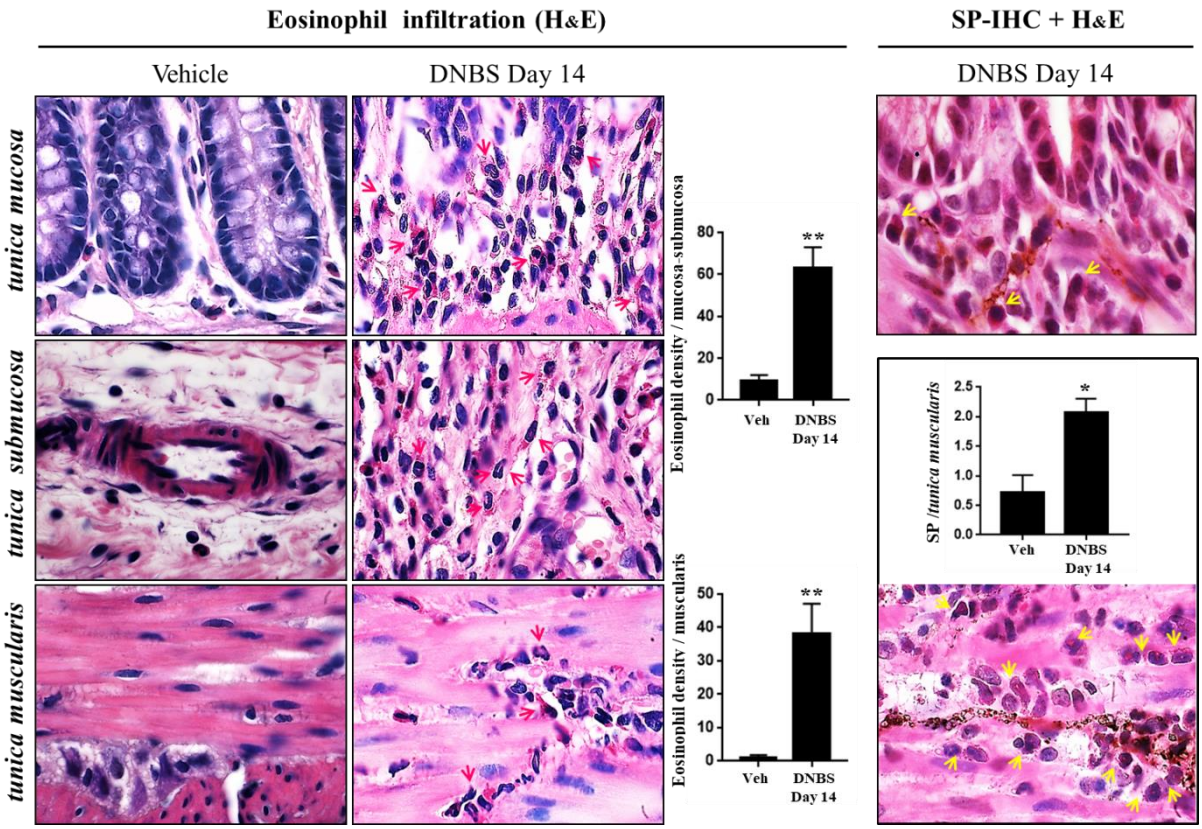



***Figure 8. Eosinophil infiltration of the colonic wall in close contiguity with SP-positive fibres***

*Representative pictures of colonic sections stained with haematoxylin/eosin and SP immunoperoxidase. The right panel shows pictures captured from tunica mucosa (up) and muscularis (down). The column graphs display the mean values of eosinophil density per mm$^2$ of colonic wall areas (cells/mm$^2$), and positive pixels percentage (PPP) of SP-reactive fibres ± S.E.M. obtained from 4 animals for each group. *P≤0.05, **P≤0.01, versus vehicle-treated animals. Original magnification: 100x.*

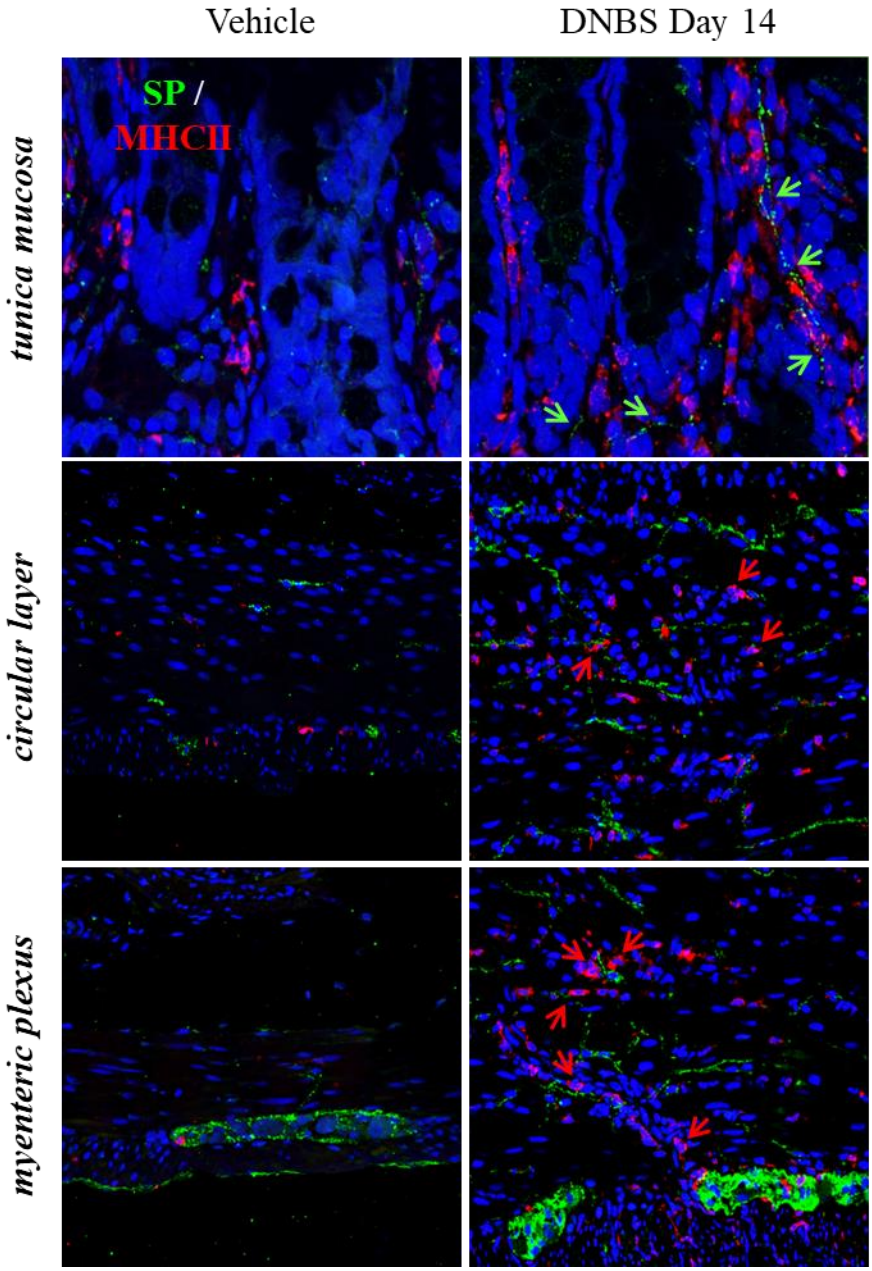



***Figure 9. MHC-II cell infiltration of the colonic wall along with SP-immunostained fibres***

*Confocal microscopy representative images of SP/MCHII double-immunolabelled colon from control rats and inflamed rats (n=4 animals for each group). Original magnification: 80x (tunica mucosa), 40x (tunica muscularis).*

### *1.7. Evaluation of glial activation in the spinal cord*

Fourteen days after DNBS injection the animals were sacrificed and the morphology and the number of astrocytes and microglial cells at spinal level was evaluated by performing an immunofluorescent analysis on lumbo-spinal sections (Fig 10 and 11). Both the dorsal and the ventral horn (Fig 10B and 11B, respectively) of DNBS treated animals were characterized by astrocyte (GFAP-positive cells) activation, highlighted by the significant increase in number (about 30%) as well as by the expansion of cellular body and processes, denoting their typical activated status (Di Cesare Mannelli et al., 2013), in comparison to control. On the other hand, microglia (Iba1-positive cells) did not change in number but underwent well-defined morphological alterations, detectable on both the dorsal and the ventral horn (Fig. 10A and 11A). The percentage of morphological activated Iba-1 positive cells was significantly increased in DNBS-treated animals in comparison to control. The activated status of microglial cells was recognized by the loss of the processes that are peculiar of resting conditions (Fernández-Arjona et al., 2017).

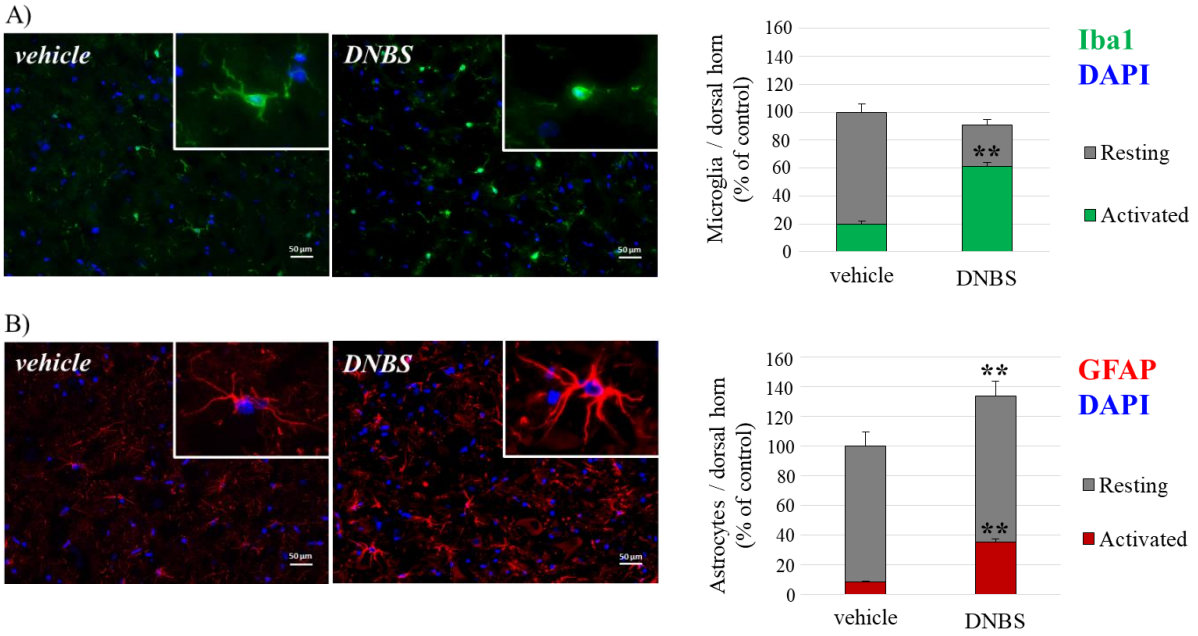



***Figure 10. Evaluation of microglia and astrocytes activation in the dorsal horn of spinal cord.*** *A) Iba1-positive cell density in the dorsal horn of the spinal cord of animals 14 days after DNBS injection; Representative images of merged Iba1-labeled microglia cells (red), plus DAPI-labeled cell nuclei (blue), scale bar: 50 µm. B) GFAP-positive cell density in the dorsal horn of the spinal cord of animals 14 days after DNBS injection; Representative images of merged GFAP-labeled microglia cells (red), plus DAPI-labeled cell nuclei (blue), scale bar: 50 µm. Each value represents the mean ± S.E.M. of 4 rats, performed by analysing 4 independent fields for both the sides of the spinal cord. **P<0.01 vs control animals.*

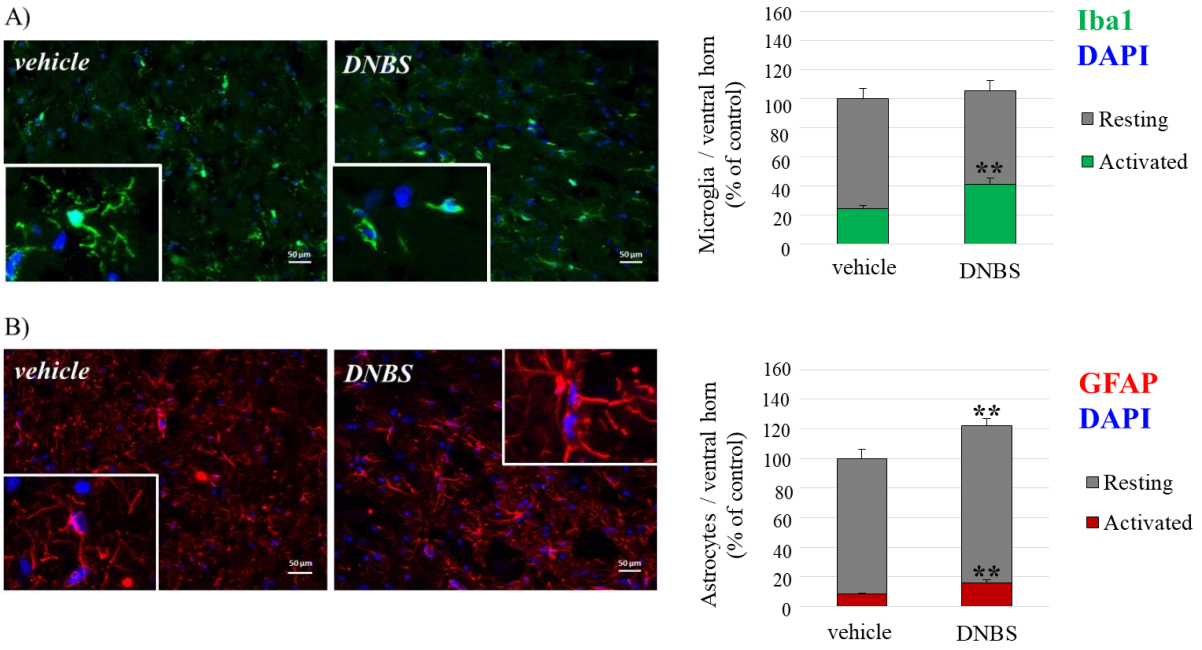



***Figure 11. Evaluation of microglia and astrocytes activation in the ventral horn of spinal cord.*** *A) Iba1-positive cell density in the ventral horn of the spinal cord of animals 14 days after DNBS injection; Representative images of merged Iba1-labeled microglia cells (red), plus DAPI-labeled cell nuclei (blue), scale bar: 50 µm. B) GFAP-positive cell density in the ventral horn of the spinal cord of animals 14 days after DNBS injection; Representative images of merged GFAP-labeled microglia cells (red), plus DAPI-labeled cell nuclei (blue), scale bar: 50 µm. Each value represents the mean ± S.E.M. of 4 rats, performed by analysing 4 independent fields for both the sides of the spinal cord. **P<0.01 vs control animals.*

## 2. *Evaluation of the efficacy of adenosine $A_3$ receptor agonists on post-inflammatory visceral pain induced by DNBS*

### *2.1. Efficacy of adenosine $A_3$AR agonist MRS5980 against visceral hypersensitivity induced by DNBS*

The viscero-motor and the abdominal withdrawal responses (VMR and AWR) to the progressive increase in colorectal distension (CRD) were measured using increasing filling volumes (highest volume: 3 ml, to avoid tissue damage). Fourteen and 21 days after DNBS injection, VMR was significantly higher in comparison to controls (vehicle + vehicle; Fig 12A and 12C) starting from 2 ml whereas AWR was already altered by 1 ml (Fig 10B and 12D). The effects of the acute i.p. administration of MRS5980 (0.3, 1.2, 2.4 µmol $kg^{-1}$) were evaluated (Fig 12). On day 14, MRS5980 dose-dependently reduced the post-inflammatory visceral hypersensitivity induced by DNBS; the magnitude of the reduction was similar for VMR and AWR. The highest dose (2.4 µmol $kg^{-1}$) completely reversed the sensitivity

alteration back to the value of control rats. MRS5980 1.2 µmol $kg^{-1}$ partially, but significantly reduced the response of the animals to CRD. The lowest dose of MRS5980 (0.3 µmol $kg^{-1}$) was ineffective in both tests (Fig 12A and 12B). On day 21, the effect of MRS5980 (0.3, 1.2, 2.4 µmol $kg^{-1}$) was confirmed (VMR and AWR to CRD, Figures 10C and 10D, respectively). Unlike VMR, the assessment of AWR allows the evaluation of DNBS-induced hypersensitivity for a long time. This behavioural response was still altered 28 and 35 days after the colonic irritation (Fig 13A and 13B, respectively), reflecting the peculiar tendency of this type of pain to become chronic. Hence, we used this test to evaluate the effect of MRS5980 on visceral pain in a more delayed phase after the initial insult. The chronic hypersensitivity in the rat is likely to be similar to the clinical condition. On Day 28 and 35, the pain-relieving effect of MRS5980 (0.3, 1.2, 2.4 µmol $kg^{-1}$) was as potent as that seen earlier. The effect on chronic hypersensitivity was dose-dependent, and with the AWR measure, the highest dose (2.4 µmol $kg^{-1}$) was again able to reverse the pain threshold of DNBS-treated animals to the value of controls (Fig 13A and 13B).

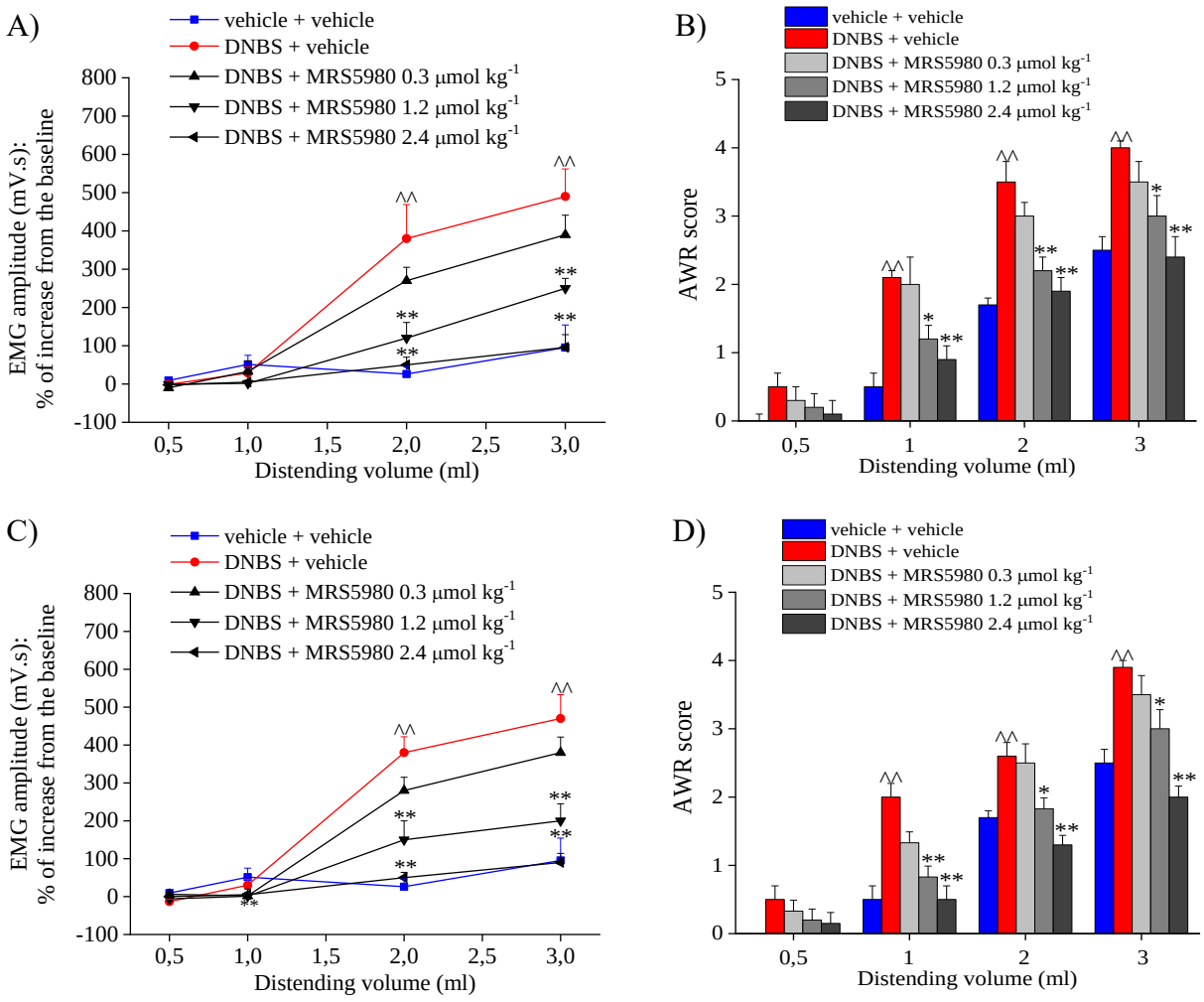


***Figure 12. Effect of MRS5980 on post-inflammatory visceral pain induced by DNBS**. Effect of MRS5980 (0.3, 1.2, and 2.4 µmol kg$^{-1}$; i.p.) on viscero-motor response (VMR) to CRD (left column) and abdominal withdrawal response (right column) on day 14 (A, B) and day 21 (C, D) after DNBS-induced colonic inflammation. Each value is the mean ± S.E.M. of 5 rats per group. ^^P < 0.01 vs. vehicle-treated normal controls (blue). *P < 0.05 and **P < 0.01 vs DNBS + vehicle treated group (red).*

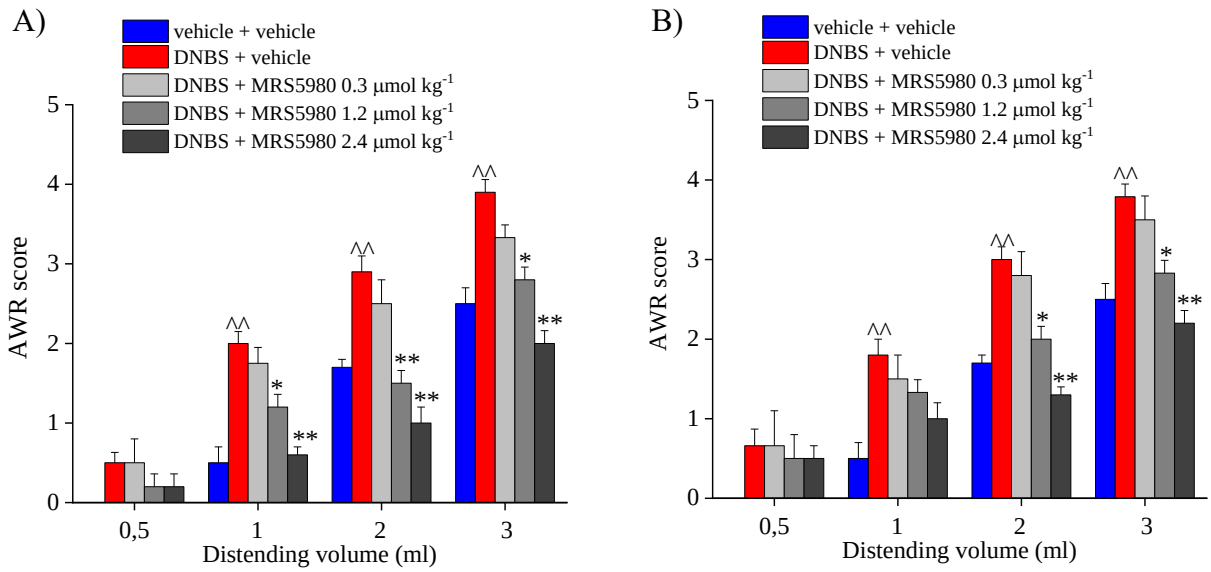



***Figure 13. Effect of MRS5980 on persistent visceral pain induced by DNBS injection**. Effect of MRS5980 (0.3, 1.2, and 2.4 µmol kg$^{-1}$; i.p.) was observed 28 days (A) and 35 days (B) after DNBS treatment. The compound was administered 15 min before the first colo-rectal distension (CRD). Visceral pain was assessed by measuring the animal abdominal withdrawal response (AWR) to CRD. Each value is the mean ± S.E.M. of 5 rats per group. ^^P < 0.01 vs. vehicle-treated normal controls (blue). *P < 0.05 and **P < 0.01 vs DNBS + vehicle treated group (red).*

### *2.2. Involvement of $A_3$A Receptor in the MRS5980 effect on visceral hypersensitivity*

Figure 14A shows the result of the pre-treatment with the selective $A_3$AR antagonist MRS1523 on the anti-hyperalgesic effect of MRS5980 on day 14. MRS1523 (5 µmol kg$^{-1}$) completely abolished the pain-relieving effect of MRS5980 (2.4 µmol kg$^{-1}$). Furthermore, the pain-relieving effect evoked by $A_3$AR stimulation was confirmed by using another selective $A_3$AR agonist, Cl-IB-MECA. On day 14, the acute administration of Cl-IB-MECA dose-dependently relieved visceral pain in DNBS treated animals, reducing their VMR to the value of controls (dosed at 1.2 µmol kg$^{-1}$, Fig 14B). The lower dose of 0.3 µmol kg$^{-1}$ evoked a

weaker effect only with the 3 ml stimulus. The Cl-IB-MECA (1.2 µmol $kg^{-1}$) effect was blocked by MRS1523 (5 µmol $kg^{-1}$) (Fig 14B).

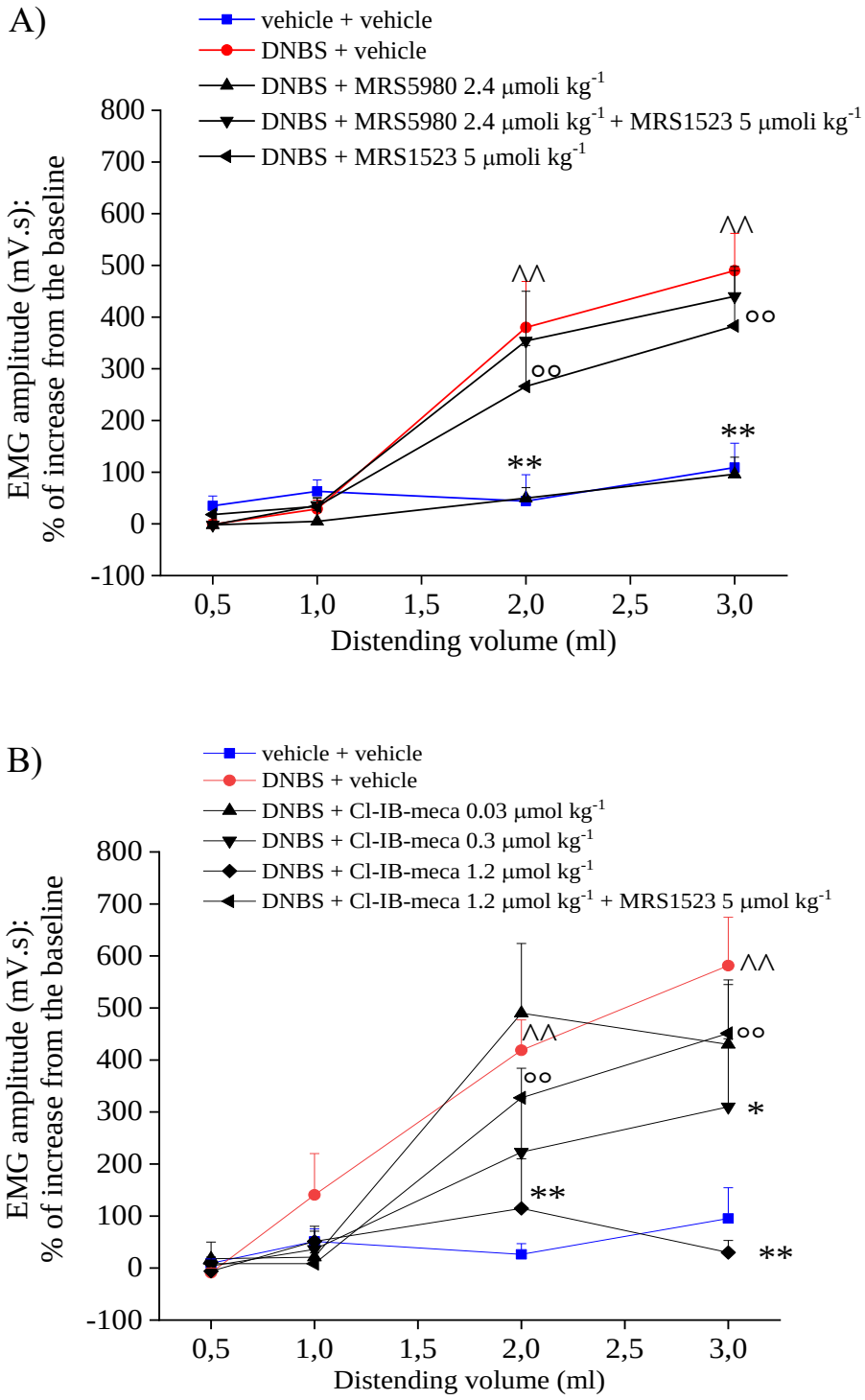



***Figure 14. Evaluation of the role of adenosine $A_3$ARs in visceral pain relief and comparison with the effect of the reference compound.*** *Tests were performed 14 days after DNBS treatment by measuring the viscero-motor response (VMR) to colo-rectal distension (CRD) after compound*

*administration. (A) Effect of pre-treatment with the selective $A_3$ antagonist MRS1523 (5 µmol kg$^{-1}$) on the visceral pain relieving effect of the highly-selective $A_3$AR agonist, MRS5980 (5 µmol kg$^{-1}$). (B) Effect of the selective $A_3$ agonist, Cl-IB-MECA (0.03, 0.3, and 2.4 µmol kg$^{-1}$; i.p.). Each value is the mean ± S.E.M. of 5 rats per group. ^^P < 0.01 vs. vehicle-treated normal controls (blue). *P < 0.05 and **P < 0.01 vs DNBS + vehicle treated group (red). °°P < 0.01 vs DNBS + MRS 5980 (2.4 µmol kg$^{-1}$, black triangles in A) or Cl-IB-MECA (1.2 µmol kg$^{-1}$, black triangles in B).*

### *2.3. Effect of N-type voltage-gated $Ca^{2+}$ channel blocking on visceral pain induced by DNBS*

$A_3$AR agonists have been recently reported to inhibit $Ca_v2.2$-mediated currents in DRG neurons, thus suggesting a possible mechanism for pain relief (Coppi et al., 2018). On this basis, we evaluated the effect of acute administration of the selective N-type $Ca_v2.2$ blocker PD173212 (Hu et al., 1999) in our visceral pain model. The test (VMR assessment) was performed on day 14 after DNBS injection. As shown in Figure 3C, PD173212 0.001-1 mg kg$^{-1}$ (0.0017 – 1.7 µmol kg$^{-1}$, i.p.) dose-dependently reduced the visceral hypersensitivity induced by DNBS. The compound's effect was first apparent at a dose of 0.01 mg kg$^{-1}$ (0.017 µmol kg$^{-1}$) and completely relieved abdominal pain when administered at a ten-fold higher dose (Fig 15).

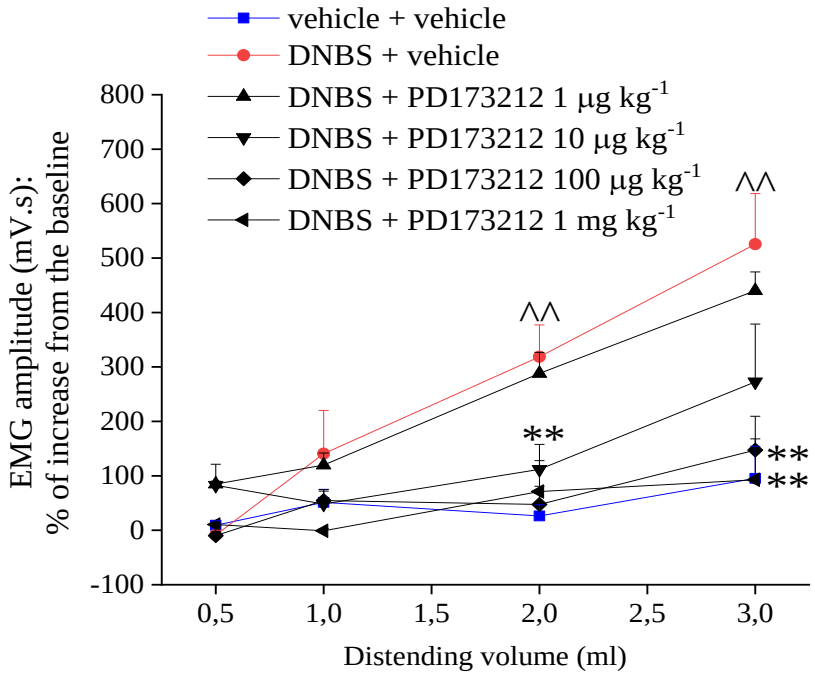



***Figure 15. Effect of blocking N-type voltage-gated calcium channels on post-inflammatory visceral pain.*** *Tests were performed 14 days after DNBS treatment by measuring the viscero-motor response*

*(VMR) to colo-rectal distension (CRD) after the administration of the selective N-type voltage-gated calcium channel ($Ca_v2.2$) blocker, PD173212 (1-1000 µg $kg^{-1}$). Each value is the mean ± S.E.M. of 5 rats per group. ^^P < 0.01 vs. vehicle-treated normal controls (blue). *P < 0.05 and **P < 0.01 vs DNBS + vehicle treated group (red).*

### *2.4. Comparative effect of the clinically-used compound linaclotide in the DNBS model*

The effects induced by MRS5980 against the post-inflammatory hypersensitivity induced by DNBS were compared with the effects of linaclotide, the reference drug in the management of pain in IBS-C patients. On day 14, 0.2 and 0.066 nmol $kg^{-1}$ (0.3 and 0.1 µg $kg^{-1}$, respectively) linaclotide, strongly reduced the VMR to CRD in DNBS-treated animals, while the ten-fold lower dose was ineffective (Fig 16).

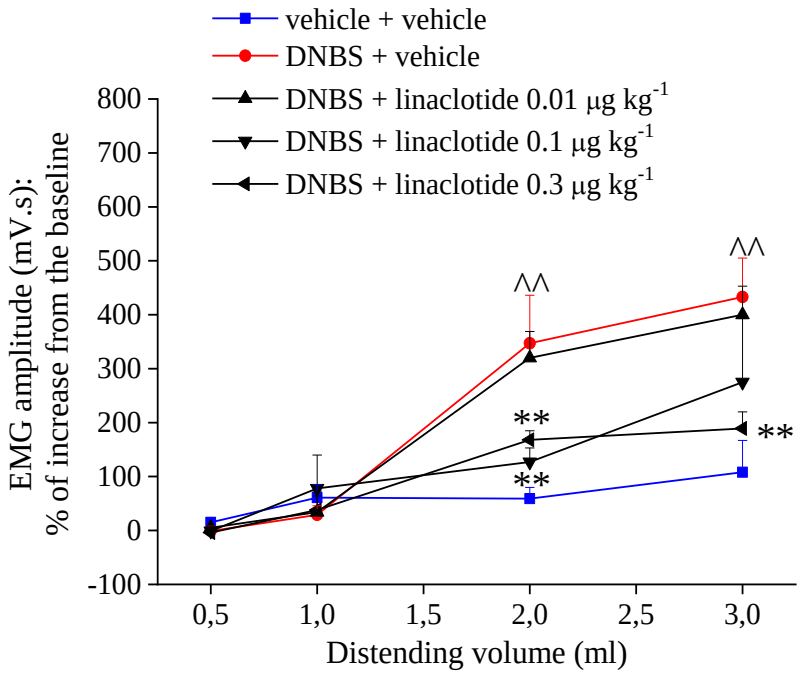



***Figure 16. Effect of the reference drug linaclotide on post-inflammatory visceral pain.*** *Tests were performed 14 days after DNBS treatment by measuring the viscero-motor response (VMR) to colo-rectal distension (CRD) after the administration of linaclotide (0.01- 0.3 µg $kg^{-1}$). Each value is the mean ± S.E.M. of 5 rats per group. ^^P < 0.01 vs. vehicle-treated normal controls (blue). *P < 0.05 and **P < 0.01 vs DNBS + vehicle treated group (red).*

### *2.5. Effect of adenosine $A_3R$ agonists on N-type voltage-gated $Ca^{2+}$ channels in DRG neurons isolated from control or DNBS-treated rats (Data not shown)*

Thanks to the collaboration with Dr. Elisabetta Coppi (Dept. of Neurofarba, University of Florence), we gained insight into mechanisms underlying the anti-hyperalgesic role of

$A_3$ARs, by exploring electrophysiological properties and Cl-IB-MECA effects in DRG primary sensory neurons isolated from vehicle-injected (control group) or DNBS-treated rats (14 days after treatment; data not shown).

Whole-cell patch-clamp recordings were performed as previously described (Coppi et al., 2018; Coppi et al., 2012). Briefly, cells were transferred to a 1 ml recording chamber mounted on the platform of an inverted microscope (Olympus CKX41, Milan, Italy) and superfused at a flow rate of 1.5 ml/min by a three-way perfusion valve controller (Harvard Apparatus) by the following extracellular solution (mM): NaCl 147; KCl 4; $MgCl_2$ 1; $CaCl_2$ 5; HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) 10; D-glucose 10 (pH 7.4 with NaOH). Borosilicate glass electrodes (Harvard Apparatus, Holliston, MA) were pulled with a Sutter Instruments puller (model P-87) to a final tip resistance of 1.5–3 ΩM. Passive membrane properties of DRG neurons isolated from control or DNBS-treated rats were investigated under physiological-like conditions by using the following K-gluconate- based pipette solution (mM): KGlu 130; NaCl 4.8; KCl 10; $MgCl_2$ 2; $CaCl_2$ 1; $Na_2$-ATP 2; $Na_2$-GTP 0.3; EGTA 3; HEPES 10 (pH 7.4 with KOH). Resting membrane potential (Vm) was measured immediately after seal break-through by switching the amplifier to the current-clamp mode. The calculated liquid junction potential for K-gluconate pipettes in our experimental conditions was 15.0 mV and Vm values reported in the present research have been corrected accordingly. Voltage-dependent $Ca^{2+}$ currents (VDCCs) were recorded by using a $Cs^+$-based pipette solution of the following composition (mM): CsCl (130); NaCl (4.8); KCl (10); $MgCl_2$ (2); $CaCl_2$ (1); $Na_2$-ATP (2); $Na_2$-GTP (0.3); EGTA (3); and HEPES (10 - pH 7.4 with CsOH). The extracellular solution was (in mM): NaCl (147); CsCl (4); $MgCl_2$ (1); and $CaCl_2$ (5); HEPES (10); D-glucose (10); pH 7.4 with NaOH. Tetrodotoxin (TTX; 1 μM) and 5-(4-butoxy-3-chlorophenyl)-N-[[2-(4-morpholinyl)-3-pyridinyl]methyl]-3-pyridinecarboxamide (A887826; 200 nM) were added to the extracellular solution to block TTX-sensitive $Na_v$1.1, 1.2, 1.3, 1.4, 1.6, 1.7 channels and TTX-resistant $Na_v$1.8, respectively. VDCC currents were evoked by a 0 mV step depolarization (200 ms) once every 30 s to minimize $Ca^{2+}$ current run down. The current-to-voltage relationship (I-V plot) of $Ca^{2+}$ currents was obtained by eliciting 10 depolarizing voltage steps (200 msec duration, 10 mV increments, 5 s interval) from -50 to +50 mV starting from a holding potential (Vh) of -65 mV. Data were acquired with an Axopatch 200B amplifier (Axon Instruments, CA), low-pass filtered at 10 kHz, and stored and analysed with pClamp 9.2 software (Axon Instruments, CA). Membrane resistance (Rm), and membrane capacitance (Cm) were routinely measured by fast hyperpolarizing voltage pulses (from -60 to -70 mV, 40 ms duration). Averaged currents were normalized to cell

capacitance and expressed as pA/pF.Cell capacitance was used to estimate neuronal diameter by assuming an approximated spherical cell shape according to the calculated Cm for all biological membranes of 1 µF/cm$^2$ and to the equation of the sphere surface: $A=4\pi r^2$ (Hille, 2001).

Concerning passive membrane properties, DNBS neurons presented a more depolarized Vm in comparison to those from the vehicle-treated animals (from - 61.4 ± 2.2 mV in control to -52.0 ± 2.6 mV in DNBS group, n=23 and n=33 respectively, P=0.0143, unpaired Student's *t*-test), whereas no obvious differences were found in Rm or Cm. Of note, VDCCs, activated by a depolarizing voltage step protocol (from -50 to +40 mV, 200 ms), showed some differences in DNBS-treated versus control rats. Although if total peak currents were unchanged, either in amplitude or time to peak, $Ca^{2+}$ currents measured at the steady-state in DNBS neurons were significantly smaller in amplitude (from -21.5 ± 8.1 pA/pF in control to -1.4 ± 4.0 pA/pF in DNBS group, n=15 and n=14 respectively, P=0.0393, unpaired Student's *t*-test). Consistent with our previous work (Coppi et al., 2019), we confirmed that Cl-IB-MECA (30 nM) inhibited PD173232-sensitive N-type $Ca^{2+}$ currents in DRG neurons isolated either from control or DNBS-treated rats and the effect was blocked by the $A_3$AR antagonist, MRS1523 (100 nM). No difference was found in the percentage of Cl-IB-MECA-inhibited $Ca^{2+}$ current in control or DNBS neurons (from 20.0 ± 4.1 to 22.5 ± 6.1 % inhibition, n=7 in both groups; *P*=0.7332 unpaired Student's *t*-test). Similar results were obtained in the presence of the newly synthetized highly-selective $A_3$AR agonist, MRS5980 (30 nM): the compound inhibited, to a similar extent, $Ca^{2+}$ currents either in control or DNBS neurons and the effect was prevented by MRS1523 and by the N-type channel blocker, PD173232 (1 µM).

## *3. Study of the role of gut microbiota in post-inflammatory visceral pain induced by DNBS*

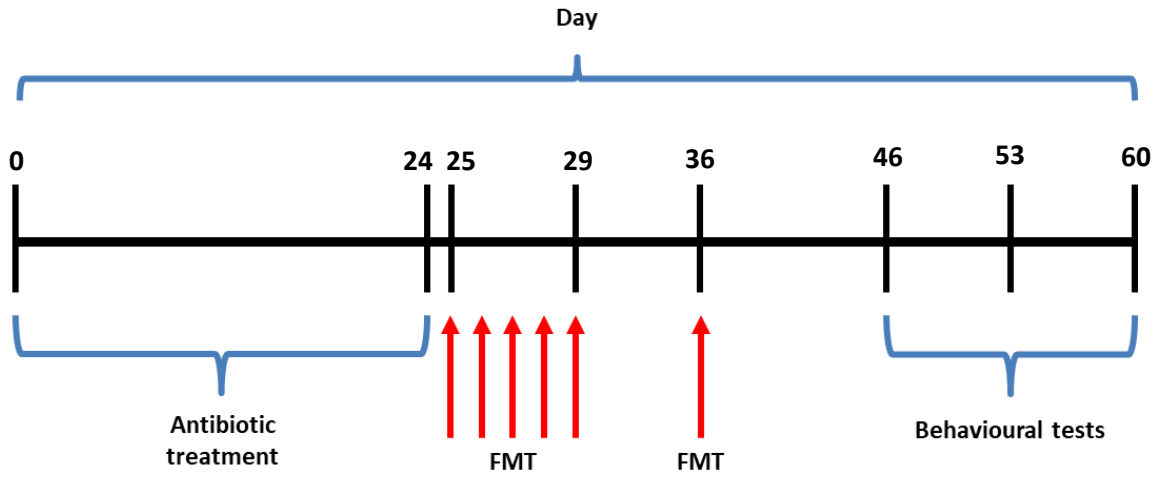
### *3.1. Effect of long-term antibiotic treatment and Faecal Microbiota Transplantation (FMT) from DNBS-treated animals on visceral sensitivity of naïve recipients*

The animals were treated as reported in the scheme below: in order to deplete the gut microbiota, rats were subjected to a combination of antibiotics for 24 days. A control group was treated with the vehicle. On day 25 the antibiotics-treated animals were divided in 2 groups, respectively receiving the control-derived faecal microbiota (FMT from CTR) or the DNBS-derived faecal microbiota (FMT from DNBS). The Faecal Microbiota Transplantation

(FMT) was performed for 5 consecutive days and repeated once after 7 days. Behavioural tests were performed 10, 17 and 24 days after the last FMT. The viscero-motor and the abdominal withdrawal responses (VMR and AWR) to the progressive increase in colorectal distension (CRD) were measured (Fig 17, respectively A and B).

Visceral pain threshold of antibiotic-treated animals appeared significantly lowered during the assessment of the abdominal withdrawal response to CRD (Fig 17B), In particular the animals treated with the antibiotics showed a greater sensitivity to the lower volume stimuli (1-2 mL) in respect to vehicle treated animals. Nevertheless, the long-term antibiotic treatment did not cause a significant increase in the viscero-motor response to CRD (Fig 17B). No difference was observed between the different groups treated with the antibiotics before FMT (Day 25, Fig 17). The transplant of the microbiota derived from controls attenuated visceral hypersensitivity induced by the long-term antibiotic regime, though the animals did not come back to the value of controls (black bars, Day 46-60). By contrast the animals receiving the microbiota of DNBS donors showed no recovery from the antibiotic-induced visceral hypersensitivity, which persists at least 24 days after the interruption of the treatments (Day 60 Fig 17B). Moreover, these animals showed an increase in the viscero motor response to CRD, which was significantly higher in respect to the other groups 10 and 17 days after the FMT (Day 46 and 53, Fig 17A). In these experimental conditions, it was difficult to discern the effect of FMT from that of the antibiotic treatment by itself.

***Experimental scheme:***



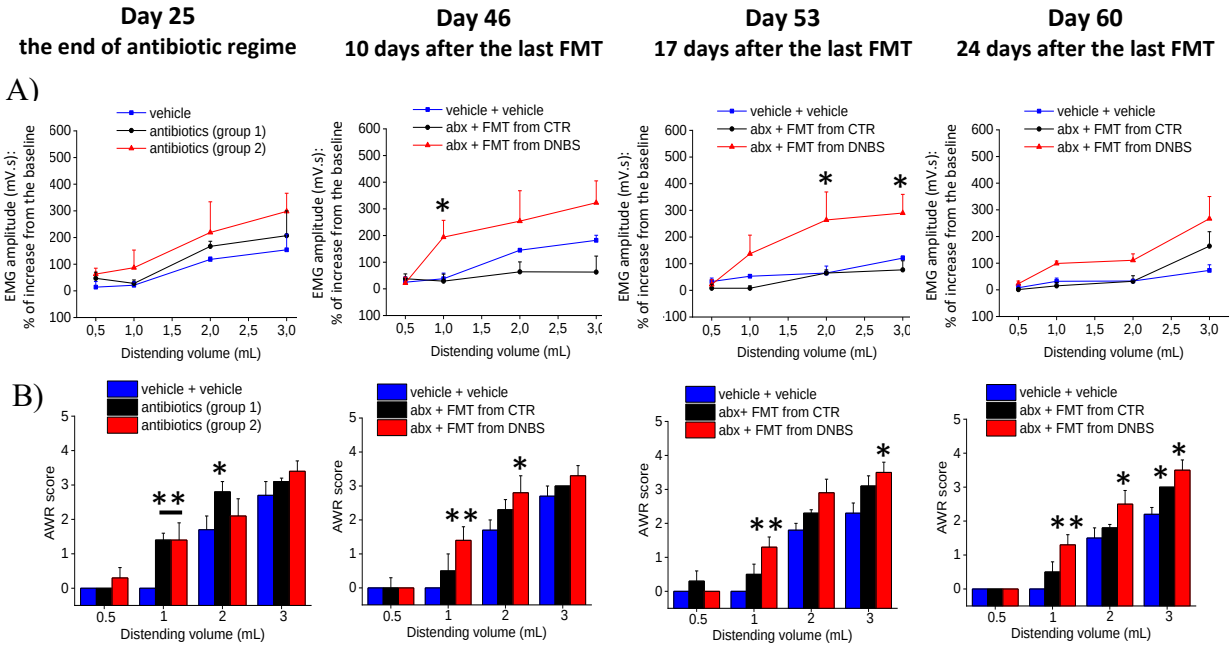



***Figure 17. Effect of long-term antibiotic treatment and Faecal Microbiota Transplantation (FMT) from DNBS-treated animals on visceral sensitivity of naïve recipients.*** *Rats were treated with a combination of antibiotics for 24 days; control group was treated with vehicle. On day 25 the antibiotics (abx- treated animals were divided in 2 groups, control-derived faecal material or DNBS-derived faecal material was respectively administered per os for five consecutive days. One week after the administration was repeated. Behavioural tests were performed 10, 17 and 24 days after the last FMT. Visceral sensitivity was assessed in the animals by measuring the Viscero-Motor Response (VMR; A) and the Abdominal Withdrawal Response (AWR; B). Each value is the mean ± S.E.M. of 5 rats per group. *P<0.05 and **P<0.01 vs vehicle or vehicle + vehicle treated animals.*

### *3.2. Effect of short-term antibiotic treatment and Faecal Microbiota Transplantation (FMT) from DNBS-treated animals on visceral sensitivity of naïve recipients*

To reduce the impact of antibiotics on visceral sensitivity we repeated the previous experiment by shortening the antibiotic treatment. With the collaboration of Dr. Di Pilato (Clinical Microbiology and Virology Unit, Florence Careggi University Hospital) we ascertained that 7 days of antibiotic regime are enough to obtain a sufficiently microbiota depletion to performed FMT (data not shown).

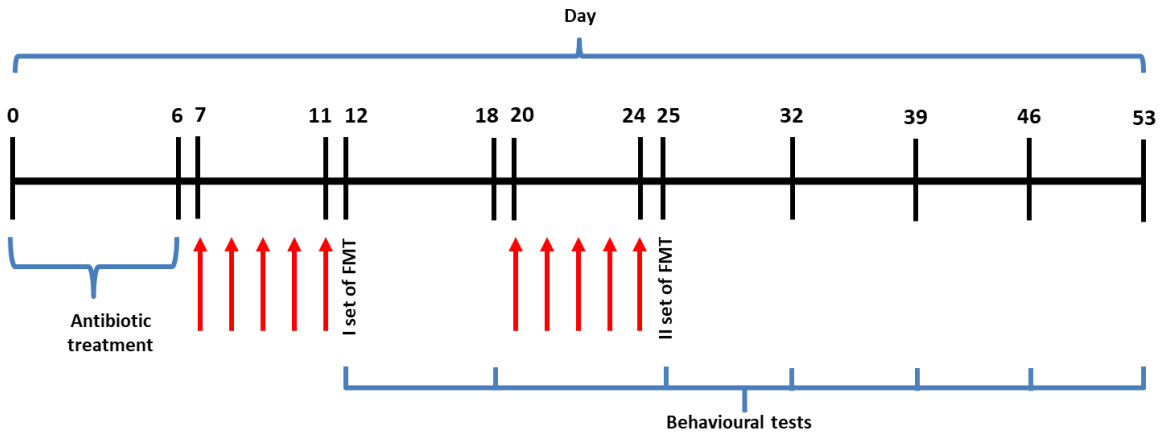
The animals were treated as reported in the scheme below: after 7 days of antibiotics, the animals were divided into 3 groups, respectively receiving the vehicle, the control-derived faecal microbiota (FMT from CTR) or the DNBS-derived faecal microbiota (FMT from DNBS). The Faecal Microbiota Transplantation (FMT) was performed every day for 5

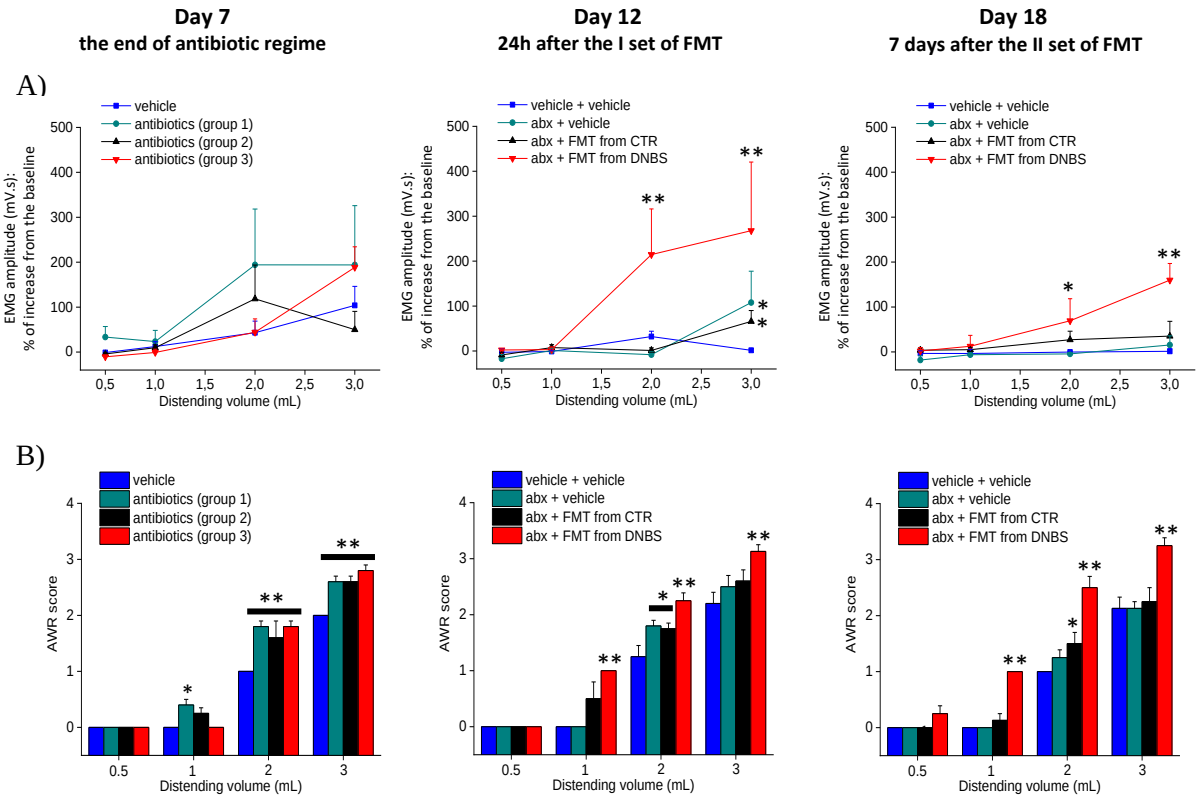
consecutive days and the same protocol was repeated one week after (I and II set of FMT). Behavioural tests were performed at the end of the antibiotic treatment, 24h and 7 days after each set of FMT and once week after that. The viscero-motor and the abdominal withdrawal responses (VMR and AWR) to the progressive increase in colorectal distension (CRD) were measured (Fig 18, respectively A and B).

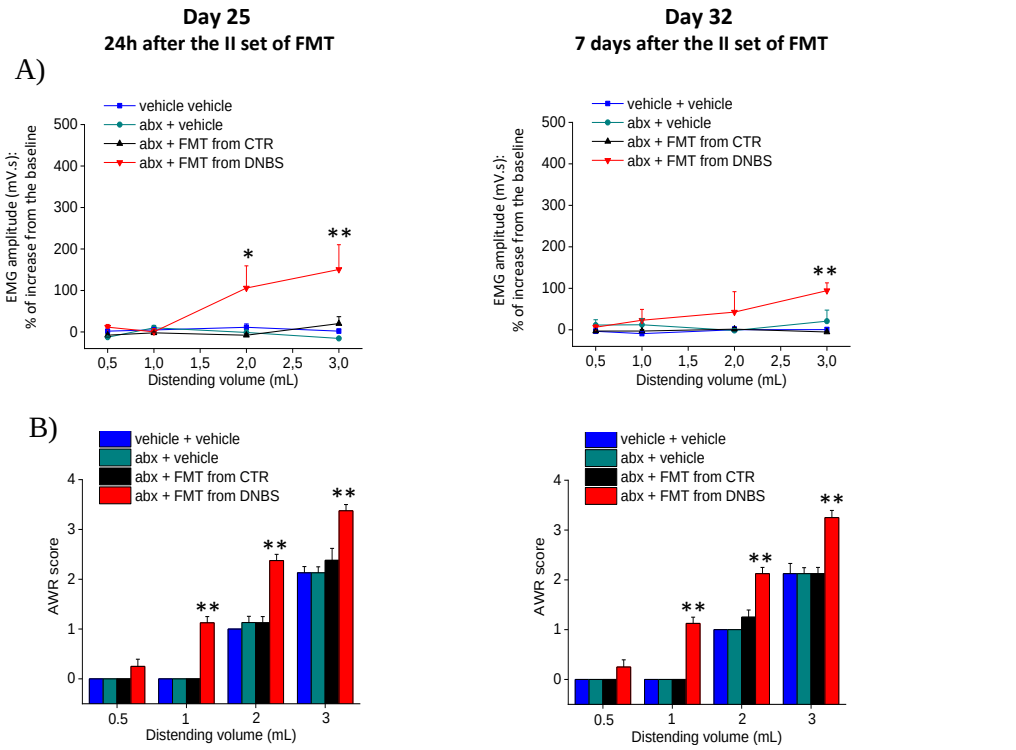
The short-term antibiotic treatment affected animals' visceral sensitivity. All the groups treated with the antibiotics showed a significantly augmented abdominal withdrawal response to CRD (Fig 18B). Also, in this case the antibiotic treatment did not cause a significant increase in the viscero-motor response to CRD (Fig 18B). No difference was observed among the different groups treated with the antibiotics before FMT (Day 7, Fig 18).

The re-colonization of the intestine with the healthy microbiota was able to restore a normal visceral pain threshold in antibiotics-treated animals. In fact, visceral hyperalgesia induced by antibiotics was remarkably reduced after the I set of FMT (Day 12-18, Fig 18) and completely reverted after the II set of FMT from CTR (Day 25-32, Fig 18). The same effect was observed by administering the vehicle (Fig 18). By contrast, the animals receiving the FMT from DNBS donors showed no recovery from the antibiotic treatment, but a further increase of both the viscero-motor response and the abdominal withdrawal response to CRD (Day 12-18, Fig 18A and B, respectively). This effect consolidated after the II set of FMT (Day 25-32, Fig 18). The animals receiving the FMT from DNBS still manifest an increased abdominal withdrawal response to CRD 2 weeks after the last FMT (Day 39, Fig 18B). On Day 46, 3 weeks after the interruption of the treatments, the effect of FMT from DNBS donors ended and the animals' visceral sensitivity came back to the value of controls (Fig 18B).

***Experimental scheme:***




Day 7
the end of antibiotic regime
Day 12
24h after the I set of FMT
Day 18
7 days after the II set of FMT
A)
B)
vehicle
antibiotics (group 1)
antibiotics (group 2)
antibiotics (group 3)
vehicle + vehicle
abx + vehicle
abx + FMT from CTR
abx + FMT from DNBS
EMG amplitude (mV.s):
% of increase from the baseline
AWR score
Distending volume (mL)


Day 25
24h after the II set of FMT
Day 32
7 days after the II set of FMT
A)
B)
vehicle vehicle
abx + vehicle
abx + FMT from CTR
abx + FMT from DNBS
EMG amplitude (mV.s):
% of increase from the baseline
AWR score
Distending volume (mL)

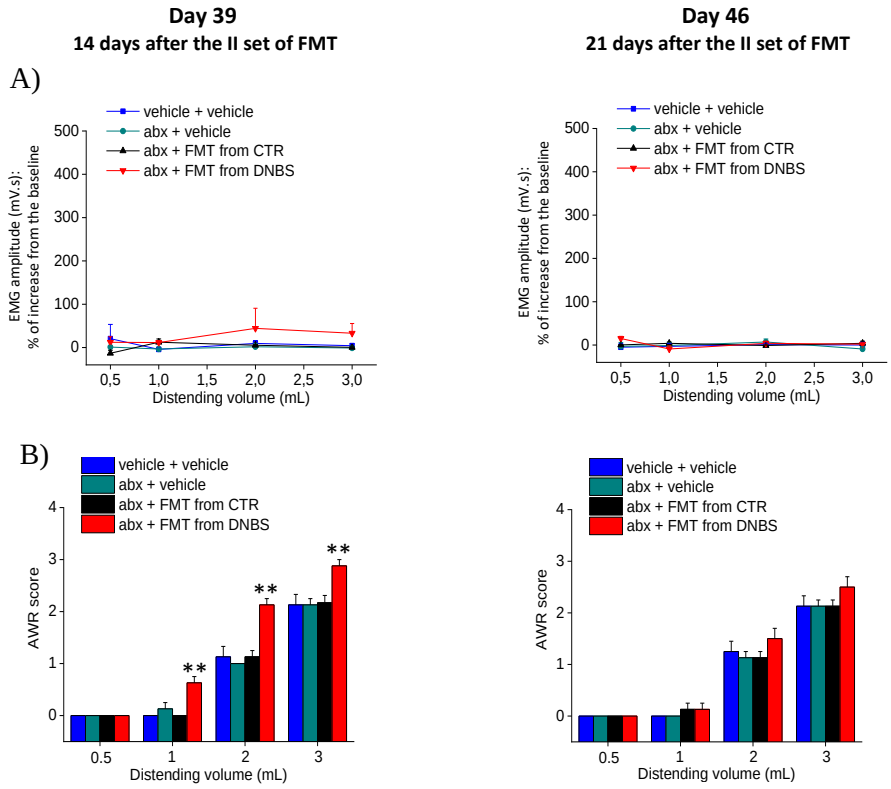



***Figure 18. Effect of short-term antibiotic treatment and Faecal Microbiota Transplantation (FMT) from DNBS-treated animals on visceral sensitivity of naïve recipients.*** *Rats were treated with a combination of antibiotics for 7 days; control group was treated with vehicle. On day 7 the antibiotics (abx)-treated animals were divided into 3 groups, FMT from CTR donors, FMT from DNBS donors or vehicle were respectively administered per os for five consecutive days. One week after the administrations were repeated. Behavioural tests were performed at the end of the antibiotic treatment, 24h and 7 days after each cycle of FMT and once week after the last treatment. Visceral sensitivity was assessed in the animals by measuring the Viscero-Motor Response (VMR; A) and the Abdominal Withdrawal Response (AWR; B) to Colo-Rectal Distension (CRD; 0.5-3 mL). Each value is the mean ± S.E.M. of 5 rats per group repeated in 2 experimental set. *P<0.05 and **P<0.01 vs vehicle or vehicle + vehicle treated animals.*

### *3.3. Detection of DNBS in faecal samples by Liquid Chromatography-Mass Spectrometry (LC-MS/MS)*

The determination of DNBS were carried out on sample solutions derived by both treated (30 mg DNBS in 0.25 mL EtOH i.r.) and control rats. Each group of samples was composed at least by three solutions obtained by different rats. The obtained LC-MS/MS chromatographic profiles were very similar for the two groups of samples (Figure 19, left). This evidence establishes that the analyte in treated samples is minor than LOD of the method (2 ng $ml^{-1}$). In order to verify the correct detection of DNBS, it was analysed a control sample spiked with 5

ng $mL^{-1}$ of analyte. The result demonstrates that the DNBS was detected and, compared with 5 ng $mL^{-1}$ calibration solution, correctly quantified (Fig 19, right).

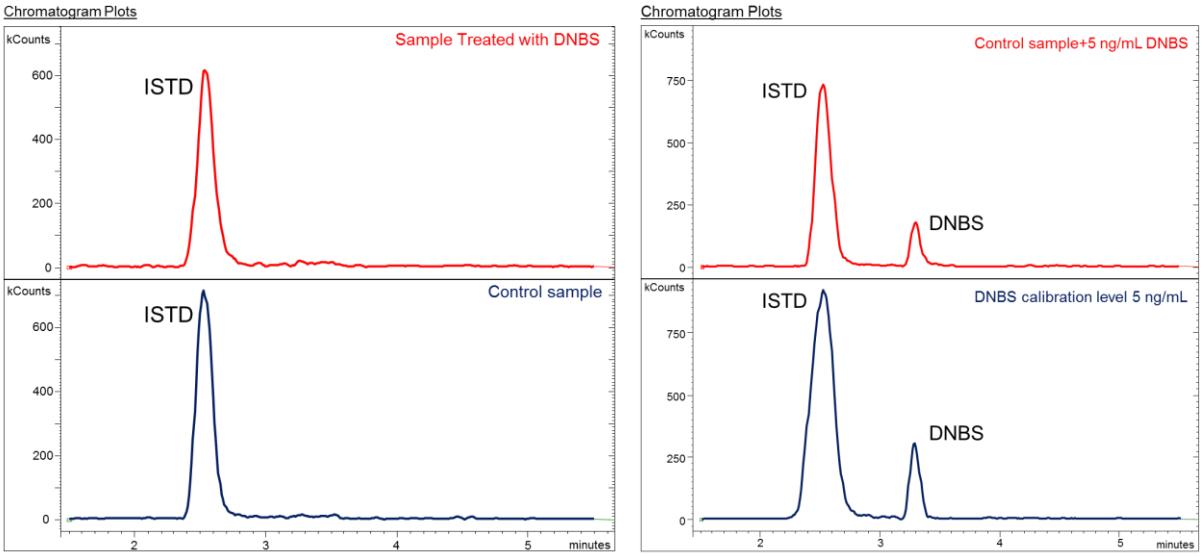



***Figure 19. Detection of DNBS in faecal samples by LC-MS/MS.*** *Left: LC-MS/MS chromatographic profiles obtained by DNBS (Above) and control (Bottom) FMT donor animals. Right: LC-MS/MS chromatographic profiles obtained by control sample added with 5 ng $mL^{-1}$ of DNBS (Above) and 5 ng $mL^{-1}$ calibration solution (Bottom) samples.*

### *3.4. Effect of short-term antibiotic treatment and Faecal Microbiota Transplantation (FMT) on colon histology*

The animals were sacrificed on Day 7 (24h after the end of antibiotic treatment), Day 32 (when the effect of FMT on pain was well established) and Day 46 (when the effect of FMT disappeared). The colon was harvested and processed for both macroscopic and microscopic analysis (Figure 20A and 20B, respectively). The Macroscopic Damage Score (MDS) was used to quantify the tissue damage degree (Fig 20A). The macroscopic examination highlight the presence of hyperaemia and a slight thickening of wall after the antibiotic treatment (Day 7). These alterations dissipated as a result of the administration of vehicle as well as the FMT from CTR donors. The recovery was slower in the animals receiving the FMT from DNBS donors (Day 32). On Day 46 no macroscopic alterations were detected among the groups (Fig 20A). These results were confirmed by microscopic analysis: an infiltration of neutrophils in mucosa and sub-mucosa were detected in the animals underwent the antibiotic treatment (Day 7; black arrow; Fig 20B). This tissue alteration found in the colon recover consequently to the interruption of antibiotic. No microscopic differences were observed in the animals receiving FMT from either CTR or DNBS donors (Day 32; Fig 20B).

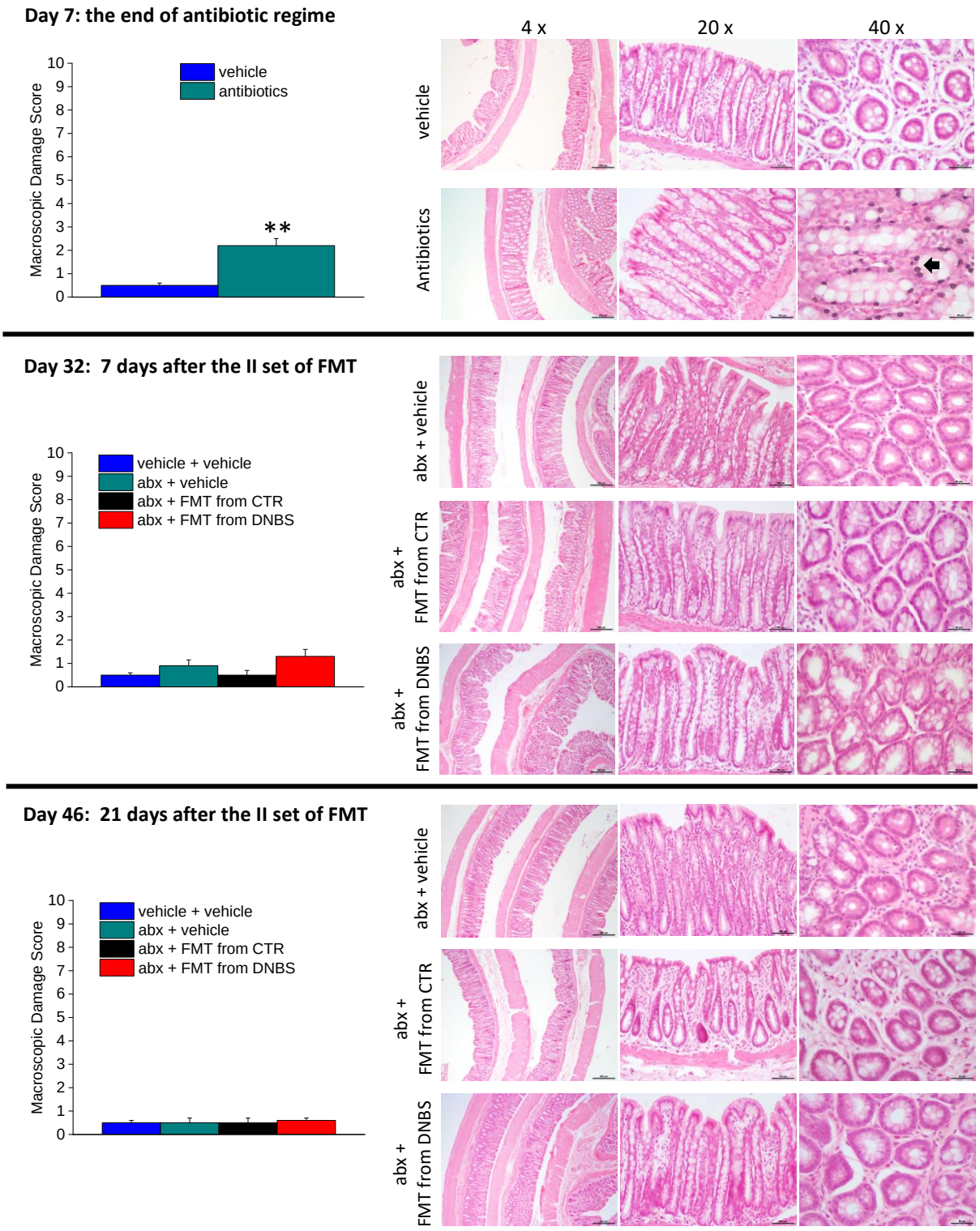


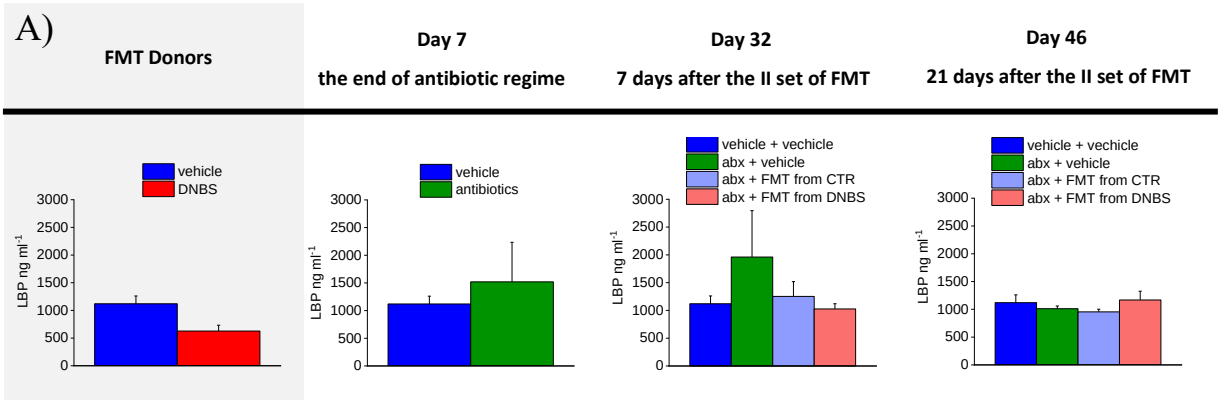

***Figure 20. Histological evaluation of colon damage after the antibiotic regime and the FMT.*** *A) Macroscopic Damage Score; B) Histological analysis on haematoxylin/eosin stained colon slices. Each value represents the mean ± S.E.M. of 5 animals per group. **P < 0.01 vs vehicle treated animals.*

### *3.5. Effect of short-term antibiotic treatment and Faecal Microbiota Transplantation (FMT) on gut permeability*

Animals' gut permeability was evaluated either by assessing the levels of Lipopolysaccharide Binding Protein (LBP) in the plasma and by measuring the expression of the tight junction related proteins, occludin and Zo-1, in the colon. The animals were sacrificed on Day 7 (24h after the end of antibiotic treatment), Day 32 (when the effect of FMT on pain was well established) and Day 46 (when the effect of FMT disappeared). Plasma and colon samples were collected from each experimental group. The results obtained in the animals underwent the FMT were compared with that obtained respectively in the CTR and DNBS donors.

Plasma was analysed for LBP using an ELISA immunoassay. No significant alteration in the plasmatic levels of LBP was found among the experimental groups, only a tendency to increase after the antibiotic treatment was appreciable (Day 7; Fig 21A). On the other hand, no divergence was found in the levels of plasmatic LBP between CTR and DNBS donors (Fig 21A).

Accordingly, We analysed the expression of tight junctions related proteins in the colon by RT-qPCR. The expression of occludin tended to increase after the antibiotic treatment (Day 7; Fig 21B). On Day 46 occludin expression appeared significantly up regulated in all the groups which had received the antibiotics (Fig 21B). Similarly, the levels of Zo-1 mRNA resulted significantly increased on Day 7 after the antibiotic regime. By contrast, the expression of occludin and Zo-1 seems not to have been influenced by the FMT. A slight derangement in Zo-1 expression was observed in DNBS donors and FMT-DNBS recipients, though this effect resulted not statistically significant (Fig 21B).



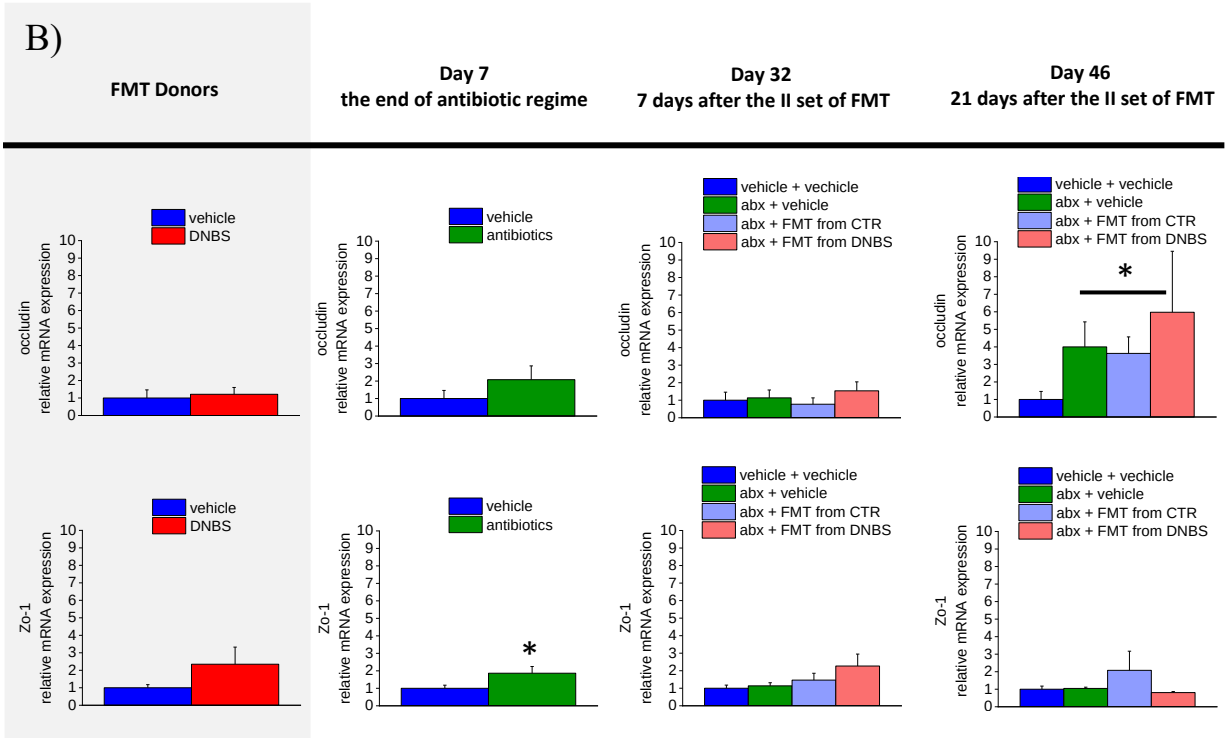



***Figure 21. Effect of antibiotic regime and FMT on gut permeability.*** *A) Elisa assay for Lipopolysaccharides Binding Protein (LBP) in plasma samples. B) Analysis of occludin and Zo-1 gene expression on colon samples by RT-qPCR. The mRNA expression was normalized to β-actin and fold changes were expressed in comparison with control group. Each value is the mean ± S.E.M. of 4-6 rats per group. *P<0.05 and **P<0.01 vs vehicle or vehicle + vehicle treated animals.*

### *3.6. Effect of short-term antibiotic treatment and Faecal Microbiota Transplantation (FMT) on gut cytokines profile*

The immune response of animals was investigated by analysing the pro- and anti-inflammatory cytokine profile in the gut. The animals were sacrificed on Day 7 (24h after the end of antibiotic treatment), Day 32 (when the effect of FMT on pain was well established) and Day 46 (when the effect of FMT disappeared) and colon samples were collected from each experimental group. The results obtained in the animals underwent the FMT were compared with that obtained respectively in the CTR and DNBS donors.

The gene expression of TNF-α, IL-6, IL-10 and Tgf-β was measured in the colon by RT-qPCR. The antibiotic regime caused a long-lasting up regulation of both pro- and anti-inflammatory cytokines expression (Day 7; Fig 22). This deregulatory effect of antibiotics was not fixed with the transplant of the microbiota (Day 32; Fig 22).

On the other hand, the cytokines profile of recipient animals did not follow the trend of the donors. In fact, the expression of both IL-6 and Tgf-β resulted augmented in FMT recipient animals, irrespective to the donors (CTR or DNBS; Day 32), though these cytokines were overexpressed only in DNBS donors (Fig 22). Unlike DNBS donors, TNF-alfa expression was drastically down-regulated in FMT-DNBS recipients, while the expression of IL-10 was remarkably increased (Day 32; Fig 22). On Day 46 all the cytokines were still upregulated in the abx + vehicle group and in the abx + FMT from CTR group. By contrast, in the abx + FMT from DNBS all the cytokines, with exception of Tgf-β, come back to the value of controls (Fig 22).

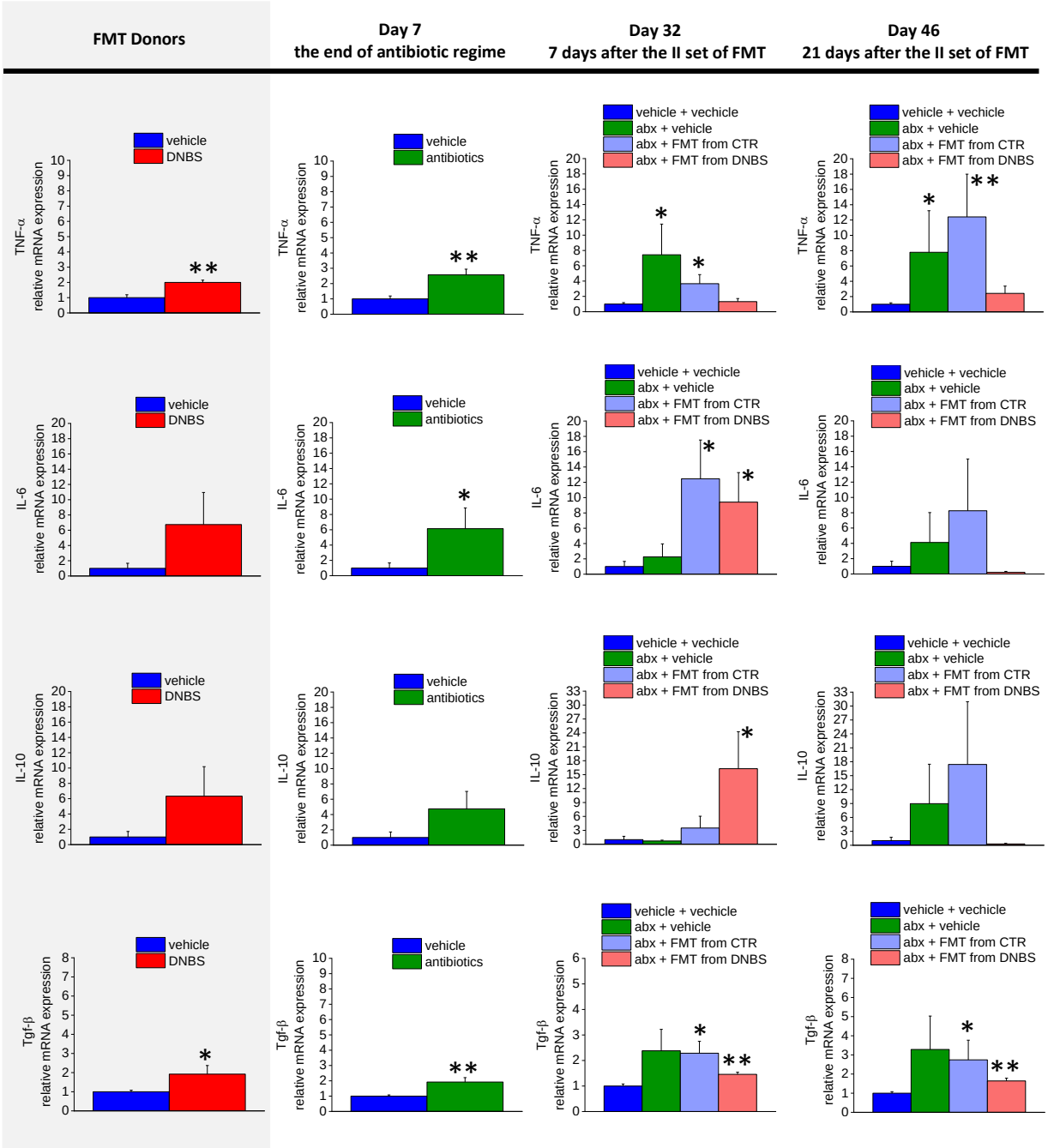


***Figure 22. Effect of antibiotic regime and FMT on gut cytokines profile.*** *Analysis of TNF-α, IL-6, IL-10 and Tgf-β gene expression on colon samples by RT-qPCR. The mRNA expression was normalized to β-actin and fold changes were expressed in comparison with control group. Each value is the mean ± S.E.M. of 4-6 rats per group. *P<0.05 and **P<0.01 vs vehicle or vehicle + vehicle treated animals.*

### *3.7. Effect of short-term antibiotic treatment and Faecal Microbiota Transplantation (FMT) on tryptophan metabolism*

The plasmatic levels of tryptophan (TRP) and kynurenine (KYN) were investigated by performing a HPLC analysis. The animals were sacrificed on Day 7 (24h after the end of antibiotic treatment), Day 32 (when the effect of FMT on pain was well established) and Day 46 (when the effect of FMT disappeared) and plasma samples were collected from each experimental group. The results obtained in the animals underwent the FMT were compared with that obtained respectively in the CTR and DNBS donors.

The levels of both TRP and KYN resulted significantly decreased in the plasma of DNBS donors in respect to controls (grey background; Fig 23). The levels of TRP were not affected by the antibiotic treatment, while the levels of KYN tended to increase (Day 7, Fig 23). Anyway, on Day 32 all the groups treated with the antibiotics, irrespective to the microbiota transplant, showed a significant reduction of TRP. On the other side, the levels of KYN did not change, leading to an increase in the TRP/KYN ratio (Day 32; Fig 23).

On Day 46 the levels of TRP were still lowered in abx + vehicle and abx + FMT from CTR animals. At the same time, the levels of KYN resulted increased in abx + vehicle animals and decreased in abx + FMT from CTR animals. Regarding the group treated with abx + FMT from DNBS, the amount of TRP rised up back to the controls value, while that of KYN significantly dropped (Day 46; Fig 23).

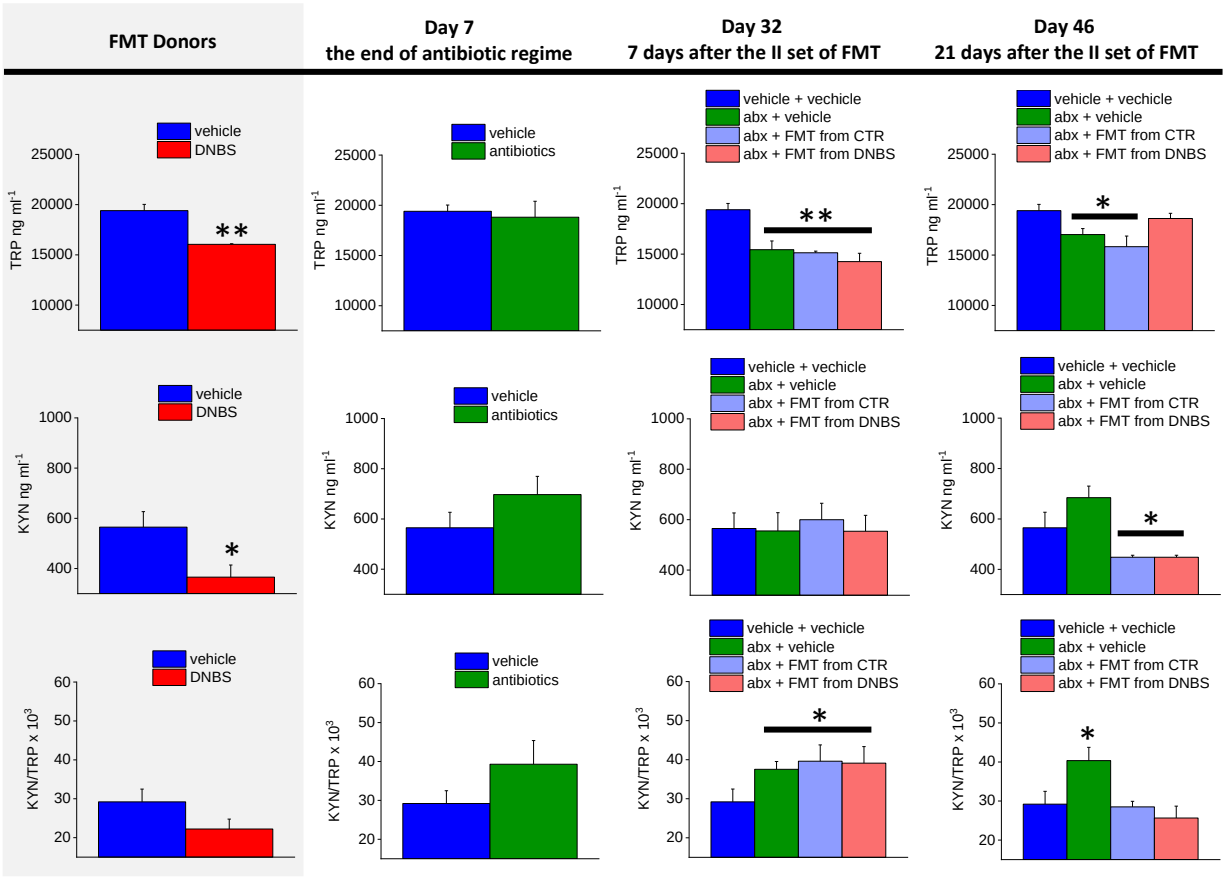



***Figure 23. Effect of antibiotic regime and FMT on tryptophan and kynurenine plasmatic levels.*** *Concentrations of tryptophan (TRP) and kynurenine (KYN) and their ratio (KYN/TRP) assessed by HPLC in plasma samples. Each value is the mean ± S.E.M. of 4-6 rats per group. *P<0.05 and **P<0.01 vs vehicle or vehicle + vehicle treated animals.*

### *3.8. Effect of short-term antibiotic treatment and Faecal Microbiota Transplantation (FMT) on gut monoamines*

The levels of monoamines in the colon were investigated by using HPLC. The animals were sacrificed on Day 7 (24h after the end of antibiotic treatment), Day 32 (when the effect of FMT on pain was well established) and Day 46 (when the effect of FMT disappeared) and colon samples were collected from each experimental group. The results obtained in the animals underwent the FMT were compared with that obtained respectively in the CTR and DNBS donors.

The amount of 5-HT, but not that of the metabolite 5-HIAA, were significantly higher in the colon of DNBS donors than controls (grey background; Fig 24A). On the other hand, the levels of dopamine (DA) were remarkably decreased in DNBS donors (Fig 24B).

The levels of 5-HT and 5-HIAA were not significantly affected by the antibiotic treatment (Day 7), neither by the microbiota transplant (Day 32, Fig 24A). Only a slight lowering of 5-HIAA was noticed in the animals underwent the FMT, irrespective to the donors (Day 32). The same framework was observed on Day 46 (Fig 24A).

Although not significant, a persistent lowering of DA was observed as a consequence of the antibiotic regime. This tendence was not reverted by FMT (Fig 24B).

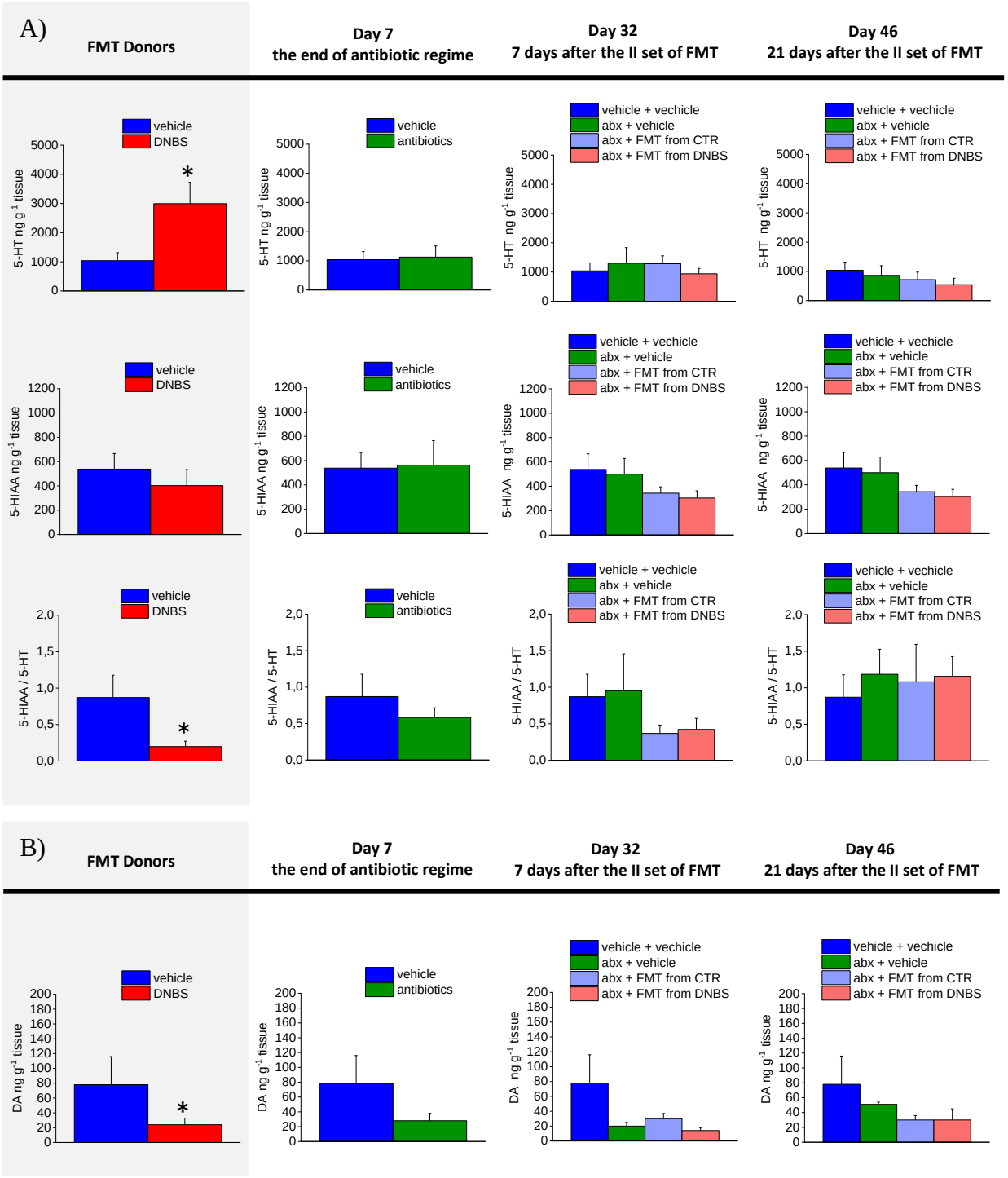


***Figure 24. Effect of antibiotic regime and FMT on monoamines levels in the gut.*** *A) Concentrations of serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) and their ratio (5-HIAA/5-HT) assessed by HPLC in colon samples. B) Concentrations of dopamine (DA) assessed by HPLC in colon samples. Each value is the mean ± S.E.M. of 4-6 rats per group. *P<0.05 and **P<0.01 vs vehicle or vehicle + vehicle treated animals.*

### *3.9. Effect of short-term antibiotic treatment and Faecal Microbiota Transplantation (FMT) on faecal monoamines*

The levels of monoamines in the colon lumen were investigated by processing the faecal pellets with HPLC. The faecal pellets were collected from each experimental group on Day 7 (24h after the end of antibiotic treatment), Day 32 (when the effect of FMT on pain was well established) and Day 46 (when the effect of FMT disappeared). The results obtained in the animals underwent the FMT were compared with that obtained respectively in the CTR and DNBS donors. In the faecal pellets, no differences in 5-HT and DA levels were found between DNBS and CTR donors (grey background; Fig 25A and B, respectively). The concentration of 5-HT was appreciably reduced as a result of the antibiotic treatment, by contrast DA amount considerably increased (Day 7, Fig 25A and B, respectively). After the FMT, the amount of 5-HT in the lumen significantly augmented, irrespective to the donor (Day 32; Fig 25A). This effect persisted on Day 46, when the levels of 5-HT were still high in both the groups receiving the FMT (Fig 25A). On the other hand, the DA increase caused by the antibiotic regime was reverted by the FMT as well as by the spontaneous re-colonization of the intestine due to the vehicle administration (Day 32; Fig 25B).

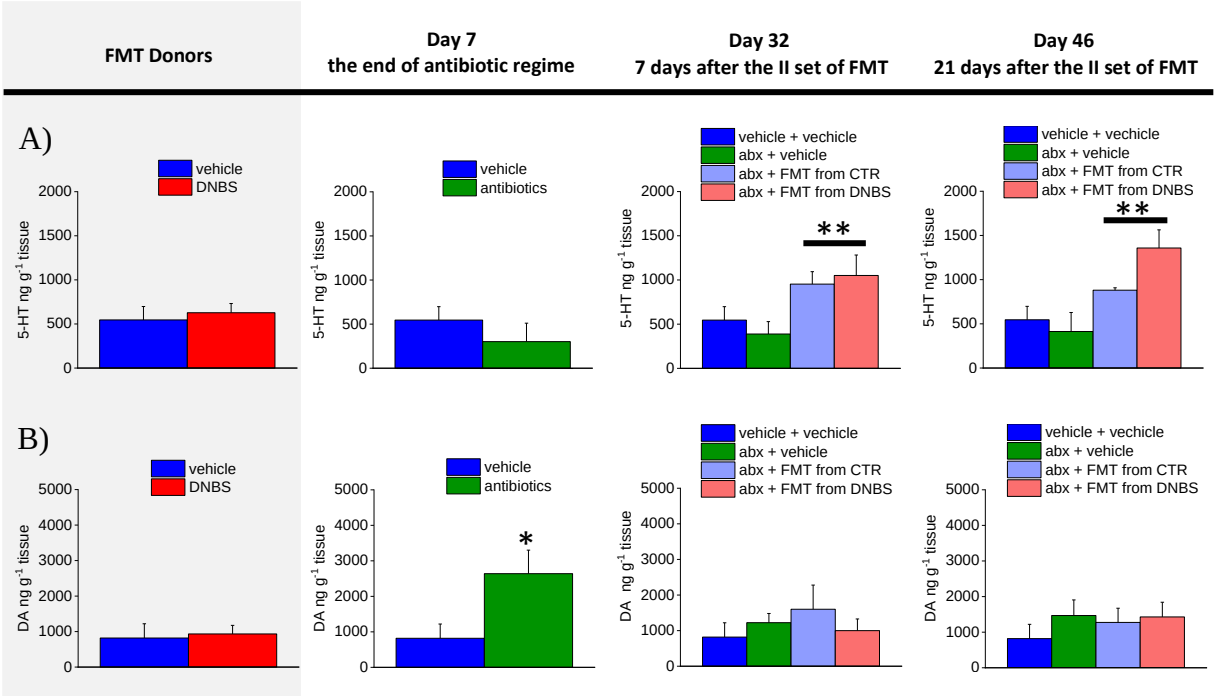


***Figure 25. Effect of antibiotic regime and FMT on monoamines levels in the faeces.*** *A) Concentrations of serotonin (5-HT; A) and dopamine (DA; B) assessed by HPLC in faecal samples. Each value is the mean ± S.E.M. of 4-6 rats per group. *P<0.05 and **P<0.01 vs vehicle or vehicle + vehicle treated animals.*

### *3.10. Effect of the antibiotic treatment and the Faecal Microbiota Transplantation (FMT) on gut microbiota composition (Data not shown)*

Thanks to the collaboration with Dr. Vincenzo Di Pilato (Dept. of Surgery and Translational Medicine, University of Florence) we performed a gut microbiota analysis.

In order to identify potential signatures in the gut microbiota of animals subjected to FMT using stools collected from both controls and DNBS-treated donors, the fecal microbiota of recipient animals was characterized through a 16S metagenomic sequencing approach. Four faecal samples of different animals for each experimental group were collected on Day 0 (before the treatments), Day 7 (24h after the last antibiotic treatment), Day 18 (7 days after the the first set of FMT) Day 32 (7 days after the second set of FMT) and on Day 46 (21 days after the last FMT). The microbiota characterization was also performed on the preparations used for the repeated fecal transfers from both CTR and DNBS donors. In particular the faecal supernatant utilized for the first and the last transfer of each set of FMT was analysed.

Fecal pellet and fecal suspensions were processed for the total DNA extraction using a commercial kit (DNeasy PowerLyzer PowerSoil Kit - Qiagen, Hilden, Germany). The gut microbiota was characterized by next generation sequencing technology using the Illumina MiSeq platform and the 16S ribosomal RNA gene as target (V3-V4 region) on amplified genomic DNA, according to the Illumina 16S Metagenomic Sequencing Library Preparation protocol (Part # 15044223 Rev. B; URL: http://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). Sequencing results were analyzed using the QIIME 2 suite (Quantitative Insights Into Microbial Ecology) (Bolyen et al., 2019).

Microbial diversity was estimated by evaluating alpha- and beta-diversity metrics using specific tools implemented in the QIIME 2 pipeline. The alpha-diversity, representing the measure of mean diversity within a sample, was analyzed evaluating the Shannon diversity index. The beta-diversity, which consists in comparison of diversities between samples, was evaluated using the Principle Coordinates Analysis (PCoA) and the Bray-Curtis metric to inspect a potential clustering of samples according to the study groups. Statistical analysis

was performed through QIIME 2 and GraphPad Prism 6 using nonparametric tests (e.g. Kruskal-Wallis, ANOSIM, PERMANOVA).

Preliminary analysis of alpha-diversity revealed an overall lower microbial diversity in samples from antibiotic-treated animals not subjected to a subsequent fecal transfer, an observation consistent with the antibiotic treatment performed to deplete the resident microbiota. Conversely, the microbial diversity was overall stable in control samples over the examined timepoints and showed an increase trend in samples from animal subjected to fecal transfer, either using stool from control or DNBS-treated animals.

The analysis of beta-diversity showed that samples from antibiotic-treated animals clustered away from controls, confirming a different structure of their gut microbiota which is maintained over time.

Differences in microbial taxonomic profiles were identified in preparations of the fecal material used for the repeated fecal transfers, obtained either from control or DNBS-treated animals, indicating that alterations of the gut ecosystem mediated by DNBS can directly impact on the resident microbial communities and persist until later stages following the acute inflammatory response.

Concerning the experimental groups of animals treated with antibiotics and then subjected to FMT, defined clusters in PCoA plots were observed for samples collected after the second series of repeated fecal transfers (Day 32), suggesting that major modifications in the composition and structure of the gut microbiota might have occurred at this stage. Evaluation of taxonomic profiles confirmed that marked differences between the animals underwent the FMT from CTR or DNBS were identifiable after the second series of repeated fecal transfers at phylum and family level for major members of the gut microbiota, including also protective microbial taxa known to be associated with gastrointestinal health.

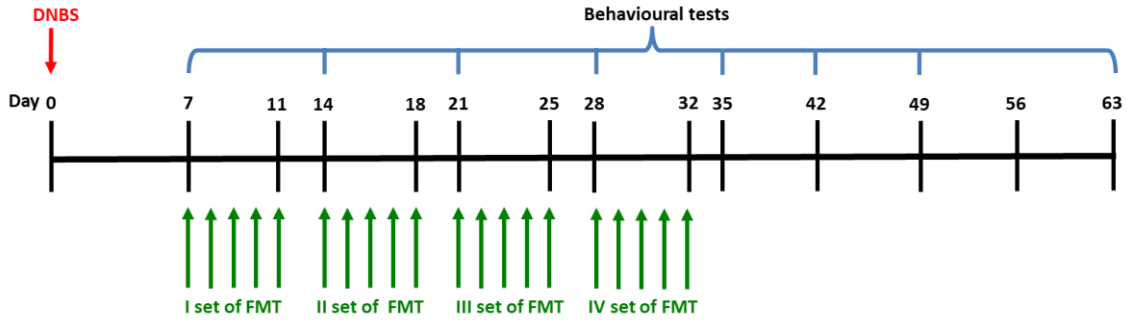
### *3.11. Therapeutic effect of Faecal Microbiota Transplantation (FMT) on post-inflammatory visceral pain persistence*

The animals were treated as reported in the scheme below. Colitis was induced in the animals by the intra rectal injection of DNBS (30 mg in 0.25 mL EtOH 50%). A control group was intra-rectally administered with saline solution. Seven day after DNBS injection the animals were split into 2 groups, respectively receiving the vehicle or the controls-derived faecal microbiota (FMT from CTR). The Faecal Microbiota Transplantation (FMT) was performed for 5 consecutive days and the same protocol was repeated for 4 weeks (I-IV set of FMT).

Behavioural tests were performed 7 day after DNBS injection, 3 days after each set of FMT and once week after the end of the treatment. The abdominal withdrawal responses (VMR and AWR) to the progressive increase in colorectal distension (CRD) was measured (Fig 26).

As expected, 7 days after the induction of the damage, the abdominal withdrawal response to CRD was significantly higher in both the groups treated with DNBS (Fig 26). The transplant of the microbiota derived from healthy donors (FMT from CTR) into DNBS treated animals led to a progressive reduction of visceral hypersensitivity which became significant after the third transplant cycle (Day 28) end consolidated after the fourth one (Day 35; Fig 26). The beneficial effect of FMT on post-inflammatory visceral pain caused by DNBS lasts up to 17 days after the treatment interruption (Day 49). Starting from Day 56, the behavioural response of animals to CRD increase again. On Day 63, irrespectively of the received treatment, both the DNBS groups showed a significantly higher visceral sensitivity in respect to controls (vehicle + vehicle treated animals), as they returned to perceive pain (Fig 26).

***Experimental scheme:***



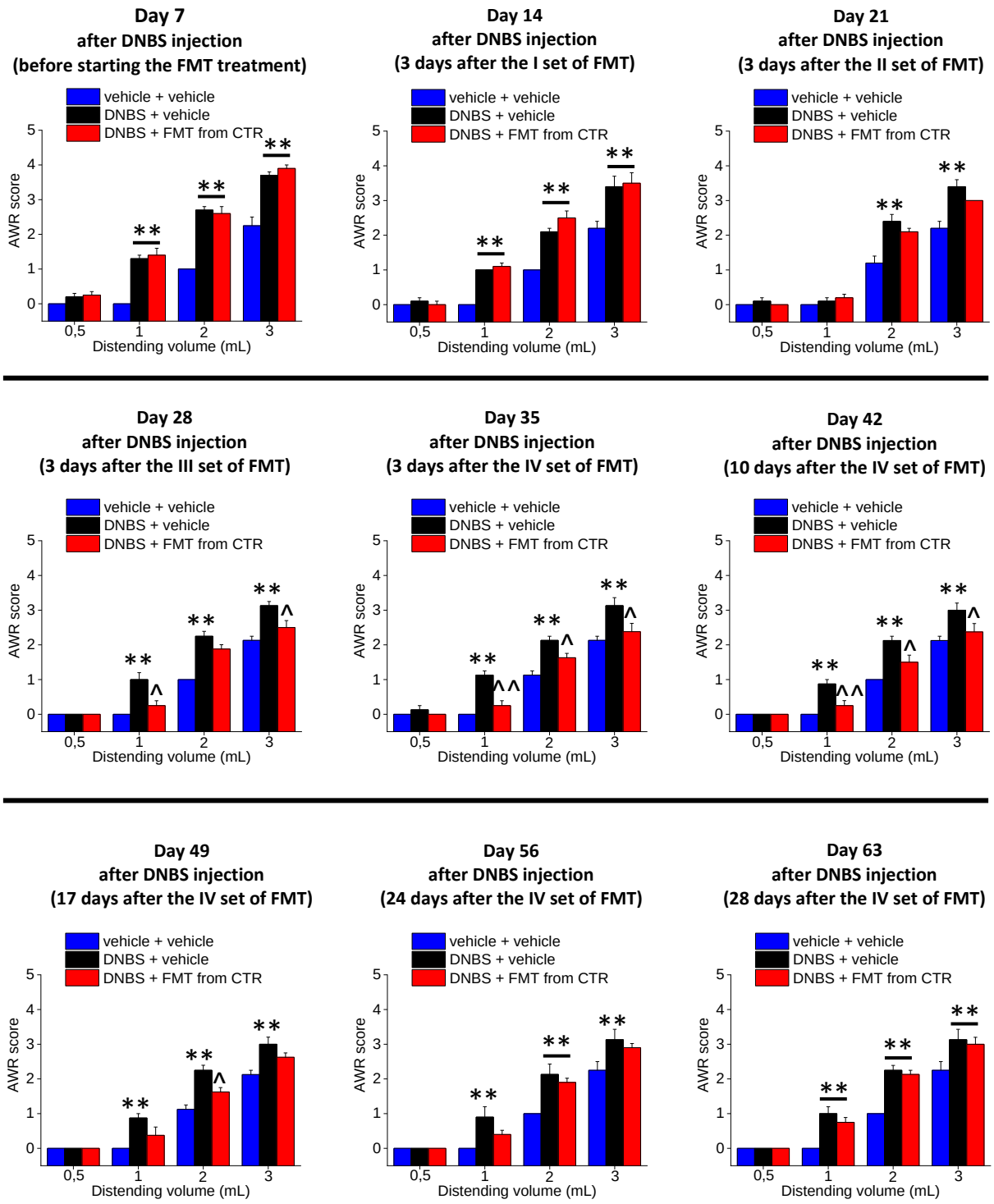



***Figure 26. Therapeutic effect of FMT on DNBS-induced post-inflammatory visceral pain.*** *Rats were intra-rectally injected with DNBS (30 mg in 0.25 mL EtOH 50%). On day 7 DNBS-animals were divided into 2 groups respectively administered with the vehicle or the FMT from CTR donors per os for five consecutive days. The FMT set was weekly repeated for 4 times. Behavioural tests were performed at the end of the antibiotic treatment, 3 days after each cycle of FMT and once week afterwards. Visceral sensitivity was assessed in the animals by measuring the Abdominal Withdrawal Response (AWR) to Colo-Rectal Distension (CRD; 0.5-3 mL). Each value is the mean ± S.E.M. of 5 rats per group. *P<0.05 and **P<0.01 vs vehicle or vehicle + vehicle treated animals. ^P<0.05 and ^^P<0.01 vs DNBS + vehicle treated animals.*

# DISCUSSION

The first part of this work provides a comprehensive pharmacological and histological characterization of the persistent visceral pain caused by intestinal inflammatory damages. We found that this kind of chronic pain is related to a persistent immune activation as well as to abnormalities in the nervous system signalling. Interestingly these alterations, which likely originate in the periphery as a consequence of the intestinal damage, reverberate at central level where they trigger mechanisms of chronicization, such as a marked activation of microglia and astrocytes.

The similarities between the animal model of post-inflammatory visceral pain induced by DNBS and the clinical manifestations showed by patients affected by both IBS and IBDs (Qin et al. 2011; Antonioli et al., 2011; Brierley and Linden, 2014; Greenwood-Van Meerveldet al., 2015; Spiller and Major, 2016) allowed us to deepen into the pathophysiological mechanisms involved in pain development and maintenance. DNBS induced a local inflammatory response with a peak between 3 and 7 days after its intra-rectal injection with a subsequent progressive remission already detectable after 2 weeks as previously reported by Ippolito et al. (2015). After colitis resolution, the animals developed a visceral hypersensitivity which persisted ~ 3 months, in accordance to previous reports by Adam et al. (2006) and Gschossmann et al. (2004).

The measure of the viscero-motor response to colo-rectal distension gave a quantitative measure of the visceral sensitivity, since the entity of the abdominal contraction is directly proportional to the intrinsic sensibility of the animals (Arvidsson et al., 2006; Christianson and Gebhart, 2007). Despite its usefulness as quantitative measure, the assessment of this viscero-motor response needed the animals to be asleep, excluding the cognitive and emotional component of pain. We complete the puzzle, by coupling this test to the measure of the abdominal withdrawal response to the colo-rectal distension in awake animals as described by Chen et al. (2014) which allowed to further analyse the different aspects of this type of pain.

Regarding the emotional aspect, we found that DNBS treated animals, along with pain, present also persistent depresive-like behaviours. By contrast, no significative anxiety or motor disorders were detected, though chronic colitis-induced visceral pain has been recently associated with increased anxiety during quiescent phase (Salameh et al., 2019). A high comorbidity of chronic pain with stress-related psychiatric disorders including anxiety and depression occurs also at clinical level, despite the mechanisms linking visceral pain with

these overlapping comorbidities remain to be elucidated (Elsenbruch et al., 2010a; Moloney et al., 2015). These behavioural disorders can be caused by the presence of spontaneous pain or discomfort in the animals. On the other hand, long term stress facilitates pain perception and sensitizes pain pathways, leading to a feed-forward cycle promoting chronic visceral pain disorders such as IBS (Greenwood-Van Meerveld and Johnson, 2017). This also explains the benefit provided by antidepressants in these patients (Camilleri and Boeckxstaens, 2017).

From a pharmacological point of view, we observed that the drugs with spasmolytic effect on the gut were effective in visceral pain relief, likely inhibiting perception of the stimulus. On the other hand, we found that anti-depressant and anti-epileptic drugs, able to restore the normal neuronal activity, as pregabalin, work better than anti-inflammatory drugs. This is similar to what usually happens in neuropathic pain conditions (Attal and Bouhassira, 2015). Nevertheless, though inflammation seems not be responsible for the persistence of visceral pain, it represents the driving force for the induction of neuroplasticity in the periphery which lead to altered motility and nociceptors sensitization (Brierley and Linden, 2014; Mawe et al., 2015).

In the study of the mechanisms it is important to consider that the emotional component has an important role in the perception of visceral pain (Elsenbruch et al., 2010b; Van Oudenhove, 2010; Schmid et al., 2015), which therefore cannot be considered only the result of a peripheral altered nervous signalling. The systemic administration of morphine was able to make DNBS-treated animals completely insensitive to the colorectal stimulus, an effect that cannot be traced back only to the muscular relaxation of the intestine walls, otherwise all the compounds with spasmolytic activity should have shown the same effect. Yet, we cannot consider this effect solely due to the inhibition of the painful signal transmission from the periphery to central nervous system. In fact, the intrathecal injection of morphine partially relief pain in awake animals but not in unconscious rats, confirming that an important regulatory component may reside in the supraspinal centres where morphine exerts a dissociative effect on pain perception (Mayer, 2000; Harton et al., 2017). Similarly amitriptyline, able to activate the descending inhibitory signal on pain pathways (Obata et al., 2017), once intrathecally administered resulted effective only in conscious animals. Conversely, pregabalin, a blocker of the alpha2-delta subunit of voltage-gated calcium channels, was more active in unconscious animals after the intrathecal infusion. Overall the pharmacological data demonstrate the complex nature of this kind of persistent pain in which inflammatory, neuropathic and emotional components intertwined.

Looking more thoroughly the colon, we found an augmented production and deposition of collage fibres in DNBS treated animals as previously reported by Ippolito et al. (2015). This process which starts as a mechanism of repair, inevitably alters the tissue physiology (Rieder and Fiocchi, 2009). On the other hand, we found a widespread increase in the production of substance P, a neurotransmitter involved in the regulation of local nervous signalling as well as in the modulation of painful perception (Von Boyen et al., 2002; Vergnolle, 2003).

As previously mentioned, the intestinal inflammation leads to significative neuroplastic alterations in the enteric nervous system resulting in the chronicization of enteric neuropathy-related symptoms, like visceral pain and intestinal dysmotility (Mawe et al., 2015). During inflammation resident and immune cells recruited to the inflamed or damaged site secrete inflammatory mediators that act on peripheral nerves innervating the affected tissue. Some of the signals triggered by the peripheral immune activation have been reported to migrate toward the dorsal root ganglia and the spinal cord, modulating pain maintenance (Raoof et al., 2018).

Among the inflammatory mediators, those mainly involved in modifying peripheral neurons activities are histamine, proteases, polyunsaturated fatty acid metabolites, and tachykinins (Corsetti et al., 2015; Wouters et al., 2016b; Cenac et al., 2015; Delvalle et al., 2018). In particular, histamine produced by MCs is increased in the gut of patients affected by IBS playing a key role in visceral sensitivity regulation, given its ability to directly enhance neuron excitability (Wouters et al., 2016b; Traina et al., 2019). Similarly, we found an increase in the number of MCs in the mucosa and in the submucosa of DNBS animals which is likely triggered by the disruption of the intestinal barrier consequent to the damage which determines the passage of luminal antigens into the mucosa. This evidence, along with that discussed below, suggests the persistence of an immune system activation after the resolution of inflammation that inevitably affect the enteric nervous signalling (Albert-Bayo et al., 2019).

In line with the hypothesis of a persistent immune activation, one interesting aspect we observed in DNBS rats at day 14 is the increase in eosinophils and MHC-II-positive macrophages detected throughout the colonic wall. Of interest, these cells accumulated consistently in the proximity of SP-positive fibres, indicating a neuro-immune interaction also during the post-inflammatory phase of colitis. Indeed, abundant eosinophils have been previously described in close apposition to enteric nerves in IBD patients, this pattern being probably mediated by the upregulated production of ICAM-1 and eotaxin-3 by enteric neurons after their damage (Smyth et al., 2013). Besides eosinophils, also activated MCH-II-

positive macrophages were previously described in inflamed intestinal tissues (Mowat and Bain, 2013). In line with this, a pivotal role of activated intestinal macrophages has been suggested in the immune-mediated pathogenesis of inflammatory-associated functional disturbances in experimental and clinical IBD (German et al., 2001, Franzè et al., 2013), as well as in enteric neuron aging (Phillips et al., 2013) and parasitic infection (Galeazzi et al., 2000), both the latter conditions being characterized by a close contiguity of these cells with intestinal dystrophic neurites. On the other hand, the MHC-class-II seems also expressed by enteroglial cells in the ganglia of the submucous and myenteric plexuses and on the enteroglial sheaths of the nerve extensions in patients with Crohn's Disease (Geboes et al., 1992). Furthermore, an inflammatory response can induce MHC-II up-regulation in both astrocytes and neuronal stem cells in vitro (Vagaska et al., 2016). So that, it becomes interesting to further characterized these neurons surrounding MHC-II positive cells.

In addition to hematogenous macrophage infiltration, other non-neuronal cells that surround the somata of sensory neurons, like the satellite glia, can react to the injury and participate to the local neuroinflammatory processes (Ippolito et al., 2015; Delvalle et al., 2018). In particular, the intestinal inflammation induced by DNBS seems to alter the functionality of the enteric nervous system by affecting the interaction between enteric neurons, nociceptors and enteric glia. In this context the activation of glia seems to be actively involved in visceral hypersensitivity (Delvalle et al., 2018). Interestingly, the glia activation persists even in the remission phase of colitis, spreading vertically to the dorsal root ganglion where it was found an increased coupling between satellite cells and neurons that was correlated with visceral pain (Hanani et al., 2010; Huang et al., 2013).

Like in the periphery, also in the central nervous system, neurons are surrounded by different type of glial cells which can be activated by excitatory neurotransmitters released from nearby neurons (Winkelstein et al., 2001; Watkins et al., 2002). Two of them, microglia and astrocytes, are particularly important because they play a strategic role in central sensitization as well as in the remodelling of synaptic plasticity responsible for the chronicization of painful state (Auld and Robitaille, 2003; Di Cesare Mannelli et al., 2013; Di Cesare Mannelli et al., 2014).

The present results showed a significant activation of both microglia and astrocytes (as well as an increased number of astrocytes) in the spinal cord dorsal horn of DNBS treated animals, where sensitive afferents reach the second order neuron in the spino-thalamic pain pathway. Anyway, a similar cellular framework was observed also in the ventral horn, indicating a diffuse activation of glia over the spinal cord. This feature has been detected in other pain

syndromes associated with hyperreflexia, as nerve injury (De Luca et al., 2016; Cirillo et al., 2016; Qian et al, 2018). Motor neurons of ventral horn project to abdominal muscles and intestine (Qian et al, 2018), so the overactivation of astrocytes and microglia in this district may be related to the increased spinal reflex that we observed in response to the colo-rectal distension as for intestinal dysmotility. It is indeed known that the dorsal and ventral horn of the spinal cord have quite different morphological and functional profiles: the dorsal horn contains interneurons and receives the sensory terminals from the peripheral nerve, while the ventral horn neurons distribute outgoing (efferent) motor impulses. The incoming information is processed at spinal level by complex circuits involving excitatory and inhibitory interneurons in the dorsal horn, and transmitted to projection neurons for relay to several brain areas. In addition, nociceptive information is conveyed to the ventral horn and contributes to spinally-mediated nocifensive reflexes (Todd et al., 2010). Interestingly, a research group of Pittsburgh recently demonstrated that extrinsic primary afferent neurons (as pain-sensing neurons) of the colon influence myenteric neuron activity through a spinal reflex in mice, providing an explanation on how pain and dysmotility co-occur in GI disorders (Smith-Edwards et al., 2019). Our results, showing a sequential activation of microglia and astrocytes along the reflex arc of the spinal cord, support the idea that glia activation along this circuit may contribute to prolong intestinal dysregulation and pain, as previously supposed in the animal model of chronic fatigue syndrome (Ji et al., 2013; Yasui et al., 2019). Moreover, this vicious circle is probably strengthened by the persisting immune activation in the periphery which can modulate signal propagation (Grace et al., 2014; Murphy et al., 2014).

In the second part of my work, I studied the role of $A_3$AR as possible new pharmacological target for relieving visceral pain. The novel $A_3$AR agonist, MRS5980, as well as the classical agonist, Cl-IB-MECA, resulted effective against post-inflammatory visceral hypersensitivity in rats.

The administration of $A_3$AR agonists in the DNBS model of post-inflammatory visceral pain showed an efficacy equivalent to that of linaclotide, an approved treatment for adult patients suffering from IBS with predominant constipation (IBS-C; Chey et al., 2012). Linaclotide, a selective agonist of the guanylate cyclase–C receptor that is expressed on the luminal surface of intestinal enterocytes, reduces intestinal pain in animal models (Potter, 2011; Eutamene et al., 2010). The use of linaclotide has been associated with the development of diarrhoea in 20% of patients. This adverse effect makes linaclotide unsuitable for patients affected by diarrhoea-predominant IBS or IBS with alternating constipation and diarrhoea (Thomas and Allmond, 2013).

Adenosinergic signalling is known to contribute to pain control (Zylka, 2011; Sawynok, 2016; Janes et al., 2016) and to modulate intestinal functionality (Antonioli et al., 2014; Asano et al., 2017; Asano and Takenaga, 2017). We were especially interested in a potential role for $A_3$AR agonists as a rapidly growing body of evidence shows that these compounds give pain relief in diverse models of chronic pain without significant adverse side effects (Fishman et al., 2012; Janes et al., 2016; Salvemini and Jacobson, 2017). This latter feature is of particular importance because of the cardiovascular side effects produced by $A_1$AR agonists (Zahn et al., 2011; Zhang et al., 2009).

The pharmacology of adenosine receptor subtypes displays significant inter-species differences. In the rat, the relative selectivity *vs* $A_3$ for Cl-IB-MECA is: $A_1/A_3$ = 848, $A_{2A}/A_3$ = 1424, and $A_{2B}:/A_3$ = <10,000 (Borea et al., 2015). It is thus unlikely but not impossible that the effects we saw with Cl-IB-MECA may have been partly due to activity at the $A_1$ and/or $A_{2A}$ receptor subtypes. However, in the rat MRS5980 is greater than 10,000-fold selective for the $A_3$ receptor subtype *vs* each of the other three subtypes and we can thus be confident that the effects seen with this agonist were entirely due to activity at the $A_3$AR. This conclusion is consistent with our data showing that both the Cl-IB-MECA the MRS5980 effects were blocked by the $A_3$AR antagonist MRS1523, whose selectivity ratios in the rat are: $A_1/A_3$ = 140 and $A_{2A}/A_3$ = 18 (the ratio for $A_{2B}$ is undetermined but is almost certain to be greater than 10,000/1) (Borea et al., 2015). The analgesic effects of $A_3$AR agonists in other pain models have been shown to be absent in $A_3$AR knockout mice (Little et al., 2015).

The efficacy of $A_3$AR agonists against visceral pain is consistent with data showing that the $A_3$AR is involved with multiple pain mechanisms (Janes et al., 2016) at peripheral, spinal and supraspinal levels (Little et al., 2015; Janes et al., 2016). There is evidence that $A_3$AR agonists are analgesic against neuropathic pain via inhibition of the astrocyte-associated neuroinflammatory response in the spinal cord (Janes et al., 2014; Janes et al., 2015), a phenomenon strongly involved in pain persistence (Di Cesare Mannelli et al., 2013; Di Cesare Mannelli et al., 2014). $A_3$AR activation has been reported to enhance the formation of anti-inflammatory cytokines (Hasko et al., 1996; Janes et al., 2014; Janes et al., 2015; Wahlman et al., 2018) and the production of glial-derived neuroprotective substances (Wittendorp et al., 2004). Furthermore, *in vitro* and *in vivo* studies demonstrate that $A_3$AR produces its effects by inhibiting the p38 MAPK and NF-κB signalling pathways (Madi et al.,2007; Varani et al., 2010; Varani et al.,2011; Janes et al.,2014). All these mechanisms may contribute to the relief of visceral pain evoked by colitis. This is consistent with the reports of Ren et al. who described that $A_3$AR activation reduced colitis-induced tissue injury by modulating the NF-

kB signalling pathway (Ren et al., 2015) and through inhibiting NLRP3 inflammasome activation and pyroptosis in colonic epithelial cells (Ren et al., 2019).

$A_3$ARs may limit excitatory neurotransmission, which is altered in neuropathic pain (Gwak et al., 2012) as well as in visceral pain (Willert et al., 2004; Lapointe et al., 2015; Gebhart and Bielefeldt, 2016). For example, the protective role of $A_3$ARs in the first phase of ischemia appears to be at least partly related to a decrease in synaptic transmission (Pugliese et al., 2003; Pugliese et al 2006; Rivera-Oliver and Diaz-Rios, 2014). Moreover, $A_3$AR activation protects against the neurotoxic intra-cellular $Ca^{2+}$ rise mediated by P2X7 or NMDA receptors (Zhang et al., 2006 and 2010).

As already demonstrated (Coppi et al., 2019), the selective stimulation of adenosine $A_3$ receptors inhibits the N-type $Ca_v2.2$ opening and decreases electrically-evoked excitation in isolated rat DRG neurons. Here, we found that both Cl-IB-MECA and MRS5980 significantly inhibited $Ca_v2.2$ activation in DRG neurons. The effect was prevented by the selective $A_3$AR antagonist, MRS1523, and by the $Ca_v2.2$ blocker, PD173212, confirming the involvement of $Ca_v2.2$ in the $A_3$AR-mediated effect. We did not measure significant difference between $A_3$AR-mediated inhibition in DRG neurons isolated from DNBS-treated *vs* vehicle-treated animals. However, alterations in passive membrane properties and steady-state $Ca^{2+}$ currents were observed between the two groups. The resting membrane potential was significantly more depolarized in DRG neurons isolated from DNBS-treated animals, suggesting a hyperexcitable state in inflamed animals. Interestingly, steady-state $Ca^{2+}$ currents, but not peak $Ca^{2+}$ currents, were markedly reduced in DRG neurons isolated from DNBS animals. In an attempt to explain the relationship between steady-state $Ca^{2+}$ current reduction in DRG neurons and visceral hypersensitivity, we hypothesize that a decrease in $Ca^{2+}$ influx into the cell can lead to membrane potential instability and depolarization through the decrease of $Ca^{2+}$-activated $K^+$ channels ($K_{Ca}$) opening (Pagadala et al., 2013; Zhang et al., 2007; for review see: Hogan, 2007). $Ca^{2+}$-activated $K^+$ channels are known to stabilize the membrane potential and participate in repolarizing neurons after action potential firing, thus avoiding bursting activity.

Moreover, a recent publication demonstrates that $Ca_v2.2$ induces a voltage-dependent, but $Ca^{2+}$ independent secretion of ATP from the soma of DRG neurons (Chai et al., 2017). ATP is a powerful mediator of pain, for example, ATP-induced activation of the P2X3 receptor is involved in visceral pain, as shown by the ability of the P2X3 antagonist, A-317491, to potently reduce hypersensitivity (Deiteren et al., 2015).

The hypothesis that $Ca_v2.2$-mediated modulation contributes to $A_3AR$-mediated anti-hyperalgesia is supported by other evidence. Modulation of $Ca_v2.2$ channel activity is seen with other pain-relieving, Gi-coupled receptor agonists, e.g., opioids, cannabinoids, neuropeptides Y, and substance P (Beedle and Zamponi, 2004; Shapiro and Hille, 1993; Soldo and Moises, 1996; Twitchell et al., 1997; Sun and Miller, 1999). Also, the neuropathic pain analgesics, gabapentin and pregabalin (Finnerup et al., 2015), inhibit synaptic transmission mediated by $Ca_v2.2$ (Sutton et al., 2002; Bauer et al., 2009). The gabapentinoids are effective in reducing visceral pain and preventing spinal neuronal activation associated with colorectal distension in animals (Ohashi et al., 2008; Ravnefjord et al., 2008; Sikandar and Dickenson, 2011) and have been suggested to be treatments for irritable bowel syndrome (Camilleri and Boeckxstaens, 2017). A direct inhibitor of $Ca_v2.2$ channel activity, ziconotide, is a clinically-approved pain therapy, although it is limited to the intrathecal route (McDowell and Pope, 2016). In our hands, the i.p. administration of the specific $Ca_v2.2$ inhibitor PD173212 (Hu et al., 1999; Ryder et al., 1999; McDowell and Pope, 2016) significantly decreased visceral hypersensitivity in DNBS-treated animals, confirming the effectiveness of modulating this channels to obtain visceral pain relief.

In the third part of the work, I studied the role of gut microbiota in the development and the persistence of post-inflammatory visceral pain induced by DNBS.

Microbial composition can shape the environment in the colon as metabolites produced by microbiota can be involved in signalling, immune system modulation or have antibiotic activity (McHardy et al., 2013; Dorrestein et al., 2014; Wu et al., 2014). The dysbiosis of gut microbiota is one of the mechanisms proposed to contribute to the initiation, exacerbation and persistence of visceral pain (De Palma et al., 2014; Hyland et al., 2014; O'Mahony et al., 2017; Foster et al., 2017). It has been demonstrated that disturbance of the gut microbiota in adult mice and rats affects the local immune response and enhances pain signalling (Verdu et al., 2006; Hoban et al., 2016; Luczynski et al., 2017). However, the most well-documented line of evidence linking disruption of gastrointestinal microbial homeostasis to the development of chronic visceral hypersensitivity comes from the literature on post-infectious IBS (Thabane et al., 2007; Klem et al., 2017).

Previous studies at clinical level showed major shifts in the gut microbial composition of patients with IBD (Morgan et al., 2012; Haberman et al., 2014; Blander et al., 2017; Imhann et al., 2018). Recently, a broad metabolic shift of microbiota has been also correlated with inflammation in these patients (Franzosa et al., 2018). Differences in microbial taxonomic profiles were identified in preparations of the fecal material used for the repeated fecal

transfers, obtained either from control or DNBS-treated animals, indicating that alterations of the gut ecosystem mediated by DNBS persist in the remission phase of colitis.

Despite all the evidences, a cause-effect relationship between dysbiosis and post-inflammatory visceral pain has not yet been proved. In this regard, Hadizadeh et al., (2018) provide evidence linking faecal microbiota composition to the occurrence of abdominal pain and its frequency, duration and intensity in the general population and, therefore proposed microbiota as risk factor for the development of functional gastrointestinal disorders. Studies in experimental animals showed that sensitivity to colonic distension of IBS patients can be transferred to rats by the fecal microbiota, without the occurrence of mucosal alterations (Crouzet et al., 2013). Additionally, alterations of visceral sensitivity and expressions of BDNF, GFAP and substance P in colon of mice in the presence or absence of IBS fecal supernatants stimulation were observed (Wang et al., 2016). In the majority of the studies, animals were transplanted with the microbiota from human, introducing another confounding factor which could be avoid using specie-specific transplants, as in our case.

We found that the transplant of the faecal microbiota derived from DNBS rats in naïve rats is able to directly transfer visceral hypersensitivity to naïve animals. This effect was not imputable to an inflammatory response, neither to changes in gut permeability or serotonin increase. Besides, the transplant of a healthy microbiota in DNBS-treated animals counteract the persistence of visceral pain after the colitis resolution, confirming an active involvement of microbiota in post-inflammatory visceral pain.

In a similar experiment, Yang et al. (2019) found a correlation between an abnormal composition of gut microbiota and susceptibility to anhedonia post spinal nerve injury (SNI) surgery. In fact, they observed that faecal microbiota transplantation from SNI rats with or without anhedonia altered pain, depression-like and anhedonia-like phenotypes in the pseudo-germ-free mice, suggesting a general control of microbiota on pain perception.

Despite all the evidences attesting a modulatory effect of faecal material transplantation on visceral sensitivity, no-one of these studies provided a strong correlation between the microbiota composition and the behavioural trends. Particularly, there are poor evidence investigating the evolution of microbiota over time after the FMT. In this work we demonstrated a strict temporal correlation between the animal's behaviour and the evolution of microbiota composition. Concerning the experimental groups of animals treated with antibiotics and then subjected to FMT, defined clusters in PCoA plots were observed for samples collected after the second series of repeated faecal transfers (Day 32) from CTR donors and DNBS donors, suggesting that major modifications in the composition and

structure of the gut microbiota might have occurred at this stage. On Day 46 (when the difference of pain disappeared) the microbiota belonging to the two different groups got closer again. Evaluation of taxonomic profiles confirmed that marked differences between the animals underwent the FMT from CTR or DNBS were identifiable on Day 32 at phylum and family level for major members of the gut microbiota, including also protective microbial taxa known to be associated with gastrointestinal health.

The fact that the effect of the transplant was not decisive, demonstrated the presence of a continuous bidirectional influence between the microbiota and the gut, which can be only temporarily modulated by FMT. Establishing and maintaining beneficial interactions between the host and its associated microbiota are key requirements for host health. On one hand, microbiota composition modulates gut physiology (Sommer and Bäckhed, 2013; Contijoch et al., 2019), on the other hand, substances released from the gut can exert a selective pressure on different microbiota bacterial strains (Hevia et al., 2015; Fung et al., 2019).

In an altered GI physiology, like that occurs after a major intestinal damage, the microbiota transplanted could somehow stem the pathogenic mechanisms underlying pain persistence, e.g., by metabolizing pain mediators or by producing analgesic substances, or even through a modulation of gene expression in the gut. This effect may last until the "new" microbiota is able to survive in the new environment. Afterwards the selective pressure exerted by the host determine further alterations in the composition of microbiota, with loss of the effects. All these hypotheses need to be tested.

Another point that need to be discussed is the employ of the antibiotics to prepare the animals to the FMT. In the first set of the experiments, we adopted a 24 days-antibiotic treatment in order to deplete the resident microbiota and therefore favor the establishment of the new one derived from CTR and DNBS animals, respectively. Although the protocols for preparing the animals to the FMT are quite heterogeneous in literature, long treatments which employ a combination of antibiotics, like this, are commonly used (Hintze et al., 2014; Staley et al., 2017). However, most of the studies have only shown the feasibly of this approach, but they have been seldom used in a disease context (Kelly et al., 2016; Routy et al., 2017). Actually, antibiotics should be used with caution, depending on the disease that is studied. For example, the use of antibiotics can be problematical when studying some diseases, as they may be improved in rodent models by the antibiotics themselves (Adachi et al., 1995; Bigorgne et al., 2008; Dapito et al., 2012).

In our case, an important bias was represented by the fact that a long antibiotic treatment directly affects visceral sensitivity, as reported in many studies (Verdu et al., 2006;

O'Mahony et al., 2014, Hoban et al., 2016; Luczynski et al., 2017), becoming a confounding factor in the study of FMT effect on visceral pain. As expected, the long antibiotic treatment increased visceral sensitivity in the animals, though the effect was significant only in the AWR test. In particular the antibiotic treatment lowered the threshold at which the animals respond to the colo-rectal stimulus. Microbial re-colonization restored normal visceral pain responsiveness and threshold, as previously observed (Luczynski et al., 2017). By contrast, the animals receiving the FMT from DNBS donors showed a partial and slow recovery from the antibiotic effect. This led us to question if the increased visceral sensitivity observed after the FMT from DNBS donors was imputed to an active effect of the established microbiota or to a slow recovery from the antibiotic effect, as the new microbiota was incapable to restore the normal gut physiology. To reduce the impact of the antibiotics on visceral sensitivity we shortened the length of the antibiotic treatment to 7 days, time needed to obtain a sufficient antibiotic depletion. Also the shortened antibiotic treatment caused a significant increase in the AWR response to CRD, confirming that visceral sensitivity is directly modulated by the resident microbiota. Anyway, in this case, the effect of the antibiotic was completely reverted by the FMT from CTR donors as well as by administering the vehicle, indicating a beneficial effect of a spontaneous re-colonization of the intestine. On the other hand, the pro-algesic effect mediated by FMT from DNBS donors was confirmed even after the shortened antibiotic treatment.

The visceral hyperalgesia induced by the transplant of the faecal material derived from DNBS animals was evident already 24h after the first set of FMT, suggesting the presence of pain mediators in the supernatant obtained from the faeces, as supposed in other studies (Buhner et al., 2019; Wang et al., 2015; Hevia et al., 2015; Wang et al., 2016). About that, we excluded the presence of residual DNBS in the faecal supernatant utilized for FMT. We cannot exclude instead the presence of cytokine, enzymes, neurotransmitters or other secondary metabolites (maybe not known yet) in these preparations. Regarding the protein content, it is likely digested through the stomach, unless the microbiota someway protects them from the enzymatic actions or continue producing them once transplanted.

The presence of pain mediators in the faecal medium might explain the acute effect of FMT. By contrast, the long-lasting hyperalgesia observed in these animals can not be imputed to this acute stimulation, as well as to a local irritation, since by colon histological analysis, no macroscopic or microscopic signs of damage were observed on Day 32 (7 days after the last FMT). These evidences increasingly strengthened the idea of an active role of microbiota in pain modulation mediated by FMT. Moreover, the fact that the visceral hyperalgesia induced

by the microbiota transplant from DNBS donors extinguished earlier in the VMR test in respect to AWR test, suggested a possible central sensitization to the painful stimulus induced by CRD. Similarly, in the animals treated with DNBS, VMR response is significantly decreased after 4 weeks, while the augmented AWR response persists up to 13 week after DNBS injection.

The integrity of the intestine was also confirmed by investigating its permeability. A slight increase in gut permeability was denotable only after the antibiotic treatment, with an increase in the expression of Zo-1. The up-regulation of Zo-1 expression and its augment at plasma level has been in fact related to a leaky gut (Sapone et al., 2006; Fasano t al., 2012; Sturgeon and Fasano, 2016). This result is in line to the previous findings showing a positive effect of microbiota on tight junctions' expression and gut barrier integrity maintenance (Kelly et al., 2015b; Desai et al., 2016; Stevens et al., 2018).

The analysis of gene expression in the gut confirmed a derangement of cytokines as a result of both the antibiotic treatment and the FMT, but no significant correlations were found between the cytokines profiles among the groups and the observed differences in visceral pain. However, it appears difficult to associate a behavioural phenotype to the alteration of few cytokines. In fact, the immune response is very finely orchestrated with many co-factors intervening. Frequently the augment of pro-inflammatory cytokines is followed by an increased release of anti-inflammatory cytokines in a circular regulatory loop where the final outcome is the result of the balance between all the actors involved, including the microbiota (Round et al., 2009; Maslowski et al., 2010; Cicchese et al., 2018). This is evident in the DNBS donors wherein an increase in both pro- and anti- inflammatory cytokines occurred. Concerning the immune response, contradictory evidences were revealed in patients affected by IBS. In fact, although the evidence for aberrant immune activation seems overwhelming in PI-IBS, various research groups found no differences in immune cell numbers or cytokine mRNA expression in mucosal biopsies of PI-IBS and IBS patients compared with healthy volunteers (Mearin et al., 2009; Braak et al., 2012; Wouters et al., 2016a; Bennett et al., 2016). Noteworthy, in the animals receiving the FMT from DNBS donors we found a decrease in TNF-α and an increase in IL-10. The opposite has been observed in the animals receiving the FMT from CTR. There are no data in literature that can explain this phenomenon, whereby it would be interesting to investigate further the inflammatory response in the gut by an extensive mRNA microarray analysis on the tissue.

Searching for other possible mechanisms, I focused the attention on tryptophan metabolism, which is one of the most important molecules at the interface between the microbiota, the gut

and the nervous system: its metabolism, which is under the strict control of the microbiota, can lead to different compounds with neuromodulator functions like serotonin and kynurenine (Agus et al., 2018). Alterations in tryptophan metabolism have been documented to be involved in different GI disorders, such as IBS-D, IBS-C and IBDs (Fitzgerald et al., 2008; Clarke et al., 2012; Lamas et al., 2016; Nikolaus et al., 2017). In the DNBS donors we found a decrease in the plasmatic levels of tryptophan that could account for an increase biosynthesis of serotonin, as confirmed by the HPLC analysis on the gut. The augment in the serotonin production and release is a characteristic consequence of intestinal damages. In fact, the enterochromaffin cells respond to a gut insult through the release of this neurotransmitter, which can directly act on visceral afferents (Nozawa et al., 2009; Cremon et al., 2011; Mawe et al., 2013; Nikolaus et al., 2017). In the animals underwent the antibiotic treatment and then subject to FMT we found a common decrease in the plasmatic levels of tryptophan, but in this case no changes in serotonin levels were observed in the gut tissue. By contrast, the animals underwent the FMT showed a massive and long-lasting increase of serotonin in the faeces, irrespective to the donor.

In the animals treated with the antibiotics+vehicle the decrease in tryptophan instead combine with an increase in the production of kynurenine. Interestingly, the metabolism of kynurenin in the different tissues leads to both neuromodulator compounds, like kynurenic acid and quinolinic acid (Stone, 2000; Vamos et al., 2009). Kynurenic acid account for the neuroprotective effects of kynurenine derivatives. Through the activation of GPR35 receptors, it also showed pain relief properties in animal models of pain (Zhao et al., 2010; Cosi et al., 2011; Resta et al., 2016), while neurotoxicity is usually ascribed to quinolinic acid (Guillemin, 2012). Preclinical evidence demonstrated direct and indirect mechanisms by which the gut microbiota can regulate tryptophan availability for kynurenine pathway metabolism, with downstream effects on gut and brain functions (Kaszaki et al., 2012; Kennedy et al., 2017). Also other metabolites of tryptophan, like aryl hydrocarbon receptors (AhR) agonists, were found to be important in neuromodulation, e.g., by the control of microglial activation (Rothhammer et al., 2018) as well as by modulating intestinal immune response (Hubbard et al., 2015; Metidji et al., 2018). Anyway, the increase in the production of these molecules and their involvement in visceral pain has to be explored yet.

As mentioned above, since the microbiota is able to directly influence the levels of 5-HT, I measured serotonin levels also in the faeces. Interestingly the faecal content did not reflect what observed in the gut tissue. In fact, in the faeces we observed a decrease in serotonin consequent the antibiotics treatment, as previously reported (Hata et al., 2017). On the other

hand, serotonin increased after the FMT. Interestingly, Fung et al. (2019) demonstrated that an increase of 5-HT in the gut lumen selectively modulate the colonization of bacteria species in the gut. Nevertheless, no difference was found between the animals receiving the FMT from CTR or from DNBS donors, demonstrating again that 5-HT is not involved in the transfer of pain mediated by FMT from DNBS donors into naïve rats. Furthermore, I found a decrease of dopamine in the gut as consequence of the antibiotic treatment and the FMT in all the experimental groups. In accordance, it was reported that the clearance of gut microbiota by antibiotic cocktail reduced synthesis of dopamine (DA) in intestines and exacerbated liver damage. Anyway, the alteration in dopamine levels were restored by recovery of gut microbiota (Xue et al., 2018; Strandwitz et al., 2018), unlike what we have observed. Physiological studies suggest that dopaminergic mechanisms are important in the regulation of gastrointestinal motility (Vieira-Coelho et al., 1993; Walker et al., 2000; Li et al., 2006). Moreover, dopamine is the metabolic precursor of noradrenaline (NA), which is another important regulator of gut functionalities (Musacchio et al., 2013; Mittal et al., 2017). The gut levels of dopamine were reduced also in DNBS donors, which reported alterations in the GI motility (Antonioli et al., 2011).

Regarding the therapeutic potential of FMT, current evidences from randomized clinical trials do not suggest a benefit of FMT for global IBS symptoms, though there remain discrepant results among the trials (Xu et al., 2019). Actually, the therapeutic effect of FMT could be empowered by adjusting the protocols. In fact, up to now it has been considered a good protocol to maintain a low number of FMT with a single donor. This protocol could not be effective in particular conditions like IBDs. Indeed, recent clinical studies highlighted the efficacy of a multiple donor and repeated treatment based therapy in patients affected by UC, chronic intestinal pseudo-obstruction and IBS (Paramsothy,et al., 2017; Gu et al., 2017; El-Salhy et al., 2019). In our animal model of colitis-induced visceral pain, we obtained a good relief from pain after 4 cycles of FMT, utilizing different donors. In fact, is likely that the "inflammatory" environment wherein the microbiota is transplanted obstacles the establishment of some species compared to others. In this case, the repetition of the FMT could force the system to accept the new microbiota.

In conclusion, this work highlighted the complex nature of post-inflammatory visceral pain whose study required a multidisciplinary approach. On one side, these findings could suggest the possibility to employ complementary therapies for modulating the different actors involved in the persistence of pain. On the other side, understanding the molecular mechanisms that regulate the interaction between the periphery and the central nervous

system in the maintenance of this hyperactivated system could represent the key to identify new therapeutic targets. Within this scenario, $A_3$AR agonists appear to be a promising resource for visceral pain management. Regarding the microbiota, the results obtained *in vivo* strengthen the hypothesis of a direct involvement of microbiota in post-inflammatory visceral pain and so encourage to further study the therapeutic effect of microbiota transplantation. Even if the question about the mechanism by which the microbiota influences visceral sensitivity remain still open, this work contributed to exclude some hypothesis. It is however necessary to continue investigating the mechanisms by which the microbiota can modulate visceral pain perception, in order to make it a suitable target for the treatment of visceral pain.

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