Alma Mater Studiorum — Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE MEDICHE GENERALI E DEI SERVIZI

Ciclo XXX°

Settore Scientifico Disciplinare: MED/09

Neurodegeneration and Neuroplasticity of the Intrinsic Innervation of the Gut in Severe Dysmotility

Presentata da: Dr. Tennekoon B. Karunaratne

Coordinatore Dottorato:

Chiar.mo Prof. Fabio Piscaglia

Supervisore:

Chiar.mo Prof. Vincenzo Stanghellini

Correlatore:

Chiar.mo Prof. Roberto De Giorgio

Esame finale anno 2018

ABSTRACT

Abnormalities in the gastrointestinal neuromuscular apparatus including interstitial cells of Cajal (ICC) is presumed to underlie a clinically heterogeneous group of disorders collectively termed gastrointestinal neuromuscular diseases (GINMDs). A subset of these patients may manifest clinically with recurrent intestinal sub-occlusive episodes, which occur in the absence of demonstrable mechanical causes, leading to numerous hospitalizations as well as useless and potentially harmful surgical interventions. Taken together clinical and radiological signs make up a condition referred to as chronic intestinal pseudo-obstruction (CIPO). This is a rare and intractable chronic digestive disease that can result from abnormalities of smooth muscle cells (effectors of contractility / relaxation), ICC (pace-makers of gut motility and regulators of neuronal input to smooth muscle cells), and neurons (either intrinsic – the enteric nervous system- or extrinsic nerve pathways).

The present thesis was thought to provide a translational view by characterizing dysmotility and establishing attendant histopathological defects in CIPO and determine whether a correlation exists between clinical features, motility patterns and neuromuscular changes, using qualitative and quantitative morphological approaches.

Summary of published and submitted papers pertinent to this thesis

In this thesis I provided an update on neurointestinal diseases, with special focus on diagnostic, therapeutic and management aspects of CIPO, which have been published during the PhD course. My senior dissertation deals with major neurogastroenterological aspects, such as those involving the most severe forms of the functional GI disorder spectrum, i.e. those patients with prominent abnormalities of gut propulsion. A better understanding of these cases will cast hope about patient management and newly applicable therapeutic intervention. Briefly:

1. Clinical Aspects of Neurointestinal Disease: Pathophysiology, Diagnosis, and Treatment

In this review we considered ENS disorders from a clinical perspective and highlighted the advancing knowledge regarding their pathophysiology. The enteric nervous system (ENS) is involved in the regulation of virtually all gut functions. Conditions referred to as enteric neuropathies are the result of various mechanisms including abnormal development, degeneration or loss of enteric neurons that affect the structure and functional integrity of the ENS. Clinical and molecular research has led to important conceptual advances in our knowledge of the pathogenetic mechanisms of these disorders.

2. Chronic intestinal pseudo-obstruction in children and adults: diagnosis and therapeutic options

Chronic intestinal pseudo-obstruction (CIPO) represents the most severe form of gastrointestinal dysmotility with debilitating and potentially lethal consequences. The present article aims to provide pediatric and adult gastroenterologists with an appraisal about clinical features, diagnosis and therapeutic options for CIPO. There is no single diagnostic test or pathognomonic finding of CIPO, thus a stepwise approach including radiology, endoscopy, laboratory, manometry and histopathology should be considered in the diagnostic work-up. Treatment of patients with CIPO is challenging and requires a multidisciplinary effort.

3. Chronic intestinal pseudo-obstruction: progress in management?

CIPO is often due to derangement of the innervation / smooth muscle / interstitial cells of Cajal with recurrent episodes of intestinal sub-occlusion mimicking a mechanical obstruction. Management is a critical aspect in CIPO patient care. Promising data indicate that percutaneous endoscopic gastro-jejunostomy (PEG-J) can be proposed as a measure for

intestinal decompression, thereby improving CIPO-associated abdominal symptoms, including pain. The present mini-review tackles management options, with a major emphasis on PEG-J, for CIPO patients.

4. Comparison between small bowel manometric patterns and full-thickness biopsy histopathology in severe intestinal dysmotility

In this original paper I contributed to assess 38 patients with severe intestinal dysmotility using intestinal manometry and a subsequent full-thickness intestinal biopsy. Patients with abnormal intestinal manometry had abnormal histopathological findings in 73% of cases. However, manometric patterns did not match with the specific neuromuscular abnormalities. The key finding was that manometrically defined small bowel dysmotility was often associated with abnormal neuromuscular findings, although this aspect did not correlate with specific histopathology.

5. Quantitative changes of enteric neurons correlate with Clinical features in patients with severe dysmotility

This original submitted / under peer review paper follows the previous one. Here I have contributed to define the role of quantitative analysis in enteric neuromuscular disorders. Specifically, the intrinsic innervation of the gut, i.e. the enteric nervous system, can reveal significant decrease of both myenteric and submucosal cell bodies in patients with severe dysmotility, even in those cases in whom a previous evaluation did not identify clear-cut histopathological abnormalities. An overall 50% decrease of myenteric and submucosal neuronal cell bodies correlated with clinical features, e.g. pain and bloating.

LIST OF ABBREVIATIONS

5-HT 5-hydroxytryptamine

ACh acetylcholine

AHPs after hyperpolarizing potentials

AN apparently normal

ARM anorectal manometry

ATP Adenosine triphosphate

BN bombesin

CALB calbindin

CCK cholecystokinin

CCSH congenital central hypoventilation syndrome

CGRP calcitonin gene-related peptide

ChAT choline acetyltransferase

CI colonic intertia

CIPO chronic intestinal pseudo-obstruction

CM circular muscle layer,

CNS central nervous system

DEG degenerative neuro-muscular alterations

ECs enterochromaffine cells

ED enteric dysmotility

EGCs enteric glial cells

ENS enteric nervous system

ESMP external submucosal plexus

GABA gamma amino butyric acid

GFAP glial fibrillary acidic protein

GI gastrointestinal

GINMDs Gastrointestinal neuromuscular diseases

GRP gastrin releasing peptide

HAEC Hirschprung associated enterlocolitis

HRM High resolution manometry

HSCR Hirschsprung disease

ICC interstitial cells of Cajal

IFANs intestinofugal afferent neurons

IHPS idiopathic hypertrophic pyloric stenosis

IND intestinal neuronal dysplasia

INF inflammatory

IPANs intrinsic primary afferent neurons

iPSCc induced pluripotent stem cells

IR immunoreactivity

IRP integrated relaxaion pressure

ISMP internal sunmucosal plexus

LES lower esophageal sphincter

LM longitudinal muscle layer,

MMC migrating myoelectric complex

MP Myenteric plexus

ND not determined

NeuN neuronal nuclei

NFP neurofilament triplet protein

NIID neuronal intranuclear inclusion disease

nNOS neuronal nitric oxide synthase

NO nitric oxide

NOS nitric oxide synthase

NPY neuropeptide Y

PACAP pituitary adenylyl-cyclase activating peptide

PVG prevertebral ganglion

SCLC small cell lung carcinoma

SD severe dysmotility

SFGID severe functional GI dysmotility

SMC smooth muscle cells

SMP submucosal plexus

SOM Somatostatin

Sox SRY-box

SP substance P

STC slow-transit constipation

UES upper esophageal sphincter

VIP vasoactive intestinal peptide

Chapter 1

GASTROINTESTINAL NEUROMUSCULAR DISEASES

Gastrointestinal neuromuscular diseases (GINMDs) are a clinically heterogeneous group of chronic conditions associated with impaired gut motility in which signs and symptoms are presumed or proven to originate from underlying dysfunction of the gastrointestinal (GI) neuromuscular compartment, including interstitial cells of Cajal (ICC)[1,2]. These conditions have a profound impact on healthcare systems since they are quite disabling and associated with high social and economic burdens due to direct and indirect costs [3,4]. The underlying neuromuscular dysfunction may result from morphological and /or functional alterations affecting the enteric nervous system (ENS) and / or the *muscularis propria* and / or ICC, with histopathologic pictures ranging from severe damage to more subtle abnormalities which may lack evident microscopic changes to neurons [5-7], enteric glial cells (EGC) [8,9], smooth muscle [10-12], and ICC [13-15]. GINMDs may encompass a wide range of pathologic conditions and they can vary largely in terms of their etiopathogenesis, clinical picture, histopathologic patterns, and region of gut involvement. These disorders are characterized mainly by clinical symptoms related to altered gut functions and patients with GINMDs represent still a clinical challenge because there are no established or widely accepted classifications so far available. A classification of GINMDs is based on morphological findings and requires signs and symptoms of impaired motor activity accompanied by demonstrable motor abnormalities, for example manometric abnormalities with or without radiological evidence of visceral dilatation [16]. Some of these disorders may occur due to relatively rare congenital defects, i.e. Hirschsprung's disease, where some causative genes have been identified and their neuropathophysiological role elucidated [17], whereas most GINMDs are acquired during lifetime as a primary (otherwise unknown) diseases or can be secondary to another established disease. Such disorders that belong to the area of GINMDs include oesophageal

achalasia [18,19], gastroparesis [20,21], enteric dysmotility (ED) [16] and chronic idiopathic intestinal pseudo-obstruction (CIPO) [22-25], slow-transit constipation (STC) [26,27] and idiopathic non-Hirschsprung megacolon [28,29]. Although they are distinct entities, for the purposes of this thesis CIPO and ED are collectively referred to as severe functional GI dysmotility (SFGID). Secondary GINMDs, clinically similar to primary disorders which arise as a complication or in association with other diseases can include CIPO occurring as a paraneoplastic syndrome, most commonly in association with small cell lung carcinoma (SCLC) [30-32], but also in cases of bronchial carcinoid [33], thymoma [34], ganglioneuroblastoma and neuroblastoma [35,36] and ovarian carcinoma [37]; Chagas' disease (South American trypanosomiasis) due to the systemic disease caused by infection with *Trypanosoma cruzi* [38,39]; connective tissue disorders such as scleroderma [40-42] and some endocrine and metabolic disorders, e.g. diabetes mellitus-related gastroenteropathy [43] and dysmotility associated with amyloidosis [44,45].

The characterisation and management of SFGID are challenging since there is limited correlation between symptoms and clinical findings or investigations. Dysmotility is a broad term without a universally accepted definition and covers a spectrum of disorders, which include ED, CIPO and pathologically defined neuromuscular disease, with significant degrees of overlap among them. Therefore, the term dysmotility is often reserved for patients presenting with severe symptoms without any objective evidence of abnormal bowel structure or function. For practical purpose, severe dysmotility can be defined as the presence of clinical morbidity (severe symptoms, nutritional compromise, poor response or refractory to treatment) with presence of abnormal manometry or transit test with or without histopathology showing intestinal neuromuscular disorder in a patient where mechanical obstruction and active mucosal pathology has been excluded.

In the next paragraphs of my thesis, I would like to provide the reader with a cellular knowledge on the neuro-muscular and ICC general aspects as a basis for a better

understanding of abnormal gut physiology and in particular motility which will be detailed afterwards.

Key points of Chapter 1:

- Gastrointestinal neuromuscular diseases (GINMDs) are a clinically heterogeneous conditions characterized by impaired gut motility
- Signs and symptoms of GINMDs may originate from underlying dysfunction of the gastrointestinal (GI) neuromuscular compartment.
- Patients with GINMDs impact profoundly on healthcare systems since their disabling disease is associated with high social and economic burdens.

Chapter 2.

MORPHOLOGICAL BASIS OF GASTROINTESTINAL MOTILITY

Nutrients constantly supply cells with substances necessary to maintain the energy balance of any living organisms. That is possible as the GI tract exerts a vast repertoire of physiological functions necessary for digestion and absorption. Muscular sphincters compartmentalize the bowel, dividing it into functionally distinct regions with different luminal environments. The upper esophageal sphincter (UES) maintains the highest resting pressure of all sphincters, preventing air from entering the esophagus. It consists of striated muscle, is under control of the swallowing center in the medulla in the brain, and relaxes during swallowing to permit food to enter the esophagus. The lower esophageal sphincter (LES) separates the esophagus and the stomach and consists of smooth muscle that relaxes during swallowing. It functions to coordinate the passage of food into the stomach after swallowing and prevent the reflux of gastric contents, including acid, into the esophagus. The pyloric sphincter separates the

stomach from the duodenum, and its resting pressure contributes to regulate gastric emptying and prevent duodenal-gastric reflux. The ileocecal sphincter is a valve-like structure that separates the ileum and cecum, preventing back flux of colonic contents into the ileum. Finally, the internal (smooth muscle) and external (skeletal muscle) anal sphincters control elimination of waste products. A constant detection of luminal contents allows for ingested material to be transported caudally at a physiological rate, allowing each region of the gut to perform their respective function. Smooth and skeletal (in the esophagus and anus) muscle contractions are thus coordinated into activity patterns, such as segmentation (small intestine) or haustration (colon) that grind, mix, and propel aborally the ingested food. Secretory mechanisms exert a pivotal role in order to maintain an appropriate pH and regulated concentrations of electrolytes, enzymes, and mucous. The pH of the highly acidic chyme in the stomach reaches 1.5 to 3.5. At this pH, the hydrogen ion concentration is around 3 million times that of the arterial blood. Secretion of hydrogen carbonate ion into the lumen of the duodenum by the exocrine pancreas neutralizes the acidic chyme delivered from the stomach to duodenum. These changes are necessary to promote digestion, absorption, and detoxification of ingested materials. A continuously regenerated semipermeable epithelial barrier separates the lumen from the internal milieu of the body. This barrier promotes absorption, but also avoid the leakage of unnecessary molecules into the intestinal lumen as well as the transit of digestive enzymes, toxins, and germs into the body from the lumen. Clearly, this highly organized structure of the GI tract (Fig. 1) and its related physiological functions require a sophisticate degree of regulation and coordination, provided by the ENS. This is also referred to as "*brain-in-the-gut*", since it exhibits control ability of a vast array of digestive functions even when severed from the central nervous system (CNS). Within the ENS, nerve cells and glia are organized in plexuses, either non-ganglionic (bundles of nerves) or ganglionic, i.e., myenteric (Auerbach's) and submucosal (Meissner's) ganglia, which are

interconnected by nerve fascicles (Fig. 2). An intact ENS is essential for body homeostasis in order to control motility, secretion / absoprtion, blood flow, cross-talk with the immune system as well as plays a role in the maintenance of epithelial barrier. Another noteworthy aspect pertains to the interplay between the ENS and the gut microbiota, i.e. the trillion of germs dispersed throughout the lumen of the gut, mainly concentrated in the most distal segments.

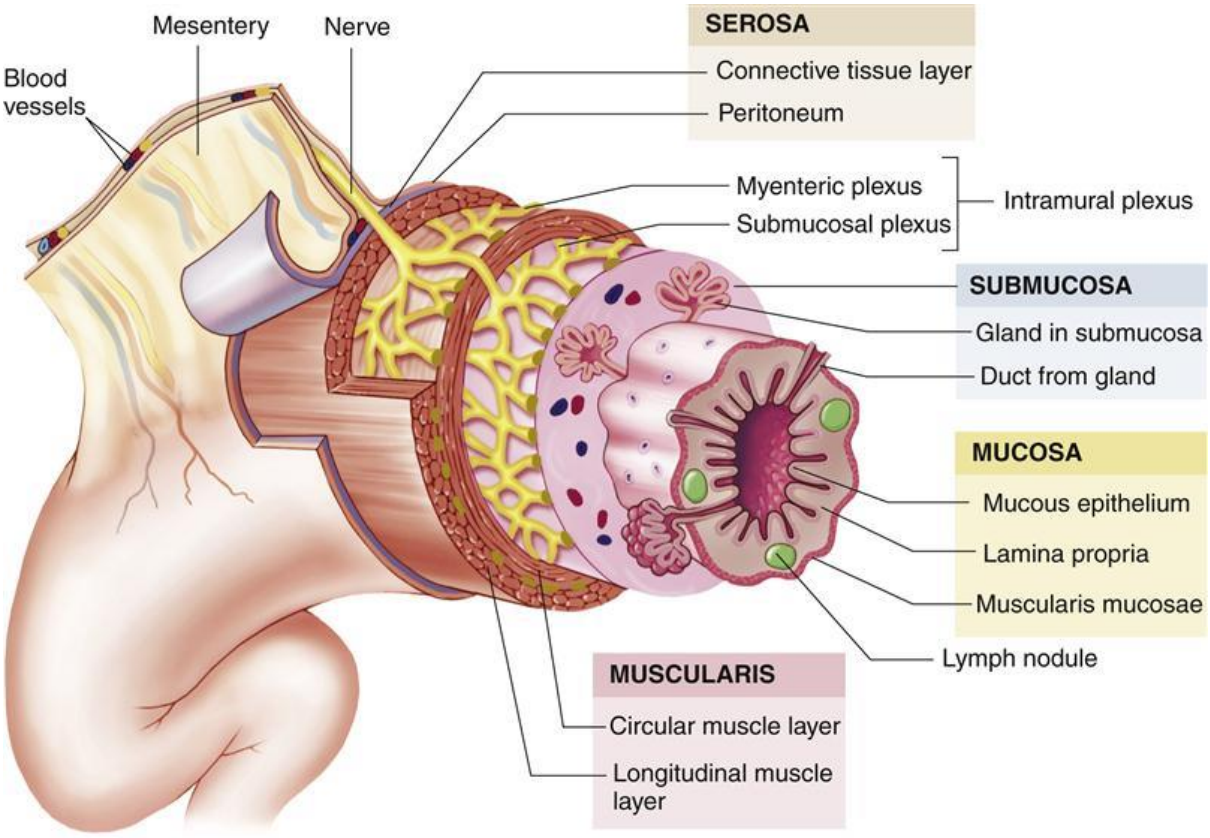


Fig. 1. **Cross-section of the intestinal wall.** The picture shows the four principal layers and associated structures: mucosa, submucosa, *muscularis* and serosa. Although different areas of the GI tract have specialized functions, the basic structure of the wall remains similar. From Patton KT, Thibodeau GA: Anatomy & physiology, 8th edition.

Thus, since the ENS contributes significantly to body homeostasis, it is not surprising that any noxae perturbing ENS maintenance and integrity can result in a variety of disorders some of them so severe to be life threatening and / or hinder significantly the patients' quality of

life. The next paragraph will detail some essential aspect of the ENS, such as structure, neurochemistry and morpho-functional aspects.

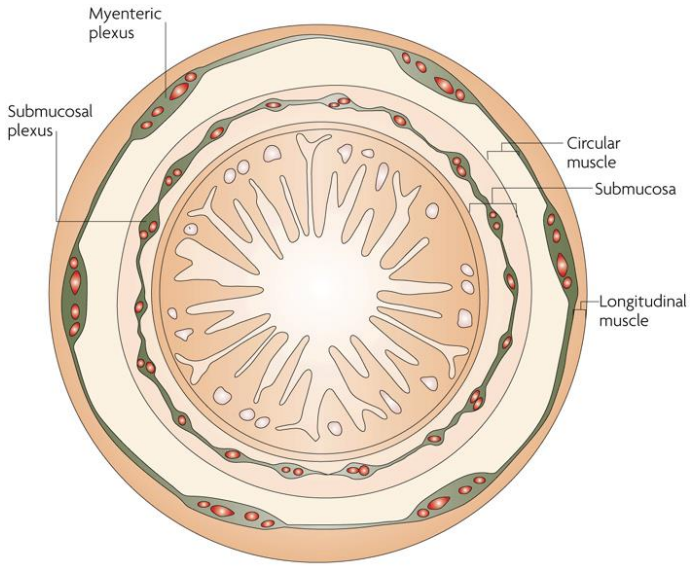


Fig. 2. Schematic representation of a transverse section through the small intestine showing the two main ganglionated plexuses. As recognizable in the drawings, enteric neurons are organized in ganglia (green background), i.e. an outer myenteric plexus, which develops first and occupies a position between the longitudinal and circular muscle layers; and an inner submucosal plexus which forms later in gestation and resides within the submucosa. From Pachnis 2007.

2.1 The Enteric Nervous System (ENS)

The entire structure of the ENS is arranged into ganglia, connected by interganglionic strands, in order to form two plexuses: the myenteric (Auerbach's) and the submucosal (Meissner's) [46,47]. The outer myenteric plexus (MP) is located between the longitudinal and circular smooth muscle layers and is (although not exclusively) involved in the control of gut motor patterns. The inner submucosal plexus (SMP) resides within the submucosa and is involved with local fluid secretion and absorption [48,49]. Taken together the network formed by the two ganglionated plxuses is constituted by about 100 million neurons in the guinea pig (likely 400-500 millions in humans) and even more supporting cells (EGC) outnumbering from 3 to 5 times enteric neurons [50].

The MP forms a network around the gut, which extends from the upper esophagus to the internal anal sphincter. The MP is embedded within the two layers of the *muscularis externa*, i.e. the circular and longitudinal coats, and composed by a neuronal cell bodies and related nerve processes [50]. The MP shows numerous differences in its morphological organization, among GI segments of different species [51-53]. In the MP, ganglia are bigger than the SMP in terms of number of neurons, and are linked by interconnecting strands (or primary strands), which constitute the primary plexus [50]. The MP are located in parallel to the circular muscle layer, although this feature can vary among species [54]; primary interconnecting strands show longitudinal course, an organization that seems to be a distinctive feature of the MP in most small and large mammals [50,55-60]. The other two components of MP are the secondary and tertiary plexuses [50]. The secondary plexus is constituted by fine bundles of nerve fibers running parallel to the circular muscle layer and primary plexus [50,61]. The tertiary plexus is made by thin interconnecting strands (the smallest in size) supplying the longitudinal muscle layer [50,55,61].

The SMP is well developed in the small and large intestine, while only a few submucous ganglia can be found in the esophagus (third inferior part) and stomach particularly in large mammals, including humans [50,62-65]. The interconnecting strands of the SMP are usually thin and SMP ganglia are small. The SMP is organized in a single layer in small mammals [66], whereas it shows a multilayered (two or three layers) organization in large mammals [67]. In these species two different ganglionated plexuses can be identified, namely the internal submucous plexus and the external submucous plexus [58,68-73]. External and internal SMP are separated by a thin connective tissue layer [69,74-76] and by submucosal blood vessels [76]. The external and internal SMPs appear different among the investigated species. Generally, they can be distinguished on the basis of their location, architecture, meshwork density, size and form of the ganglia, and light microscopic appearance [77-81]. The external SMP shows the most

irregularly organized nerve meshwork, while the internal meshwork is smaller and more regular compared to the external SMP [61,69,75,82,83]. Additionally, the two compartments of SMP neurons also show differences in the content of neurochemical messengers / transmitters, being the external SMP more similar to that of myenteric neurons [84,85]. In addition to the mucosa, some neurons of the external SMP also supply the muscular layer [48,66,73,86-88]. The internal SMP neurons supply the mucosa and only a small number have projections to the muscle layers [66,88]. Thus, in conclusion the external and internal SMP neurons overlap in terms of functional control of fluid movements, local blood flow and sensory functions, while the external SMP can also affect gut motility [83].

Enteric neurons are able to synthesize and release about 30 different mediators / bioactive substances that may act as messengers, i.e. neurotransmitters, neuromodulators and neuropeptides (Table 1). Studies over the years have clearly shown that each subclass of enteric neurons can be characterized on the basis of the combination of messengers. This property is known as *neurochemical coding* and turned to be a feature of the ENS that is maintained through most animal species [89].

Primary neurotrasmitters exert the same role in different species and along the GI tract. These substances include acetylcholine (ACh) and tachykinins in enteric excitatory motor neurons, and nitric oxide (NO), vasoactive intestinal polypeptide (VIP) and others messengers (see below) in inhibitory motor neurons. Inhibitory motor neurons may be immunohistochemically identified by the presence of the neuronal nitric oxide synthase (nNOS), the neuronal isoform of the enzyme synthesizing NO, the primary neurotransmitter released by inhibitory motor neurons, while excitatory motor neurons may be immunohistochemically identified by the presence of the synthesizing enzyme choline acetyltransferase (ChAT). Secondary neurotransmitters or modulators include substances which may vary among different groups of neurons depending on the GI tract and the species

considered [50]. Research performed in the last thirty years has shown that classification of the several classes of enteric neurons is the result of a combination of various technical approaches and criteria. These features include: neuronal shape; histochemical and immunohistochemical staining; projections; and electrophysiological and functional properties.

Table 1. Multiple transmitters of functionally distinct enteric neurons in the gut.

Type of neuron	Primary transmitter	Secondary transmitters, modulators	Other neurochemical markers
Enteric excitatory muscle motor neuron	ACh	Tachykinin, enkephalin (presynaptic inhibition)	Calretinin, γ-aminobutyric acid
Enteric inhibitory muscle motor neuron	Nitric oxide	VIP, ATP or ATP-like compound, carbon monoxide	PACAP, opioids
Ascending interneuron	ACh	Tachykinin, ATP	Calretinin, enkephalin
ChAT, NOS descending interneuron	ATP, ACh	ND	Nitric oxide, VIP
ChAT, 5-HT descending interneuron	ACh	5-HT, ATP	ND
ChAT, somatostatin descending interneuron	ACh	ND	Somatostatin
Intrinsic sensory neuron	ACh, CGRP, tachykinin	ND	Calbindin, calretinin, IB4 binding
Interneurons supplying secretomotor neurons	ACh	ATP, 5-HT	ND
Noncholinergic secretomotor neuron	VIP	PACAP	NPY (in most species)
Cholinergic secretomotor neuron	ACh	ND	Calretinin
Motor neuron to gastrin cells	GRP, ACh	ND	NPY
Motor neurons to parietal cells	ACh	Potentially VIP	ND
Sympathetic neurons, motility inhibiting	Noradrenaline	ND	NPY in some species
Sympathetic neurons, secretion inhibiting	Noradrenaline	Somatostatin (in guinea pig)	ND
Sympathetic neurons, vasoconstrictor	Noradrenaline, ATP	Potentially NPY	NPY
Intestinofugal neurons to sympathetic ganglia	ACh	VIP	Opioid peptides, CCK, GRP

Abbreviations: 5-HT, 5-hydroxytryptamine; ACh, acetylcholine; CCK, cholecystokinin; ChAT, choline acetyltransferase; CGRP, calcitonin gene-related peptide; GRP, gastrin releasing peptide; ND, not determined; NPY, neuropeptide Y; NOS, nitric oxide synthase; PACAP, pituitary adenylyl-cyclase activating peptide; VIP vasoactive intestinal peptide. From Furness 2012.

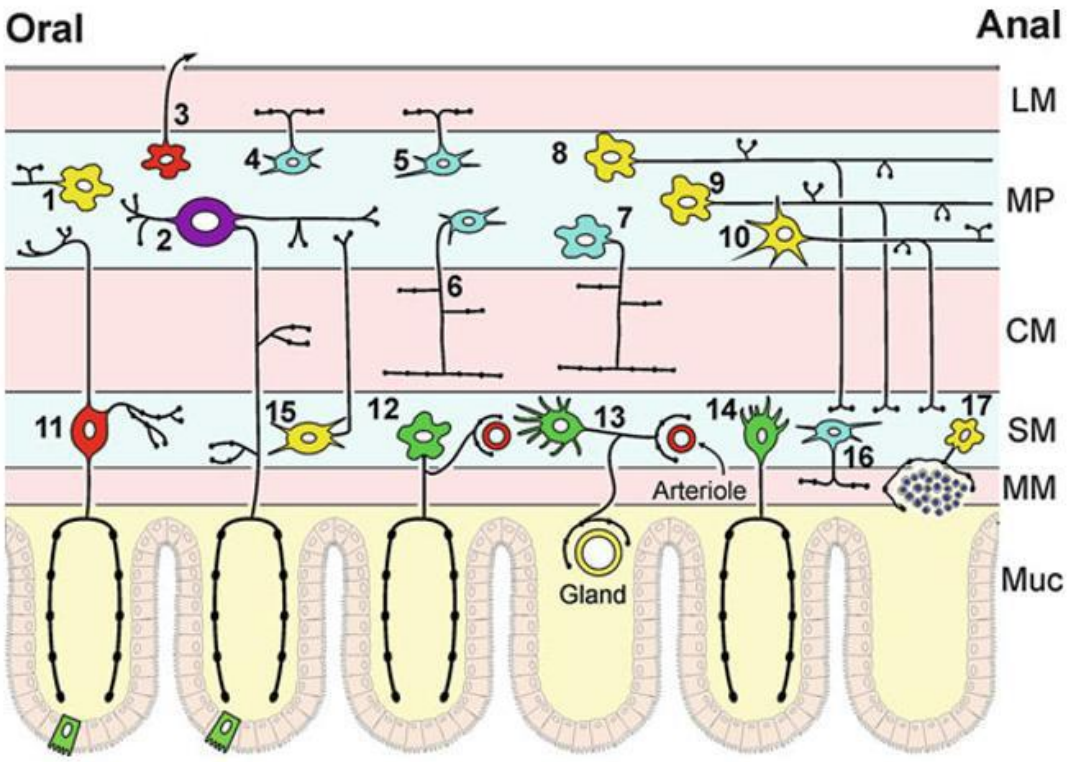


Fig. 3. Types of neurons in the small intestine. The types of neurons in the small intestine, all of which have been defined by their functions, cell body morphologies, chemistries, key transmitters, and projections to targets. Neuron Types: Ascending interneurons (1); Myenteric intrinsic primary afferent neurons (IPANs) (2); Intestinofugal neurons (3); Excitatory longitudinal muscle motor neurons (4); Inhibitory longitudinal muscle motor neurons (5); Excitatory circular muscle motor neurons (6); Inhibitory circular muscle motor neurons (7); Descending interneurons (local reflex) (8); Descending interneurons (secretomotor and motility reflex) (9); Descending interneurons (migrating myoelectric complex) (10); Submucosal IPANs (11); Non-cholinergic secretomotor/ vasodilator neurons (12); Cholinergic secretomotor/vasodilator neuron (13); Cholinergic secretomotor (non-vasodilator) neurons (14); Uni-axonal neurons projecting to the myenteric plexus (15); motor neuron to the muscularis mucosa (16); innervation of Peyer's patches (17). Abbreviations: LM longitudinal muscle, MP myenteric plexus, CM circular muscle, SM submucosal plexus, Muc mucosa. From Furness 2012.

According to their shape, enteric neurons were classified by Dogiel in three types, referred to as Dogiel type I, II and III [90]. Many other authors proposed additional classifications based on individual neuronal morphology revealed by silver staining, immunoreactivity for neurofilaments and / or other markers [91-95]. Taken together, these studies confirmed and extended the Dogiel's classification from three up to seven types of neurons in addition to 'mini-neurons'. The possibility to correlate the morphological appearance of a given neuron

to its neurochemical code, as well as bioelectrical / functional properties has also refueled the importance of studying the morphology of the enteric nerve cells.

From a functional standpoint, enteric neurons can be divided in motor neurons, interneurons, intrinsic primary afferent neurons (IPANs) [47,96], and intestinofugal afferent neurons (IFANs) [97] (Fig. 3).

Motor neurons. This includes excitatory and inhibitory neurons directed to the GI musculature; secretomotor / vasodilator neurons are able to regulate mucosal secreting cells and vasodilation / vasoconstriction of the intestinal vasculature. Furthermore, another subset is represented by secretomotor neurons and neurons innervating entero-endocrine cells [96]. The motor neurons innervating the smooth muscle of digestive tract are located within the MP of rodents [98,99] and within myenteric and external and internal submucous plexuses of large mammals [MP > external SMP > internal SMP] and humans [MP > external SMP > internal SMP] [83,86-88,100]. These neurons could be distinguished in circular muscle motor neurons, longitudinal muscle motor neurons, and motor neurons innervating the muscularis mucosae. According to the peristaltic reflex, the excitatory motor neurons are especially localized aborally to the innervated circular and longitudinal muscle [101,102], while the inhibitory motor neurons are generally localized orally to the innervated circular and longitudinal muscle [102-108]. However, the polarized projection patterns of enteric neurons are apparently species- and region-dependent. Based on the distance from their target(s), motor neurons can be distinguished in short- and long-axon (both excitatory and inhibitory) cell types [101,109]. Other well preserved features of enteric motor neurons can be found in their neurochemical code. In fact, it is well established that the primary transmitter of excitatory muscle motor neurons include Ach often colocalized with tachykinins (especially substance P, SP) [110-113]. The NO is considered the main transmitter of inhibitory muscle motor neurons;

however it is clear that different transmitters are implicated in the inhibitory neurotransmission [96] including Adenosine triphosphate (ATP) [114,115], VIP [116,117], NO [62,87,88,118-124], pituitary adenylate cyclase-activating polypeptide (PACAP) [125,126] and carbon monoxide [127] often colocalized in the same neuronal cells. Finally, many similarities exist in the ENS organization of small and large mammals. Muscle motor neurons with analogous functions can release the same neurotransmitters in different species, however important differences in the distribution of these cells can be observed by comparing the same gastrointestinal region in different mammals.

This neuronal organization represents a general rule applicable to the ENS of all studied species. For example, concerning the swine and horse ileum and the human descending and sigmoid colon, (segments with high tone and propulsive functions) the number of cholinergic neurons is higher than that of nitrergic ones [53,95,128-131]. In contrast, in the human ascending colon (a segment with a low tone and accommodation and mixing function) [128,129], the MP contains a number of nitrergic neurons greater than the cholinergic ones [131].

Two important subgroups of motor neurons include secretomotor and vasomotor neurons. Hens et al. showed that in all the ganglionated plexuses of the pig small intestine there are mucosal projecting neurons (internal SMP 78% > external SMP 10% ≈ MP 12%) [85]. Dogiel type II cells exhibiting calcitonin gene-related peptide (CGRP) immunoreactivity (-IR) (MP>ESMP>ISMP) are probably afferent cells; however other mucosal projecting neurons, showing different morphology and phenotype, are present in MP and in both divisions of SMP. The majority of internal SMP neurons projecting to mucosa are ChAT/SP- or VIP/Galanin-IR minineurons [85]; [66,132], whereas in both external SMP and MP most of neurons projecting to mucosa are multidendritic in morphology and displaying ChAT/Somatostatin (SOM)-IR [85]. Hens et al. showed that in human jejunum the highest proportion of mucosal projecting neurons is located in the ESMP (ESMP 54% > MP 23% > internal SMP 10%) [100].

However, in both species most of these neurons are located in the SMP, while in rodents, mucosal projecting neurons are more equally distributed between the SMP and MP [61% SMP and 39% MP in the mouse; 50% SMP and 50% MP in the guinea-pig [133].

Considering the dense network of SP-IR nerve fibers found in the mm, as well as pharmacologic data showing an involvement of SP as mediator in excitatory response of the mm [134-137], it has been suggested that SP-IR small neurons could mainly be involved in the mm innervation, rather than of the epithelium [8]. However, SP is also widely distributed in the nerve fibers in relationship to the cores of the villi, intestinal glands, and muscular sheath of blood vessels [53,138-141]. The anatomical distribution of a dense network of VIP-IR nerve fibers in the intestinal villi, together with the evidence of its secretory actions in porcine intestine [142,143], suggests that VIP minineurons are secretomotor neurons. SOM-IR nerve fibers in the lamina propria have been described in both human [144] and pig [83] small intestine. Pharmacological and physiological evidence suggests an antisecretory role for SOM-IR neurons [145,146]. Furthermore, the presence of specific SOM binding sites on the basolateral membranes of enterocytes indicates a direct action of SOM onto the epithelium [147-149]. Although mucosal projecting neurons show different topographical organization between small and large mammals, these cells seem to show a certain degree of consistency in their neurochemical code. In fact, in all studied species, small neurons expressing VIP-IR are mucosal projecting neurons that are likely to be involved in secretory processes [100,150].

The distribution of SP and VIP has been widely studied in some other large mammals, while SOM has been less studied than the other two substances. In the horse and cattle intestine, both SP and VIP neurons have been especially detected in the SMP; furthermore a rich network of SP and VIP nerve fibers has been also detected in the *muscularis mucosae*, the *lamina propria* of the villi and the intestinal glands [51,52,58,61,151-153]. These data reflect a possible secretory role for these neurons.

Interneurons. These enteric neurons show the longest projections. These cells, which have been identified with certainty only in the guinea-pig [47], mouse, rat, and human are mainly localized in the MP. Interneurons form long functional chains of ascending and descending elements through which information travel for short or long distances [154]. Interneuron projections extend up to 14 mm anally and up to 136 mm orally in guinea pig small intestine [99]. It is worth noting that interneurons may also function also as mechanoceptors [155-158]. So far, four types of interneurons have been identified in the guinea-pig small intestine: one ascending and three types of descending interneurons. The ascending interneurons are MP Dogiel type I cholinergic neurons [96,109] and may contain also calretinin, SP, neurofilament triplet protein (NFP), and enkephalin [105]. Descending MP interneurons (5% of all ENS cells in the guinea-pig) are phenotypically cholinergic neurons further distinct into three types based on their immunoreactivity for NOS/VIP, SOM and 5 hydroxytryptamine (5HT) [99,109,159]. nNOS / VIP / ChAT positive Dogiel type I interneurons can contain also neuropeptide Y (NPY) [160], gastrin releasing peptide (GRP), bombesin (BN) [109] and the enzyme alkaline phosphatase [161]. SOM/ChAT immunoreactive Dogiel type III MP neurons [99,159] project to other MP ganglia and also to SMP ganglia [109]. Also, 5HT/ChAT immunoreactive Dogiel type I MP neurons send their projections to MP and SMP and seem to have significant roles in excitatory pathways regulating both motility and secretion [162].

Data on human interneurons are scanty but it seems that MP interneurons project up to 36 mm anally and up to 70 mm orally. In the human colon, 90% of orally projecting interneurons contain ChAT alone, whereas the others are ChAT- and nNOS-negative; three main types of anally projecting interneurons have been identified: neurons co-expressing ChAT and NOS, neurons immunoreactive for NOS alone, and neurons immunoreactive for ChAT-IR alone [163]. Descending NOS interneurons of the human gut may co-express VIP [94].

Intrinsic primary afferent neurons (IPANs). The IPANs are the first neurons of the reflex pathways in the intestine [164]. They are involved in the control of physiological functions as motility, blood flow and secretion, being responding to several stimuli, such as distention, luminal chemistry and mechanical stimulation of the mucosa [50]. IPANs have typical electrophysiological properties. In fact, they have broad action potentials that are related to both sodium and calcium currents and are followed by early and late (slow) after hyperpolarizing potentials (AHPs) [164]. Their targets are represented by mucosa and other MP and SMP neurons [165-168]. Cell bodies of multi-axonal IPANs are 10-30% of neurons in SMP and MP ganglia of the small and large intestine; no IPANs are detected in the esophagus and they are rare in the stomach, where motility is primary controlled by vagal efferents [169]. All the IPANs in the intestine of the guinea pig and other species show Dogiel type II morphology (non-dendritic, multi-axonal type II neurons) [166,170-173]. A large percentage (82-84%) of myenteric IPANs of the guinea pig ileum expresses the calcium-binding protein calbindin (CALB) [89,174,175] and almost all MP and SMP IPANs express cytoplasmic neuronal nuclei (NeuN) [176,177]. Furthermore, all of them show positivity for ChAT [178,179]. Notably, only 30% of submucosal IPANs of the guinea pig ileum appear to be positive for CALB [133,175,180], and that CALB is not confined to the IPANs since it is also localized in 50% of submucosal calretinin-IR secretomotor / vasodilator neurons [180]. Many researchers studied CALB also in other species, with the aim to establish whether CALB could be considered a marker for IPAN. In the pig small intestine, CALB cannot be considered a marker of IPANs, being mainly localized in interneurones and intestinofugal neurons [132,181]. Dénes and Gábriel described CALB containing myenteric neurons in the rabbit small intestine [182]. These cells showed Dogiel type I morphology, ChAT labeling and were identified as interneurons. Also in the mouse colon, CALB cannot be considered a good marker of IPANs, while CGRP immunolocalization is the most specific feature of these cells. Non-dendritic multiaxonal type

II neurons involved in mucosal innervations have been demonstrated also in porcine [85] and human [100] small intestine. Unlikely from guinea-pig, mouse, porcine and human IPANs express CGRP, which has been considered a specific marker of type II neurons in these species [55,71,76,85,183,184]. However, Brehmer et al. showed that in human small intestine only a minority of type II neurons displayed distinct reactivity for CGRP, while most of them were immunoreactive for SOM, calretinin and SP[181]. Notably, in the human small intestine this neurochemical code is common also to Dogiel type V neurons; thus, the morphological distinction has an important role to recognize IPANs.

In pig small intestine, Krammer et al. and Balemba et al. showed that also anti-neurofilament 200 KDa (NF200) antibody identifies type II neurons [76,185]. A difference between small and large mammals is in the localization of neurons with mucosal projections: in guinea pig and mouse intestine these neurons are localized approximately in equal amount in the SMP and MP. In the pig intestine IPANs are especially localized in internal SMP (about 80%) [85], whereas in the human intestine most of these neurons are localized in the external SMP [100]. Another important difference between small and large mammals is in the proportion of IPANs and secretomotor neurons. In fact, almost all neurons with mucosal projections in the MP of the guinea-pig small intestine exhibit type II morphology, and are thought to be IPANs [167]. In both pig and human small intestine, the number of IPANs in the external SMP and MP represents only a minority (less than 30%) of the total number of neurons [66].

Intestinofugal primary afferent neurons (IFANs). Intestinofugal neurons represent a unique subset of enteric neurons with their cell body located in the myenteric ganglia and projections giving off the intestinal wall [186]. Most of them show a Dogiel type I morphology, whereas a minority have Dogiel type II features [123,187]. IFANs act as mechanoceptors, being able to detect changes in volume and to respond to the stretching (but not to the tension) of the smooth muscle cells [188-190]. Once activated, IFANs usually release Ach in the in the

prevertebral ganglion (PVG) thereby evoking nicotinic fast postsynaptic potentials [191]. A subset of IFANs responds to colonic distention by releasing gamma amino butyric acid (GABA), which facilitate Ach release from cholinergic IFANs in the PVG. Because of IFANs activation, the response of the entero-PVG reflex circuitry is the release of noradrenaline by sympathetic neurons in GI wall. This effect modulates smooth muscle contraction or myenteric neuron activity [192-194]. The functional significance of this reflex arc is to counteract large increases in tone and intraluminal pressure during filling [186].

Enteric glia. The term 'enteric glial cell' was used for the first time by Giorgio Gabella in his original research work made along 10 years [195-197]. Other studies, which have used immunohistochemical markers to locate enteric glia, revealed that these cells are common in the ganglia and nerve fiber bundles. Enteric glia express glial fibrillary acidic protein [198,199] and the S-100 Ca^{2+}-binding protein [200], both of which are typical of CNS astrocytes. Glial cells in other autonomic ganglia do not contain glial fibrillary acidic protein [198]. Electron microscopy studies also confirmed that glial cells are reminiscent of the astrocytes in the CNS and distinct from Schwann cells of other peripheral ganglia or nerve trunks [195-197,201.203]. Specifically, glial cells partly surround nerve cell bodies and axons in the ganglia, leaving bare large areas of neuronal membrane at the surface of ganglia; also, their processes contain bundles of gliofilaments and are surrounded by a single basement membrane (none of these aspects are detectable in Schwann cells). There is a marked difference in the glial cell-to-axon relationship between small (guinea pig, rat) and large mammals (such as cat or human). In small mammals, glial cell processes fail to penetrate into the interstitium between nerve cell bodies as well as axons in the neuropil [196,203]. In fact, many nerve processes show direct membrane-to-membrane contacts with each other; the glial cells only separate them into groups and rarely form a sheath around an individual axon. In contrast, in enteric ganglia of human and monkey, axons are separated from one another by intervening glial cell processes

[204]. It is noteworthy that Auerbach (1864) had recognized the different relationships between neurites and supporting cells in humans as compared to those of other mammals. Glial cells in nerve strands of the myenteric plexus of small mammals give rise to radiating lamellae which divide the axons into large bundles, and up to 600 neurites may be associated with one glial cell [197]. The ratio of glial to nerve cells numbers increases with species size, e.g. from 1.1 to 4.5 in the MP and from 0.6 to 1.5 in SMP of mice and sheep, respectively [205]. In the human SMP and MP the glia-to-neuron ratio ranges from 1.3 to 1.9 and from 5.9 to 7.0, respectively [203,206,207]. Glial cell nuclei can readily be distinguished from those of neurons. The glial nuclei have conspicuous clumps of chromatin, particularly adjacent to the nuclear envelope, and the nuclear surface often displays deep invaginations. The most prominent distinguishing feature of the majority of enteric glial cells is the numerous 10 nm gliofilaments. The cytoplasm also contains smooth and rough endoplasmic reticulum, numerous free ribosomes, mitochondria, lysosomes, and microtubules. Groups of gliofilaments criss-cross the cell bodies and run parallel to the long axes of the cell processes. The gliofilaments appear to be anchored to dense aggregations of material adjacent to the surface membrane. There are sites of apposition between glial cells and between glial cells and neurons where small areas of cytoplasmic density can be identified inside each plasma membrane. Gap junctions between enteric glia are not often seen, but dye filling indicates that they are coupled to each other [208].

Compared with other peripheral glial cells (e.g., Schwann cells), enteric glial cells do not form basal laminae and they ensheath nerve bundles and not individual axons [209]. In addition to the previously mentioned glial fibrillary acidic protein (GFAP) and S100b, other available immunohistochemical markers for glial cell labeling in the adult gut include and Sox (SRY-box) 8/9/10, the first two being the most frequently used [206,207]. Recently, marker expression analysis showed that the majority of glia in the myenteric plexus co-express GFAP, S100b,

and Sox10 [210]. However, a considerable fraction (up to 80%) of glia outside the myenteric ganglia, did not show labeling for these markers. The alternative combinations of markers reflect dynamic gene regulation rather than lineage restrictions, revealing an extensive heterogeneity and phenotypic plasticity of enteric glial cells [211].

Enteric glial cells have long been thought to exert a mere mechanical property by supporting neurons. Many evidence, however, indicates that these cells exhibit a number of functions, ranging from support to neurotransmission to enteric neuronal maintenance and survival [211-214]. In fact, glial cells are involved in many crucial tasks, such as synthesis of neurotransmitter precursors, uptake and degradation of neuroligands (i.e., detoxification of glutamate and g-aminobutyric acid), and expression of neurotransmitter receptors, thereby contributing to neuron-glia cross talk and neurotransmission [212].

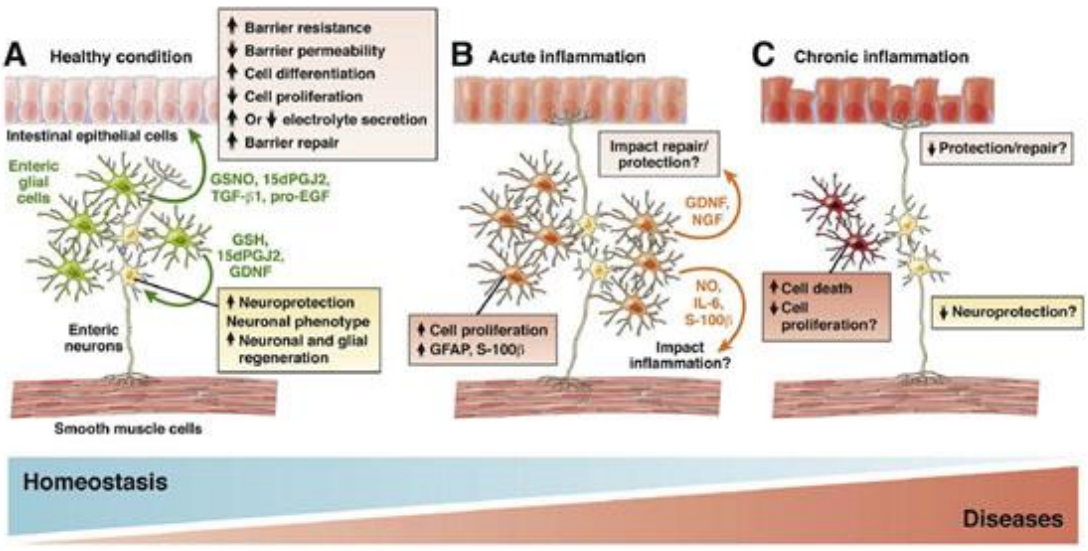


Fig. 4. Enteric glial cells are central regulators of gut homeostasis and can play a role in gut diseases. (A) In normal conditions, glia control a number of neuronal aspects, i.e. neuroprotection, neuromediator expression, or neuronal renewal via liberation of different mediators. In addition, glia exhibit a key role in intestinal epithelial barrier integrity through the release of function-specific messengers (gliomediators). Taken together these features indicate that enteric glia possess protective and reparative properties in the gastrointestinal tract. (B) In pathological conditions, such as inflammation or bacterial stimulation, a phenomenon, known as reactive enteric gliosis (similar to astrogliosis in the brain) can occur. This contributes to the development of intestinal inflammation, but also participate in protection/repair of intestinal epithelial barrier/neuronal lesions evoked by these mechanisms. (C) Enteric glia death (induced by specific viruses or pathogens) or abnormal enteric gliosis could contribute to neuronal degeneration or barrier dysfunctions observed in some chronic intestinal or extraintestinal diseases. From Neunlist et al. 2014.

Furthermore, glial cells exhibit immunological properties [206,215,216], participate in epithelial barrier functions [217-222] and evoke neuroprotection [214] (Fig. 4). In addition, enteric glial cells have neurogenic potential being capable of generating enteric neurons in response to injury [223,224]. Recent works also demonstrated that enteric glial cells can respond to neurotransmitters by changes in intracellular Ca2+, such as purinergic (ATP) [213,225,226] serotonergic and cholinergic signaling mechanisms [227].

2.2 Smooth muscle electrical activity and regulatory mediators

Gut smooth muscle cells form a morpho-functional syncytium throughout the length of the GI tract. They exhibit two types of electrical activity: *i*) 'slow waves' that set the basic electrical rhythm; and *ii*) spike potentials. The ICC cells initiate the slow waves in the smooth muscle cells, which spreads via the gap junctions in aboral direction. Motility patterns depend on the slow waves of the smooth muscle cells except the giant contractions, which are independent of the electrical slow waves and are exclusively controlled by the ENS [228]. The frequency of the slow waves and the intestinal contractions decreases from the duodenum (the highest frequency) down to the ileum (the lowest), and this phenomenon is associated with an increase of the resistance to the electrical spread of the slow waves along the intestine [228].

Concerning bioactive substances able to affect gut motility, the enterochromaffine cells (ECs) found in the mucosal layer play a key role in transmitting chemical or mechanical stimulation of the mucosa to ENS. The ECs release serotonin / 5-HT, triggered by the mechanical and chemical stimuli, in the basolateral compartment from where 5-HT eventually activates underneath neurons, e.g. the IPANs, displaying specific serotonin receptors, and synaptically connected with ascending and descending interneurones, and thereby activating

motorneurons which orchestrate the initiation of peristalsis (see below). Excitatory motor neurons release Ach and SP, evoking a muscle contraction proximal to the stimuli, whereas inhibitory motor neurons release of inhibitory neurotransmitters e.g. NOS and VIP, evoking relaxation distal to the stimuli [50,169]. Other paracrine and endocrine hormonal mechanisms including motilin, opioids, peptides and SOM, are intimately involved in short and long-term regulation of contractile patterns albeit to varying degrees and in different regions of the intestine. CCK and gastrin stimulates the peristaltic activity whereas neurotensin mainly produces stationary segmenting contractions.

Key points:

- The regulatory system through which the gut exhibits its functions is given by a number of specialized cells, i.e. neurons, glia, interstitial cells of Cajal (ICC), smooth muscle cells and neuroendocrine cells of the mucosal lining.
- Among these cells, neurons are at the top of the herarchy as they provide control on virtually all gut functions, including motility.
- Current knowledge indicates that intrinsic innervation of the gut is supplied by the enteric nervous system (ENS), roughly constituted by 500 million neurons distributed in two major ganglionated (i.e., myenteric and submucosal) plexuses.
- Functionally distinct classes of myenteric and submucosal neurons include, e.g. motorneurons, interneurons and intrinsic primary afferent neurons and other subtypes, each exhibiting a peculiar neurochemical cocktail.
- Enteric neurons are outnumbered by a significant amount of glial cells, which are now recognized being endowed with many regulatory functional effects.

Chapter 3.

PERISTALSIS AND MAIN MOTOR PATTERNS OF GI MOTILITY

GI motility is a major aspect of the digestive and absorptive processes, governed by a multitude of cooperating mechanisms. In 1899, using isolated segments of small bowel / colon of the dog, two eminent English physiologists, namely William Bayliss and Ernest Starling showed the existence of a basic intestinal behaviour that they name it 'law of the intestine' [229]. Bayliss and Starling showed that mechanical stimulation of the intestinal wall resulted in an oral contractile response associated with a relaxation of the aboral segment. Since this phenomenon occurred regularly in the presence of identical stimulation, they were keen to refer to it as a 'law' as detectable by a segment of the intestine completely disconnected by any extrinsic neural input [230]. Both Bayliss and Starling were well aware of the existence of an intrinsic innervation supplying the gut, thus the brilliantly inferred what they observed as a result of a neurally mediated activity. Later on, other distinguished physiologists, such as Paul Trendelenburg developed a special set up to better investigate the law of the intestine and to our knowledge he was first to use the term 'peristalsis' (from the ancient Greek περιστελλο, meaning 'push all around') [231]. Like the heartbeat, peristalsis is fundamental for life and many highly integrated mechanisms can contribute to its genesis and maintenance, including neuro-immune mechanisms as recently demonstrated [232]. Investigations during the last 35 years allowed for a better understanding of at least the basic neural mechanisms underlying peristalsis as previously outlined (see section 2.2) and summarized in Fig. 5.

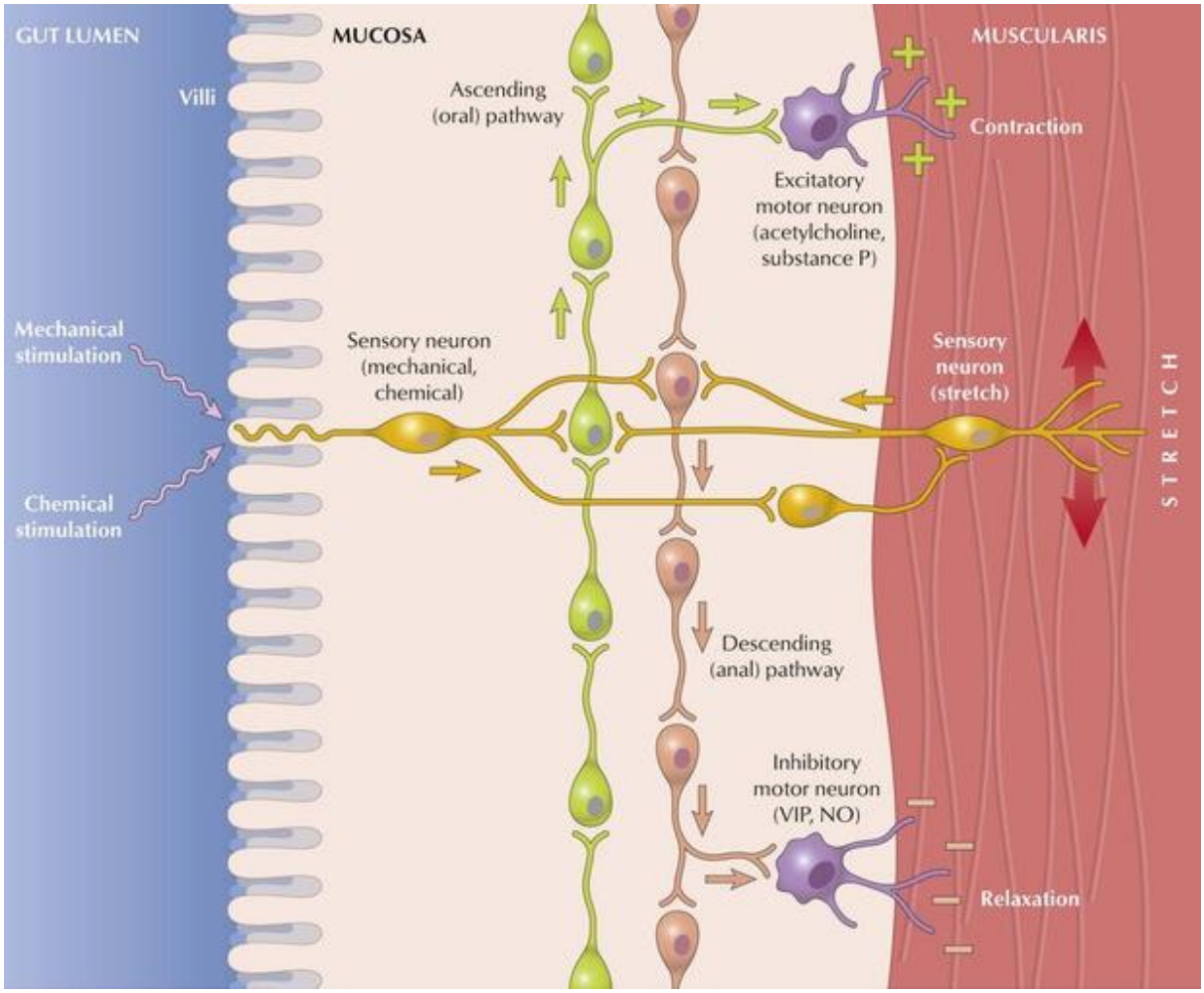


Fig. 5. **Regulation of peristalsis**. Chyme causes smooth muscle contraction above the bolus and relaxation below, so that a peristaltic wave moves chyme in aboral direction. The ENS controls peristalsis and can work independently, but complex digestive process requires the ENS and CNS coordination. Adapted from Perkins JA. Netter's Illustrated Pharmacology, Updated Ed. Philadelphia, PA: Elsevier, 2014.

3.1 Motor patterns of the small intestine

There are various types of motility pattern in the gut (Fig. 6). Stationary segmenting contractions are the non-propulsive annular contraction of the circular muscle layer, which is a common type of mixing motility seen predominantly in the small intestine. Together with alternating contraction and relaxation of the LM, it provides effective mixing of its content through its squeezing action. Clusters of contractions also known as the migrating myoelectric complex (MMC), are waves of fluctuating contractions and relaxations that cause

back and forth movement to facilitate the transportation of chyme from the stomach, through the small intestine, past the ileocecal sphincter, and into the colon in a regular cycle during fasting.

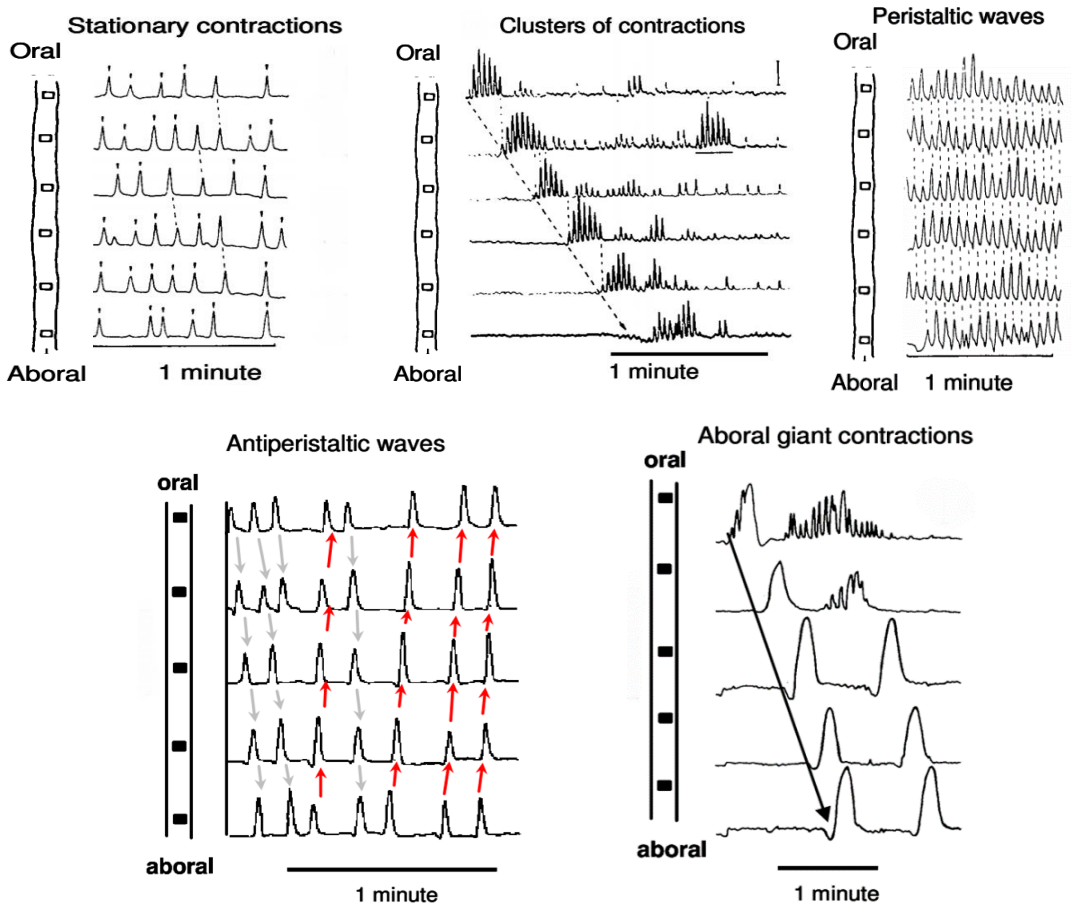


Fig. 6. Types of contractile and motor patterns of the small intestine. Different contractile patterns recordable in the human small bowel. Adaptded from "The Moving Gut" by Hans Jörg Ehrlein and Michael Schemann, Technische Universität MünchenLehrstuhl für Humanbiologie.

Giant contractions (or stripping waves) occur in ileum during the fasting or interdigestive period in which slowly propagating contractions with large amplitude and long duration completely occlude the intestinal lumen and push the luminal contents distally in an aboral direction cleaning the intestine. The giant contractions when occurring in the postprandial period represents a pathological contractile pattern and often the motor precursor of vomiting. Retropulsion or anti-peristaltic waves are a pathological contractile pattern occurring seldom

causing expulsion of noxious substances from the system through vomiting [46]. In addition to that, tonic contractions in sphincters of the gut allow the luminal contents to pass at appropriate times. Further discussion of these patterns in the context of small intestine function is provided below.

3.2 Postprandial motility of the small intestine

The small intestine produces different muscular contractile patterns in response to extrinsic and intrinsic factors. Entry of gastric chyme into the proximal small intestine cause it to distend and this stimulates a mixture of stationary segmenting contractions, clustered contractions and short peristaltic-like waves (mostly confined to the upper small bowel) which are modulated by the nutrient content in the chime (Fig. 7).

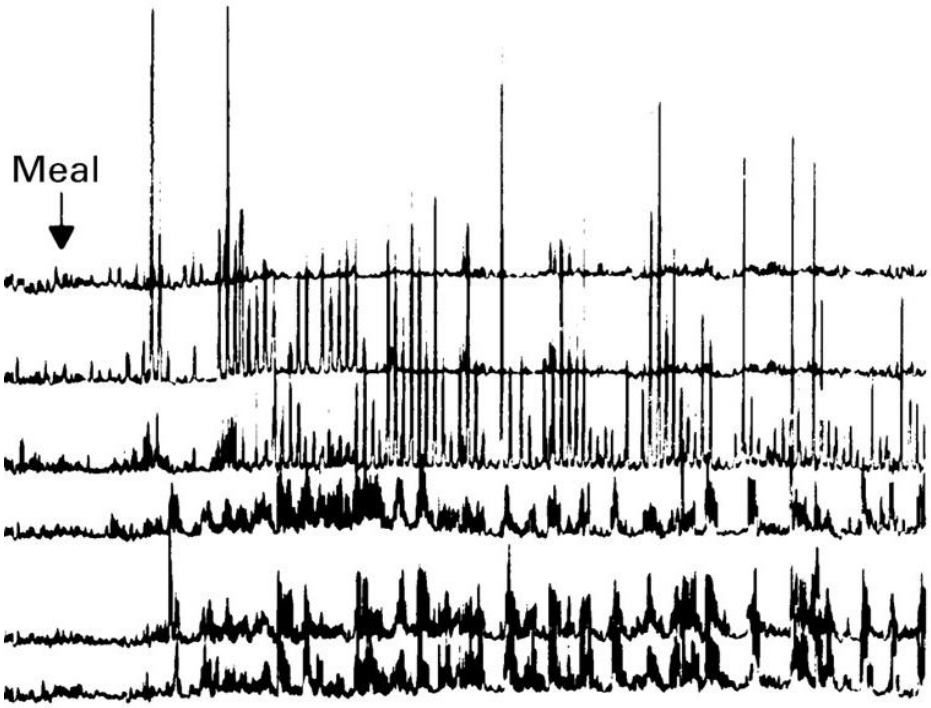


Fig. 7: **Postprandial contractile patterns of the small intestine.** Ingesting meal convert the fasting pattern to postprandial pattern characterized by the sustained but irregular contractile phasic pressure activity

As the chyme start to fill the distal intestinal segments the proximal intestine motility is inhibited, and the number of peristaltic waves decreases while the number of stationary segmenting contractions increases. This feedback-regulation initiated by the nutrients entering the distal small intestine reduce gastric emptying and the flow rate of chyme along the gut.

3.3 Interdigestive motility of the small intestine

In normal healthy subjects the digestion and absorption process take about 12 hours and during the remaining period the stomach and small intestine are remain empty. This interval period is referred to as the interdigestive period and during this time a specific pattern of propulsive motor activity in the stomach and small intestine can be observed (Fig. 8). This pattern is characterized by rhythmically recurring cycles of motor activity which are called the interdigestive motility that migrate from stomach to distal ileum. The interdigestive motility cycle consists of a period of motor quiescence (phase I), followed by a period of irregular electrical and mechanical activity (phase II), and finally by a short phase of striking intestinal contractile pattern, MMC (phase III). The duration of the interdigestive cycles differs among species and the frequency of MMC varies with the time of day and activity, being slowest in sleep and more active during the day.

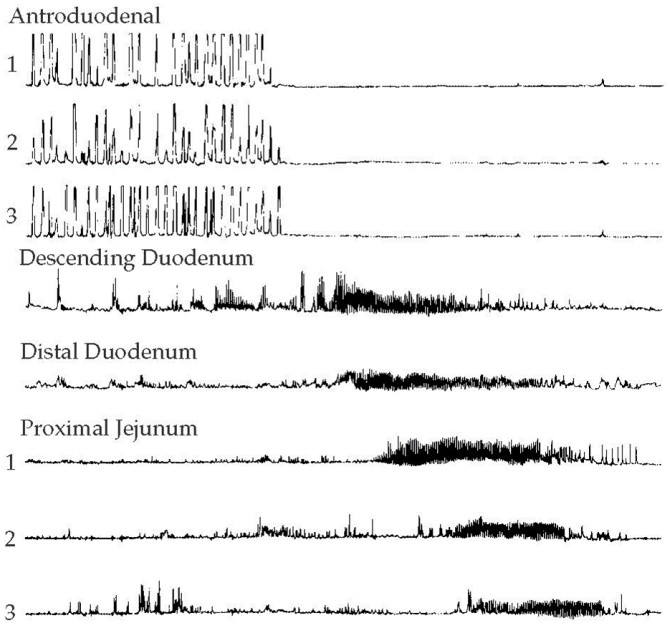


Fig. 8: **Normal interdigestive motility pattern.** The phase III of the interdigestive motility or migrating motor complex (MMC) represents a c complex contractile pattern consisting of long peristaltic waves that migrates aborally.

In average phase III occurs about every 90 min and travel through the intestine at a rate of approximately 5 cm/m (velocity declines along the intestine). The phase III originates simultaneously at the stomach and duodenum. The MMC of the antrum is characterised by 1-3 forceful tonic contractions per minute (1- 3 cpm) occurring at intervals of 2-3 min. During the lumen occluding antral contraction, antroduodenal co-ordination cause the wide opening of the pylorus the relaxation of the duodenal bulb. These powerful gastric contractions and the antroduodenal co-ordination force the chyme and secretions into the duodenum. When a MMC originates at the duodenum, the contractions in the stomach cease and the peristaltic waves occurring at 11 to 12 cpm in the duodenum slowly migrates aboral along the entire small intestine to the ileum sweeping the lumen clean as it moves. In addition to the cleaning activity enhanced by the secretion of gastric and bilio-pancreatic juice occurring immediately before its onset, the MMC also prevents a bacterial overgrowth in the small intestine.

The activities of the alimentary tract are controlled via combination of hormones secreted by specialized cells in the gut and the neuronal control mediated by ENS with the cooperation of the CNS. This complex modulation of the GI tract and its accessory organs is referred to as neurohumoral regulation. Hormones secreted by cells in GI segment are either exclusively endocrine, exclusively neural or both and they serve regulatory functions with the digestive system. Although the ENS can act independently, it receives afferent and efferent input from both sympathetic and parasympathetic arms of the autonomic nervous system allowing the CNS to exert control over GI functions and the GI system to communicate alimentary tract status to the brain. In general, parasympathetic stimulation tends to enhance nearly all functions of the alimentary tract while sympathetic activity tends to inhibit nearly all functions of the alimentary tract including motility and secretion.

Key points:

- Effective propulsion is the main aspect chraterizing all mammalian species, humans included. Peristalsis is by far the best recognized propulsive pattern. Lack of peristalsis, as it occurs in aganglionosis of the ENS, is incompatible with life.
- A retrograde peristalsis, as in vomiting, represents an ontogenetically defensive motor behaviour of major mammals. Pendular (or mixing) movements of the small bowel may contribute to enhance absorption.
- Other motor patterns include migrating motor complex of the small bowel, high amplitude propagated contractions (or ‘mass movements’) of the colon and other coordinated patterns.

Chapter 4.

IN VIVO ASSESSMENT OF INTESTINAL MOTILITY

The accurate assessment of small intestinal motility constitutes one of the most important issues in gastroenterology and the small intestine still represents a clinical challenge due to the restricted access to this organ. The current standard technique for the evaluation of small intestinal motility is the intestinal manometry [233,234] but other techniques such as transit tests, MRI and wireless capsule endoscopy are also used to assess and diagnose motor functions of the gut. The current available tests to evaluate intestinal motility are generally complementary to each other. The application of small intestinal manometry in diagnosing the small intestinal motility has been restricted to few referral centres around the world owing to the complexity of the procedure and the lack clinical expertise demanded in the interpretation of the results. High resolution manometry (HRM) represents a significant technological advancement for the evaluation of motor patterns along the alimentary canal that has already allowed to overcome limitations of conventional manometry, thus leading to improved knowledge of esophageal, anorectal and colonic physiologic and pathologic motor patterns [235-237]. In comparison to conventional manometric catheters, HRM catheters have closely placed sensors that are more sensitive to detect the frequency and direction of intestinal contractile activity [238]. However, the application of HRM to the small bowel has not been intensively investigated and currently no data are available on small intestinal motor abnormalities in healthy individuals and patients with small intestinal motility disorders. Transit tests such as breath tests, scintigraphy and Smartpill can be used to indirectly estimate intestinal motility. These tests are relatively inexpensive and widely available and thus they tend to be used frequently to facilitate the clinical diagnosis small intestinal motility evaluation since the testing with manometry is not readily available. Manometry usually follows transit tests to identify possible dysmotility patterns and may be most helpful in

excluding dysmotility as a cause of symptoms. Average small intestinal transit time in healthy subjects has shown a wide inter- and intra subject variability and for this reason only extreme values can be considered abnormal and diagnostic. Dynamic imaging with MRI technology has also being applied to measure the motor function of the small intestine as it is able to measure accommodation, wall motion and emptying at the same time [239,240]. In addition to the static imaging, recently the cine-MRI technology has also shown to be useful in detecting the reduced intestinal contractility in CIPO patients [241-243]. Wireless capsule endoscopy is a small, weight less pill like device using imaging technology to visualize the GI tract and has shown to reliably detect intestinal dysmotility. This non-invasive tool can measure and quantify contractile events, non-contractile patterns, type of content and motion of wall [244-246]. The clinical utility of this standardized radiation free method is yet unclear and its use in severe dysmotility disorders is cautioned due the risk of capsule retention.

4.1 Small intestinal manometry

Intestinal manometry was introduced to clinical practice several decades ago [247,248] and still the current gold standard technique for the evaluation of small intestinal motility. The diagnosis of small intestinal motor disorders currently relies on manometric findings that can provide clinically meaningful information to help differentiate neuropathic from myopathic dysfunction [249,250]. This invasive but still well tolerated test may be performed with a water-perfused manometric catheter introduced orally under fluoroscopic and/or endoscopic guidance and placed it laying just beyond the ligament of Treitz or about 20 cm length in small intestine to adequately evaluate the antral and duodenal activities. In difficult cases, catheter placement can be done using a guidewire with endoscope. Catheter placement assisted by endoscopy requires the administration of sedative medications that may alter gut

motor activity. In such cases midazolam can be given intravenously without significant interference with intestinal motility. The standard manometric catheter incorporates six manometric ports spanned at 10-cm intervals in comparison to the HRM catheter which incorporates 24 water perfused channels with recording sites spaced at 1 cm intervals. These ports register the amplitude and duration of phasic pressure waves produced by the intestinal contractions and the pressure changes will be displayed as traditional pressure tracings or contour plots. The studies are performed after an overnight fast. Medications that affect GI tract motility must be withheld for 48 hours prior to the test. The total recording time for stationary manometry is at least 8 hours; 6 hours for fasting period followed by standard meal ingestion and next 2 hours for studying post-prandial period. The meal should be at least 400 kcal. and it may be required to the adjust meal according to the digestive capacities of patients. Some variations on the protocol procedure have been introduced by various specialized groups such as use of longer or shorter recordings, including ambulatory or overnight recordings. The solid-stat catheter has the microtransducer sensors which are vary in the sensor number and the spacing of each sensor and these solid-state systems are highly portable and more comfortable for patients. Thus, they can be used for monitoring with long observation periods such as 24-hour ambulatory manometry.

4.2 Normal antroduodenal manometry findings

The criteria for analysing and interpreting manometric tracings have been developed from observation of normal patterns in healthy subjects and contrasting patterns observed in patients with clinical proven intestinal dysmotility. Several specialized patterns occur during the fasting and postprandial periods. During fasting, the presence of 3 distinct phases of interdigestive period described above can be identified and the interpretation of the test

analyse their presence, propagation and duration. The most characteristic and the more easily recognized pattern is the phase III contraction. The fasting period is terminated immediately after meal ingestion and the distinct fasting pattern is converted to the post-prandial contraction pattern or fed-response. The fed response consists high amplitude and irregular contractions in gastric antrum which represent the mixing and grinding of solid food and irregular contractions with lower amplitude in duodenum. The duration of this phase is dependent on food calories and consistency and the fed period ends when the phase III MMC returns.

4.3 Abnormal antroduodenal manometry findings

Motor patterns that differ from the normal motor patterns should be recognized carefully since they usually carry clinical and pathological significance. These abnormal motor patterns include: propagated contractions, ultra-rapid contractions, propagated clustered contractions, bursts of contraction, hypocontractility, hypercontractility (bursts and sustained uncoordinated pressure activity) and a failure to convert to the fed response [248,251]. Three abnormal patterns have been recognized in patient with intestinal dysmotility: neuropathic patter, myopathic pattern and occlusive pattern.

In intrinsic neuropathy, the loss of inhibitory motor neurons leads to un-coordinated or disorganized configuration or propagation of MMC or phase III but with preserved normal or even increased amplitude contractions (Fig. 9). In contrast, patients with in the extrinsic (autonomic) neuropathy, impairments in the fed response and postprandial antral hypomotility are observed [252]. In addition, other neuropathic patterns described in CIPO include bursts of non-propagated phasic pressure activity (duration > 2 minutes, amplitude > 20 mmHg, frequency > 10 waves/minute) during fasting period and/or fed state, sustained in

coordinated fasting pressure activity (duration > 30 minutes) and inability of an ingested meal to convert fasting into fed pattern 253.

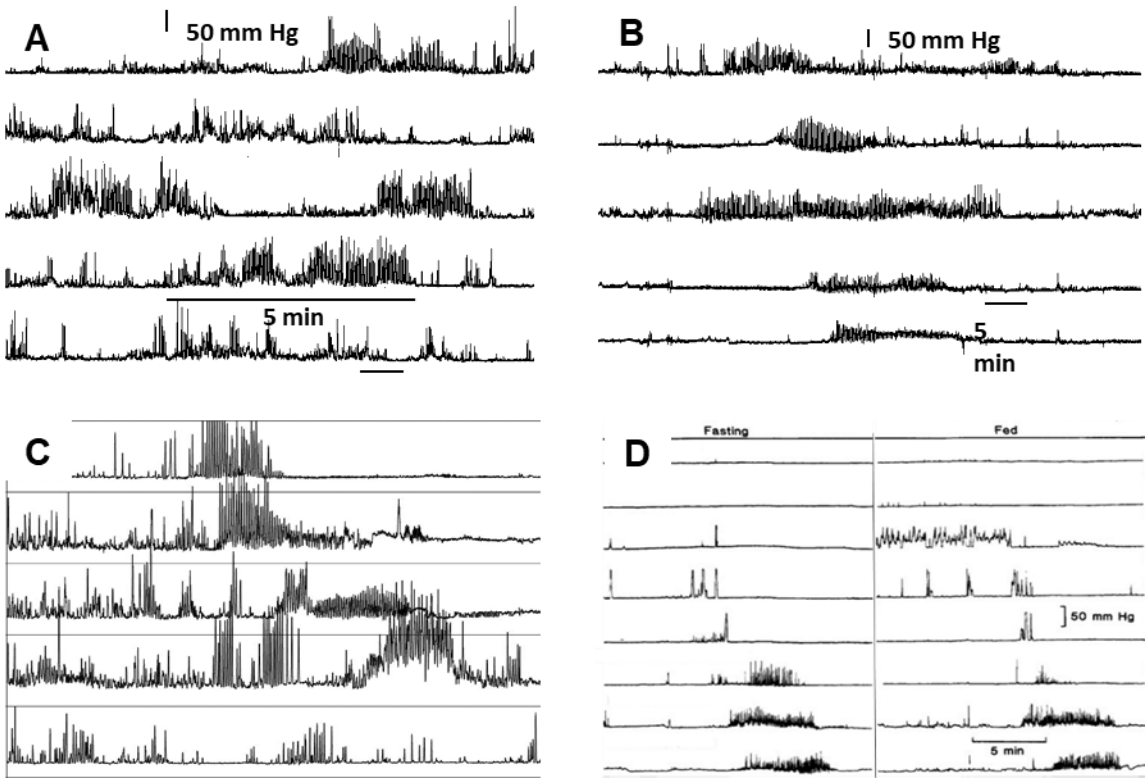


Fig. 9. Manometric patterns suggestive of underlying neuropathy. A. During the phase of motor incoordination (phase II) intestinal motor activity is characterized by periods of hypercontractility of >2 min duration (i.e., bursts). During the phase III activity, MMC can be propagated in aboral sense (**B)** and the in this setting the MMC appears abnormal for conformation, uneven speed and durability (**C)**. Extrinsic neuropathy is characterized by the inability of the meal to convert the fasting in to fed pattern (**D)**.

In patients with visceral myopathy, the MMC is often preserved but manometry demonstrates a decrease in frequency and very low amplitude contractions (< 20 mmHg) of the affected segment in both fed and fasting states and in the CIPO patients with myopathy there are co-ordinated low amplitude pressures (<10 mm Hg) (Fig. 10) activities in early and mild stages and complete absence of contractions in advanced stages of the disease [254]. However, in advanced stage of severe visceral myopathy, the normal MMC pattern may be absent or difficult identify due to very low amplitude.

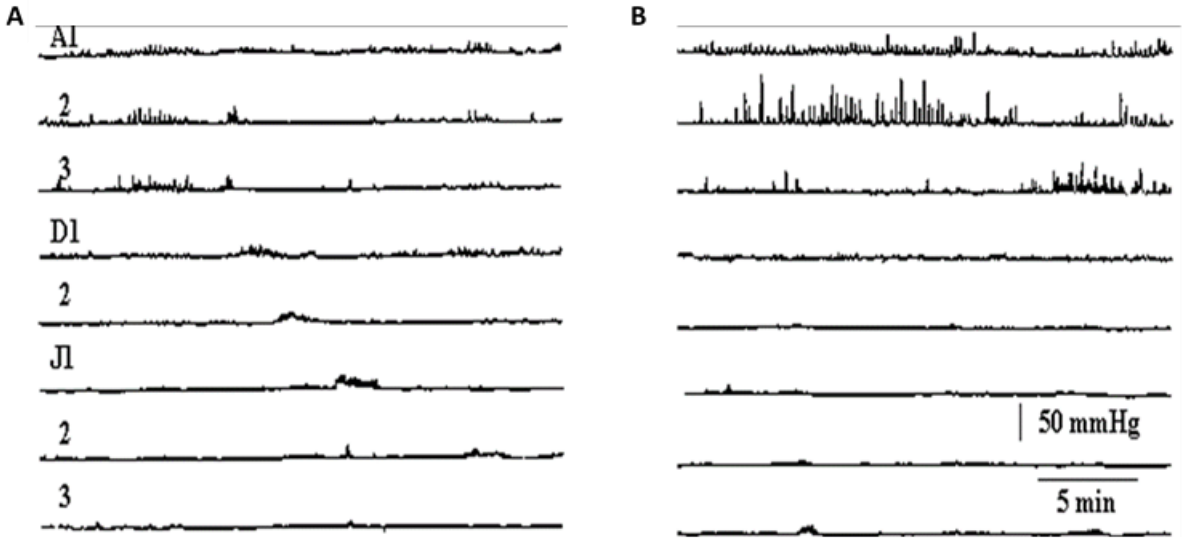


Fig. 10. Manometric patterns suggestive of underlying myopathy. Normal propagation of MMC, **A,** and normal fed response, **B,** but with decreased contraction amplitude due to the dilated bowel loops do not allow occlusive contractions recordable by manometry.

The manometric patterns of mechanical obstruction described include: periodic short bursts of non-propagated and prolonged contractile activity every 1–3 min in postprandial period (also named clustered contractions), giant prolonged contractions occurring simultaneously in recording sites at least 20 cm apart and mix of these patterns (Fig. 11).

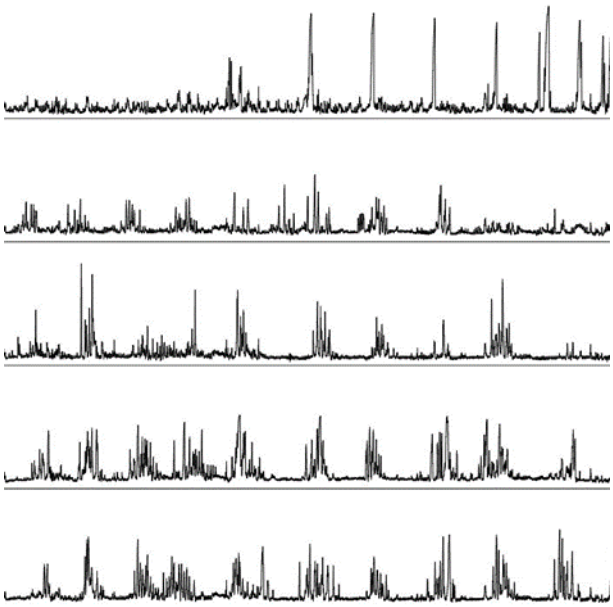


Fig. 11. Manometric patterns suggestive of mechanical obstruction. During the period of motor incoordination (phase II), record periods of 20 min >, characterized by springs of contractions regularly interspersed with motor quiescence (clustered contractions)

Key points:

- In vivo assement of gut motility can be performed by stationary / ambulatory (24-hr) GI manometry in patients with severe digestive symptoms or recurrent sub-occlusive episodes.
- Other non invasive methods to investigate patients with presumed or demonstrated dysmotility are cine-MRI and capsule endoscopy (the latter being carefully used in patients with severe digestive symptoms / sub-occlusive episodes).
- A number of established manometric patterns have been identified to document possible underlying gut neuromuscular abnormalities (i.e., neuropathy, myopathy, occlusive patterns, and others).

Chapter 5.

CLINICAL ASPECTS OF NEUROINTESTINAL DISEASE: PATHOPHYSIOLOGY, DIAGNOSIS, AND TREATMENT

Modified from

Clinical Aspects of Neurointestinal Disease: Pathophysiology, Diagnosis, and Treatment

Allan M. Goldstein, Nikhil Thapar, Tennekoon Buddhika Karunaratne
and Roberto De Giorgio

Dev Biol. 2016 Sep 15;417(2):217-28.

Introduction

The principal function of the gastrointestinal (GI) tract is to ensure the proper digestion and absorption of nutrients and the expulsion of undigested residue and unwanted waste. This complex process requires the coordinated propulsion of endoluminal contents along the length of the GI tract, which in turn relies on the activity of specialized cells, including smooth muscle cells (SMC; final effectors of contraction and relaxation), interstitial cells of Cajal (ICC; gut pacemakers and regulators of neuronal input to the SMC), and a hierarchy of intrinsic and extrinsic neurons to regulate the motor programs [255]. While the propagation, absorption, and excretion of food and waste have long been appreciated as key aspects of gut function, the GI tract also has a myriad of other critical roles necessary for maintaining health and homeostasis. These include its capacity to sense and respond to its luminal environment, its role in controlling immune activation (i.e. distinguishing nutrients and commensal bacteria from toxins, allergens, and pathogens) and initiating inflammation, as well as its ability to

monitor and control microbiota composition. All of these aspects of GI function share a common feature: they rely on the intestinal tract's intrinsic network of neuronal and glial cells, referred to as the enteric nervous system (ENS).

The ENS is a morphologically and functionally complex system that controls, largely independent of central and peripheral nervous system input, virtually all gut functions [50]. It is comprised of a very large number of neuronal and glial cells (about the same number as are in the mammalian spinal cord) that arise from the embryonic neural crest and become organized into two major plexuses: the myenteric (Auerbach's plexus), which extends along the entire length of the GI tract, and the submucosal (Meissner's plexus), which is present from the stomach to the rectum. Enteric neurons can be classified into functionally distinct subpopulations (e.g. intrinsic primary afferent neurons, motor neurons, and interneurons) synaptically linked in reflex circuitries [50]. The central nervous system (CNS) can modulate some intrinsic reflexes, particularly in the esophagus, stomach and rectum, via sympathetic and parasympathetic pathways [46]. However, the ENS has the ability to control most gut functions independent of CNS input, including secretion, absorption, vascular tone, and motility. Any injury to the ENS, whether congenital or acquired, thus results in intestinal neuropathies that can cause clinical symptoms and lead to significant morbidity.

This review focuses on neurointestinal diseases, which are those conditions associated with abnormalities of enteric innervation. From a biologic perspective, neuronal defects in the GI tract can arise from various etiologies. Developmental disorders represent one cause, in which any aspect of enteric neural crest cell development might be perturbed, including cell migration, proliferation, survival, differentiation, or patterning. Disordered ENS development typically gives rise to congenital enteric neuropathies and manifests in early childhood. Hirschsprung disease is the best characterized of these developmental conditions and therefore the first disease discussed in this review. Many other neurointestinal diseases are

acquired, and these are generally believed to be caused by neuronal degeneration, immune-mediated inflammation, or infection. These factors, however, are often intertwined. Neurodegeneration can be associated with prominent inflammation in the enteric ganglia, whether as the cause of the inflammation or as its consequence. Infection can lead to autoantibodies against enteric neuronal antigens and subsequent loss of neurons. Understanding the normal biology of ENS development and maintenance, and defining the pathophysiology that underlies the various neurointestinal diseases, are essential in order to develop improved therapies for these conditions.

It is important to note that the emphasis on the ENS in this review is not intended to undervalue the critical contributions of the SMC, ICC, or extrinsic innervation (autonomic nervous system, spinal cord and brain) to GI health and disease. Rather, it is only intended to focus the review on this specific area of interest. The reader is referred to excellent reviews on those topics [256,257]. We have also taken a neuro-centric approach, which is not meant to ignore the importance of the enteric glia, but rather reflects our limited current understanding of their normal function and their role in intestinal disease. Thus, the broad objective of this review is to provide an update on the molecular and clinical features of neurointestinal diseases, to give an overview of therapeutic options for their management, and to present the potential future use of neuroregenerative approaches for their treatment. We hope that by presenting the basic biology of these diseases in a clinical context, the reader will appreciate the significant unmet needs faced by clinicians caring for patients with any form of neurointestinal disease and consequently identify areas where further fundamental research might contribute to improving clinical care.

Hirschsprung Disease

Hirschsprung disease (HSCR) is a congenital enteric neuropathy that causes functional bowel obstruction as a result of distal intestinal aganglionosis [258]. First described by Harald Hirschsprung in 1888, HSCR occurs in approximately 1 in 5000 births with a male predominance (4:1 male to female ratio in short-segment disease) [259]. HSCR is characterized by the absence of enteric ganglion cells in the submucosal and myenteric plexuses along variable lengths of the distal bowel. In most cases, children present in the neonatal period with delayed passage of meconium beyond the first 24-48 hours of life. Other symptoms and signs are those of complete or partial intestinal obstruction, including feeding difficulties, abdominal distension, vomiting (which may be bilious), and constipation (or obstipation). The most common form is short-segment, affecting approximately 80% of cases and involving no more than the rectosigmoid colon. Less commonly, the aganglionic segment is longer, extending proximal to the rectosigmoid. The aganglionosis can involve the entire colon in 8-10% of patients, or, rarely, the entire intestine [260].

HSCR is a classic example of a neurocristopathy, a disease arising from abnormalities of neural crest-derived cells. In HSCR, enteric neural crest-derived cells fail to complete their rostrocaudal migration along the length of the intestine, leaving variable lengths of distal gut without ganglion cells [261]. The failure to complete migration can be caused by defects in neural crest cell migration, proliferation, survival, or differentiation. HSCR is most commonly an isolated finding, but can occur along with other disorders, most commonly Down syndrome, which is present in about 10% of cases [17]. Since neural crest cells also give rise to melanocytes, abnormalities of pigmentation occasionally co-exist with HSCR. Shah-Waardenburg syndrome, caused by mutations in *SOX10*, *EDNRB*, or *ET3*, is a neurocristopathy characterized by abnormal pigmentation of the hair, skin, and eyes,

intestinal aganglionosis, and congenital hearing loss due to absence of neural crest-derived cells in the cochlea of the inner ear [262]. Congenital central hypoventilation syndrome (CCHS), in which autonomic control of spontaneous breathing is impaired, can co-exist with HSCR in a condition called Haddad syndrome [263]. This neurocristopathy is associated with mutations in *PHOX2B*, a transcription factor expressed by neural crest cells, which give rise not only to the ENS but also to parts of the autonomic nervous system, accounting for the respiratory defects in this disease. Mowat-Wilson is a syndrome characterized by mental retardation, epilepsy, delayed motor development, and HSCR and has been associated with mutations in *ZFHX1B*, a zinc finger homeobox gene that encodes Smad-interacting protein 1 [264].

Several observations initially indicated the genetic origin of the disease, including: (*i*) average risk of recurrence in siblings of 3-4%, about 200-fold higher than in the normal population; (*ii*) increased prevalence in males; (*iii*) association with other genetic diseases and chromosomal abnormalities; and (*iv*) presence of genetic models of aganglionosis with a specific mode of inheritance. The high proportion of sporadic cases (~80%), variable clinical expression (i.e. variable lengths of aganglionosis among relatives), and incomplete penetrance (i.e. some mutation carriers do not have aganglionosis) support a multigenic model to explain the predominantly non-Mendelian inheritance pattern of non-syndromic cases. Linkage analyses in large HSCR families led to the identification of the *RET* gene (10q11.2) as the first gene shown to be involved in HSCR [265]. Coding sequence mutations in *RET* account for 15-35% of patients with sporadic HSCR and 50% of familial cases [261]. Recent data suggest that non-coding mutations in *RET* within a conserved enhancer-like sequence in intron 1 confer susceptibility to HSCR, whereas a variant in the 3'-untranslated region confers a "protective" haplotype that is underrepresented in HSCR [266,267]. In addition to *RET*, mutations have been identified in over a dozen other genes, but these account for a minority of cases [261,268]. While RET mutations are the major risk factor in this disease,

evidence suggests that they may not be sufficient on their own to result in aganglionosis [261]. The majority of cases appear to be multigenic, comprising a combination of RET mutations with genetic abnormalities at other loci, with these interactions impacting the incidence and severity of the aganglionosis. These types of interactions have been observed for RET and EDNRB signaling and for EDNRB and SOX10 [269,270]. An important role has also been described for modifier genes. These are genes that, when mutated, do not result in a phenotype but, when present with a mutation in another gene, they worsen the effect. Examples of modifier genes include neuregulin 1 (NRG1) and L1CAM [268]. These modifier genes may help to explain the phenotypic variability and incomplete penetrance characteristic of HSCR.

The diagnosis of HSCR should be considered in neonates presenting with the delayed passage of meconium, or in older infants and children with severe constipation. The gold standard for the diagnosis of HSCR is histological assessment of tissue obtained by deep rectal biopsy (suction biopsy or full-thickness biopsy) showing the complete absence of submucosal (and myenteric) ganglion cells. The presence of hypertrophied acetylcholinesterase-positive nerve trunks, representing projections of extrinsic nerve fibers, in the muscularis mucosae and lamina propria provides additional confirmation [271], as does the absence of calretinin-positive nerve fibers in the lamina propria and submucosa [2,271,272]. It is important to note that a physiologic zone of hypoganglionosis is normally present in the distal 1-3 cm of the rectum and may lead to false-positive results if that distal segment is biopsied [273]. In contrast, if taken too proximally, rectal biopsy may miss a very short segment of clinically important aganglionosis [274].

Anorectal manometry (ARM) should not be used as a sole diagnostic tool for HSCR in neonates and infants, but in older children may provide a useful screening test in patients presenting with symptoms suggestive of HSCR. Whereas the presence of a rectoanal

inhibitory reflex on ARM would reasonably exclude HSCR in older children, its absence should lead to a rectal biopsy to definitively make the diagnosis. Contrast enemas are useful radiologic studies that often suggest the diagnosis in the first place by demonstrating a tonically contracted distal colon, reflecting the aganglionic segment, and possibly also giving a rough indication of the length of aganglionosis prior to surgery.

The standard treatment for HSCR involves surgical removal of the aganglionic bowel and transition zone, which refers to the relatively hypoganglionic bowel proximal to the aganglionosis. The normoganglionic intestine is then anastomosed to the rectum or anus, depending on the specific procedure performed. A number of techniques have been described to fashion the distal anastomosis, and these are named for their surgical pioneers, most notably Soave, Swenson, and Duhamel. For short-segment aganglionosis, a single-stage transanal operation is commonly performed [275].

Surgical treatment of HSCR is undoubtedly life-saving and allows many children to live normal lives. For many patients, however, the long-term outcomes following surgery are far from perfect, with the main problem being fecal incontinence and the significant psychosocial impact that this can have [276-279]. Severe constipation can also occur after surgery, possibly related to residual abnormalities in the remaining, presumably "normoganglionic" bowel, or to the failure of normal internal anal sphincter relaxation, which is associated with HSCR. Children with HSCR are also at risk for a potentially life-threatening inflammatory process termed Hirschsprung-associated enterocolitis (HAEC), which can occur before or after surgical removal of the aganglionic segment [280]. HAEC, which usually manifests with abdominal distension and diarrhea, often accompanied by fever and vomiting, can progress to sepsis. HAEC is usually treated with antibiotics and rectal irrigations to evacuate stool from the distal bowel. Since RET signaling is essential for formation of Peyer's patches (aggregates of lymphoid tissue in the gut wall), patients with

HSCR and a *RET* mutation may have defects in intestinal immunity, which might contribute to the risk for HAEC [281,282]. Several studies implicate alterations in the fecal microbiome as contributing to the pathogenesis of HAEC [283,284]. This notion is supported by a recent prospective, multicenter, randomized controlled trial showing that administration of probiotics reduces both the frequency and severity of HAEC [285].

Esophageal Achalasia

Achalasia is a disorder of the esophagus characterized by the absence of peristalsis and impaired swallow-induced relaxation of the lower esophageal sphincter (LES), resulting from loss of intrinsic inhibitory neurons in the myenteric plexus. The clinical manifestation is characterized by dysphagia for both solid food and liquids, regurgitation of undigested food, respiratory complications, chest pain, and weight loss. The condition is characterized by a defect of intrinsic inhibitory neurons, which release nitric oxide (NO) and vasoactive intestinal polypeptide (VIP), both in the esophageal body and LES [286]. The resulting imbalance between defective inhibitory innervation and apparently spared excitatory (cholinergic/ tachykininergic) neuronal components likely underlies the pathogenesis. It remains unclear why esophageal myenteric neurons are preferentially targeted and irreversibly damaged by the immune system, although several lines of evidence indicate that infectious and immune factors could underlie the etiopathogenesis of this neuropathy [19]. These factors likely act independently and their influence may be different in different subsets of patients. The myenteric plexus in patients with end-stage achalasia has been found to have an extensive reduction in ganglion cells in association with lymphocytic and eosinophilic infiltrates [287]. A follow-up study examined the histopathological features of esophageal specimens from patients with early-stage achalasia undergoing surgical myotomy. Inflammation was found in both stages, but fibrosis was only seen in end-stage disease,

suggesting a spectrum of histopathological changes during the course of the disease [288]. These results are consistent with other reports that have shown myenteric inflammatory infiltrates (hence the term "myenteric ganglionitis") represented by CD3+ and CD8+ T cells in both early- and end-stage achalasia, as well as a normal number of myenteric ganglion cells in the early stage of the disease [289,290].

Infectious agents, possibly viruses, might trigger an immune response to esophageal myenteric neurons. This was demonstrated by previous work showing herpes zoster virus in patients with the sporadic form of achalasia, although this has been debated [291,292]. A causal link between viral infections and neuropathological alterations has not been definitively demonstrated yet, although an increase in lymphocytic proliferation has been noted in esophageal biopsies of achalasic patients after exposure to herpes simplex 1, supporting a role for an immune response against viral agents infecting esophageal neurons in predisposed patients [293].

Other studies have suggested that circulating autoantibodies might cause immune-mediated neuronal damage in patients with achalasia [294], although the autoantibodies could certainly be the consequence of neuronal injury and the expression of normally occult neuronal antigens. Serum of achalasic patients has been shown to contain anti-neuronal antibodies that react against rodent enteric neurons [295,296]. In both studies, the immunohistochemical pattern was characterized by immunoreactivity toward the nucleus and cytoplasm of myenteric and submucosal neurons, although targets of this immune response are unknown. Intriguingly, the immunolabelling was specific for enteric neurons and was not detected in neurons of the spinal cord, sensory ganglia, or superior cervical ganglia [296]. Another study of sera from 18 achalasic patients showed that applying the sera to gastric fundus muscle strips of healthy individuals led to an abnormal neurochemical code with reduced nitrergic/VIPergic neurons, increased cholinergic neurons and altered relaxation response when electrical stimuli were

applied [297]. These observations suggest that other factors, other than antineuronal antibodies, are present in the sera of achalasic patients and may contribute to the ENS dysfunction [298].

Although achalasia is mainly sporadic in origin, a genetic predisposition has been suggested. Achalasia can be found in association with rare syndromic disorders with an autosomal recessive inheritance, including familial esophageal achalasia (OMIM 200400) and the achalasia microcephaly syndrome (OMIM 200450). Causative genes for these two conditions have not been identified yet, but for achalasia-addison-alacrima, also referred to as Allgrove syndrome (AAAS, OMIM 231550), the genetic basis has been elucidated. AAAS was originally reported in two pairs of siblings with achalasia of the cardia and glucocorticoid deficiency. Other clinical signs included defective tear formation (alacrima) and other signs of dysautonomia. The causative *AAAS* gene has been mapped to chromosome 12q13, encoding the protein ALADIN. Several *AAAS* mutations have been identified in patients with a broad spectrum of clinical presentations, while patients with sporadic achalasia do not carry *AAAS* mutations, indicating that different mechanisms can contribute to the pathogenesis of idiopathic achalasia [299]. Based on the evidence collected to date, the current view suggests that idiopathic achalasia can be defined as an inflammatory/autoimmune disease of unknown etiology with loss of inhibitory neurons in the esophageal myenteric plexus.

Patients presenting with dysphagia and regurgitation, classic hallmarks of achalasia, should be investigated with high-resolution esophageal manometry, which will show a lack of peristalsis and incomplete LES relaxation. According to the Chicago classification for esophageal motility dosorders, three manometrically distinct types of achalasia exist: type I, the “classic form" (usually with a markedly dilated esophagus), is characterized by an integrated relaxation pressure (IRP) above the upper limit of normal and complete lack of peristalsis throughout the esophagus; type II is characterized by esophageal compression

(mean IRP > upper normal limit), abnormal peristalsis and pan-esophageal pressurization with ≥20% of swallows; and type III, formerly referred to as "vigorous achalasia," also exhibits mean IRP > upper limit of normal and abnormal peristalsis, with preserved fragments of distal peristalsis or premature (spastic) contractions with ≥20% of swallows [300]. Although quite technical, this classification carries clinically meaningful implications, including certainty of diagnosis, treatment response with either dilatation or surgery, and the natural evolution of the disease [301,302]. In clinical practice, fluoroscopic imaging during ingestion of a contrast agent will show the classic "bird beak" narrowing at the LES and also identify whether esophageal dilatation exists and to what extent. Because manometry and imaging allow for safe and accurate diagnosis in virtually all cases, histopathological examination of biopsies, other than for research protocols, is not indicated. An exception to this may occur in the small subset of patients with a suspected underlying paraneoplastic syndrome in whom myenteric ganglionitis might be identified at the tissue level. In those rare cases, a search for circulating antineuronal autoantibody should be undertaken, as well as imaging to search for a possible occult malignancy [289].

In adults and children, medical treatment for patients with achalasia has limited efficacy and is used only as a temporizing strategy while the patient awaits either dilatation or surgery. Medical therapy is, however, appropriate for subjects deemed unsuitable for an interventional procedure. Pharmacological options include nitrates (nitroglycerin and isosorbide dinitrate), phospho-diesterase-5 inhibitors (e.g., sildenafil), calcium-channel blockers (e.g., nifedipine), and direct injection of botulinum toxin into the LES [303]. Surgical myotomy, which involves longitudinally dividing the LES, or pneumatic dilatation to physically stretch the LES, remain the definitive treatments. A recent double-blind controlled trial showed that surgery (laparoscopic Heller myotomy) was not superior to pneumatic dilatation at a mean follow-up of 43 months in an international cohort of 210 patients with achalasia [304]. In children, in

whom achalasia is much less common than in adults, a recent systematic review concluded that available data could not determine whether surgical myotomy or pneumatic dilatations were superior [305]. It is important to note that while both interventions effectively reduce LES pressure in most patients, they do not restore esophageal peristalsis, which can continue to cause significant symptomatic dysphagia.

Chagas disease

Chagas disease is caused by the parasite *Trypanosoma cruzi*, a member of the Trypanosomatidae family, and is transmitted to humans by triatomine insects called Reduviidae beetles or "kissing bug" [306]. The disease is endemic in South and Central America and causes >15,000 deaths annually [307]. Acute symptoms go largely unattended and the infection subsides without treatment. However, some patients develop chronic infection, leading to cardiomyopathy and motor dysfunction of the gastrointestinal tract. Dilation of the esophagus (megaesophagus) and colon (megacolon) are the most common gastrointestinal findings, although the disease may progress to any segment of the bowel and extrahepatic biliary tract [306,307]. Typical gastrointestinal symptoms include dysphagia, constipation, and abdominal pain. A progressive degeneration and loss of the intrinsic innervation of the gut represents the neuropathological hallmark of Chagas disease. This is thought to result from an immune cross-reactivity between the *T. cruzi* flagellar antigen Fl-160 (a surface protein of 160-kilodalton) and a structurally similar 48-kilodalton protein expressed by mammalian axons and myenteric neurons [308]. The histopathologic analysis shows an immune infiltrate in the myenteric plexus, which leads to enteric neuronal degeneration and loss in a fashion similar to that observed in idiopathic cases of enteric ganglionitis (see below). In addition to an immune cell–mediated response, patients with chronic Chagas disease show high titers of circulating antibodies directed against the type 2 muscarinic acetylcholine receptor, which is

widely expressed on smooth muscle cells, but not on neurons. These autoantibodies play a pathogenetic role in Chagasic achalasia as they bind to muscarinic receptors and evoke muscle contraction [309]. Treatment options for chronic Chagas disease have not shown satisfactory results, with both nifurtimox and *o*-benznidazole yielding eradication of the infection in only 10% of treated patients. Surgical resection is still the mainstay of therapy in patients with end-stage megaesophagus or megacolon [310].

Gastroparesis

Gastroparesis, meaning "paralyzed stomach," results in a significant delay in the emptying of solids and liquids from the stomach. Symptomatic gastroparesis is characterized by nausea, vomiting, bloating, early satiety, and epigastric pain. In adults, gastroparesis can be divided into two common forms: idiopathic gastroparesis, accounting for 50% of cases, and diabetic gastroparesis [311,312]. In children, 70% of cases appear to be idiopathic, with the remainder accounted for by drugs, viral infections, or post-surgical [313,314].

Nearly any pathological condition that disrupts the neuromuscular function of the stomach can result in gastroparesis. Alterations in the extrinsic neural supply to the stomach are known to contribute to its pathophysiology. While the pathogenesis of idiopathic gastroparesis is unknown, it has been proposed that abnormalities of intrinsic gastric innervation can also be causative [315,316]. Recent data based on histopathological characterization of laparoscopic biopsies showed ICC depletion or abnormalities in 39% and 48% of idiopathic and secondary (diabetic) gastroparesis cases, respectively, with neuropathic changes observed in 14% and 17% of cases [317]. Tissue analysis of a patient who underwent total gastrectomy showed hypoganglionosis with features suggestive of neuronal dysplasia. In this report, abnormalities of myenteric and intramuscular ICC, along with neuronal alterations, were described, suggesting a more generalized process. Neuronal

inflammation, manifested as myenteric ganglionitis, has also been described in gastroparesis. Several conditions, including paraneoplastic syndromes, are associated with inflammatory neurodegenerative alterations and gastroparesis [318]. Cases of idiopathic inflammatory neuropathies with gastroparesis have also been described. For example, a patient with intractable vomiting had a dense lympho-plasmacellular infiltrate in the gastric myenteric plexus [319]. Treatment with steroids improved the symptoms, suggesting an important role for immunity and supporting the use of anti-inflammatory therapy in patients with proven myenteric ganglionitis [319]. Additional causes include post-viral gastroparesis and mitochondrial disease, which were identified in 18% and 8%, respectively, of children with gastroparesis in a recent review [320].

A diagnosis of gastroparesis can be made based on clinical assessment that includes (1) symptom evaluation (postprandial fullness, early satiety, nausea, vomiting, upper abdominal bloating), (2) exclusion of mechanical causes for delayed gastric emptying, such as hypertrophic pyloric stenosis in children, and (3) objective evidence from a gastric emptying assay performed using either ^{99}Tc-scintigraphy or ^{13}C octanoic acid breath test. Additional tests can be used to determine possible causes for the gastroparesis, such as electrolyte abnormalities or diabetes mellitus, which are among the most common secondary forms of gastroparesis [321]. Gastric biopsies are not included in the diagnostic work-up for gastroparesis.

The most common therapeutic strategies include dietary modification, nutritional supplements, and prokinetic drugs. The two most commonly used drugs, metoclopramide and domperidone (the latter not commercially available in the U.S.), have been shown to be effective in ameliorating symptoms in patients with gastroparesis [322]. Both compounds, however, should be used with caution because of their extrapyramidal side effects

(metoclopramide) and increased risk of sudden cardiac death (domperidone). Promising results have been obtained using a ghrelin agonist (relamorelin), newly developed motilin agonist (camicinal), 5-HT_4 receptor agonists (velusetrag), and neurokinin-1 receptor antagonists (aprepitant). These medications all deserve further investigation in clinical trials [323]. In medically refractory cases, operations, including pyloroplasty or gastrojejunostomy, can be performed to accelerate gastric emptying, but have been met with variable results [303]. The evidence of a predominant reduction of ICC, rather than a neuropathy, in a considerable number of patients may provide a cellular basis for the efficacy of gastric pacing, which is generally reserved for the most severe and refractory cases [324]. Interestingly, gastric pacing results in symptom control, but without improvement in gastric emptying time.

Idiopathic hypertrophic pyloric stenosis

Failure of the stomach to empty properly also occurs in a condition referred to as idiopathic hypertrophic pyloric stenosis (IHPS). This disease presents at around one month of age and affects approximately 1 in 500 children. The hallmark of the disease is the lack of nitric oxide synthase (nNOS) in myenteric ganglia of the pyloric region [325]. The absence of muscle relaxation due to the inhibitory denervation results in a continuous tonic contraction of the pylorus followed by muscle hypertrophy, thereby impeding gastric emptying. The pathogenic role of deficient nitrergic innervation is supported by the $Nos1^{-/-}$ mouse model, which exhibits gastric obstruction similar to that occurring in the human disease [326]. IHPS can be either sporadic or associated with several conditions, including Turner syndrome, phenylketonuria, and trisomy of chromosome 18 [327]. Although the hypertrophic muscle forms a palpable epigastric mass, the force exerted by antral contractions may be sufficient to overcome the malfunctioning pylorus and propel some gastric contents into the duodenum, at

least in the early phase of the disease. Surgery is the only therapeutic option currently available.

Neuronal intranuclear inclusion disease (NIID)

NIID is a progressive neurodegenerative disorder affecting the three main neuronal systems, including central, peripheral and enteric neurons. The neuropathological hallmark, represented by eosinophilic inclusions typically detectable within neuronal nuclei, is associated with neuronal loss of variable degree. Molecular studies reveal that these eosinophilic inclusions contain expanded polyglutamine tracts and are positive for ubiquitin and small ubiquitin-like modifier-1 (SUMO-1) [328]. These factors post-translationally modify numerous proteins via processes called ubiquitination and sumoylation, respectively, altering protein localization, metabolism, and function. Abnormalities in these processes may account for the accumulation of intraneuronal protein aggregates (ie inclusion bodies) seen in this disease. Neurological manifestations usually start in childhood, although adult onset has also been reported. The clinical expression depends on the predominant central, peripheral, or enteric neuronal loss [329]. When autonomic dysfunction characterizes the clinical picture, then the whole gastrointestinal tract can be affected with severe impairment of gut motility, including dysphagia, gastroparesis, and CIPO in combination with urinary abnormalities, such as neurogenic bladder [330]. NIID is usually sporadic, although familial cases have been reported. Rectal or colonic biopsies with histopathological identification of eosinophilic inclusions in the submucosal ganglion cell bodies may help in the diagnostic work-up. Currently, there are no specific treatment options available other than general supportive measures.

Ganglioneuromatosis in MEN2B

MEN2B is a rare autosomal dominant syndrome that affects 1 in 30,000 individuals and is characterized by an early-onset of an aggressive form of medullary thyroid carcinoma. Pheochromocytomas (50% of cases) and a marfanoid (tall and thin) body build are also seen in MEN2B. Mucosal neuromas involving lips (‘‘blubbery lips’’) and eyelids (leading to thickening and eversion) represent other features in these patients [331]. Gastrointestinal symptoms, including constipation, megacolon, or CIPO, beginning in infancy or early childhood occur in up to 80% of patients, with diffuse ganglioneuromatosis of the gastrointestinal tract occurring in about 40% of cases [332]. A specific germ-line point mutation (methionine to threonine) in exon 16 of the *RET* gene (M918T) occurs in 95% of patients [333]. Other rarer mutations involve exon 15 (A833F) [334] or codon 691 (G691S) leading to isolated ganglioneuromatosis [335]. Patients with intestinal ganglioneuromatosis involving both myenteric and submucosal plexus should be tested for *RET* mutation. In these positive cases, prophylactic thyroidectomy is indicated. Adrenal gland surveillance with ultrasound scanning and urinary fractionated catecholamines is recommended to identify the presence of a pheochromocytoma [336].

Intestinal Neuronal Dysplasia (IND)

IND represents a hotly debated entity, largely due to disagreement regarding its existence as a pathologic condition [337] and disagreement regarding its histopathology [338]. IND type B, which comprises about 98% of described cases, presents in infants or older children as severe constipation. It can occur in isolation or proximal to the aganglionic segment in a patient with HSCR [339]. Diagnostic criteria vary regarding the details, but broadly speaking are based on the presence of neuronal hyperplasia specifically in the submucosal plexus. One set of criteria includes the presence of giant submucosal ganglia, defined as containing >8 ganglion cells

per ganglion, in at least 20% of submucosal ganglia examined [340], and often also the presence of ectopic ganglia in the lamina propria [341,342]. Interestingly, later descriptions of IND require that the child be >1 year of age for this diagnosis because of the recognition that the submucosal ganglia of infants contain a larger number of cells per ganglion due to their immaturity [340]. Importantly, the diagnosis of IND applies only to patients with these histologic features in the large intestine, not the small intestine, where the importance of these findings is unknown. Most children with IND are treated conservatively for their constipation, with surgical removal of the abnormal colon reserved for the most severe cases.

No definitive animal model of IND exists. Rats with a heterozygous mutation of *EDNRB* have histologic features reminiscent of IND, with larger and denser submucosal ganglia present in the colon [343]. However, while the histology is consistent with IND, the rats have no intestinal dysmotility and develop normally. A recent study in avian embryos showed that inhibition of Shh signaling led to the development of large and ectopic submucosal ganglia [344], suggesting the possibility that Shh may be associated with this condition. Since IND has been identified proximal to the aganglionic segment in HSCR [339], a search for mutations in HSCR-related genes (*RET*, *GDNF*, *EDNRB*, and *ET3*) was performed in 20 patients with IND, but failed to uncover any abnormalities [345].

Chronic constipation

Chronic constipation is a functional disorder of the gastrointestinal tract characterized by difficult and infrequent bowel movements. Depending on the definition used, estimates indicate that about 10-15% of adults and up to 30% of children in the general population suffer from constipation [346,347]. Although in children no specific gender, age or socioeconomic associations are evident, in adults the condition most commonly affects women (female:male ratio of 2-3:1), elderly, non-whites, and those with a lower socio-

economic status [347-349]. From a pathophysiologic standpoint, three main subtypes of constipation exist: (1) normal transit (also referred to as functional constipation), (2) slow transit, and (3) obstructed defecation (also called dyssynergic defecation when no mechanical obstruction is present) [350].

Data regarding neuromuscular abnormalities in chronic constipation have been obtained in the minority of patients who undergo surgery for the most severe forms of the disease, predominantly slow transit constipation (STC) with colonic inertia (CI), which mainly affects young women and has also been described in children without an obvious sex bias [351]. Recent studies have shown that tissue analysis in STC/CI shows features indicative of an underlying enteric neuropathy, a finding previously predicted using gastrointestinal manometry to assess bowel motility in these patients [352,353]. Alterations of colonic innervation in STC/CI were initially characterized by silver staining, showing a reduction in the total number of argyrophilic neurons and structural alterations in both neuronal perikarya and axons [354,355]. Other reports described a reduction of enteric neural elements (cell bodies and/or processes) as a common feature in patients with STC/CI who underwent surgery [356-358]. Furthermore, these neuronal abnormalities are often associated with reduced ICC and enteric ganglion cells [14,359,360]. In a cohort of patients with a severe and intractable form of STC, it has been found that: 1) ICCs were significantly decreased; 2) enteric neuronal loss was present, partly ascribed to apoptosis; and 3) enteric glial cell number was markedly reduced in both the submucosal and myenteric plexuses [361]. However, in the same study, the examination of the terminal ileum of patients with intractable STC/CI revealed only a reduction of enteric glial cells, while neuronal and ICC alterations were not detected at this level [362]. In a study of children with severe STC, abnormal contractile patterns present on high resolution colonic manometry were significantly associated with histopathological abnormalities primarily involving enteric neurons, but also included changes in ICCs and smooth muscle [351]. Giorgio

et al [351] showed that a failure of motor quiescence between bisacodyl-induced high amplitude propagating sequences reliably predicted neuromuscular pathology. Other results suggest that colonic dysmotility in STC/CI patients can be attributable to more subtle imbalances of enteric neurotransmitters and neuropeptides. An excessive production of NO in the colonic myenteric plexus of patients with STC has been suggested as a pathophysiological mechanism eliciting a constant inhibition of propulsive contractile activity [363]. However, this possibility is still debated, since other studies investigating mediators like VIP, substance P, neuropeptide Y, or serotonin gave contradictory results in tissue specimens of patients with STC [364-367]. A major limitation to finding a consistent histopathological diagnosis and likely contributing to the wide spectrum of neuromuscular abnormalities reported is the large variability in disease phenotype, anatomic source of the sample, morphological methods for tissue evaluation, and the lack of normative data to detect subtle changes in enteric neuronal, glial, or muscular structure [368]. The field would benefit from improved diagnostic methods and the development of a clear and comprehensive nosology to more accurately define subtypes of constipation.

Current management of severe constipation primarily consists of laxatives, given orally or as suppositories. Many types of laxatives are used, based on their mechanism of action, including the following classes of agents (with examples): bulk-formers (fiber), stool softeners (docusate), lubricants (mineral oil), hyperosmotic agents (polyethylene glycol or lactulose), and stimulants (bisacodyl or senna). Other agents include serotonin agonists (prucalopride), which stimulate motility by activating 5-HT_4 receptors on enteric neurons, or chloride channel activators (lubiprostone) or guanylatecyclase-C agonists (linaclotide), which soften stool by increasing fluid secretion by the gut epithelium. Other agents, including newer 5-HT_4 agonists (naronapride and velusetrag), the ileal bile acid transporter A3309 (elobixibat), and ghrelin agonists (relamorelin), represent the forefront of treatment [369]. In

children there is limited experience with many of these newer medications. A recent multicentre double-blind placebo-controlled trial of prucalopride in children with functional constipation failed to show any benefit, although it may be of value in some cases of severe constipation [370]. Although lubiprostone showed some efficacy in pediatric constipation in an open-label study, this remains to be shown in a larger multicentre trial [371]. When medical management fails, surgery can be considered in carefully selected patients. Subtotal colectomy has long been the standard approach for intractable constipation [372]. In children, and possibly in adults, antegrade enemas via a catheterizable appendicostomy or a cecostomy tube have proven to be effective [373,374]. In the most severe cases, total proctocolectomy with ileoanal anastomosis or diverting ileostomy have been used [375]. A better understanding of the underlying causes of severe constipation is needed in order to develop improved pharmacotherapies that can target specific abnormalities in a given individual.

Future therapies

One of the most promising areas of research in neurointestinal disease is the development of novel cell-based therapies. Recent advances in cell biology and molecular genetics have enhanced the identification and isolation of stem cells from a variety of adult tissues, including the gut. In the human gastrointestinal tract, multipotent stem cells have been found to be present, not only during development, but also postnatally, well into late adult life [376]. The identification of enteric neural stem cells represents an exciting opportunity to test new therapies for the ENS [377]. Human enteric neural stem cells can be collected by relatively noninvasive techniques, such as routine endoscopy, and have been transplanted *ex vivo* into strips of aganglionic intestinal muscle, where they differentiate into enteric neurons and glial cells [378].

Autologous cell transplantation offers the possibility of using a patient's own cells to replace missing or injured enteric neurons in a variety of neurointestinal diseases. Autologous cells would eliminate issues around immunologic rejection and ethical concerns raised by other sources of stem cells. Hotta et al [379] recently successfully isolated isogenic enteric neuronal progenitors from the ganglionated bowel of Ednrb-deficient mice and transplanted them into the postnatal aganglionic rectum of Ednrb-deficient hosts. In considering autologous transplantation as a potential therapy, one needs to consider that ENS stem cells obtained from HSCR patients might be impaired in their biological function [380]. However, in HSCR the "abnormal" enteric neural crest-derived cells are able to migrate a significant distance along the bowel, from foregut to distal hindgut in the majority of cases, failing to colonize only the very distal end. In fact, in the recent study of isogenic cell transplantation into Ednrb-deficient mice, transplanted cells demonstrated a normal capacity for self-renewal and neuronal differentiation [379]. However, this was an issue for mice carrying the monoisoformic Ret51 (miRet51) mutation, in which only the Ret51 isoform is expressed, resulting in a HSCR-like phenotype with distal colonic aganglionosis and intestinal obstruction [381]. Enteric progenitors harvested from these mice show delayed differentiation compared to wild-type controls [381]. These cells could be genetically modified to restore a normal phenotype, for example by restoring the Ret9 isoform within the miRet51 ENS progenitors, which has been shown to rescue the differentiation anomalies [381].

An alternative source of autologous cells for transplantation is induced pluripotent stem cells (iPSC). Transfection of key transcription factors into a patient's somatic cells (e.g. fibroblasts or peripheral blood leukocytes) allows these cells to reprogram into a multipotent-like state, and these cells are referred to as iPSC [382]. For cell therapy this technology offers the possibility of overcoming the need for harvesting ENS stem cells from the gut and instead

using an easily accessible source of autologous cells, such as the skin. iPSC can be expanded *in vitro* and genetically engineered, either by introduction of a normal gene to replace a mutant allele or by gene editing to correct a known mutation 383. iPSC were recently successfully induced to enteric neural crest progenitors, and subsequently differentiated into enteric neurons [384]. These cells were able to migrate and differentiate appropriately in embryonic aneural chick intestine and to colonize adult mouse colon [384]. In patients with a known mutation, a variety of techniques can be used to correct the mutation using homologous recombination or a genome editing tool, such as CRISPR, TALENs, or ZFNs [385]. Recent publications have demonstrated successful genome modification of iPSC harboring known disease-causing mutations in several genetic diseases, including sickle cell disease [386], beta-thalassemia [387], and spinal muscular atrophy [388].

While it appears plausible that cell-based approaches could be used to treat enteric neuropathies, many challenges remain, including identification of the most appropriate diseases to treat, optimal cell sources, strategies for maximizing cell expansion, methods for efficient and targeted cell delivery, and issues with immune response. Addressing these challenges will usher in a new era in the treatment of enteric neuropathies and offer hope to patients with these difficult conditions.

Take-home messages of this review article:

- Enteric neurons arise from neural crest cells and regulate gastrointestinal function.
- Congenital neurointestinal diseases result from abnormal development of enteric neurons.
- Acquired neurointestinal diseases are due to neurodegeneration, inflammation, or infection.

- Treatment of neurointestinal diseases represents an unmet need for patients and clinicians alike due to incomplete understanding of underlying pathophysiology.
- Therapeutic options include nutritional support, prokinetics and antibiotics (for underlying bacterial overgrowth). Specific treatments may be advocated in very particular cases, e.g. anti-inflammatory drugs in case of ganglionitis / leiomyositis; surgery in HSCR; stem cells represent the forefront of future therapies.

Chapter 6.

CHRONIC INTESTINAL PSEUDO-OBSTRUCTION

CIPO represents the most severe form of GI dysmotility with diabling and potentially lethal consequences. Symptoms are non-specific, and as a result this condition is usually diagnosed incorrectly or too late with consequences for morbidity and even mortality. Although pediatric and adult CIPO have many common clinical features, distinctive features can also be identified. There is no single diagnostic test or pathognomonic finding for CIPO. Therefore, a multidisciplinary stepwise approach including radiology, endoscopy, laboratory, manometry and histopathology should be considered in the diagnostic work-up. Treatment of this condition is challenging and often requires a collaborative effort with participation of appropriately experienced gastroenterologists, pathologists, dieticians, surgeons, psychologists, and other subspecialists.

6.1 Diagnosis and therapeutic options

Modified from

Chronic intestinal pseudo-obstruction in children and adults: diagnosis and therapeutic options

Giovanni Di Nardo, Carlo Di Lorenzo, Augusto Lauro, Vincenzo Stanghellini, Nikhil Thapar, Tennekoon B. Karunaratne, Umberto Volta and Roberto De Giorgio

Neurogastroenterol Motil. 2017 Jan;29(1).

Introduction

Chronic intestinal pseudo-obstruction (CIPO) is a rare condition characterized by a severe impairment of gastrointestinal (GI) propulsion, which results in symptoms suggestive of partial or complete intestinal obstruction in the absence of any lesion restricting or occluding the intestinal lumen.[24,254,389-391] CIPO can involve any segment of the GI tract (although the small bowel and colon are mainly affected) and represents the most severe form of GI dysmotility with potentially lethal consequences.[24,390] Symptoms can be non-specific with CIPO mistaken for other diseases and consequently not diagnosed for long periods of time.[392,393] The chronicity of the severe digestive symptoms, the inability to maintain an adequate nutritional status without specialist support, the suboptimal efficacy of medical treatments, and the limited knowledge of the syndrome by physicians constitute some of the main factors contributing to the poor quality of life and the high morbidity and mortality rate of patients with CIPO.[392-395]

Much like other rare diseases with poorly defined diagnostic criteria, CIPO has a largely unknown prevalence and incidence.[24] One nationwide survey in the U.S. reported that about 100 infants were born with CIPO every year.[396] A more recent nationwide survey in Japan found a prevalence of 3.7 in one million children (1 in 270,000 children younger than 15 years of age) with equal sex incidence.[397] These studies most likely underestimate the true number of new cases per year, as they do not include patients who develop symptoms of CIPO later in life. In another survey of 378 institutions belonging to the Japanese Society of Gastroenterology, CIPO prevalence in adult patients was estimated to be 1.0 and 0.8 cases per 100,000 males and females, respectively. The incidence in the same population was 0.21 and 0.24 cases per 100,000 males and females, respectively.[398]

The purpose of this paper is to provide a broad review of CIPO ranging from the pediatric to adult age selectively highlighting the main clinical aspects such as symptoms / signs,

diagnosis and therapeutic options in the different age groups. Deliberately, we did not address the putative mechanisms underlying severe gut dysfunction as well as the attendant histopathological features and the reader is referred to published comprehensive articles on these topics. In Table 2 summarize the classification of CIPO in relation to etiopathogenetic factors.

Disease spectrum

CIPO is not a single clinical entity, rather an umbrella term for a range of different diseases leading to severe, end-stage gut motor failure. The most severe cases of the CIPO spectrum are those involving pediatric patients with antenatal (*in utero*) evidence of multivisceral dilation of the hollow viscera (e.g. digestive and urinary systems), often characterized by inability to tolerate enteral feeding and poor prognosis (Fig. 12 A-B).[394,399-401] This clinical subset represents the most common group of pediatric CIPO patients with diffuse involvement of the GI tract. In these cases, the neuromuscular abnormalities (either genetic or acquired) of the GI tract do not preclude birth, but can be severe enough to generate the onset of symptoms in the early newborn period with reported mortality rates ranging from 10% to 32%.[394,399,400]

More rarely, some cases appear to be acquired after birth, being characterized by a variable period of normality followed by rapid progression to intestinal failure with bowel (and often urinary tract) dilatation. In some of the most aggressive forms of acquired CIPO, the histopathological analysis may detect a massive inflammatory (mainly lymphocytic) neuro-muscular infiltrate, reminiscent of the autoimmune pancreatic 'insulitis' underlying insulin-dependent diabetes mellitus of the childhood [402] (Fig. 12 C-D). These cases of CIPO may respond to an immunosuppressive treatment if the immune-mediated insult has not

completely damaged regulatory cells, i.e. enteric nerves, interstitial cells of Cajal (ICC), and smooth muscle.[403-408]

Other cases of pediatric and adult CIPO may occur in patients who experience more insidious mild and nonspecific symptoms (either 'irritable bowel syndrome' - or 'dyspepsia'- like) thought not to carry a risk to evolve into severe dysmotility. Nonetheless, some of these patients do progress on to a classic CIPO phenotype over time (Figure 1E-G). We consider them the 'dark-side' of adult CIPO spectrum, i.e. cases in whom a number of factors, including an altered gut microbiota, intestinal epithelial barrier dysfunction, immune dysregulation and other poorly defined mechanisms may operate, individually or in concert, to impair the neuro-muscular homoestasis.[392] Patients with acute onset of CIPO after abdominal surgery, i.e. mimicking a prolonged postoperative ileus, remain a largely unexplained subset. Other striking examples are those cases occurring after ileal bypass performed to treat morbid obesity, suggesting that surgical manipulation may by itself evoke neuro-myoelectrical abnormalities in predisposed alimentary tracts.[409] A synoptic view of this section is reported in Fig. 13.

Clinical findings

CIPO may involve any segment of the GI tract and therefore symptoms can vary from patient to patient based on the location and the extent of the gut segment involved. Also, extra-intestinal manifestations and malnutrition contribute to the clinical features. [1,391,393,410-412] Prenatal signs are only detected in about 20% of cases, whereas 50-70% of patients show clinical signs perinatally (i.e. within the first month of age). Most patients (80%) show clinical manifestations by the first year of age, while the remaining 20% have sporadic onset during the first two decades of life. [394,395,397,399,400] One study indicated that the median age of symptom onset in adults is 17 years.[406]

Both pediatric and adult CIPO shares many clinical aspects, although distinctive features can be identified (Table 3). In any age group, the clinical picture tends to be dominated by abdominal pain and distension (80%), which are particularly severe during acute episodes of pseudo-obstruction.[406] Associated symptoms include nausea and vomiting (75%), constipation (40%), and diarrhea (20%).[392,406] Between acute episodes, patients can be minimally symptomatic, or continue to experience severe proximal (anorexia, early satiety, nausea and vomiting) and distal digestive symptoms (constipation, diffuse abdominal pain and / or distension).[392,394] Prevalence and severity of acute episodes that recur at irregular intervals vary from patient to patient. Malnutrition is another significant clinical aspect in any patient with CIPO. This is due to both limited oral intake, because ingestion of food generally aggravates symptoms, and intestinal malabsorption related to the altered gut transit, often associated with dilated bowel loops. In about 30% of CIPO patients small intestinal bacterial overgrowth (SIBO) occurs as a result of intestinal stasis and can cause diarrhea and steatorrhea.[392] Gastroparesis and urinary bladder dysfunction (with or without megacystis and megaureter) are co-morbidities that are likely to share similar pathophysiological mechanisms with those underlying CIPO.[24] Also, because of the frustrating ineffectiveness of most therapeutic interventions, patients with CIPO may develop depression or other psychological disorders.[413,414]

In children, there is a higher risk of colonic and small bowel volvulus secondary to severe dysmotility and gut dilatation, congenital adhesions or concurrent malrotation.[394,400,415-417] Urological involvement is commonly identified in patients with familial and congenital forms of CIPO, particularly in the myopathic subgroup, ranging from 36 to 100%.[394,418-421] Findings include urinary retention secondary to bladder atony, hydronephrosis, vesicoureteral reflux, and recurrent urinary tract infections. Megacystis on antenatal ultrasound has been reported in up to 59% of CIPO and this finding may require delivery by caesarean section.[420,421]

Megacystis can be associated with a microcolon, a phenotype referred to as megacystis-microcolon-intestinal-hypoperistalsis syndrome.[4] Other syndromic form of CIPO are represented by the mitochondrial disorders which in a large adult series account for 19% of all CIPO patients. They are characterized by severe intestinal dysmotility, poor nutritional status and neurologic manifestations, e.g. peripheral neuropathy (with mild to moderate hypoesthesia), proprioceptive ataxia, progressive external ophthalmoplegia with ptosis and hearing loss.[2] GI manifestations are common at presentation and positive family history together with the progressive neurological and nutritional deterioration should alert clinicians to search for mitochondrial disorders.[422-425]

Secondary systemic forms (i.e. related to underlying conditions) of CIPO are more common in adult patients in whom they occur at much older age.[24] A proximal muscle weakness may indicate the presence of polymyositis and dermatomyositis. Scleroderma is usually associated with skin abnormalities, while the suspicion of a paraneoplastic syndrome should prompt investigations aimed at uncovering an occult malignancy of the lung, ovary and breast. Finally, forms of CIPO associated with Chagas' disease are common in Latin America and are characterized by combination of dysphagia and cardiomyopathy. [319,392,393,396,402,426,427]

Diagnosis

The diagnosis of CIPO is mainly clinical. The diagnostic work u.p of both children and adults with suspected CIPO has the following goals: 1) rule out mechanical causes of bowel obstruction. This can be achieved by abdominal CT or plain X-ray; 2) identify any underlying diseases by an accurate laboratory test profile; 3) evaluate the possibility of a drug-induced CIPO-like presentation (e.g. opioids, tricyclic antidepressants, anti-cholinergic agents, anti-Parkinsonian agents, phenothiazines); and 4) understand the pathophysiological features that may improve management or bear prognostic information in selected cases (particularly by

performing GI manometry in cases without bowel dilation). Thus, a stepwise approach (as outlined above) is commonly recommended in CIPO and includes radiology, endoscopy, laboratory, manometry and histopathology.

Radiology

A plain radiograph of the abdomen usually identifies the typical signs of intestinal obstruction, i.e. air-fluid levels and dilated bowel loops.[24, 397,400] Air-fluid levels are better visualized in the upright position, but lateral views can be useful. In symptomatic patients without these radiographic findings, other conditions (e.g. chronic constipation, irritable bowel syndrome and functional dyspepsia) should be considered.

Fluoroscopic studies should be performed using water-soluble contrast solutions in order to avoid complications related to barium concretions and, at the same time, enhance hydration and transit of intestinal contents. Upper GI series with small bowel follow-through can unravel dilated bowel loops (often involving the stomach and duodenum) with very slow transit, although the latter finding may not be detectable in some pediatric cases. Intestinal malrotation may be identified in up to a third of children with CIPO.[394] Less common findings include diverticulosis of the small intestine (in 53% of patients with mitochondrial neurogastrointestinal encephalomyopathy, MNGIE, and 42% of scleroderma), and intestinal pneumatosis.[24]

Contrast medium radiologic tests have been recently superseded by dedicated enterography with high-resolution CT or MRI, which more accurately detect mechanical obstruction and intestinal adhesions. Cine-MRI is emerging as a non invasive, radiation-free method for assessing and monitoring GI motility particularly in the pediatric population. [241,243,428]

Excretory urograms should be performed in patients with urinary symptoms, since neuro-myopathies may affect both the GI tract and urinary system. A chest CT may be necessary to

exclude small cell lung cancer in adult patients with suspected paraneoplastic CIPO. Finally, imaging of the brain can identify leukoencephalopathy in CIPO related to MNGIE.[423-425]

Endoscopy

Upper GI endoscopy may be useful to exclude a mechanical occlusion of the proximal small intestine as well as to collect duodenal biopsies in cases where celiac disease or eosinophilic gastroenteropathy are suspected.[389] Colonoscopy can be used to rule out mechanical obstruction and decompress the large intestine, although this manoeuvre rarely provides long-term satisfactory results.[429] The wireless motility capsule measures intraluminal pH, temperature, and pressure; however, the role of this technique in the diagnosis of CIPO has not been established yet and its use is thought to be even hazardous when a mechanical obstruction has not been definitely ruled out.[430]

Laboratory tests

Laboratory exams are aimed to unveil secondary causes of CIPO. Blood tests for diabetes mellitus (i.e. hemoglobin A1C and / or postprandial blood glucose concentration), celiac disease (anti-tissue transglutaminase IgA and anti-deamidated gliadin peptides IgG), connective tissue and skeletal muscle disorders (anti-nuclear antibody, anti-double-stranded DNA and SCL-70, creatine phosphokinase, aldolase), and hypothyroidism should be performed. Other tests include serology for Chagas' disease, urinary catecholamines for pheochromocytoma, and enteric neuronal autoantibodies (anti-Hu or type 1 anti-neuronal nuclear antibodies) in patients with suspected paraneoplastic syndrome. Urinary porphyrins should be assayed in patients with severe, otherwise unexplained abdominal pain. A complete blood cell count, electrolytes, albumin, liver enzymes, vitamin B12, fasting cortisol, inflammatory indexes (C-reactive protein and erythrocyte sedimentation rate) are useful in all

cases.[414] Patients receiving parenteral nutrition (PN) should be carefully monitored with particular attention to fluid, electrolytes and circulating levels of trace elements. In patients with symptoms and signs suggestive of an underlying mitochondrial disorder, serum lactate and thymidine phosphorylase activities should be performed. If thymidine phosphorylase activity is markedly reduced / absent and nucleosides are increased, then thymidine phosphorylase (*TYMP*) (in MNGIE), and polymerase DNA-gamma (*POLG*) (in sensory ataxic neuropathy dysarthria and ophthalmoparesis) gene mutations should be tested.[424,425]

Manometry

Intestinal manometry can be useful to define the pathophysiological (neuro-muscular) mechanisms involved in CIPO (e.g. neuropathy or myopathy), although it has a low diagnostic specificity and in some pediatric and adult patients does not influence treatment. Nonetheless, intestinal manometry can differentiate mechanical from functional forms of sub-occlusion, provided that the organic cause is at an early stage.[392,393,410,411,431-435] In fact, the presence of postprandial, prolonged, high pressure, non-propagated contractions is a pattern suggestive of a recently occurred mechanical obstruction.[5,7,10,11] A neuropathy is characterized by contractions showing a normal amplitude, although with uncoordinated pattern [391,393,396] (Fig. 14), whereas coordinated contractions with a reduced amplitude are indicative of an enteric myopathy. However, a careful interpretation is mandatory since low-amplitude contractions could be secondary to the inability of the manometric catheter to register non-occlusive contractions when bowel loops are dilatated.[24] Antroduodenal manometry has been applied extensively in children in order to assess prognosis, response to treatment and tolerance to oral feeding.[431-433] In children with chronic symptoms suggestive of CIPO, a normal manometry essentially excludes CIPO and should lead to the consideration of emotional or factitious disorders (e.g. Munchausen syndrome by proxy).[434,435] Amiot et al.

have identified an abnormal esophageal manometry in 73% (51% with severe ineffective dysmotility) adult CIPO cases.[436] Similar findings have been also documented in paediatric cases of CIPO.[400] Notably, only esophageal motor disorders had a significantly negative predictive value in terms of survival, home PN requirement, and inability to maintain sufficient oral feeding, suggesting the presence of a more generalized disease.[436] Also, esophageal manometry can be useful in CIPO associated with scleroderma. Anorectal manometry is indicated when the clinical picture is characterized by intractable constipation and marked colonic dilatation, suggestive of Hirschsprung's disease.[24] A careful manometric assessment of the entire GI tract, including the colon, has been deemed helpful in helping to plan for an isolated or a multivisceral transplantation in the most severe forms of pediatric CIPO.[396]

Histopathology

Collection of gut full-thickness biopsies is aimed at providing clinicians with a histopathological correlate which may unravel abnormalities related to: *a*) extrinsic and/or intrinsic neurons controlling gut functions; *b*) the ICC networks; and *c*) enteric smooth muscle cells. Changes affecting these cellular systems are tightly linked to the pathophysiology of CIPO and may have prognostic and sometimes therapeutic implications.[437,438] Minimally invasive procedures, e.g. laparoscopic surgery or - very recently - endoscopic approaches (for example natural-orifice translumenal endoscopic surgery), have shown a high diagnostic yield and safety.[439] In addition, guidelines proposed by the Gastro 2009 International Working Group have helped to find consensus about technical aspects (including tissue collection and processing) and histopathological reporting of results in a variety of gut neuro-muscular disorders, including CIPO.[2,440]

In the absence of clinical guidelines, we suggest that ideal CIPO patients who should be referred to laparoscopic surgery for full-thickness biopsies fall into these two major categories: 1) idiopathic cases characterized by an acute onset likely of post-infectious origin; 2) patients with progressive, rapidly evolving forms of CIPO who are not under treatment with opioids and do not respond to any therapeutic options. In contrast, CIPO patients with severe pain, not uncommonly treated with opioids, should not undergo intestinal biopsy. In those cases it is advisable to taper down opioids and change with other non-opioidergic analgesic compounds. This measure is directed to avoid useless and often misleading histopathological analysis. In CIPO cases with a clear origin (i.e. secondary forms of CIPO), tissue sampling may be less clinically meaningful as many systemic diseases are known to affect the gut neuro-muscular layer.

Natural history

A severe clinical course can be expected for both pediatric and adult patients with CIPO when an underlying treatable disease is not identified.[392,410-412,419] In a single-centre study of 59 adult patients with idiopathic CIPO followed up for a long time (13 years), it was demonstrated that the average time between the first sub-occlusive episode and the diagnosis of CIPO was 8 years, with 88% of patients undergoing an average of 3 unnecessary surgical procedures.[392] Similar rates of questionable surgery are seen in the pediatric setting.[400] The digestive symptoms worsened over time with abdominal pain becoming intractable or responsive only to major analgesics (e.g. opioids - always used parsimoniously and with extreme caution in our own experience) in approximately 25% of cases.[392] Most patients had oral feeding restrictions, while 30%-50% of patients needed long term PN.[392,410,411] A study by Amiot et al., which examined all patients with CIPO on PN at home, revealed that the lowest mortality was associated with the ability to restore oral feeding and with the presence

of symptoms before 20 years of age, while an increased mortality was associated with the presence of scleroderma.[410]

Manometric parameters, such as inadequate or absent motor response to meals, absence of migrating motor complex (MMC) during fasting, and generalized hypomotility have been shown to be predictive of poor outcome in patients with CIPO.[410,433] Finally, detection of esophageal dysmotility in CIPO seems to have negative prognostic implications in terms of mortality and need for home PN.[436] In children with CIPO, a myopathic etiology, coexisting urinary involvement, and concurrent intestinal malrotation are poor prognostic factors.[394] In both adults and children, the risk of death is increased by the absence of a specialist team and in the early phases after the diagnosis of intestinal failure has been established.[441]

Therapy

Treatment of patients with CIPO is challenging and requires a multidisciplinary effort. The management of both children and adults with CIPO should be directed to avoid unnecessary surgery, restore fluid and electrolyte balance, maintain an adequate caloric intake, promote coordinated intestinal motility, and treat complications e.g. sepsis, SIBO and associated symptoms. In general, current therapeutic approaches are not very effective; however, recent advances in nutritional, pharmacological and surgical treatment has helped to improve the management of patients with CIPO. [24,390,391,393]

Nutritional support

Patients with CIPO are often malnourished, due to malabsorption and insufficient food intake. Patients with sufficient intestinal absorption capacity should be encouraged to take small, frequent meals (5-6 / day), with an emphasis on liquid calories and protein intake, while avoiding high-fat and high-residue (fiber containing) foods. Carbohydrate containing

foods, rich in lactose and fructose, may worsen abdominal bloating and discomfort.[389] Vitamin levels, i.e. A, D, E and K as well as B12 and folic acid, should be supplemented when needed. In cases of inadequate oral intake, enteral nutrition with standard non-elemental formula may be considered. In children, extensively hydrolysed and elemental formulas are often empirically used to facilitate intestinal transit and absorption[390,391,442]. Before a permanent feeding tube is placed, a trial with a nasogastric or nasojejunal feeding tube should be attempted using an enteral formula at a rate sufficient to provide an adequate caloric support [441,443,443]. When delayed gastric emptying is present, bypassing the stomach and directing the feeding into the small intestine is generally preferred. Jejunal feeding tubes were tolerated in all patients with manometrically detectable MMC *vs.* 33% of those without. Enteral nutrition should be started with a slow infusion given continuously or, preferably, in a cyclical manner (during the night).[389] In the most severe cases, PN is necessary to maintain nutritional support and an adequate level of hydration.[443,444] If adult patients are exclusively dependent on PN, they should receive approximately 25 kcal / kg / day, and lipids should approximately supply 30% of total parenteral calories with 1.0-1.5 g / kg / day of proteins and dextrose covering the remaining caloric amount.[443,444] Complications of PN, including liver failure, pancreatitis, glomerulonephritis, thrombosis and sepsis, are frequent causes of morbidity and mortality in any form of pediatric and adult CIPO.[441,443] Individualized PN formulations with minimal amount of intravenous lipids can help reducing metabolic complications. A long-term PN does not seem to be associated with a significant increase in morbidity and mortality in CIPO.[443] A retrospective analysis of 51 adult patients receiving PN for an average of 8.3 years showed 180 episodes of catheter-related sepsis, 9 of acute pancreatitis, 5 encephalopathy, and 4 patients progressing to cirrhosis.[410] Oral intake was a major independent factor associated with better survival; thus, patients receiving PN should be allowed and encouraged to maximize oral intake as tolerated.[443,445]

Pharmacologic therapy

The main aim of pharmacological treatment in patients with CIPO is to promote GI propulsive activity, thereby improving oral feeding, decreasing symptom severity, and minimizing SIBO[24,389,390].

The efficacy and main features related to various drugs, such as erythromycin, metoclopramide, domperidone, somatostatin analogues (octreotide and lanreotide), cholinesterases inhibitors (neostigmine and pyridostigmine), serotoninergic agents (such as 5-hydroxytryptamine receptor 4 - 5-HT_4 - agonists, e.g. prucalopride), prostaglandins and gonadotropin-releasing hormone analogues (leuprolide), have been reported in a number of pediatric and adult CIPO studies (Table 4).[446-462] Erythromycin is a macrolide antibiotic mimicking the prokinetic hormone motilin that induces phase III of the GI MMC. It has shown to be effective (at a dose of 1.5-2 g/day in adults or 3-5 mg/kg/dose in children) in accelerating gastric emptying and improving symptoms of CIPO in case reports.[390,446] Metoclopramide and domperidone are two orthopramides which exert their prokinetic effects via type 2 dopamine receptor antagonism and increasing acetylcholine release from myenteric neurons. Although widely used in patients with functional gut disorders,[393] there are no clinical data of for their use in CIPO. In addition, metoclopramide has a boxed warning by the Food and Drug Administration given the risk of tardive dyskinesia.[447] Octreotide, a long-acting analogue of somatostatin, is known to evoke phase III of the MMC in the small intestine of patients with scleroderma-related CIPO.[448] Subcutaneous octreotide at a dose of 50 mcg / day resulted in a significant beneficial effect by relieving bacterial overgrowth in those patients.[448] Further studies confirmed its efficacy showing reduction of nausea, vomiting, bloating, and abdominal pain, in a subset of idiopathic CIPO.[449] The acetylcholinesterase inhibitor neostigmine has proven efficacy in colonic decompression in

adult and pediatric acute colonic pseudo-obstruction.[450] Repeated use was successful in an adult patient with colonic pseudo-obstruction, although chronic use in children with CIPO has not been reported.[451] The longer acting pyridostigmine has also been used with success in adult patients with CIPO.[452] Among the newly discovered highly selective 5-HT_4 receptor agonists, prucalopride has shown high bioavailability and lack of major interactions with other drugs as it is not metabolized by the cytochrome P3A4.[453] Prucalopride exerts a significant enterokinetic effects [454] and a recent randomized, controlled trial on CIPO patients (3 patients had visceral myopathy; 1 visceral neuropathy; all treated with 2-4 mg once daily and followed for 48 weeks), showed beneficial symptomatic effects and lower use of analgesic drugs.[455] Although the sample size was very small, the results of this study lend support to future multicenter controlled trials.

SIBO is known to cause mucosal inflammation, which further impairs GI motility thereby contributing to nausea, bloating and abdominal distension.[24,456.461] Various antibiotic regimens have been recommended.[459] The treatment of choice usually involves the use of non-absorbable antibiotics, such as rifaximin.[460] Nonetheless, most clinicians use 1- to 2-week rotation of broad-spectrum antibiotics such as amoxicillin and clavulanic acid, gentamicin, and metronidazole, often combined with an antifungal compound (e.g., nystatin or fluconazole) followed by antibiotic-free periods.[24] Notably, amoxicillin-clavulanate has been shown to combine antibiotic and enterokinetic properties in children.[461]

Non-narcotic pain modulators, such as tricyclic antidepressants and serotonin-norepinephrine reuptake inhibitors, should be used with caution in CIPO patients due to their significant side effects (i.e. constipation and / or drowsiness). Starting with low doses followed by gradual increase is advisable to optimise the beneficial / side effect ratio. Gabapenentinoids (gabapentin and pregabalin) as well as peripherally acting μ-opioid receptor antagonists (PAMORAs) may represent promising alternatives to antidepressants; however studies on

CIPO patients are lacking. If visceral pain is untreatable, an extremely careful and cautionary use of opiates may be attempted. In children with CIPO and significant abdominal pain, transdermal buprenorphine (5 mcg / h), a μ- partial agonist and κ- and δ-opioid receptor antagonist, has shown adequate pain relief in 3 out of 4 patients.[462]

In CIPO characterized by histopathological signs of marked inflammation / immune response within myenteric ganglia or throughout the neuromuscular tract, immunosuppressive drugs (e.g., steroids and azathioprine), might be an effective therapeutic options.[403,404,407] Treatment of secondary forms of CIPO is mainly directed toward the underlying diseases (e.g., scleroderma, paraneoplastic syndrome, endocrine related disorders and many others) in addition to gut-directed therapy such as antibiotics, prokinetics and laxatives.

Endoscopic and surgical therapy

Decompression of distended GI segments via intermittent nasogastric suction, rectal tubes or endoscopy in both adults and children is an important therapeutic target.[24,389,463] In some cases a "venting" enterostomy,[464] typically placed endoscopically in the intestine, may be necessary. Recently, repetitive colonoscopic decompression has been successfully used as a bridge therapy before surgery in a pregnant woman with CIPO.[465] In adult patients needing multiple endoscopic decompression, percutaneous endoscopic colostomy has been recently proposed as a feasible therapeutic option since it leads to durable symptom relief without risk of surgical intervention.[466] The role of surgery in CIPO has been debated over the years. Although its use may be indicated as a tool to collect gut biopsy specimens for histopathology, the need for surgical therapy is sometimes required in emergency situations (massive bowel distension and perforation / ischemia). In a retrospective study on 63 adult patients there was an overall postoperative mortality rate of 7.9%, while CIPO-related re-operation rate was 66% at 5 years.[467,468] In children palliative procedures such as feeding /

venting gastrostomies and jejunostomies are mainly used to relieve symptoms in half of the patients.[395, 469,470] It is important to keep in mind that when a surgical procedure is performed in a CIPO patient, full-thickness biopsies should be obtained and processed in dedicated centres.

Transplantation

Intestinal transplantation (isolated or multivisceral) is thought a reasonable therapeutic alternative for patients with CIPO who experience serious complications of PN (e.g. liver failure or recurrent sepsis arising from a central venous catheter). Other indications to transplantation are the inability to obtain venous access for PN and a poor quality of life while on PN.[471] Patients with CIPO account for approximately 9% of the total intestinal transplants in both adults and children.[472-475] Patients with CIPO should be evaluated for urological abnormalities and antibiotic prophylaxis for urinary tract infections is often required following transplant in these patients. The development of new immunosuppressive agents such as tacrolimus and novel induction agents (basiliximab, alemtuzumab, daclizumab and anti-lymphocyte globulins) have been associated with an increased overall survival rate and reduced graft rejection rate.[476,477] Complications include acute rejection, opportunistic infections, e.g. cytomegalovirus and Epstein-Barr virus, and surgical morbidity including wound infections, stoma-related complications, graft ischemia, intestinal perforations, delayed gastric emptying, intestinal obstruction and biliary tract dilatation.

In the presence of gastric involvement, modified multivisceral transplantation (stomach-duodenum-pancreas plus small bowel) should be performed, even though some reports described the possibility to overcome this issue by transplanting the bowel and modifying surgically the stomach in order to facilitate gastric emptying.[477] In children, PN-related liver failure represents an indication for combined transplant (liver and bowel), but a full

multivisceral transplantation (modified multivisceral graft plus liver) is rarely required. While isolated small bowel and liver-bowel transplantation have reasonable long term-results, multivisceral transplant should be performed only in very selected cases and in experienced centers. A study by the University of Miami including 98 adult patients undergoing multivisceral transplantation showed patient and graft survival rates of 49% and 47%, respectively, at a 5-year follow up.[475] Italian experiences in adult patients with CIPO showed that isolated intestinal transplantation performed with different surgical approaches (in order to reduce the delayed post-surgical gastric emptying) had patient survival rates of 70% at 5 years.[477] These results are comparable to those of intestinal transplant in patients with underlying diseases distinct from CIPO.[478-479] The same data have been reported by the United Network for Organ Sharing in children transplanted for CIPO with a 5-year survival rate of 57%, which is comparable to the overall survival rates for intestinal transplant.[473] These data were also recently confirmed by an Italian study with a reported survival rate for recipient of intestinal grafts, in any combination, of 75% at 2-year follow up.[480] Notably, the catch-up growth seen in children following liver transplantation has not been demonstrated in children with intestinal transplantation. This is probably related to the severity of the illness at the time of presentation and the use of intense immunosuppression, including long-term steroids.[472]

Concerning extra-digestive organ transplant, the allogeneic haematopoietic stem cell transplantation (AHSCT) can be used to restore thymidine phosphorylase enzyme function which is the key biochemical deficit in patients with MNGIE. However, so far the results of AHSCT at a 10-year follow up show that only about 30% of transplanted MNGIE patients are alive. This finding prompted research to focus on alternative tissue source of TP.[481] Based on recent data displaying that the liver has high thymidine phosphorylase expression, we have recently transplanted a MNGIE patient with promising results.[482]

Table 2. Etiology and classification of CIPO.

Primary	Secondary	Familial forms
• No demonstrable etiopathogenetic causes	• Neurological disorders • Metabolic diseases • Paraneoplastic syndromes • Neurotropic viruses • Autoimmune disorders • Celiac disease • Neuro-muscular disorders • Radiation enteritis • Endocrinological disorders • Drugs	• **Autosomal dominant** ✓ *SOX 10** • **Autosomal recessive** ✓ *RAD21** ✓ *SGOL1** ✓ *TYMP** ✓ *POLG** • **X-Linked** ✓ *FLNA** ✓ *L1CAM**

Notes: *, denotes mutation(s) to the indicated genes. *FLNA*, filamin; L1CAM, L1 cell adhesion molecule; *POLG*, polymerase DNA gamma; *RAD21*, cohesin complex component; *SGOL1*, shugoshin-like 1; *SOX10*, SRY-BOX 10; *TYMP*, thymidine phosphorylase.

Table 3. Main similarities and differences of CIPO in children and adults.

	Children	Adults
Etiology	Mainly idiopathic	Half of cases are secondary to acquired diseases.
Histopathology	Myopathies and neuropathies	Mainly neuropathies.
Symptom onset	In utero, from birth or early infancy with 65-80% of patients symptomatic by 12 months of age	Median age of onset at 17 years.
Clinical features	Occlusive symptoms at birth and/or chronic symptoms without free intervals Urological involvement is commonly encountered ranging from 36-100% pediatric case series High risk of colonic and small bowel volvulus secondary to severe gut dilation, dysmotility, congenital bridles or concurrent malrotation	Chronic abdominal pain and distension with superimposed acute episodes of pseudo-obstruction. Urinary bladder involvement not so often reported
Natural history	Myopathic CIPO, urinary involvement and concurrent intestinal malrotation are poor prognostic factors	The ability to restore oral feeding and the presence of symptoms < 20 years of age is associated with a low mortality; while, systemic sclerosis and severe/diffuse esophageal and intestinal dysmotility are associated with a high mortality
Diagnostic approach	Specialized tests (e.g. intestinal manometry) often difficult to perform; non-invasive, radiation-free imaging tests are warranted	Various methodological approaches usually starting from endoscopy and radiological tests up to more sophisticated functional exams
Nutritional therapy	To ensure normal growth extensively hydrolysed and elemental formulas are often empirically used to facilitate intestinal absorption	To improve nutritional status and prevent malnutrition
Pharmacological therapy	Small number / sample size controlled trials	Small number / sample size controlled trials; few conclusions can be drawn for most drugs.
Surgical therapy	Venting ostomies (although characterized by high complication rates) possibly helpful; surgery as a 'bridge' to transplantation may be indicated in highly selected cases	Venting ostomies can be helpful; resective surgery may be indicated in accurately selected patients (i.e. cases with proven segmental gut dysfunction)

Table 4. Compounds used for the treatment of CIPO in isolated cases or in small series of pediatric and adult patients.

Drug and study	Type of study	Number and type of patients	Dose and period of treatment	Results	Side effects
Erythromycin ***Emmanuel et al. APT 2004***	Retrospective case series	15 adults	1.5-2 g/day, oral or i.v.	6 responders (4 myopathy)	Not reported
Metoclopramide	No reported studies	----------	----------	----------	--------
Domperidone	No reported studies	----------	----------	----------	--------
Octreotide ***Soudah et al. N Eng J Med 1991***	Case series	5 adults with scleroderma related CIPO and SIBO	100 mcg/day sc	reduces SIBO and improved symptoms	Not reported
Verne et al. Dig Dis Sci 1995	Prospective case series	14 adults	50 mcg/day sc, 20-33 weeks in association with erythromycin	5 responders	Not reported
Neostigmine ***Calvet et al. Am J Gastroenterol 2003***	Case report	1 adult with chronic colonic pseudo-obstruction with autonomic paraneoplastic neuropathy	2 mg i.v. every 6 hours	Improvement of abdominal distension and discomfort and enteral diet could be resumed	Not reported
Pyridostigmine ***O'Dea et al. Colorectal Dis 2010***	Case series	7 adults	10 mg b.i.d. and increased if required (max 30 mg) orally	7 responders	Not reported
Prucalopride ***Emmanuel et al. APT 2012***	RCT cross-over trial	4 adults	2-4 mg	3 responders	Not reported
Antibiotics	No reported studies	----------	----------	---------	----------
Non-narcotic pain modulators	No reported studies	----------	----------	---------	----------
Buprenorphine ***Prapaitrakool et al. Clin J Pain 2012***	Case Series	4 children	10-15 mcg/h, transdermal, 1-3 years	3 responders	Pruritus and erythema on the application site

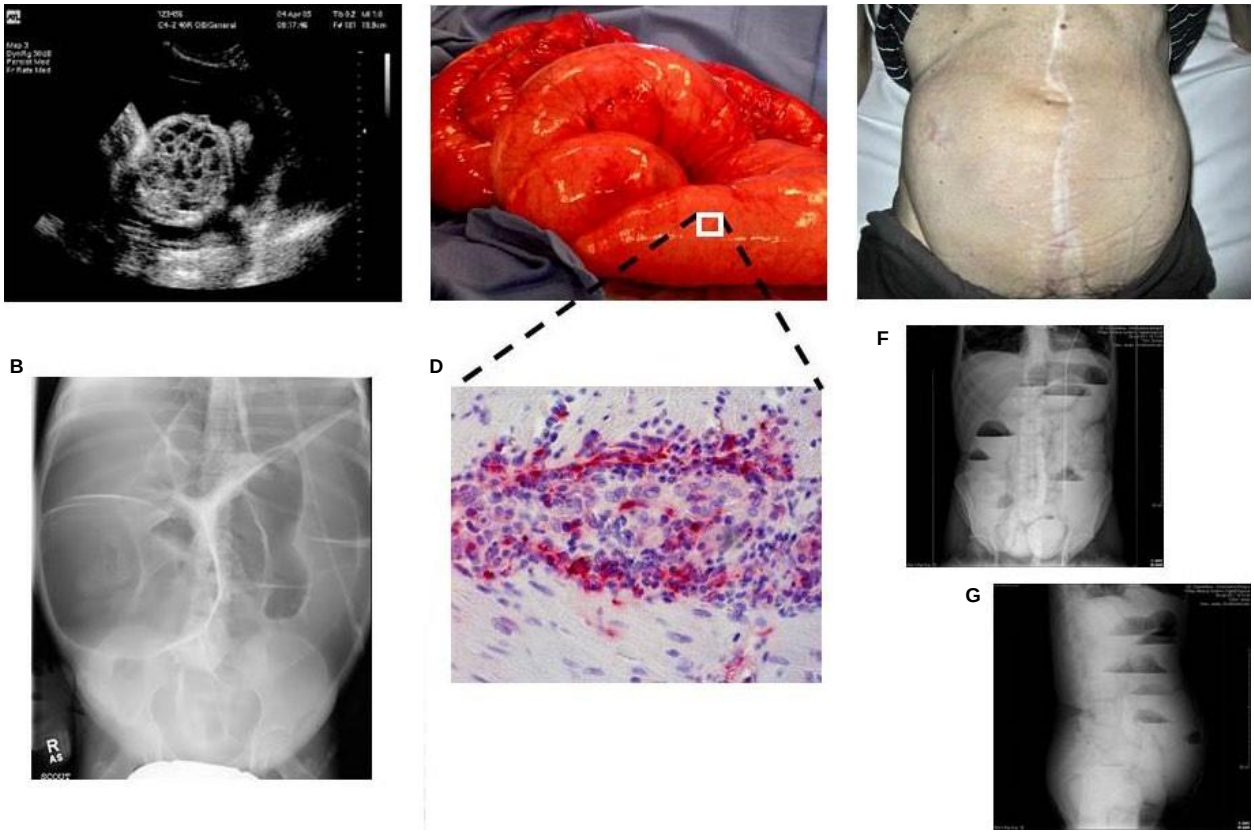


Fig. 12. **Synoptic view of the CIPO spectrum.**
A-B: illustrate the most severe pediatric cases with antenatal (*in utero*) evidence of multivisceral dilation (ultrasound picture A - from ref 60) i.e. often gut (B) and urinary system, commonly associated with an extremely poor prognosis. C-D: exemplify the CIPO phenotype with a rapid progression to intestinal dilatation (± urether / bladder) and failure often occurring as a result of an anamnestically reported gastroenteritis. Specifically, C depicts one of such case with massive bowel dilatation at the operative room and histopathology (inset to figure D) revealed an intense inflammatory (mainly lymphocytic) neuropathy (hence, myenteric ganglionitis. Alkaline phosphatase antialkaline phosphatase immunohistochemical technique using specific anti-CD8 monoclonal antibodies to identify a subset of T lymphocytes). E-G: are representative examples of another phenotype of the syndrome which may be observed in patients who experience more insidious mild and nonspecific symptoms progressing up to a classic CIPO over time. E shows a markedly distended abdomen of a 32-year old male patient who presented with a sub-occlusive episodes after some years of unspecific (dyspeptic-/irritable bowel syndrome-like) symptoms. Note the evident air-fluid levels detectable in up-right position at a conventional plain abdominal X-ray (F and G, antero-posterior and latero-lateral view, respectively).

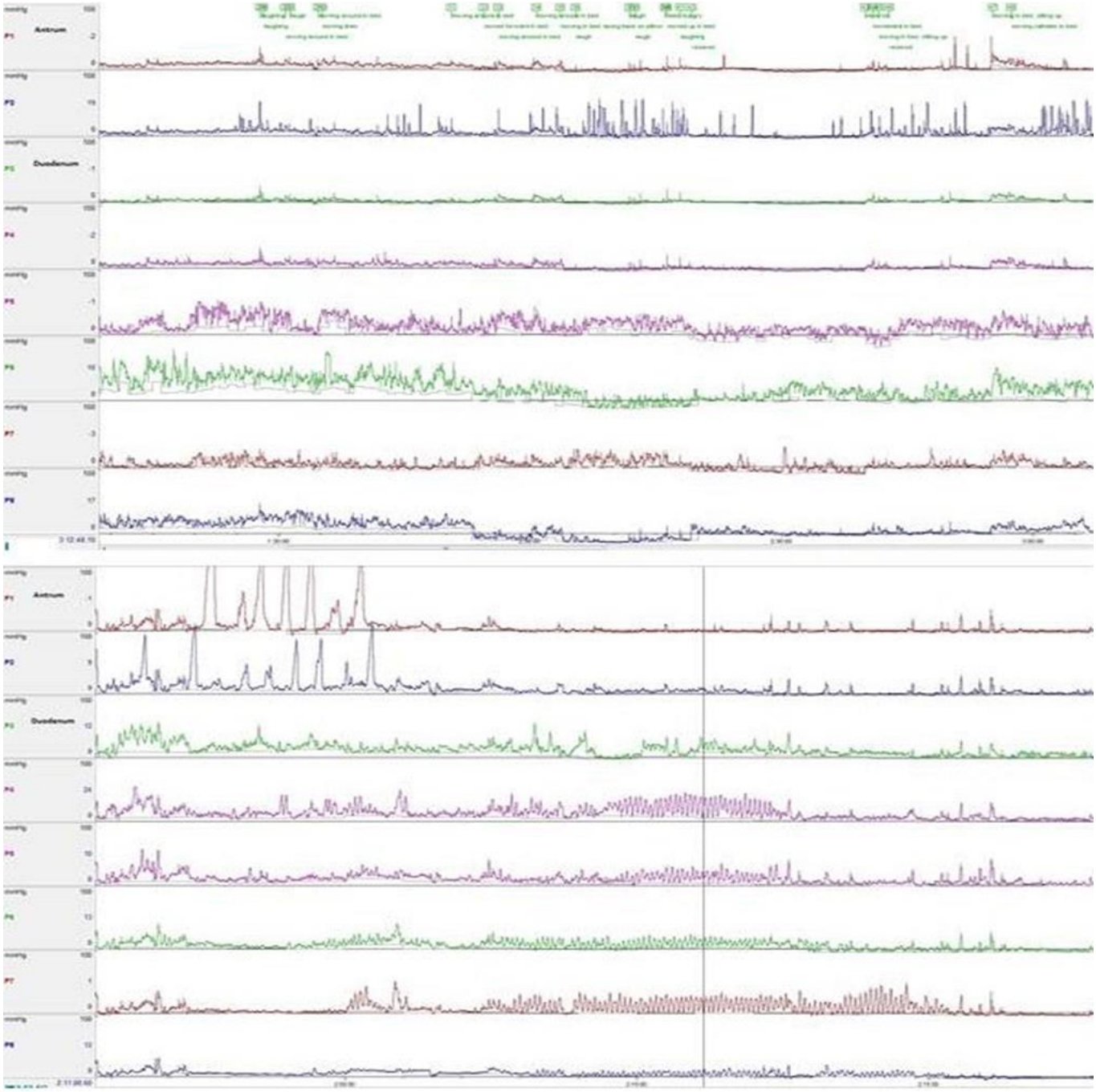


Fig. 13. Manometric findings in CIPO.
A: illustrates a monotonous pattern of phasic and tonic contractions in the small bowel of a 3-year old boy with a congenital CIPO. No migrating motor complexes (MMC) were detected during a 4-hour fasting study. B: shows an abnormal migration of the phase 3 of the MMC with a simultaneous component in the duodenum in a 7-year old girl affected by a non-congenital form of CIPO.

Take home messages:

- There is no single diagnostic test or pathognomonic fidings for CIPO. The diagnostic work up aims to: *i*) exclude bowel mechanical obstruction; *ii*) identiy underlying diseases; *iii*) understand the underlying pathophysiological features.
- Treatment is challenging and requires a multidisciplinary effort.
- Key objectives in the management of patints with CIPO are to avoid unnecessary surgery, restore fluid and electrolyte balance, maintain an adequate caloric intake, promote coordinated intestial motiity, and treat bacterial overgrowth.

6.2 Management

Modified from

Chronic intestinal pseudo-obstruction:

Progress in management?

Giovanni Di Nardo, Tennekoon B. Karunaratne, Simone Frediani and

Roberto De Giorgio

Neurogastroenterol Motil. 2017 Dec;29(12).

Introduction

Chronic intestinal pseudo-obstruction (CIPO) is a rare and debilitating condition affecting about 100 infants per year and with an estimated incidence of 0.2-0.24 per 100,000 adults per year.[24,25,390,392,398] CIPO patients show severe impairment of gastrointestinal (GI) propulsion ensuing symptoms / signs suggestive of partial or complete intestinal obstruction in the absence of any lesion occluding the intestinal lumen.[24,25,390] Abnormalities in the GI neuro-muscular region, including neuropathy (either intrinsic or extrinsic), myopathy and / or interstitial cells of Cajal (ICC) network changes, individually or in combination, contribute to severe dysmotility in CIPO. CIPO is still a challenge for clinicians and surgeons because of several reasons. First, most physicians fail to recognize CIPO patients early due to their limited experience; secondly, symptoms shown by CIPO patients are non-specific, thus they can be mistaken with other functional GI disorders. This drawback may lead patients to be subjected to inadequate management consist of ineffective, and potentially dangerous, surgical procedures; thirdly, CIPO is an 'umbrella term' covering a wide heterogeneity of patients, i.e. idiopathic, with no apparent cause underlying dysmotility, *vs.* secondary to

metabolic / endocrinological, neurological and paraneoplastic disorders; fourthly, the majority of CIPO patients have a sporadic disease, while few have a genetically-related background (latter presents with a syndromic phenotype, i.e. in addition to the GI tract, other organs, such as heart, brain and hematopoietic system, can be affected); finally, most CIPO patients show a variable outcome, i.e. some cases may remain stable over time, whereas others rapidly worsen to unavoidable parenteral nutrition as a unique measure to contrast severe malnutrition and death. Taken together these challenges and intrinsic difficulties surrounding CIPO hinder thorough phenotyping of patients, mechanistic studies and, more importantly, patients' management.[4,5] Nonetheless, recent data by Ohkubo et al., in the current issue of Neurogastroenterology and Motility, propose percutaneous endoscopic gastro-jejunostomy (PEG-J) as a method to alleviate symptoms (including pain) and nutritional consequences related to intestinal distension, a key feature of CIPO.[483]

Prompt by the promising results of Ohkubo et al., the purpose of this minireview is to provide the readers with an update on clinical, diagnostic and management aspects of CIPO patients.

Clinical features and diagnostic aspects

Usually CIPO patients show severe symptoms and signs.[413] Abdominal pain and distension are reported / detected in most (80%) cases. Although predominantly chronic in nature, these symptoms worsen during acute sub-occlusive episodes.[24,392] Nausea and vomiting occur in 75% and 40-50% of those cases can be associated with test-proven gastroparesis; constipation occurs in 40%, while diarrhea (rarely steatorrhea) occurs in about 20-30% of cases.[24,406] The latter can be related to small intestinal bacterial overgrowth (SIBO) due to intestinal stasis.[24,25392] Malnutrition, often requiring parenteral nutrition, is another significant clinical aspect in most CIPO cases.[24,392,413] Esophageal dysmotility has been reported in approximately 70% of CIPO patients.[436] Urinary bladder dysfunction (with or without

megacystis and megaureter) is a co-morbidity of CIPO, more commonly detectable in children with an underlying myopathic derangement of the GI tract.[24,25,392,406] Finally, CIPO patients may develop depression and / or other psychological disorders as a consequence of the disabling nature of this condition as well as of the frustrating ineffectiveness of most prokinetic drugs.[413]

So far, there is no single diagnostic test or pathognomonic finding indicative of this condition. A stepwise diagnostic approach, aimed to rule out mechanical causes of bowel obstruction, identify underlying diseases and understand the pathophysiological features, is recommended. Evidence of air-fluid levels and dilated bowel loops in a plain radiograph of the abdomen with patients in upright position is mandatory to suspect CIPO. In current clinical practice, computerized tomography (CT) scan of the abdomen is more accurate than conventional radiology to demonstrate air-fluid levels, while ruling out mechanical causes as well as intestinal wall adhesions. Transit time examination with contrast medium has been largely replaced by dedicated enterography with high-resolution CT or magnetic resonance imaging (MRI).[243,42,] Cine-MRI is an emerging, non-invasive, radiation-free method to assess and monitor GI motility. Using cine-MRI, Fuyuki et al. studied 33 patients and showed that the mean luminal diameter and contraction ratio in the CIPO group differed significantly from healthy volunteers. Their data suggest that cine-MRI is useful in detecting even subtle contractile impairment of the gut in patients with CIPO.[242] Upper and lower GI endoscopy can contribute to exclude mechanical occlusions and collect routine biopsies. Mucosal sampling in the duodenum can help to rule out those very rare cases in which an underlying celiac disease can be associated with dysmotility, whereas biopsies throughout the upper and lower gut can be useful to unravel an eosinophilic gastroenteropathy.[389] Mucosal biopsies can also be exploited to obtain the submucosal layer with associated neural plexuses.[484] This approach may become of aid to show neural changes in patients with severe dysmotility.

Finally, wireless motility and endoscopy capsules have been proposed for evaluating patients with functional bowel disease.[245,430] However, the role of these techniques in the diagnosis of CIPO has not yet been established and their use can be even potentially hazardous if a mechanical obstruction has not been firmly excluded. When imaging and endoscopy fail to show causes of intraluminal or extraluminal mechanical obstruction, secondary causes of potentially treatable pseudo-obstruction should be excluded. Screening tests for diabetes mellitus, neurotropic viruses (e.g., cytomegalovirus or Epstein-Barr virus), celiac disease, connective tissue and skeletal muscle disorders (antinuclear antibody, anti-double-stranded DNA and SCL-70, creatine phosphokinase, aldolase), and thyroid function should be performed. Other tests include serology for Chagas' disease, urinary catecholamines and porphyrins to rule out pheochromocytoma and porphyria, respectively, and enteric neuronal autoantibodies (e.g. anti-nuclear neuronal antibodies – ANNA-1 – also referred to as anti-Hu antibodies based on molecular target) for paraneoplastic syndrome.[24,25]

Small bowel manometry provides pathophysiologically relevant information on the mechanisms underlying dysmotility in CIPO patients (e.g. neuropathic *vs.* myopathic patterns).[24,392,413] In some exceptional cases with an apparently unremarkable imaging, an accurate manometric assessment may reveal a pattern (i.e., discrete clustered contractions) suggestive of a recent stenosis of the gut not yet accompanied by bowel loop dilatation above the blockade.[24,25,392] Manometric findings does not affect management strategies in CIPO patients, although the evidence of a propulsive pattern (i.e. migrating motor complexes) predicts successful adaptation to jejunal feeding.[433] Esophageal manometry can be useful to predict survival, home parenteral nutrition requirement and inability to maintain sufficient oral feeding.[436] Anorectal manometry is indicated when the clinical picture is characterized by intractable constipation and marked colonic distension in order to exclude Hirschsprung's disease.[24] A manometric assessment of the entire GI tract, including colon, has been deemed

helpful to plan for isolated or multivisceral transplantation in the most severe forms of pediatric CIPO.[25]

Minimally invasive procedures, e.g. laparoscopic surgery or endoscopic approaches have contributed to refuel interest for histopathological analysis of full-thickness biopsies.[485] Recently, Valli et al. used endoscopic, full-thickness resection (eFTR) with a full-thickness resection device under moderate propofol sedation in four CIPO patients with suspected neuromuscular gut disorders. Large colonic full-thickness tissue samples of excellent quality did identify neuro-muscular changes in all four patients with no adverse events. These data suggest that eFTR allows safe and minimally invasive collection of full-thickness biopsies suitable for histological analysis in patients with neuromuscular gut disorders.[486] Guidelines proposed by an international working group have helped standardizing technical aspects (tissue collection and processing) and histopathological reporting of a variety of gut neuromuscular disorders, including CIPO.[2]

Management

The management of CIPO patients is aimed to avoid unnecessary surgery, restore fluid and electrolyte balance, maintain an adequate caloric intake, promote coordinated intestinal motility, and treat SIBO and associated symptoms (i.e. abdominal pain and distension). As experienced in daily practice, current therapeutic approaches are not very effective and this generate frustration in patients and physicians. The following are some indications recommended to improve the management of patients with CIPO.

Patients with adequate intestinal absorption should be encouraged to take small, frequent liquid meals (5-6 / day), while avoiding high -fat, -residue (delaying gastric emptying) and high -lactose / -fructose (evoking bloating / discomfort) foods. Vitamins A, D, E and K as well as B12 and folic acid should be supplemented when needed. Elemental feedings and

dietary supplements with medium-chain triglycerides can be used in combination with aforementioned dietary changes.[24,25,389,390] In cases with inadequate oral intake, enteral nutrition with standard, non-elemental formula should be considered. Before placing a permanent feeding tube, a trial of nasogastric or nasojejunal feeding should be attempted using an enteral formula at a rate sufficient to provide an adequate caloric support. When delayed gastric emptying is present, bypassing the stomach and directing the feeding into the small intestine is recommended. Enteral nutrition starting with a slow infusion and continuous feeding or cyclical feeding (e.g. overnight) is preferred to large bolus feedings.[24,25,389,445] In most severe cases, total parenteral nutrition (TPN) is necessary to maintain nutritional support and an adequate level of hydration. Complications of TPN, including liver failure, pancreatitis, glomerulonephritis, thrombosis and sepsis, are frequent causes of morbidity and mortality in CIPO. [24,25,389,445] Personalized TPN formulations, with minimal intravenous lipid infusion, can help reducing metabolic complications. In any case, a long-term TPN does not seem to be associated with a significant increase in morbidity and mortality in CIPO as compared to other conditions requiring TPN.[410,441]

Pharmacological treatment should be one of the most important options for patients with CIPO since it is aimed to restore gastrointestinal propulsion, thereby leading to tolerate oral feedings, while decreasing symptom severity and the occurrence of SIBO. Unfortunately, due to limited number of trials, most of which based on few patients, the overall efficacy of prokinetics yielded unsatisfactory results.[24,25] Nonetheless, some data should be mentioned as a basis to help clinicians in practice. Erythromycin, a macrolide antibiotic, showed efficacy at a dose of 1.5-2 g / day in adults, or 3-5 mg / kg / day in children, in accelerating gastric emptying and ameliorating symptoms of CIPO.[390,446] Metoclopramide and domperidone are known to exert their prokinetic effects via type 2 dopamine receptor antagonism and by increasing acetylcholine release in the enteric nervous system. Although widely used in most

dysmotilities, clinical data for their use in CIPO are lacking. Metoclopramide should be used only for short-term period, as its chronic administration leads to a significant risk of tardive dyskinesia.[447] Octreotide is a long-acting analogue of somatostatin that at a dose of 100 mcg subcutaneously / day resulted in a significant beneficial effect by relieving bacterial overgrowth and reducing pain, nausea and bloating in scleroderma-related CIPO patients.[448] Repeated intravenous use of acetylcholinesterase inhibitor neostigmine (at a dose of 8 mg / day) was successful in an adult patient with chronic colonic pseudo-obstruction.[451] The oral formulation of acetylcholinesterase inhibitor, pyridostigmine (at a starting dose of 20 mg / day), has also been used with success in some adult CIPO patients.[452] Prucalopride, a highly selective 5-hydroxytryptamine-4 (5-HT_4) receptor agonists lacking cardiotoxicity (which caused cisapride to be withdrawn), exerts significant enterokinetic effects. In a recent randomized controlled trial with CIPO patients, prucalopride showed beneficial effects on symptoms.[455] In practice, the association of different prokinetic drugs and / or their rotation may be a strategy useful to increase therapeutic efficacy, while minimizing tachyphylaxis and side effects. Various antibiotic regimens have been recommended for SIBO treatment.[459] Non-absorbable antibiotics, such as rifaximin, are nowadays the first choice, although broad-spectrum antibiotics, such as amoxicillin and clavulanic acid, gentamicin, and metronidazole, often with an antifungal compounds (e.g., nystatin or fluconazole), can be used for 1- to 2-week cycles alternated with antibiotic-free periods.[34,389,459] Recently, amoxicillin-clavulanate has been demonstrated to accelerate intestinal transit in children, thus representing an interesting therapeutic option combining antibiotic and prokinetic effects.[461]

Visceral pain is major concern in patients with CIPO. The ideal treatment would be the use of non-narcotic pain modulators, such as tricyclic antidepressants, serotonin-norepinephrine reuptake inhibitors and GABA analogues, that anyway require caution because of their significant side effects, such as constipation and / or drowsiness. Starting with low dose

followed by gradual increase is recommended to optimise the beneficial effect. In patients with chronic and unsustainable visceral pain, physicians may carefully consider the use of opiate drugs paying attention to the known anti-motility effects of these compounds.[25]

Since bowel distension is commonly associated with pain and other symptoms in CIPO patients, decompression therapy represents one of the key aspects in CIPO management. Apart from neostigmine (mentioned above) proved to be useful in reducing dilatation in patients with acute colonic pseudo-obstruction (also referred to as Ogilvie's syndrome),[487] so far there are no pharamacological remedies to achieve an effective, non-invasive decompression in CIPO. Current therapeutic strategies include conventional methods, i.e. intermittent nasogastric suction, rectal tubes or colonoscopic decompression, and surgical procedures, such as feeding / venting gastro- / jejunostomies (or other intestinal 'ostomies'), which overall evoke symptom relief in about half of the patients.[429,464,465,468,469] However, conventional methods are limited by temporary efficacy in GI decompression, whereas surgery shows a high rate of stoma prolapse along with a considerable risk of dehydration due to enteric fluid loss.[468,469] Nonetheless, recent data in this issue of Neurogastroenterology and Motility provide new insights into the management of patients with CIPO by using percutaneous endoscopic gastrostomy (PEG), a commonly applied method for long-term home enteral nutrition.[483,488] However, PEG is often associated with aspiration pneumonia, therefore current management suggests a gastro-jejunostomy tube insertion via PEG (hence, PEG-J) as a measure to prevent such a life-threatening complication.[489] Furthermore, both PEG / PEG-J have been exploited to decompress patients with mechanical occlusion of the bowel, such as cases with malignant bowel diseases.[488] Based on this background, Ohkubo and co-workers tested whether PEG-J could be an effective measure for intestinal decompression and symptom improvement in CIPO patients. The authors enrolled seven CIPO patients with a severe symptom profile / manifestations refractory to any

pharmacological treatment. All patients required at least once nasogastric tube or trans-nasal small intestinal tube insertion for intestinal decompression. PEG tube (24F caliber) was placed using the introducer method.[483,488] If distended small bowel loops were interposed between the stomach and the abdominal wall, a preceding decompression was obtained via trans-nasal small intestinal tube. After 7 days of gastropexy, a PEG-J tube (with a caliber of 24F and length of 60 cm) was inserted through the PEG fistula under fluoroscopy upon removal of the PEG button. Evaluation of subjective symptoms (number of days without abdominal symptoms in a month) and the assessment of objective nutritional status (body mass index and serum albumin level) were evaluated in each patient before and 3 months after PEG-J. PEG placement and PEG-J tube insertion were performed without major procedure-related complications and both were well tolerated by all patients. Also, oral intake was well maintained or improved in all patients after the procedure. A significant decrease in the number of days without abdominal symptoms was observed in 6 out of 7 patients, along with the improvement of wasting and malnutrition in all patients. Plain abdominal radiographs demonstrated a reduction of abdominal distension, whereas the total volume of the small intestine did not significantly change compared to pre-PEG-J status. One patient developed severe reflux esophagitis, while another had a chemical dermatitis around the PEG-J fistula during follow-up and in both cases conservative therapy with proton-pump inhibitors and ointment therapy, respectively, was effective. None of the treated CIPO patients had ulcer formation and perforation due to the tube placement at 1-year follow-up after the procedure. PEG-J is therefore suggested as a safe and minimally invasive method to improve abdominal symptoms, including pain, and nutritional status in CIPO.[6] Depending on symptom fluctuation, the use of PEG-J can be modulated (open *vs.* closure intervals) ensuing control of fluid output and avoiding dehydration, a common complication of conventional jejunostomy / ileostomy. Clearly, this study, based on few cases of CIPO, requires further

investigation. First, a better characterization of CIPO patients is required. For example, the study by Ohkubo et al. does not specify which patient category is more suitable to PEG-J insertion based on underlying pathology (e.g., most severe cases - myopathic CIPO - may have a worse outcome than neuropathic CIPO to the procedure). Second, a thorough and better symptom and objective data assessment is highly recommended. Overall, patients with CIPO are unlikely to show a complete remission of symptoms, therefore new studies using this endoscopic approach should require symptom questionnaire and severity score to determine the actual beneficial effect due to PEG-J. Finally, long-term trials designed on a larger cohort of CIPO patients are necessary to minimize the 'heterogeneity effect' of CIPO, which may hinder the plausible efficacy of PEG-J.

Take homes messages:

- Since most drugs failed to restore gastrointestinal coordinated motility, nutritional support, fluid/electrolyte replacement, and antibiotics are still mandatory as life-saving measures.
- Promising data indicate that percutaneous endoscopic gastro-jejunostomy (PEG-J) can be proposed as a measure for intestinal decompression in CIPO, thereby improving symptoms and preventing malnutrition.

Chapter 7.

COMPARISON BETWEEN SMALL BOWEL MANOMETRIC PATTERNS AND FULL-THICKNESS BIOPSY HISTOPATHOLOGY IN SEVERE INTESTINAL DYSMOTILITY

Modified from

Comparison between small bowel manometric patterns and full-thickness biopsy histopathology in severe intestinal dysmotility

Malagelada C[1,2,§], Karunaratne TB[3,§], Accarino A[1,2], Cogliandro RF[3], Landolfi S[4], Gori A[3], Boschetti E[3], Malagelada JR[1,2], Stanghellini V[3], Azpiroz F[1,2,*], De Giorgio R[3,*]

§ these authors share co-first authorship

* these authors share co-last authorship

Neurogastroenterol Motil. 2017 Sep 22. doi: 10.1111/nmo.13219.

ABSTRACT

Background: Intestinal manometry is the current standard for direct evaluation of small bowel dysmotility. Patients with abnormal motility can either be diagnosed of pseudo-obstruction when there are radiological findings mimicking mechanical intestinal obstruction or of enteric dysmotility when these findings are absent. The aim of the present study was to prospectively compare small bowel manometric abnormalities with histopathological findings in intestinal full-thickness biopsies in patients with severe dysmotility disorders.

Methods: We investigated 38 patients with intestinal manometry and a subsequent full-thickness intestinal biopsy. Manometric recordings were read by 4 investigators and a diagnostic consensus was obtained in 35 patients. Histopathological analysis, including

specific immunohistochemical techniques of small bowel biopsies was performed and compared to manometric readings. **Key Results.** Patients with abnormal intestinal manometry had abnormal histopathological findings in 73% of cases. However, manometric patterns did not match with the specific neuromuscular abnormalities. Among patients with a neuropathic manometry pattern and abnormal histopathology, only 23% had an enteric neuropathy, whereas 62% had neuromuscular inflammation and 15% an enteric myopathy. On the other hand, patients with a myopathic manometry pattern all had abnormal histopathology, however none of them with signs of enteric myopathy.

Conclusion & Inferences: Small bowel dysmotility detected by intestinal manometry is often associated with abnormal neuromuscular findings in full-thickness biopsies. However, there is no correlation between the specific manometric patterns and the histopathological findings.

INTRODUCTION

Intestinal manometry remains the current standard for recognition of gastrointestinal motility disorders, although other methods such as transit scintigraphy, functional magnetic resonance and endoluminal image motility analysis are further expanding diagnostic capabilities and general understanding of these conditions.[244,245,490-492] Beyond the classic clinical picture of chronic intestinal pseudo-obstruction (CIPO), characterized by recurrent episodes or sustained radiological findings mimicking mechanical obstruction,[393] intestinal manometry has documented an additional group of patients suffering from disabling abdominal pain and other severe abdominal symptoms in the absence of clinical signs of mechanical bowel obstruction. This group of patients with abnormal contractile activity has been named enteric dysmotility (ED).[16,411] The clinical presentation of ED corresponds to that of severe functional gastrointestinal disorders (SFGID).[413] Both CIPO and ED are associated with

enteric neuromuscular abnormalities shown by histological and immunopathological analysis of full-thickness biopsies of the small bowel usually obtained via laparoscopy.[1,485]

Neither intestinal manometry nor small bowel neuromuscular pathology are fully standardized diagnostic methods and both, technical details and criteria for interpretation vary from center to center.[25,493] To some extent, this uncertain status results from the relative rarity of patients with a clinical expression of gastrointestinal dysmotility severe enough to justify invasive diagnostic procedures such as intestinal manometry and full-thickness biopsy. It also derives from the relative sophistication and complexity of both technologies, manometry and neuromuscular pathology, that confines them to a relatively small cluster of university medical centers around the world. Moreover, only a handful of published studies over the last 15-20 years have attempted to correlate intestinal dysmotility patterns, recorded manometrically, with morphological changes subsequently investigated in full-thickness bowel biopsies.[11,392,485,494,495]

The aim of the present study was to prospectively compare small bowel manometric abnormalities with subsequent histopathological findings in full-thickness intestinal biopsies. The former were evaluated by consensus, among an international group of academic gastroenterologists routinely applying intestinal manometry for diagnostic purposes. Enteric neuromuscular abnormalities were assessed by a group of experienced gut neuromuscular pathologists at a single medical center. The comparison between manometric findings and neuromuscular pathological findings was performed on a group of patients presenting with severe and chronic clinical manifestations of gastrointestinal dysmotility. Our hypothesis was that abnormal manometric patterns would predict histopathological abnormalities found on subsequently obtained intestinal tissue specimens.

MATERIAL AND METHODS

Patients

Thirty eight patients (16 to 65 years old; 32 females and 6 males) with severe and chronic clinical manifestations of gastrointestinal dysmotility participated in the study. Patients were recruited and clinically evaluated at the Vall d'Hebron University Hospital in Barcelona between November 1999 and November 2009. During this time span, all consecutive patients who underwent a manometric study and subsequently a full-thickness biopsy were included in the study. Full-thickness intestinal biopsy was obtained only in patients with a clinical diagnosis of an idiopathic severe intestinal motility disorder. Therefore, high severity was the enforced selection criterion that delimited the consecutive series of patients included in this study. Patients with motility disorders secondary to infectious, neurological, metabolic, systemic-autoimmune and paraneoplastic conditions were excluded. Anorexia nervosa was specifically excluded in all patients with malnutrition by a psychiatric examination.

The clinical diagnosis of CIPO was established on the basis of a chronic (>3 months), severe symptom complex mimicking mechanical small bowel obstruction, as well as (at least on one occasion) radiological evidence of air-fluid levels or dilated small bowel loops. Thus, patients presented with abdominal pain and distension associated with nausea, vomiting, bloating and malnutrition. These clinical manifestations occurred episodically or continuously. Presence of mechanical obstruction had been excluded by conventional imaging studies or by prior exploratory abdominal surgery.

The clinical diagnosis of SFGID with inability to maintain normal body weight was established on the basis of chronic (>3 months) persistent or recurrent symptoms in the absence of episodes mimicking mechanical intestinal obstruction and of associated organic, systemic or metabolic diseases potentially responsible for the digestive manifestations.

Inability to maintain a normal body weight was defined by a BMI <18.7 Kg m^{-2} (in women) or <20.1 Kg m^{-2} (in men).

The study protocol was approved by the Ethics Committee of the University Hospital Vall d'Hebron and all patients gave written informed consent.

Intestinal manometry

All study patients underwent intestinal manometry according to a standard stationary procedure. After an overnight fast, a manometric tube (9012X1106 Special Manometric Catheter; Medtronic, Skovlunde, Denmark) was orally introduced into the jejunum under endoscopic guidance. Eight water-perfused manometric ports (five at 10 cm intervals and the proximal three at 1 cm intervals) were positioned from the proximal duodenum to the mid-jejunum with fluoroscopic assistance. Recordings were obtained for 3 hours during fasting and 2 hours after ingestion of a solid-liquid meal (450 kcal). Patients with gastroparesis or unable to finish the meal alternatively received a liquid meal (Ensure HN; Abbott, Zwolle, The Netherlands; 1 kcal/mL) infused through the most proximal intestinal manometric port at 2 kcal/min throughout the two-hour postprandial period.

Manometric recordings were made available to each of 4 investigators for review in both paper and electronic format. All 4 investigators are academic gastroenterologists (F.A., JR.M., R.C., V.S.) whose names and institutions are provided in the heading as co-authors. Investigators read each manometric recording without prior knowledge of patient's identity and clinical details. All assigned investigators reviewed the entire group of manometric recordings and classified each case as neuropathic, myopathic, occlusive, indeterminate or normal based on the established criteria (listed in Table 5). The occlusive pattern was considered indicative of intestinal neuropathy in the absence of mechanical obstruction.[496]

Intestinal tissue samples

Full-thickness biopsy specimens were obtained from the small bowel either by laparoscopy or laparotomy subsequently to the performance of the intestinal manometric studies. The time interval between the manometric study and collection of intestinal biopsies at surgery ranged from 3 to 38 months, median 10 months.

In each patient a single circular segment (5 cm long) was obtained from the proximal jejunum, except in those cases with segmental dilatation in whom the biopsy was obtained from the most dilated segment. Immediately after resection, tissue samples were placed in 10% formalin to later be embedded in paraffin wax according to standard protocol for tissue processing. Histopathological review was conducted by one of the investigators (R. De G) and his team (A. G. and E. B.) at the University of Bologna, Italy. Histopathological material was examined without prior information about clinical and motility test outcomes. All biopsies were first analyzed to exclude associated pathological findings such as malignancy, dysplasia, villous atrophy or mucosal inflammation. Specific enteric neuromuscular evaluation was performed on the hematoxylin and eosin (H&E)-stained sections to assess the overall structure of ganglia, myenteric and submucosal neuronal cell bodies and fibers, glia and smooth muscle cells. When needed, other standard histological stains (e.g. Masson trichrome) were applied to detect additional tissue changes such as fibrosis. Finally, immunohistochemical techniques to analyze structural markers of the enteric neuromuscular component were used. We used specific antibodies to: neuron specific enolase (NSE) and synaptophysin (both general neuronal markers), BCL-2 (as a marker of cell survival), S100□ (glial cells), c-Kit (interstitial cells of Cajal, ICC), alpha-smooth muscle actin (□-SMA) (smooth muscle cells), CD45 (leukocytes), CD3 (general T lymphocyte marker), CD4 and CD8 (markers of T lymphocyte subsets, i.e. “helper” and “suppressor”, respectively). Finally, an anti-tryptase antibody was used to detect mast cells (all antibodies are listed in the Table

6). The immunohistochemical techniques used in this study have been performed according to standardized and widely validated protocols.[440]

Four histopathological patterns, identified according to the London classification were recognized.[2] Main findings for each of these patterns are detailed:

- Apparently normal: no detectable changes in the neuromuscular layer using conventional histochemical staining and immunohistochemistry;
- Myopathy: muscular degeneration with cell swelling, vacuolization or atrophy; fibrotic replacement of smooth muscle (detectable by conventional staining, such as H&E, and immunohistochemical approaches, e.g. alpha-SMA immunoreactivity abnormalities);
- Neuropathy: overt ganglionic abnormalities as identified by traditional tinctorial staining (H&E) and immunohistochemical approaches (pan-neuronal markers); altered neuronal survival was identified by assessing BCL-2 expression in perikarya and nerve fibers (a feature which has been already associated with increased neuronal apoptosis);[497]
- Inflammatory neuro-myopathy: characterized by lymphocytic infiltrate in muscle layers (leiomyositis) and / or neuronal ganglia (ganglionitis) (visualized by anti-CD3, -CD4 / -CD8 immunolabeling); or mast cell or eosinophil infiltration throughout the neuromuscular structures (detected by tryptase immunolabeling or via conventional H&E staining, respectively).[402]

Statistical analysis

Statistical analysis was performed using the SPSS 12.0 for Windows statistical package. Distribution of abnormalities between groups was evaluated by the chi-square test. Predictive values (with 95% exact confidence intervals) were calculated as the probability of abnormal

histopathology when manometry was abnormal (positive predictive value) and of normal histopathology when manometry was normal (negative predictive value). Concordance between the outcomes of manometry and histology was evaluated with Cohen's Kappa (κ) agreement analysis. Differences were considered significant at a *P* value < 0.05.

RESULTS

Clinical features

Patients were classified into two categories depending on the predominant clinical pattern: 21 patients (18 females; age range: 16-77) presented with symptoms and signs of CIPO and the remaining 17 (14 females; age range: 24-61) manifested a variety of symptoms compatible with SFGID, i.e. patients that could not maintain a normal body weight along with severe upper and lower GI symptoms (Table 7).

Manometric study

Manometry recordings obtained from the 38 patients included in the study were reviewed by each of the expert investigators. Investigators were instructed to read each patient manometric tracing according to the strict criteria described in Table 5. A diagnostic consensus was obtained in 35 of the 38 patients (92% agreement). These 35 patients were included in the subsequent comparison manometry vs. histopathology results.

We found no apparent correlation between manometric features and clinical presentation. As shown by Table 3, there was a similar proportion of patients with a neuropathic or an undetermined manometric pattern in both the CIPO and SFGID clinical groups. The myopathic or occlusive patterns were only observed in patients with CIPO. It should be noted

that all 3 patients who showed a myopathic pattern on manometry had dilated small bowel loops on imaging studies.

Histopathological findings

Ten of the study patients (7 females; age range: 24-61) showed an apparently normal small bowel histopathology. Another group of 5 patients (3 females; age range: 32-70) showed histological features compatible with enteric neuropathy (Fig. 14) and other 4 patients (3 females; age range: 29-53) showed features of myopathy (Fig. 15). All remaining 16 patients (12 females; age range: 16-77) had an inflammatory neuro-myopathy with a predominantly lymphocytic or eosinophilic or mast cell infiltrate in the neuro-muscular layer (Fig. 16 and 17).

There were no differences in terms of pathological findings between CIPO and SFGID groups (Table 7).

Comparison between manometric findings and histopathological features

Among the 21 patients with a neuropathic pattern on manometry (Fig. 18), there were 3 patients with histological evidence of enteric neuropathy, 8 patients with inflammatory neuro-myopathy and 2 patients with myopathy. The remaining 8 had an apparently normal architecture of the neuromuscular layer at the histopathological evaluation.

Two of the 3 patients with a myopathic motility pattern on manometry (Fig. 19) showed histopathological features of neuropathy and the third patient showed an inflammatory neuro-myopathy. Furthermore, all 3 patients with an occlusive manometric pattern (Fig. 20) also showed an inflammatory neuro-myopathy at histology.

All 3 patients with an indeterminate manometry had abnormal histopathology: 1 myopathy and the other 2 an inflammatory neuro-myopathy. Finally, among the 5 patients who

displayed a normal manometric pattern there was a mixture of histopathological features: 2 patients were apparently normal, 2 showed inflammatory neuro-myopathy, and the remaining patient showed myopathy.

Cross tabulation between manometric and histopathological findings is shown in Table 8. The degree of agreement between both techniques was non-significant by Cohen's κ test (κ = 0.09, p = 0.541). The positive predictive value of manometry to detect abnormal histopathology was 73% (66-79), whereas the negative predictive value was 40% (12-77).

DISCUSSION

The present study addresses a relevant clinical question, that is, what is the value of manometric findings to predict abnormal histopathology on a subsequent full-thickness biopsy performed in patients with a major chronic intestinal motor disorder.

The results suggest that about three quarters of patients with a definitely abnormal intestinal manometry will show an abnormal intestinal histopathology if biopsied. This observation may be particularly useful when clinicians ponder whether to biopsy a patient with a symptomatic motility disorder given the fact that full-thickness intestinal biopsy, be it performed via laparoscopy or laparotomy, is an invasive procedure with significant morbidity. On the other hand, the small number of patients with a normal manometry in our study is insufficient to either support or challenge Törnblom et als observation that patients with or without abnormal manometry show similar histological degrees of inflammatory neuromyopathy.[6]

We found no correlation between the manometric pattern and the histopathological findings. The so called myopathic manometric pattern did not correspond with a myopathic condition

in any of the three patients in whom it was observed. The predictability of the neuropathic pattern was not more specific since it corresponded with a mixture of histopathological patterns: myopathy, neuropathy, or inflammatory neuro-myopathy. The present observations suggest that the various manometric patterns currently accepted as indicative of abnormal intestinal motility do not consistently match each of the various histopathologic patterns observed in biopsy tissue as currently defined. However, one may argue that manometry only records occlusive contractions and thus the so called myopathic pattern may simply reflect the inability of overdistended bowel loops to produce occlusive contractions, which is not necessarily due to a muscular disorder. On the same line, a clear cut enteric neuropathy was detected at histopathology in only a minority (about 14%) of patients with a neurogenic manometric pattern. Nonetheless, it is possible that neuro-muscular inflammation may affect neuronal activities and thereby induce neuropathic patterns on manometry. Clustered contractions have been recorded not only in mechanical obstructions, but also in severe forms of irritable bowel syndrome.[498,499] These data may link with our observation of inflammation at histology in all 3 patients with an occlusive manometric pattern.

Concerning inflammation, a special consideration goes to the mast cell infiltrate detected in tissue biopsies of about half of the investigated cases. Since mast cells play an important role in mucosal inflammation and immune activation, their localization within the gut neuro-muscular compartment provides support to gastrointestinal sensory-motor dysfunction and symptom generation. A predominant mast cell infiltration in the deeper layers of the gut has been reported in patients with severe gut dysmotility, including those with severe slow transit constipation undergoing surgery.[500]

It is of interest to examine the relevance of clinical features in relation to both manometric patterns and histopathology. Manometric data gathered by Cogliandro et al. suggest that CIPO patients exhibited more frequently a manometric pattern characterized by intestinal

hypomotility, abnormalities in activity fronts and an inadequate response to the meal by comparison to patients with SFGID.[9] However, our study does not show substantial differences between CIPO and SFGID clinical groups. Indeed, all the reported abnormal manometric patterns and indeterminate patterns of uncertain significance were observed in similar proportions. Clinical features also did not seem to reflect specific histological findings. Both CIPO and SFGID groups had similar proportions of patients with each of the four characteristic morphological patterns identified in the present study.

We recognize some weaknesses in our study. First, the small number of patients included due to the relative rarity of gut motility disorders, even at established referral centers, with a high enough degree of clinical severity and quality of life impairment to warrant invasive diagnostic procedures such as those applied in our patient cohort, could have contributed to a "limited challenge bias". Moreover, the rarity of some conditions in adulthood, for instance degenerative myopathies (4 cases in our study), could make our sample size insufficient to assert the predictive value of manometry in these small groups of patients. Second, to standardize the manometric procedure employed in our patient cohort, we included only patients studied in one of the participating centers. We do not know whether other intestinal manometry protocols, such as 24-hour ambulatory manometry, would provide similar information or produce different outcomes. In addition, contrary to the consensus approach adopted for assessment of manometric evidence, histopathology was evaluated by a single investigator, albeit supported by a team of pathology associates. Finally, histopathological analysis, although supported by immunohistochemical techniques, did not include quantitative, e.g. assessment of number of perykaria / ganglion in each section for each biopsy. The quantitative approach was beyond the purpose of the present study which will be the objective of further studies.

Even accepting the above shortcomings, our data suggests that intestinal manometry helps to objectively diagnose intestinal dysmotility in patients with severe and long-standing abdominal symptoms of uncertain origin. In addition, an abnormal manometry implies a relatively high probability of subsequently finding neuro-muscular histological abnormalities on full-thickness intestinal biopsies.

Table 5. Diagnostic criteria for abnormal intestinal manometry.

NEUROPATHIC PATTERN

- Abnormal configuration of Phase III: Tonic rises of baseline pressure over 30 mmHg amplitude ≥ 3 minute duration.
- Abnormal propagation of Phase III: Simultaneous or retrograde propagation over ≥ 20 cm segment of small bowel.
- Inability of an adequate meal to induce a normal feed pattern: No changes in activity during postprandial period (no fed pattern) or phase III-like activity during postprandial period (while disregarding the first 20 minute after beginning of meal).
- Bursts: At least one period of ≥3 minutes, or two periods of ≥2 minute duration with continuous high amplitude (≥20 mm Hg) and high frequency (10 -12/min) phasic pressure activity not followed by motor quiescence.
- Sustained contractions: Prolonged (≥30 minutes duration), high amplitude (≥20 mm Hg) and high frequency (10/min) phasic pressure activity that occurs in a segment of intestine while normal or reduced contractility is simultaneously recorded at other levels.

MYOPATHIC PATTERN

- Hypomotility: Low amplitude contractions (i.e., contractions with amplitude <10 mm Hg) or contractions with amplitude <20 mm Hg also during phase III.

OCCLUSIVE PATTERN

- Minute rhythm: intermittent periodic activity (shorts bursts repeating every 1-3 min) occurring simultaneously in recording sites at least 20 cm apart, at least during 30 minutes in postprandial period.
- Prolonged contractions (PC): prolonged (>20 sec duration) contractions occurring simultaneously in recording sites at least 20 cm apart, at least during 30 minutes in postprandial period.

INDETERMINATE PATTERN

- Not fulfilling above criteria but with some abnormal findings (i.e.: isolated PC, postprandial hypomotility, hypermotility, etc).

Table 6. Antibodies used for immunohistochemical analysis of small bowel full-thickness biopsies.

Primary Antibody	Host	Code	Dilution	Source
Neuron-specific enolase	Rabbit	PA1-28217	Ready to use 1:1	Thermo Fisher Scientific°
Synaptophysin	Rabbit	A0010	1:100	DAKO*
BCL-2 oncoprotein (clone 124)	Mouse	M0887	1:100	DAKO
S100b	Rabbit	Z0311	1:400	DAKO
C-Kit / CD117	Rabbit	A4502	1:400	DAKO
Smooth muscle actin	Mouse	M0851	1:400	DAKO
CD45R0 (clone OPD4)	Mouse	M0834	1:40	DAKO
CD8 (clone C8/144B)	Mouse	M 7103	1:50	DAKO
CD3 (clone F7.2.38)	Rabbit	A0452	1:25	DAKO
Mast cell tryptase (clone AA1)	Mouse	M7052	1:800	DAKO

Notes: °Thermo Fisher Scientific, Rockford, USA; *Dako, Glostrup, Denmark

Table 7. Clinical, manometric and histopathological features in CIPO and SFGID.

	CIPO (n=20)	SFGID (n=15)	P value
Clinical features			
◆ Esophageal involvement	3 (15.0%)	2 (13.3%)	0.889
◆ Gastroparesis	5 (25.0%)	7 (46.7%)	0.181
◆ Small intestinal bacterial overgrowth	10 (50.0%)	6 (40.0%)	0.557
◆ Urinary tract involvement	2 (10.0%)	2 (13.3%)	0.759
◆ Psychiatric comorbidities	5 (25.0%)	2 (13.3%)	0.393
◆ Enteral nutrition	4 (20.0%)	1 (6.7%)	0.265
◆ Parenteral nutrition	4 (20.0%)	7 (46.7%)	0.093
Manometric pattern			
◆ Neuropathy	10 (50.0%)	11 (73.3%)	0.163
◆ Myopathy	3 (15.0%)	0 (0.0%)	0.117
◆ Occlusive	3 (15.0%)	0 (0.0%)	0.117
◆ Indeterminate	1 (5.0%)	2 (13.3%)	0.383
◆ No abnormalities	3 (15.0%)	2 (13.3%)	0.889
Histological subgroup			
◆ Neuropathy	4 (20.0%)	1 (6.7%)	0.265
◆ Myopathy	1 (5.0%)	3 (20.0%)	0.167
◆ Inflammatory neuro-myopathy	10 (50.0%)	6 (40.0%)	0.557
◆ Apparently normal	5 (25.0%)	5 (33.3%)	0.589

Table 8. Cross-tabulation of manometric findings and histopathological features.

	Histological subgroup			
Manometric subgroup	Neuropathy	Myopathy	Inflammatory	Apparently normal
Neuropathic	3	2	8	8
Myopathic	2		1	
Occlusive			3	
Indeterminate		1	2	
Normal		1	2	2

Values indicate number of patients per subgroup.

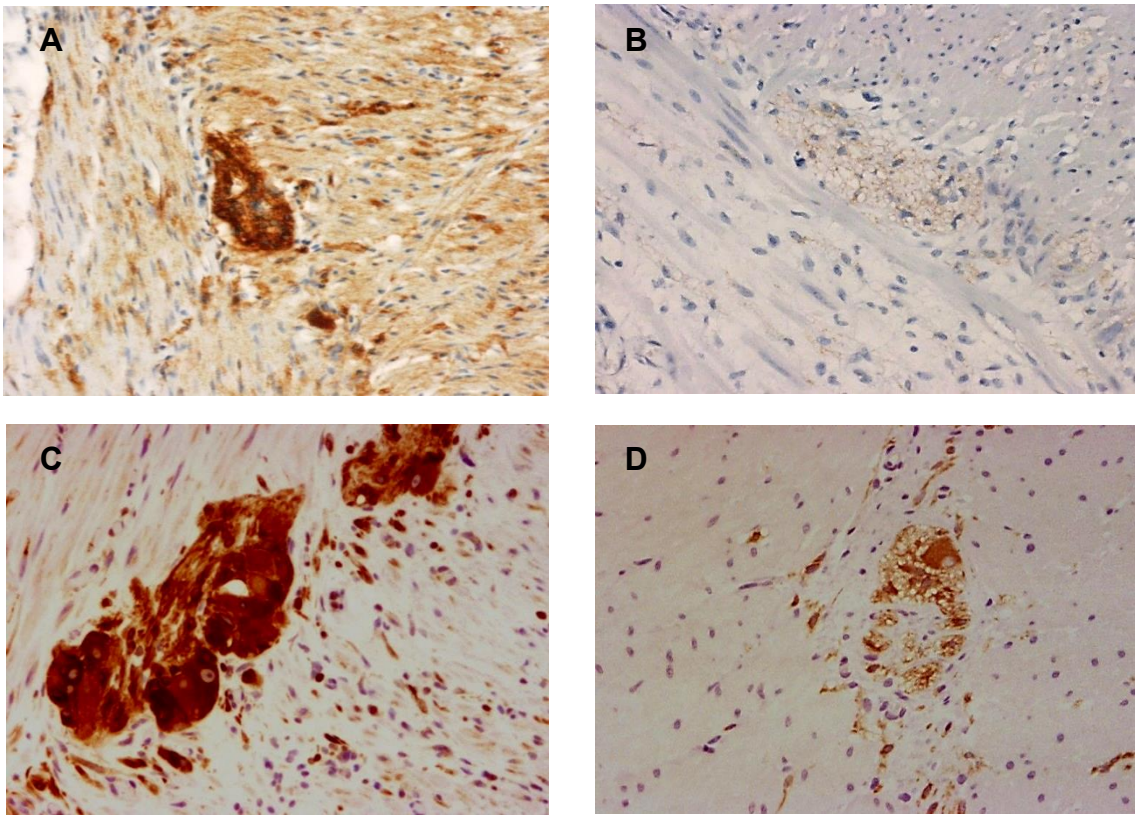


Fig. 14. Representative photomicrographs showing NSE (A and B) and Bcl-2 (C and D) immunolabeling in the neuromuscular component of a jejunal biopsy. Compared to controls (A and C), note the clear-cut reduction of the NSE (B) and Bcl-2 (D) immunoreactivity in a patient with severe dysmotility; both features support an underlying neuropathy. Also, note that myenteric neurons and nerve fibers are much less detectable in both photomicrographs, either labeled by NSE (B) or Bcl-2 (D) further supporting degenerative mechanisms leading to neuronal impairment. Original magnification: 200x in A-D.

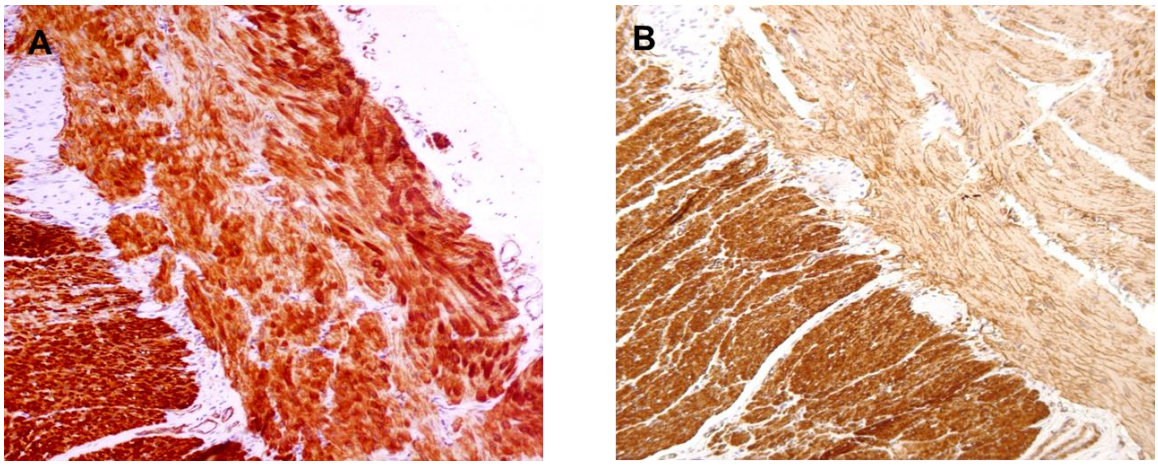


Fig. 15. Representative photomicrographs showing smooth muscle α-actin (SMA) immunostaining in a jejunal biopsy. Compared to control (A), note the marked reduction of SMA immunostaining in the circular layer of a patient with severe intestinal dysmotility (B). Original magnification: 100x in A and B.

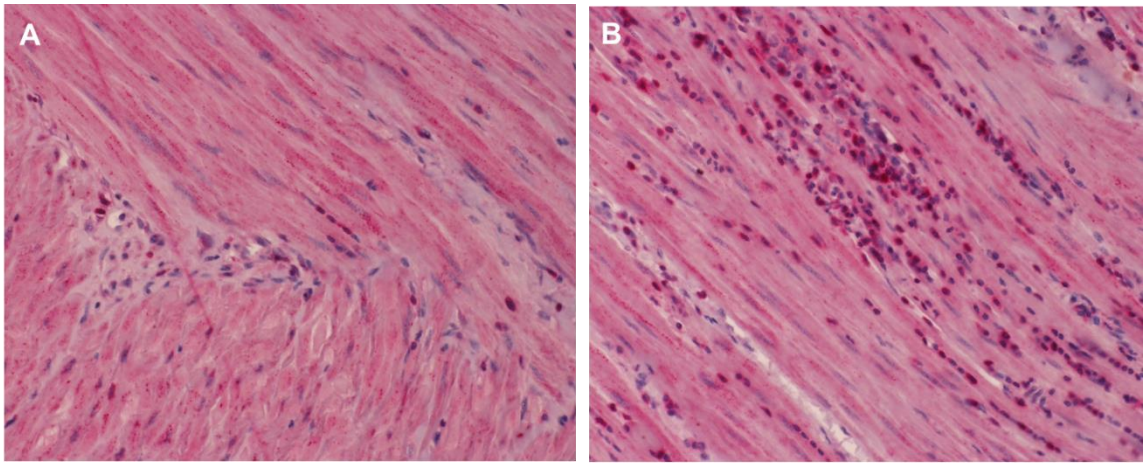


Fig. 16. Representative photomicrographs showing CD3 immunolabeling in the neuromuscular component of a jejunal biopsy. Pictures show a dense CD3 positive lymphocytes within a myenteric plexus (hence the term of myenteric ganglionitis) (A) and throughout the circular muscle adjacent to nerve fibers (axonitis) (B). Original magnification: 200x in A and B.

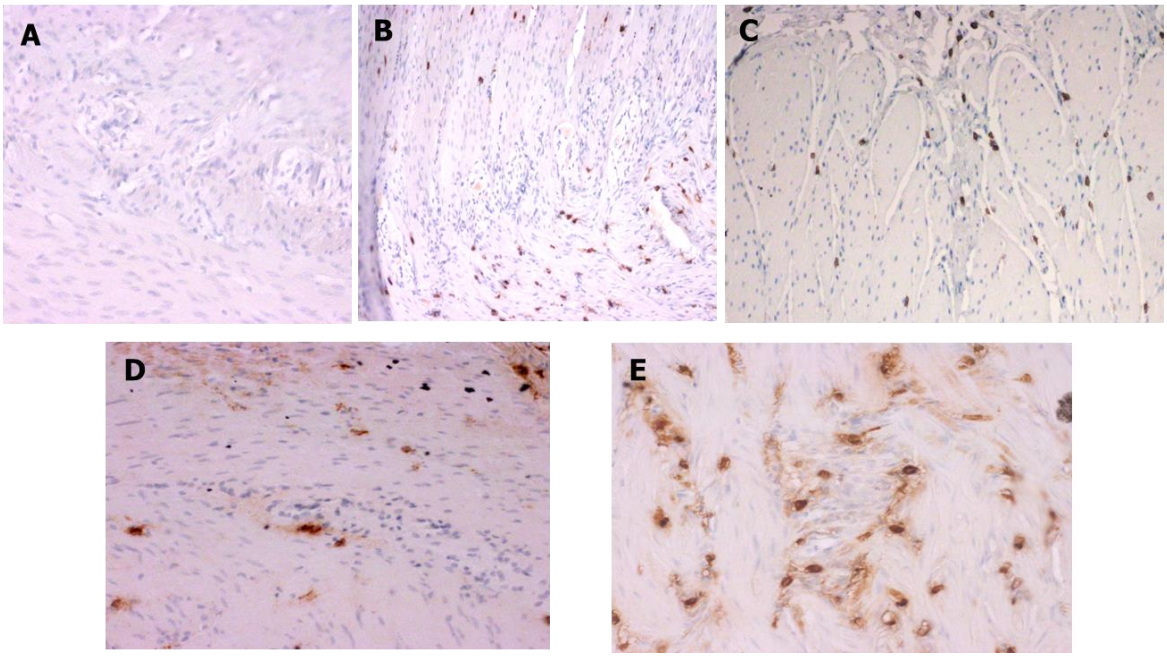


Fig. 17. Representative photomicrographs showing tryptase immunolabeling in the jejunal neuromuscular layer. Compared to a control subject (A), note the presence of tryptase immunoreactive mast cells infiltrating longitudinal (B) and circular (C) muscle layer, surrounding (D) and within (E) a myenteric plexus of patients with intestinal dysmotility. Original magnifications: 100x in A and C; 200x in D and E.

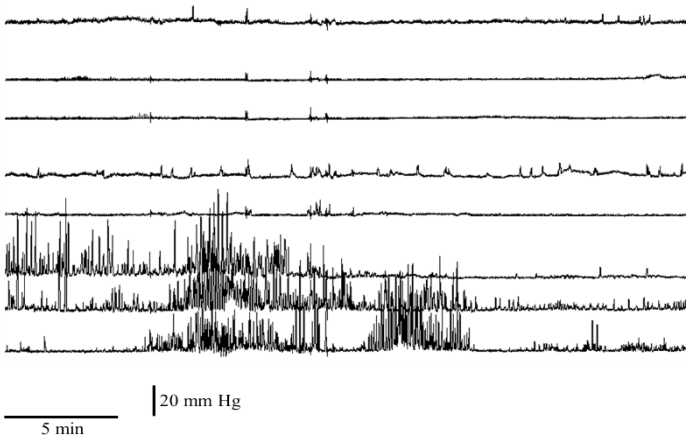


Fig. 18. **Intestinal manometry: neuropathic pattern.** Bursts of unpropagated activity are observed in the last three channels. Histopathology of full-thickness small bowel biopsies showed signs of lymphocytic neuro-myositis.

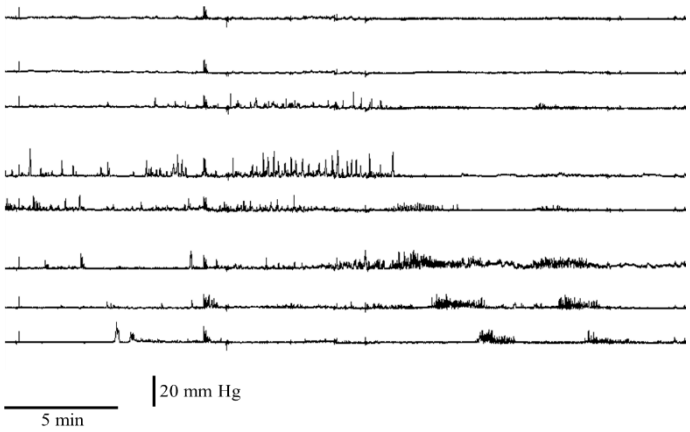


Fig. 19. **Intestinal manometry: myopathic pattern.** Note reduced amplitude of intestinal contractions during phase III activity fronts. Small bowel full-thickness biopsies revealed an underlying intestinal neuropathy.

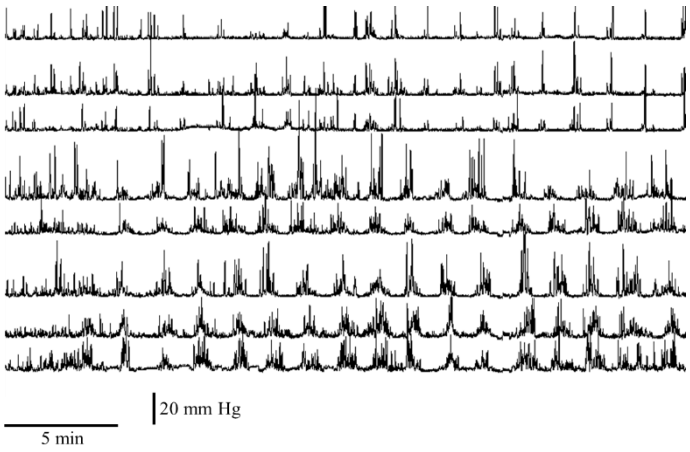


Fig. 20. **Intestinal manometry: occlusive pattern.** The manometric recording shows a minute rhythm activity characterized by short bursts of contractions repeating every 1-2 min during the postprandial period. An extensive mast cell infiltration was detected in the neuromuscular layers of the small bowel wall.

Take home messages:

- Intestinal manometry and / or histopathology are the current standard for the diagnosis of severe small bowel dysmotility disorders. However, the degree of correlation between the two methods is unknown.
- Results of this study indicate that more than two-thirds of these severe clinical cases with abnormal manometry also have abnormal histopathology underlying gut dysmotility.
- Nonetheless, we detected no correlation between the specific manometric patterns and histopathological findings.

Chapter 8.

QUANTITATIVE CHANGES OF ENTERIC NEURONS CORRELATE WITH CLINICAL FEATURES IN PATIENTS WITH SEVERE DYSMOTILITY

Modified from

Quantitative changes of enteric neurons correlate with clinical features in patients with severe dysmotility

Boschetti E[1*], Karunaratne TB[1*], Gori A[1*], Malagelada C[2,3], Accarino A[2,3], Azpiroz F[2,3], Malagelada JR[2,3], Bianco F[1], Giancola F[1], Bonora E[1], Clavenzani P[4], Mazzoni M[4], Sternini C[5], Stanghellini V[1] and De Giorgio R[6]

* These authors share co-first authorship

Submitted / under revision

ABSTRACT

Severe gastrointestinal symptoms are often associated with markedly perturbed enteric motility, a finding related to underlying enteric neuropathies. Current methods used to demonstrate enteric neuropathies are mainly based on classic qualitative histopathological/immunohistochemical evaluation. This standard approach, however, is

hampered by data interpretation, inter-observer variation and lack of expertise among pathologists. Objective: We assessed quantitatively the enteric innervation in patients with severe dysmotility (SD) and correlate the data with clinical features. Methods: Jejunal full-thickness biopsies were collected from 32 well characterized SD patients (16-77 years; 22 F); and from n=8 controls (47-73 years 4F). A symptom questionnaire was fulfilled prior to surgery. Patients were subdivided according to a previous qualitative histopathological evaluation: n=10 with an apparently normal (AN) neuro-muscular layer; n=14 with inflammatory (INF) changes throughout the neuromuscular layer; and n=8 with degenerative neuro-muscular alterations (DEG). Myenteric plexus (MP) and submucosal plexus (SMP) neurons were stained using neuron specific enolase antibody and neuronal cell bodies/ganglion were counted in at least three sections by 3 independent and skilled operators. Mean numbers of neuronal cell bodies/ganglion were analyzed by student's t-test and the correlation with symptoms/signs via Spearman correlation test. Results: The final concordance among the 3 operators was 80%. MP and SP neuronal cell bodies were decreased in SD vs. controls ($P<0.001$). Also MP and SP neurons decreased in AN, INF and DEG vs. controls ($P<0.0001$ in MP and $P<0.05$ in SP). Furthermore INF and DEG showed less MP (but not SP) neuronal cell bodies compared to AN ($P=0.0224$ and $P=0.0044$). Both the reduced MP and SP neuronal cell bodies correlated with abdominal distension/pain, early satiety, constipation and gastroparesis ($P<0.05$). Conclusions: The proposed method, showing a low discordance rate (20%), identified an overall 50% decreased of MP and SP neuronal cell bodies implying a critical loss of the neuronal mass. The 50% neuronal reduction correlated with a variety of symptoms / signs of SD patients. Notably, quantitative neuronal abnormalities can be demonstrated in patients with AN histopathology.

INTRODUCTION

Dysmotility represents an important clinical subgroup of the gastrointestinal (GI) neuromuscular disorders characterized by changes in the speed, strength or coordination of the muscles in the GI tract. Abnormal motility in the small intestine can lead to symptoms / signs suggestive of partial or complete intestinal obstruction.[1] The most severe form of intestinal dysmotility is represent by chronic intestinal pseudo-obstruction (CIPO) which typically shows a severe and recurrent episodes of impaired GI propulsion with sustained radiological findings mimicking mechanical obstruction.[24,25,393] Enteric dysmotility (ED) encompass an additional group of patients with abnormal intestinal motor activity and severe symptoms but no radiological signs of pseudo-obstruction and the clinical presentation of ED corresponds to that of severe functional GI disorders (SFGID).[16,411,413]

Both CIPO and ED are associated with abnormalities in enteric neurons, interstitial cells of Cajal (ICC) networks and enteric smooth muscle cells and their interactions with other cell types such as cells in the immnue system[5-6]. These alterations in the neuro-muscular systems are tightly linked to the contractile abnormalities detected in intestinal dysmotility. Intestinal manometry can be used to assess the contractile activity of the gut over long periods of time and to define the pathophysiological mechanisms involved in dysmotility (e.g. neuropathy or myopathy)[1,485]. Recently we published a study prospectively comparing small bowel manometric abnormalities with subsequent histopathological findings in full-thickness intestinal biopsies.[501] Notably, 38% of the patients who presented a neuropathic manometric pattern in that study showed apparently normal histopathology in the full-thickness biopsies. However, currently used methods to demonstrate pathological abnormalities of the enteric neuromusculature in full-thickness biopsies are not fully standardized and the technical details and criteria for interpretation may be subjected to observer variation. In particular,

these methods use a qualitative approach which misses the quantitative metrics in the histologic tissue. In a complex condition like severe dysmotility, total histologic evaluation for subtle alteration in the microanatomy, rather than defining biopsies exclusively on broader microscopic architecture, may impact diagnosis. This may be especially true in neuropathy, since for an optimal neurological function, it is imperative to have the neurons in good quality as well as in a good quantity.

Clearly, whole-mount preparations represent the most suitable approach for neuronal cell count,[502,503] however, formalin-fixed paraffin-embedded tissue specimens are easier to prepare and routinely collected in histopathology archives. Therefore these specimens represent the only material available for examination and are precious resources for retrospective clinical studies.[503,504] Accordingly, standard methodological criteria must be established for accurate evaluations of neuron cell populations in paraffin-embedded intestinal samples. In an attempt to address this issue, in the present study we analyzed the number of neurons per ganglion in sub mucosal plexus (SP) and myenteric plexus (MP) in a group of patients with severe and chronic clinical manifestations of GI dysmotility and another group of individuals who severed as controls. Subsequently, we evaluated the possible correlation between the severity of symptoms and the number of neurons per ganglia in SP and MP, our hypothesis being that as the number of neurons per ganglia in SP and / or MP reduces, dysmotility symptoms will become more severe.

MATERIAL AND METHODS

Patients

Thirty eight patients with severe dismotility (SD) of the GI tract were recruited and clinically evaluated at the Vall d'Hebron University Hospital in Barcelona between November 1999 and November 2009. During this time span a subgroup of thirty two patients (16 to 77 years

old; 22 females and 10 males) underwent full-thickness biopsy. Patients with motility disorders secondary to infectious, neurological, metabolic, systemic-autoimmune and paraneoplastic conditions were excluded. Anorexia nervosa was specifically excluded in all patients with malnutrition by a psychiatric examination.

The clinical diagnosis of SD with inability to maintain normal body weight was established on the basis of chronic (>3 months) persistent or recurrent symptoms in the absence of episodes mimicking mechanical CIPO and of associated organic, systemic or metabolic diseases potentially responsible for the digestive manifestations. Inability to maintain a normal body weight was defined by a BMI <18.7 Kg m^{-2} (in women) or <20.1 Kg m^{-2} (in men). Within the n=32 SD patients n=19 had recurrent sub-occlusive episodes. The clinical diagnosis of CIPO was established on the basis of a chronic (>3 months), severe symptom complex mimicking mechanical small bowel obstruction, as well as (at least on one occasion) radiological evidence of air-fluid levels or dilated small bowel loops. Thus, patients presented with abdominal pain and distension associated with nausea, vomiting, bloating and malnutrition. These clinical manifestations occurred episodically or continuously. Presence of mechanical obstruction had been excluded by conventional imaging studies or by prior exploratory abdominal surgery.

Controls (CTR) patients n=8 (47-73 years old; 4 females and 4 males) underwent abdominal surgery for non complicated neoplastic tumor. Full thickness biopsies were collected in the flanking jejunal tissue without infiltration and preserved architecture.

For patients and controls a symptoms questionnaire was fulfilled prior to the surgical intervention. Particularly the following data were collected: age; sex, BMI, CVC and related infections; the requirement of parenteral nutrition; the number of sub-occlusive episodes, the presence of abdominal distension; the presence and entity of the abdominal pain; the incidence of nausea, vomiting, fullness; the sensation of early satiety; the presence of

constipation and / or diarrhea according to the Bristol stool scale; the possible esophageal involvement; the occurrence of gastroparesis; the presence of small intestinal bacterial overwrought (SIBO), the occurrence of urinary symptoms; and finally the age of symptoms onset.

The study protocol was approved by the Ethics Committee of the University Hospital Vall d'Hebron and all patients gave written informed consent.

Intestinal tissue samples

Full-thickness biopsy specimens were obtained from small bowel either by laparoscopy or laparotomy. In each patient a single circular segment (5 cm long) was obtained from the proximal jejunum, except in those cases with segmental dilatation in whom the biopsy was obtained from the most dilated segment. Immediately after resection, specimens were fixed in 10% formalin overnight, embedded in paraffin and sectioned (4□m) according to standard protocol for tissue processing.

Histopathological review was conducted by one of the investigators (R. De G) and his team (A. G. and E. B.) at the University of Bologna, Italy. Histopathological material was examined without prior information about clinical and motility test outcomes. All biopsies were first analyzed to exclude associated pathological findings such as malignancy, dysplasia or villous atrophy. Specific enteric neuromuscular evaluation was performed on the hematoxylin and eosin (H&E)-stained sections to assess the overall structure of ganglia, myenteric and submucosal neuronal cell bodies and fibers, glia and smooth muscle cells. When needed, other standard histological stains (e.g. Masson trichrome) were applied to detect additional tissue changes such as fibrosis. Finally, immunohistochemical techniques to analyze structural markers of the enteric neuromuscular component were used. We used specific antibodies to: neuron specific enolase (NSE) and synaptophysin (both general

neuronal markers), BCL-2 (as a marker of cell survival), S100β (glial cells), c-Kit (interstitial cells of Cajal, ICC), alpha-smooth muscle actin (α-SMA) (smooth muscle cells), CD45 (leukocytes), CD3 (general T lymphocyte marker), CD4 and CD8 (markers of T lymphocyte subsets, i.e. "helper" and "suppressor", respectively). Finally, an anti-tryptase antibody was used to detect mast cells (antibodies feature and experimental dilution are listed in Table 9). The immunohistochemical techniques used in this study have been performed according to standardized and widely validated protocols.[440]

Three histopathological patterns, were recognized according to the London classification.[2] Main findings for each of these patterns are detailed:

Apparently normal (AN): (n=10; age range 24-61; 3 male) no detectable changes in the neuromuscular layer using conventional histochemical staining and immunohistochemistry;

Inflammatory neuro-myopathy (INF): (n=14; age range 16-77; 4 male) characterized by lymphocytic infiltrate in muscle layers (leiomyositis) and / or neuronal ganglia (ganglionitis) (visualized by anti-CD3, -CD4 / -CD8 immunolabeling); or mast cell or eosinophil infiltration throughout the neuromuscular structures (detected by tryptase immunolabeling or via conventional H&E staining, respectively).[497]

Degenerative neuro-myopathy (DEG): (n=8; age range 29-70; 3 male) overt ganglionic abnormalities as identified by traditional tinctorial staining (H&E) and immunohistochemical approaches (pan-neuronal markers); altered neuronal survival was identified by assessing BCL-2 expression in perikarya and nerve fibers (a feature which has been already associated with increased neuronal apoptosis);14muscular degeneration with cell swelling, vacuolization or atrophy; fibrotic replacement of smooth muscle (detectable by conventional staining, such as H&E, and immunohistochemical approaches, e.g. α-SMA immunoreactivity abnormalities);

Table 9. Antibodies used for immunohistochemical analysis of small bowel full-thickness biopsies.

Primary Antibody	Host	Code	Dilution	Source
Neuron-specific enolase	Rabbit	PA1-28217	1:1	Thermo Fisher Scientific°
Synaptophysin	Rabbit	A0010	1:100	DAKO*
BCL-2 oncoprotein (clone 124)	Mouse	M0887	1:100	DAKO
S100b	Rabbit	Z0311	1:400	DAKO
C-Kit / CD117	Rabbit	A4502	1:400	DAKO
Smooth muscle actin	Mouse	M0851	1:400	DAKO
CD45R0 (clone OPD4)	Mouse	M0834	1:40	DAKO
CD8 (clone C8/144B)	Mouse	M 7103	1:50	DAKO
CD3 (clone F7.2.38)	Rabbit	A0452	1:25	DAKO
Mast cell tryptase (clone AA1)	Mouse	M7052	1:800	DAKO

Notes: °Thermo Fisher Scientific, Rockford, USA; *Dako, Glostrup, Denmark. Immunoperoxidase secondary detection system (IHC Select HRP/DAB), (Millipore, Canada) was performed using chromogen substrates to obtain the typical brown-dark product indicative of immunolabeling. Furthermore, alkaline phosphatase anti-alkaline phosphatase immunohistochemical technique was used to detect the lymphocytic infiltrate by applying anti-CD3, -CD4 and -CD8 rabbit and mouse antibodies.

MP and SP neuronal cell count

MP and SP neurons per ganglion were counted at 400X final magnification with a light transmission microscope (Olympus AX 70; Olympus, Melville, NY, USA) by three investigators (E.B, A. G. and R. De G. unaware of the diagnosis of the assessed slides). At least three jejunal cross sections from one specimen per patient and control were examined. Focusing along the neuromuscular ridge of the myenteric ganglia and submucosa, all the consecutive microscopic fields (at least 20) were considered for each analyzed section. Each operator counted the total amount of neurons NSE immunoreactive per ganglion. The final number of neuron per ganglion per patient was compared among the three operators to assess the inter observer variability of this technique. The concordance among the three operators was reached when the deference between counted averages was ≤ 5 neurons, otherwise the sample was considered discordant. The final concordance of 80% was reached in analyzed cases (n=40). Figure 1 shows an example of the operative magnification of the neuronal count in the neuromuscular layer (Fig. 21 A) and in the submucosa (Fig. 21 B).

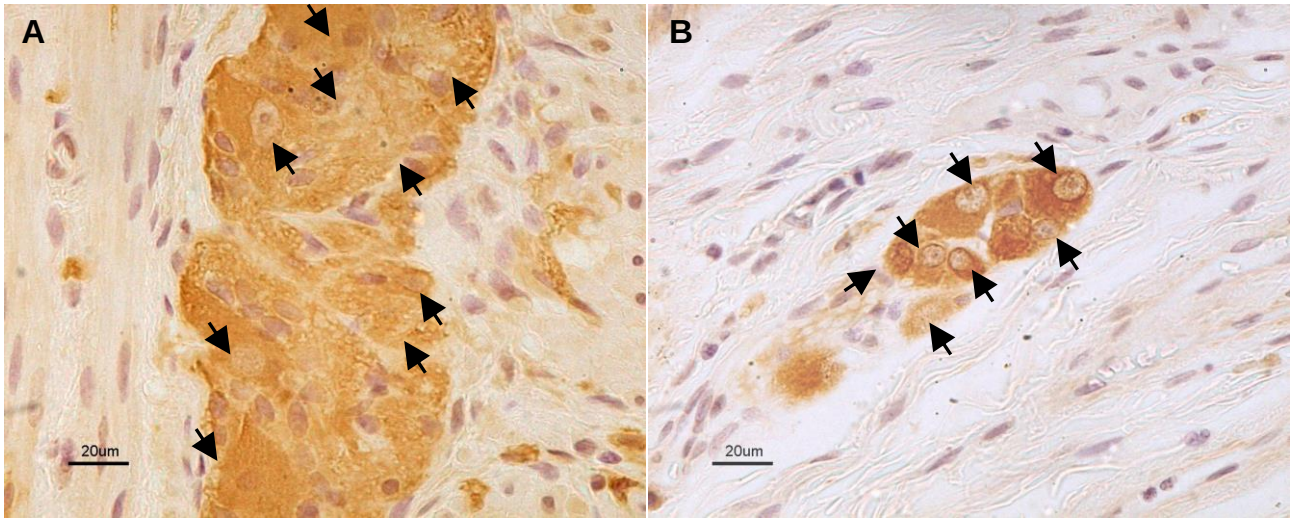


Fig. 21. Neuronal count in MP and SP. The two pictures are a representative view of the operative neuronal count on a CTR sample. Black arrow indicates neurons identified with NSE staining in **A)** MP and **B)** SP.

Statistical analysis

The non parametric student's t-test was used to define differences between means (± SD) of MP and SP neurons from ED patients (together or divided in the tree subgroups) and control specimens; and also to evaluate distribution of patients with CIPO episodes among the whole cohort. In both cases the analysis of variance was calculated by a two tailed ANOVA test. The correlation among the number of MP or SP neurons and symptoms was performed using the non parametric correlation test of Spearman. Where possible, the linear regression and the 95% confidence interval were calculated.

RESULTS

MP and SP neuronal cell count in SD and SD subgroups vs CTR

Number of neurons per ganglion measured in MP (Fig. 22 A) and SP (Fig. 22 B) was decreased in all SD patients ($P<0.001$ both) *vs* CTR. Subdividing patients into the three IHC subgroups identified, neurons in MP were drastically decreased in AN, INF and DEG vs CTR ($P<0.0001$). Furthermore, INF and DEG showed less MP neurons compared to AN ($P=0.0224$ and $P=0.0044$ respectively). In SP, neurons in AN, INF and DEG were decreased equally *vs* CTR ($P= 0.0024$; $P=0.0005$; $P=0.0366$ respectively).

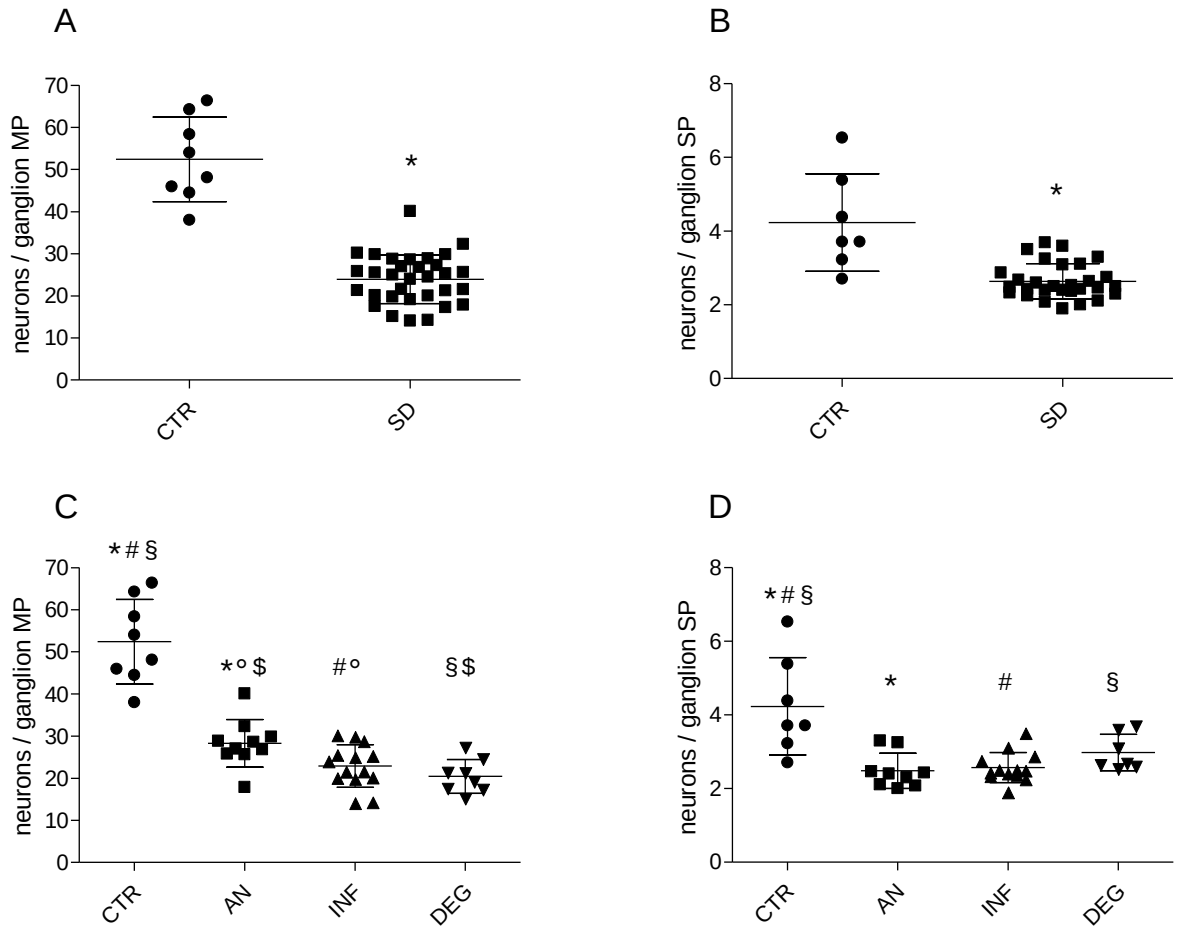


Fig. 22. MP and SP neuronal cell count in SD and SD subgroups vs CTR. A) MP neuron counted in the total SD group *P<0.0001; **B)** SP neuron counted in the total SD group *P<0.0001; **C)** MP neuron counted diving SD in AN, INF and DEG *$^{\#\S}$=P<0.0001; $^{\$}$P= 0.0044 and °P=0.0224; ANOVA P<0.0001 **D)** SP neuron counted diving SD in AN, INF and DEG *P<0.0024; $^{\#}$P= 0.0005 and §P=0.0366; ANOVA P<0.0001.

Distribution of patients with CIPO episodes among total SD patients

59% of SD patients had CIPO episodes. CIPO patients were distributed in the 3 subgroups with a non-significant statistic trend to increase from AN (40%) to INF (64%) and DEG (75%) (Fig. 23 A). The number of episodes had a trend to increase among groups, but without statistic significance. Only the 30% of AN patients showed more than 2 episodes, otherwise in INF the percentage increased to 56% and the totality of DEG cases with CIPO had more than 2 episodes (75%) (Fig. 23 B). Patients with 3 or more sub-occlusive episodes were 10% in AN, 36% in INF and 37.5% in DEG (Fig. 23 C).

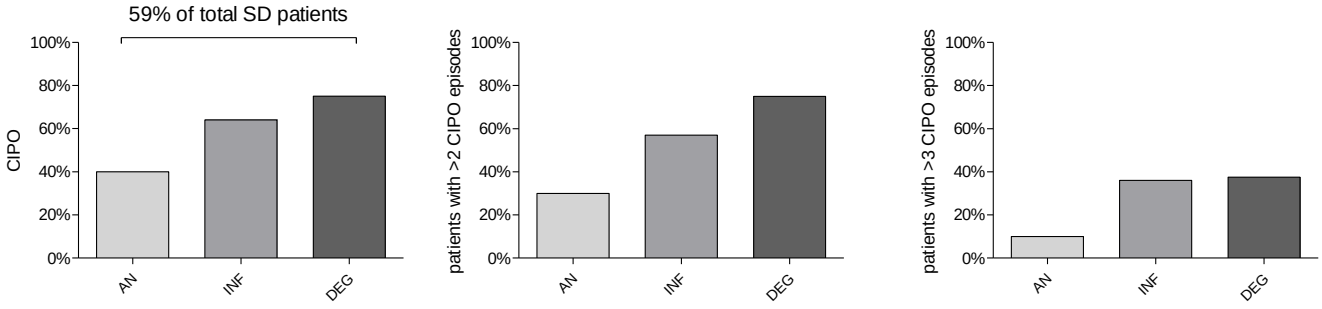


Fig. 23 Distribution of sub occlusive episodes among the tree groups of SD patients. A) Percentage of patients with almost one sub occlusive episode **B)** Percentage of patients with 2 or more sub occlusive episodes **C)** Percentage of patients with tree or more sub occlusive episode.

MP and SP neuronal cell count in SD with or without sub occlusive episodes vs CTR

Since SD group encompass patients with a severe impairment of GI motility with or without sub-occlusive episodes, the neuronal count was performed also by subdividing the cases as patients without CIPO episodes (SD-NON-CIPO) and patients with one or more CIPO documented episodes (SD-CIPO). Fig. 24 shows that neuron equally decreased between SD-NON-CIPO and SD-CIPO *vs* CTR.

Correlation between the number of MP and SP neurons and reported symptoms.

There is a direct correlation among the decrease of MP and SP neurons (P=0.0062 R=0.4418 Fig. 25 A). The MP neuronal decrement did not showed a trend for association with the severity of CIPO episodes (P=0.0574 R=-0.3030 Fig. 25 B). Symptoms that increase with the MP neurons reduction (Fig. 25 C) are: abdominal distension (P<0.0001 R=-0.6238); abdominal pain (P=0.0037 R=-0.4490); early satiety sensation (P=0.0264 R=-0.3509); constipation (P=0.0490 R=-0.3132) and gastroparesis (P=0.0128 R=-0.3901). Symptoms that increase with the SP neurons decline are abdominal distension (P=0.0012 R=-0.5124); abdominal pain (P=0.0033 R=-0.4709), and constipation (P=0.0152 R=-0.3961).

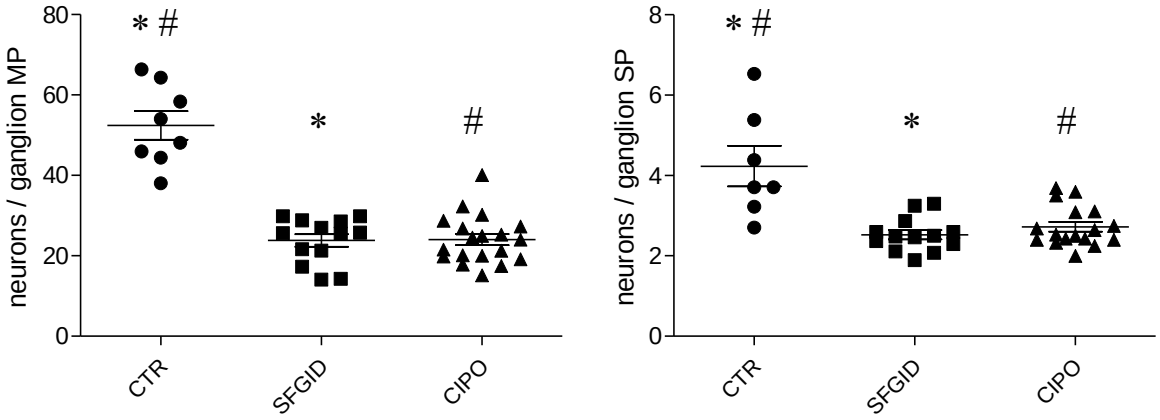
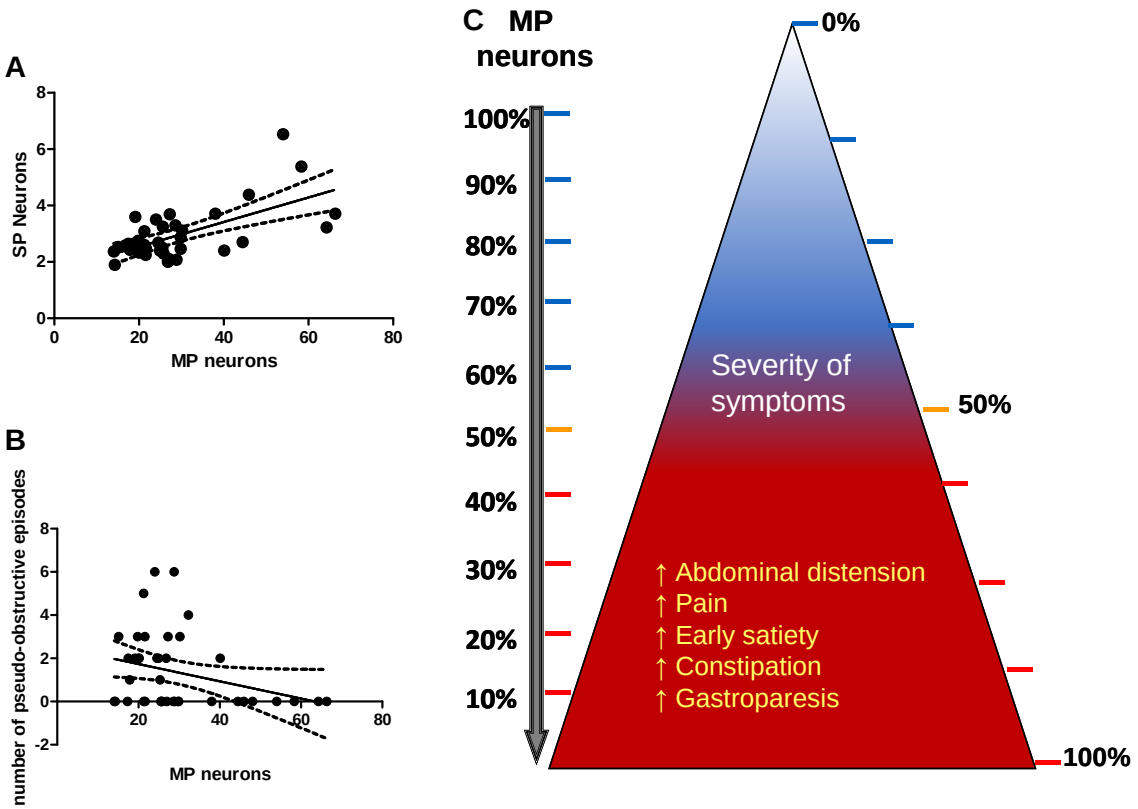


Fig. 24 MP and SP neuronal cell count in SD with or without sub-occlusive episodes vs CTR. Neurons counted in patients subdivided considering the presence or the absence of pseudobstruction episodes in A) MP *#P<0.0001 and ANOVA P<0.0001; and in B) SP *#P=0.0004 and ANOVA P<0.0001.



Fig, 25 Association among MP neurons and symptoms. A) Linear regression illustrating the quantitative correlation among MP and SP neurons Spearman P=0.0062 R=0.4418. **B)** Linear regression illustrating the trend (not significant) among MP neurons and the number of sub-occlusive episode Spearman P=0.0574 R=-0.3030. **C)** Illustration that highlights symptoms that worsens when MP neurons decrease.

DISCUSSION

Minimally invasive procedures such as laparoscopic surgery and natural orifice trans luminal endoscopic surgery have enabled, with a relative safety, the demonstration of derangements occurring in the enteric neuro-muscular system in full-thickness biopsies of patients with severe dysmotility. Despite these technical and safety advancements, often biopsying patients with severe GI dysmotility has been discouraged by reasons such as an 'apparently normal' neuro-muscular layer reported even in cases characterized by severe symptoms / signs and / or manometric abnormalities. Degeneration of neurons in the ENS is still not completely understood and therefore in the present study we used quantification of neurons in SP and MP ganglions to divulge neuronal degeneration in severe intestinal dysmotility, especially in cases where apparently normal neuro-muscular architecture was observed with standard staining methods.

The quantitative results in this study show that the group of patients who were labeled AN has significant reduction in number of neurons in the enteric ganglions. The loss of approximately a half of neurons per ganglion in both SP and MP may explain the clinical manifestations and the abnormal manometric patterns detected in the AN group, which we have published previously.[7] The significant difference in neuronal counts per ganglion between the AN and DEG or between AN and INF hints that the AN group may be at the initial stage of the neurodegeneration or inflammation process and may develop into either inflammatory or degenerative neuropathy / myopathy identifiable with standard staining methods in histopathology as the neuronal count declines. Further experiments are warranted in order to clarify the biochemical pattern of the three groups with the aim to find predictive molecules that may stratify CIPO patients, and possibly reveal if AN really represent the

early stage of INF and DEG. In this line, it would be possible to identify potential biomarker to develop a targeted therapy.

Furthermore, the differences in neuronal counts among histological subgroups highlight the importance of number of neurons in the generation of sub-occlusive episodes and dysmotility symptoms. In fact, we could identify an increase in number of sub-occlusive episodes as the neuronal count declines. Though we could not establish a statistically significant correlation between number of sub-occlusive episodes and the neuronal count with our data, it could be inferred that a significant correlation may be reached if the sample sizes are increased. Abdominal pain and distension, two very important clinical manifestations of SD, positively correlated with the declined neuronal count. In our clinical assessment of these SD patients, we did not consider the subjective severity of the pain according to a pain scale or the distention. Therefore, with our data we cannot conclude on variation of abdominal pain and distension according to the neurons per ganglion in the ENS, although this could have put some emphasis on the neuronal count as a predictive marker. However, based on our results we could tentatively propose the number of neurons per ganglion in the SP and MP as a predictive marker for symptom / sign generation and their severity.

One limitation of the neuronal count technique is the inter-observer-variability. The discordance among evaluators of neuronal count in this study was 20% and the differences between the averages more than five neurons. Among the patients who had discordance in their neuronal counts, only one patient had a difference in average neuronal counts which was greater than 10 while in all others, the differences were less than 9. The differences between the counted average of CTR and any group of CIPO were ≥ 24 neurons. Even if this technique is influenced by operators, the estimated discordance is considered to be very low

to affect the results. We used a pan neuronal marker, therefore we were unable to identify if the decrement involved one or more neuronal subtypes. The use of specific staining for different neuronal classes will clarify if the neuronal loss is generalized or affect a specific neuron subtype.

Our data indicate that altered GI propulsion and the generation of dysmotility symptoms are associated with at least 50% of neuronal loss in the small intestine. Furthermore, it appears that the clinical manifestations and their severity may increase as the neuronal count decrease in the ENS. This finding in the gut reflects a neuronal impairment characteristic of other pathological conditions involving central nervous system, such as Parkinson's or Alzheimer diseases.

Take home messages:

- Qualitative approach used to demonstrate pathological abnormalities of the enteric neuromusculature in full-thickness biopsies are not fully standardized and does not account for the quantitative metrics in the histologic tissue.
- The quantitative results in this study show that the group of patients who had no detectable changes in the neuromuscular layer using qualitative approach had significant reduction in number of neurons in the enteric ganglions.
- The loss of approximately a half of neurons per ganglion in both submucosal and myenteric plexus correlated with a variety of symptoms / signs of SD patients

CONCLUSIONS

The ENS serves a vast array of regulatory functions through which the physiology of the GI tract occurs to preserve body homeostasis and ultimately life. However, mounting evidence clearly indicate that the ENS is reminiscent of the CNS organization, not only for the neuro-bioelectrical properties and function, but also for the important glial cell component reminiscent of astrocytes of the CNS. Any changes that could alter this finely tuned and highly integrated system could result in gut dysfunction. The diagnosis of enteric neuromuscular disorders has seen many advances since the first identification of an enteric neuropathic disorder by the Danish physician Harald Hirschsprung in 1886. These technical advances achieved in microscopy and enteric histopathological staining techniques have made it possible to identify subtle neuropathies, myopathies or ICCpathies that underlie the most severe intestinal motility disorders, such as CIPO. This condition is part of GINMDs with still unclear pathophysiological aspects, which impact on the clinical practice in terms of their diagnosis and treatment. The physiological characterization of CIPO currently relies on manometric findings that are suggestive of underlying neurogenic or myogenic pathological conditions. However, none previous studies have reported on manometric finds and histopathology and small intestinal dysmotility in CIPO. In this thesis, I tackled this question by investigating patients with severe dysmotility using both standard manometry and intestinal full thickness biopsy samples. I showed that there is no apparent correlation between the manometric patterns and the underlying qualititative histopathological pictures although the abnormal manometry most likely suggests an underlying pathology detectable by histochemical / immunohistochemical approaches. Three histopathological subgroups have been identified: a) degenerative feature of neurones and / or muscle; b) inflammatory with an infiltrate spreading throughout the neuromuscular layer; and c) an appatently normal neuromusculat architecture (at elast at a qualitative assessment by expert pathologists). The

latter group was very intriguing since they present with severe symptoms and manometric abnormalities. Therefore, I further considered these subgroups using quantitative method to count the number of neurones present within myentric and submucosal ganglia. In this study I found that in all three histological subgroups number of neurones per ganglia was reduced by at least half compared to that in controls. Furthermore, I also showed the correlation of predominant symptoms (e.g. abdominal pain and distention) with the diminishing neuronal count in the ganglia. The data presented in this thesis therefore could infer neuronal dengeneration in the ENS not visible by the currently available qualititative histopathological techniques along with pushing forward the concept of a 'critical neuronal mass' below which symptoms arise in patients with severe gut dysmotility, i.e. CIPO.

At last, I would state strongly that the present thesis clearly manifests my willgness to continue to investigate pathophysiological mechanisms that impact on diseases affecting gut homeostasis. Over the years, I learned that perseverance and enthusiasm in research pave the way to better patients' management and treatment.

REFERENCES

1. De Giorgio R, Sarnelli G, Corinaldesi R, Stanghellini V. Advances in our understanding of the pathology of chronic intestinal pseudo-obstruction. Gut. 2004; 53(11): 1549-1552.

2. Knowles CH, De Giorgio R, Kapur RP, Bruder E, Farrugia G, Geboes K, Lindberg G, Martin JE, Meier-Ruge WA, Milla PJ, Smith VV, Vandervinden JM, Veress B. Wedel T. The London Classification of gastrointestinal neuromuscular pathology: report on behalf of the Gastro 2009 International Working Group. Gut. 2010; 59(7): 882-887.

3. Talley NJ. Functional gastrointestinal disorders as a public health problem. Neurogastroenterol Motil. 2008; 20(S1): 121-129.

4. Bryant RV, van Langenberg DR, Holtmann GJ, Andrews JM. Functional gastrointestinal disorders in inflammatory bowel disease: impact on quality of life and psychological status. J Gastroenterol Hepatol. 2011; 26: 916–923.

5. Schuffler MD, Jonak Z. Chronic idiopathic intestinal pseudo-obstruction caused by a degenerative disorder of the myenteric plexus: the use of Smith's method to define the neuropathology. Gastroenterology 1982; 82(3): 476-86.

6. Törnblom H, Lindberg G, Nyberg B, Veress B. Full-thickness biopsy of the jejunum reveals inflammation and enteric neuropathy in irritable bowel syndrome. Gastroenterology 2002; 123: 1972–1979.

7. Barnett JL, McDonnell WM, Appelman HD, Dobbins WO. Familial visceral neuropathy with neuronal intranuclear inclusions: diagnosis by rectal biopsy. Gastroenterology 2004; 102: 684–691.

8. Bassotti G, Villanacci V, Fisogni S, Rossi E, Baronio P, Clerici C, Maurer CA, Cathomas G, Antonelli E. Enteric glial cells and their role in gastrointestinal motor

abnormalities: introducing the neuro-gliopathies. World J Gastroenterol. 2007; 13: 4035-4041.

9. Selgrad M, De Giorgio R, Fini L, Cogliandro RF, Williams S, Stanghellini V, Barbara G, Tonini M, Corinaldesi R, Genta RM, Domiati-Saad R, Meyer R, Goel A, Boland CR, Ricciardiello L. JC virus infects the enteric glia of patients with chronic idiopathic intestinal pseudo-obstruction. Gut 2008; 14: 6717-6721.

10. Smith VV, Milla PJ. Histological phenotypes of enteric smooth muscle disease causing functional intestinal obstruction in childhood. Histopathology 1997; 31(2): 112-122.

11. Knowles CH, Silk DB, Darzi A, Veress B, Feakins R, Raimundo AH, Crompton T, Browning EC, Lindberg G, Martin JE. Deranged smooth muscle alpha-actin as a biomarker of intestinal pseudo-obstruction: a controlled multinational case series. Gut 2004; 53(11): 1583-9.

12. Wedel T, van Eys G, Glènisson W, Waltregny D, Vanderwinden JM. Novel smooth muscle markers reveal abnormalities of the intestinal musculature in severe colorectal motility disorders. Neurogastroenterol Motil. 2006; 18: 526–538.

13. Huizinga, JD. Neural injury, repair, and adaptation in the GI tract. IV. Pathophysiology of GI motility related to interstitial cells of Cajal. Am J Physiol. 1998; 275(3 Pt1): G381-386.

14. He CL, Burgart L, Wang L, Pemberton J, Young-Fadok T, Szurszewski J, Farrugia G. Decreased interstitial cell of cajal volume in patients with slow-transit constipation. Gastroenterology 2000; 118(1): 14-21.

15. d G. Interstitial cells of Cajal in health and disease. Neurogastroenterol Motil. 2008; 20(S1): 54-63.

16. Wingate D, Hongo M, Kellow J, Lindberg G, Smout A. Disorders of gastrointestinal motility: towards a new classification. J Gastroenterol Hepatol. 2002; 17: S1-14.

17. ult TA, Pachnis V. Enteric nervous system development and Hirschsprung's disease: advances in genetic and stem cell studies. Nat Rev Neurosci 2007; 8(6): 466-479.

18. Hertz AF. Achalasia of the Cardia (so-called Cardio-spasm). Proc R Soc Med. 1915; 8(Clin Sect): 22-25.

19. Gyawali CP. Achalasia: new perspectives on an old disease. Neurogastroenterol Motil. 2016; 28(1): 4-11.

20. Shellito PC, Warshaw AL. Idiopathic intermittent gastroparesis and its surgical alleviation. Am J Surg. 1984; 148(3): 408-12.

21. Navas CM, Patel NK, Lacy BE. Gastroparesis: Medical and Therapeutic Advances. Dig Dis Sci. 2017; 62(9): 2231-2240.

22. Dudley HA, Sinclair IS, McLaren IF, McNair TJ, Newsam JE. Intestinal pseudo-obstruction. J R Coll Surg Edinb. 1958; 3(3): 206-217.

23. Stanghellini V, Camilleri M, Malagelada JR. Chronic idiopathic intestinal pseudo-obstruction: clinical and intestinal manometric findings. Gut. 1987; 28(1): 5-12.

24. De Giorgio R, Cogliandro RF, Barbara G, Corinaldesi R, Stanghellini V. Chronic intestinal pseudo-obstruction: clinical features, diagnosis, and therapy. Gastroenterol Clin North Am. 2011; 40(4): 787-807.

25. Di Nardo G, Di Lorenzo C, Lauro A, Stanghellini V, Thapar N, Karunaratne TB, Volta U, De Giorgio R. Chronic intestinal pseudo-obstruction in children and adults: diagnosis and therapeutic options. Neurogastroenterol Motil. 2017; 29(1).

26. Preston DM, Lennard-Jones JE. Severe chronic constipation of young women: 'idiopathic slow transit constipation'. Gut 1986; 27(1): 41-48.

27. Tillou J, Poylin V. Functional Disorders: Slow-Transit Constipation. Clin Colon Rectal Surg. 2017; 30(1): 76-86.

28. Bodian M, Stephens FD, Ward BC. Hirschsprung's disease and idiopathic megacolon. Lancet 1949; 1(6540): 6-11.

29. O'Dwyer RH, Acosta A, Camilleri M, Burton D, Busciglio I, Bharucha AE. Clinical Features and Colonic Motor Disturbances in Chronic Megacolon in Adults. Dig Dis Sci. 2015; 60(8): 2398-2407.

30. Sodhi N, Camilleri M, Camoriano JK, Low PA, Fealey RD, Perry MC. Autonomic function and motility in intestinal pseudoobstruction caused by paraneoplastic syndrome. Dig Dis Sci. 1989; 34(12): 1937-1942.

31. Lucchinetti CF, Kimmel DW, Lennon VA. Paraneoplastic and oncologic profiles of patients seropositive for type 1 antineuronal nuclear autoantibodies. Neurology 1998; 50(3): 652-657.

32. Badari A, Farolino D, Nasser E, Mehboob S, Crossland D. A novel approach to paraneoplastic intestinal pseudo-obstruction. Support Care Cancer. 2012; 20(2):425-428.

33. Gerl A, Storck M, Schalhorn A, Müller-Höcker J, Jauch KW, Schildberg FW, Wilmanns W. Paraneoplastic chronic intestinal pseudoobstruction as a rare complication of bronchial carcinoid. Gut 1992; 33(7): 1000-1003.

34. Pande R, Leis AA. Myasthenia gravis, thymoma, intestinal pseudoobstruction, and neuronal nicotinic acetylcholine receptor antibody. Muscle Nerve 1999; 22(11): 1600-1602.

35. Schobinger-Clement S, Gerber HA, Stallmach T. Autoaggressive inflammation of the myenteric plexus resulting in intestinal pseudoobstruction. Am J Surg Pathol. 1999; 23(5): 602-606.

36. Martin A, Messineo A, Lionetti P, Volta U, Stanghellini V, Barbara G, Cogliandro R, Corinaldesi R, De Giorgio R. A case of paraneoplastic inflammatory neuropathy of the gastrointestinal tract related to an underlying neuroblastoma: successful management with immunosuppressive therapy. J Pediatr Gastroenterol Nutr. 2008; 46(4): 457-460.

37. Lee HR, Lennon VA, Camilleri M, Prather CM. Paraneoplastic gastrointestinal motor dysfunction: clinical and laboratory characteristics. Am J Gastroenterol. 2001; 96(2): 373-379.

38. Ferreira-Santos R. Aperistalsis of the esophagus and colon (megaesophagus and megacolon) etiologically related to Chagas' disease. Am J Dig Dis 1961; 6: 700-726.

39. Pérez-Molina JA, Molina I. Chagas disease. Lancet. 2017; pii: S0140-6736(17): 31612-31614.

40. Treacy WL, baggenstoss AH, slocumb CH, code CF. Scleroderma of the esophagus. A correlation of histologic and physiologic findings. Ann Intern Med. 1963; 59: 351-356.

41. Cohen S, Fisher R, Lipshutz W, Turner R, Myers A, Schumacher R. The pathogenesis of esophageal dysfunction in scleroderma and Raynaud's disease. J Clin Invest 1972; 51(10): 2663-2668.

42. Battle WM, Snape WJ Jr, Wright S, Sullivan MA, Cohen S, Meyers A, Tuthill R. Abnormal colonic motility in progressive systemic sclerosis. Ann Intern Med 1981; 94(6): 749-752.

43. Kashyap P, Farrugia G. Diabetic gastroparesis: what we have learned and had to unlearn in the past 5 years. Gut. 2010; 59(12): 1716-1726.

44. Legge DA, Wollaeger EE, Carlson HC. Intestinal pseudo-obstruction in systemic amyloidosis. Gut 1970; 11(9): 764-767.

45. Liapis K, Michelis FV, Delimpasi S, Karmiris T. Intestinal pseudo-obstruction associated with amyloidosis. Amyloid. 2011; 18(2):76-78.

46. Furness JB. The enteric nervous system and neurogastroenterology. Nat Rev Gastroenterol Hepatol 2012; 9(5): 286-294.

47. Costa M, Brookes SH. Architecture of enteric neural circuits involved in intestinal motility. Eur Rev Med Pharmacol Sci 2008; 12(S1): 3-19.

48. Furness JB, Bornstein JC, Smith TK. The normal structure of gastrointestinal innervation. J Gastroenterol Hepatol. 1990; 5(S1): 1-9.

49. Brehmer A, Stach W, Krammer HJ, Neuhuber W. Distribution, morphology and projections of nitrergic and non-nitrergic submucosal neurons in the pig small intestine. Histochem Cell Biol. 1998; 109(1): 87-94.

50. Furness JB. The enteric nervous system. 2006. Oxford, Blackwell.

51. Vittoria A, Costagliola A, Carrese E, Mayer B, Cecio A. Nitric oxide-containing neurons in the bovine gut, with special reference to their relationship with VIP and galanin. Arch Histol Cytol. 2000; 63(4): 357-368.

52. Domeneghini C, Radaelli G, Arrighi S, Bosi G, Dolera M. Cholinergic, nitrergic and peptidergic (Substance P- and CGRP-utilizing) innervation of the horse intestine. A histochemical and immunohistochemical study. Histol Histopathol. 2004; 19(2): 357-370.

53. Chiocchetti R, Bombardi C, Mongardi Fantaguzzi C, Russo D, Venturelli E, Montoneri C, Spadari A, Romagnoli N, Grandis A. Intrinsic innervation of the ileocaecal junction in the horse: preliminary study. Equine Vet J. 2009; 41(8): 759-764.

54. Irwin DA. The anatomy of Auerbach’s plexus. Am. J. Anat. 1931; 49: 141-166.

55. Scheuermann DW, Stach W, Timmermans JP. Three-dimensional organization and topographical features of the myenteric plexus (Auerbach) in the porcine small intestine: scanning electron microscopy after enzymatic digestion and HCl-hydrolysis. Acta Anat (Basel). 1986; 127(4): 290-295.

56. Gabella G. The number of neurons in the small intestine of mice, guinea-pigs and sheep. Neuroscience. 1987; 22(2): 737-752.

57. Santer RM, Baker DM. Enteric neuron numbers and sizes in Auerbach's plexus in the small and large intestine of adult and aged rats. J Auton Nerv Syst. 1988; 25(1): 59-67.

58. Pearson GT. Structural organization and neuropeptide distributions in the equine enteric nervous system: an immunohistochemical study using whole-mount preparations from the small intestine. Cell Tissue Res. 1994; 276(3): 523-534.

59. Freytag C, Seeger J, Siegemund T, Grosche J, Grosche A, Freeman DE, Schusser GF, Härtig W. Immunohistochemical characterization and quantitative analysis of neurons in the myenteric plexus of the equine intestine. Brain Res. 2008; 1244: 53-64.

60. Bódi N, Battonyai I, Talapka P, Fekete E, Bagyánszki M. Spatial pattern analysis of nitrergic neurons in the myenteric plexus of the duodenum of different mammalian species. Acta Biol Hung. 2009; 60(4): 347-358.

61. Balemba OB, Mbassa GK, Semuguruka WD, Assey RJ, Kahwa CK, Hay-Schmidt A, Dantzer V. The topography, architecture and structure of the enteric nervous system in the jejunum and ileum of cattle. J Anat. 1999; 195(P1): 1-9.

62. Lefebvre RA, Smits GJ, Timmermans JP. Study of NO and VIP as non-adrenergic non-cholinergic neurotransmitters in the pig gastric fundus. Br J Pharmacol. 1995; 116(3): 2017-2026.

63. van Ginneken C, Weyns A, van Meir F, Ooms L, Verhofstad A. Intrinsic innervation of the stomach of the fetal pig: an immunohistochemical study of VIP-immunoreactive nerve fibres and cell bodies. Anat Histol Embryol. 1996; 25(4): 269-275.

64. Teixeira AF, Vives P, Krammer HJ, Kühnel W, Wedel T. Structural organization of the enteric nervous system in the cattle esophagus revealed by wholemount immunohistochemistry. Ital J Anat Embryol. 2001; 106(2 S1):313-321.

65. Izumi N, Matsuyama H, Yamamoto Y, Atoji Y, Suzuki Y, Unno T, Takewaki T. Morphological and morphometrical characteristics of the esophageal intrinsic nervous system in the golden hamster. Eur J Morphol. 2002; 40(3): 137-144.

66. Timmermans JP, Hens J, Adriaensen D. Outer submucous plexus: an intrinsic nerve network involved in both secretory and motility processes in the intestine of large mammals and humans. Anat Rec. 2001; 262(1): 71-78.

67. Brehmer A, Rupprecht H, Neuhuber W. Two submucosal nerve plexus in human intestines. Histochem Cell Biol. 2010; 133(2): 149-161.

68. Schabadasch A. Intramurale Nervengefl echte des Darmrohrs. Z. Zellforsch. 1930; 10: 254-319.

69. Gunn M. Histological and histochemical observations on the myenteric and submucous plexuses of mammals. J Anat. 1968; 102(Pt 2): 223-239.

70. Messenger JP, Furness JB. Projections of chemically-specified neurons in the guinea-pig colon. Arch Histol Cytol. 1990; 53(5): 467-495.

71. Timmermans JP, Scheuermann DW, Barbiers M, Adriaensen D, Stach W, Van Hee R, De Groodt-Lasseel MH. Calcitonin gene-related peptide-like immunoreactivity in the human small intestine. Acta Anat (Basel). 1992; 143(1): 48-53.

72. Timmermans JP, Scheuermann DW, Stach W, Adriaensen D, De Groodt-Lasseel MH. Functional morphology of the enteric nervous system with special reference to large mammals. Eur J Morphol. 1992; 30(2): 113-122.

73. Timmermans JP, Adriaensen D, Cornelissen W, Scheuermann DW. Structural organization and neuropeptide distribution in the mammalian enteric nervous system, with special attention to those components involved in mucosal reflexes. Comp Biochem Physiol A Physiol. 1997; 118(2): 331-340.

74. Christensen J, Rick GA. Intrinsic nerves in the mammalian colon: confirmation of a plexus at the circular muscle-submucosal interface. J Auton Nerv Syst. 1987; 21(2-3): 223-231.

75. Hoyle CH, Burnstock G. Neuronal populations in the submucous plexus of the human colon. J Anat. 1989; 166: 7-22.

76. Balemba OB, Grøndahl ML, Mbassa GK, Semuguruka WD, Hay-Smith A, Skadhauge E, Dantzer V. The organisation of the enteric nervous system in the submucous and mucous layers of the small intestine of the pig studied by VIP and neurofilament protein immunohistochemistry. J Anat. 1998;192 (Pt 2): 257-267.

77. Stach W. Differentiated vascularization of the Dogiel cell types and the preferred vascularization of type I cells in the ganglia of plexus submucosus externus (Schabadasch) of the swine. Z Mikrosk Anat Forsch. 1977; 91(3): 421-429.

78. Stach W. The external submucous plexus (Schabadasch) in the small intestine of the swine. I. Form, structure and connections of ganglia and nerve cells. Z Mikrosk Anat Forsch. 1977; 91(4): 737-755.

79. Stach W. Structure of neurons and architecture in the plexus submucosus externus (Schabadasch) of the duodenum. Verh Anat Ges. 1977; 71(Pt 2): 867-871.

80. Stach W. The vascularization of the submucous external plexus (Schabadasch) and the submucous internal plexus (Meissner) in the small intestine of swine and cat. Acta Anat (Basel). 1978; 101(2): 170-178.

81. Scheuermann DW, Stach W. Fluorescence microscopic study of the architecture and structure of an adrenergic network in the plexus myentericus (Auerbach), plexus submucosus externus (Schabadasch) and plexus submucosus internus (Meissner) of the porcine small intestine. Acta Anat (Basel). 1984; 119(1): 49-59.

82. Scheuermann DW, Stach W, Timmermans JP. Topography, architecture and structure of the plexus submucosus internus (Meissner) of the porcine small intestine in scanning electron microscopy. Acta Anat (Basel). 1987; 129(2): 96-104.

83. Timmermans JP, Scheuermann DW, Stach W, Adriaensen D, De Groodt-Lasseel MH. Distinct distribution of CGRP-, enkephalin-, galanin-, neuromedin U-, neuropeptide Y-, somatostatin-, substance P-, VIP- and serotonin-containing neurons in the two submucosal ganglionic neural networks of the porcine small intestine. Cell Tissue Res. 1990; 260(2): 367-379.

84. Scheuermann DW, Stach W, Timmermans JP 1988 Morphology and immunocytochemistry of the enteric nervous system in the porcine small intestine. Acta Gastroenterol Belg 1988; 51(Pt 2): (A3).

85. Hens J, Schrödl F, Brehmer A, Adriaensen D, Neuhuber W, Scheuermann DW, Schemann M, Timmermans JP. Mucosal projections of enteric neurons in the porcine small intestine. J Comp Neurol. 2000; 421(3): 429-436.

86. Sanders KM, Smith TK. Motoneurones of the submucous plexus regulate electrical activity of the circular muscle of canine proximal colon. J Physiol. 1986; 380: 293-310.

87. Timmermans JP, Barbiers M, Scheuermann DW, Stach W, Adriaensen D, Mayer B, De Groodt-Lasseel MH. Distribution pattern, neurochemical features and projections of nitrergic neurons in the pig small intestine. Ann Anat. 1994; 176(6): 515-525.

88. Porter AJ, Wattchow DA, Brookes SJ, Costa M. Projections of nitric oxide synthase and vasoactive intestinal polypeptide-reactive submucosal neurons in the human colon. J Gastroenterol Hepatol. 1999; 14(12): 1180-1187.

89. Costa M, Brookes SJ, Steele PA, Gibbins I, Burcher E, Kandiah CJ. Neurochemical classification of myenteric neurons in the guinea-pig ileum. Neuroscience. 1996; 75(3): 949-967.

90. Dogiel A. Über den Bau der Ganglien in den Geflechten des Darmes und der Gallenblase des Menschen und der Sa¨ugetiere. Arch Anat Physiol (Leipzig) (Anat Abt Jg) 1899; 130-158.

91. Stach W. A revised morphological classification of neurons in the enteric nervous system. In: Singer MV, Goebell H (Eds) Nerves and the gastrointestinal tract. MTP Press Dordrecht;1989: 29-45.

92. Brehmer A, Schrodl F, Neuhuber W. Morphological classifications of enteric neurons--100 years after Dogiel. Anat Embryol (Berl). 1999; 200(2): 125-135.

93. Stach W, Krammer HJ, Brehmer A. Structural organization of enteric nerve cells in large mammals including man. in: Krammer HJ, Singer MV (Eds.) Neurogastroenterology from the Basics to the Clinics. Kluwer Academic Publishing, 2000: 3-20.

94. Brehmer A, Schrödl F, Neuhuber W. Morphological phenotyping of enteric neurons using neurofilament immunohistochemistry renders chemical phenotyping more precise in porcine ileum. Histochem Cell Biol. 2002; 117(3): 257-263.

95. Brehmer A, Schrödl F, Neuhuber W, Tooyama I, Kimura H. Co-expression pattern of neuronal nitric oxide synthase and two variants of choline acetyltransferase in myenteric neurons of porcine ileum. J Chem Neuroanat. 2004; 27(1): 33-341.

96. Furness JB, Koopmans HS, Robbins HL, Lin HC. Identification of intestinofugal neurons projecting to the coeliac and superior mesenteric ganglia in the rat. Auton Neurosci. 2000; 83(1-2): 81-85.

97. Furness JB. Intestinofugal neurons and sympathetic reflexes that bypass the central nervous system. J Comp Neurol. 2003; 455(3): 281-284.

98. Wilson AJ, Llewellyn-Smith IJ, Furness JB, Costa M. The source of the nerve fibres forming the deep muscular and circular muscle plexuses in the small intestine of the guinea-pig. Cell Tissue Res. 1987; 247(3): 497-504.

99. Song ZM, Brookes SJ, Ramsay GA, Costa M. Characterization of myenteric interneurons with somatostatin immunoreactivity in the guinea-pig small intestine. Neuroscience. 1997; 80(3): 907-923.

100. Hens J, Vanderwinden JM, De Laet MH, Scheuermann DW, Timmermans JP. Morphological and neurochemical identification of enteric neurones with mucosal projections in the human small intestine. J Neurochem. 2001; 76(2): 464-471.

101. Brookes SJ, Steele PA, Costa M. Identification and immunohistochemistry of cholinergic and non-cholinergic circular muscle motor neurons in the guinea-pig small intestine. Neuroscience. 1991; 42(3): 863-878.

102. Michel K, Reiche D, Schemann M. Projections and neurochemical coding of motor neurones to the circular and longitudinal muscle of the guinea pig gastric corpus. Pflugers Arch. 2000; 440(3): 393-408.

103. Brookes SJ, Song ZM, Steele PA, Costa M. Identification of motor neurons to the longitudinal muscle of the guinea pig ileum. Gastroenterology. 1992; 103(3): 961-973.

104. Brookes SJ, Chen BN, Hodgson WM, Costa M. Characterization of excitatory and inhibitory motor neurons to the guinea pig lower esophageal sphincter. Gastroenterology. 1996; 111(1): 108-117.

105. Brookes SJ, Meedeniya AC, Jobling P, Costa M. Orally projecting interneurones in the guinea-pig small intestine. J Physiol. 1997; 505(Pt2): 473-491.

106. Pfannkuche H, Reiche D, Sann H, Schemann M. Different subpopulations of cholinergic and nitrergic myenteric neurones project to mucosa and circular muscle of the guinea-pig gastric fundus. Cell Tissue Res. 1998; 292(3): 463-475.

107. Yuan S, Brookes SJ. Neuronal control of the gastric sling muscle of the guinea pig. J Comp Neurol. 1999; 412(4): 669-680.

108. Yuan S, Costa M, Brookes SJ. Neuronal pathways and transmission to the lower esophageal sphincter of the guinea Pig. Gastroenterology. 1998; 115(3): 661-671.

109. Brookes SJ. Classes of enteric nerve cells in the guinea-pig small intestine. Anat Rec. 2001; 262(1): 58-70.

110. Vassileva P, Stoyanov I, Loukanov Y. Neurotransmitted responses of smooth-muscle strips of complex sheep stomach after electrical field stimulation. Acta Physiol Pharmacol Bulg. 1978; 4(1): 11-18.

111. Veenendaal GH, Woutersen-van Nijnanten FM, Van Miert AS. Responses of goat ruminal musculature to substance P in vitro and in vivo. Vet Res Commun. 1982 Nov;5(4):363-367.

112. Wong MH, McLeay LM. In vitro spontaneous motility of gastric smooth muscles of the sheep. Q J Exp Physiol. 1988; 73(4): 521-531.

113. Bornstein JC, Costa M, Grider JR. Enteric motor and interneuronal circuits controlling motility. Neurogastroenterol Motil. 2004; 16(S1): 34-38.

114. Yamanaka K, Furukawa K, Kitamura K. The different mechanisms of action of nicorandil and adenosine triphosphate on potassium channels of circular smooth muscle of the guinea-pig small intestine. Naunyn Schmiedebergs Arch Pharmacol. 1985; 331(1): 96-103.

115. Bian X, Bertrand PP, Bornstein JC. Descending inhibitory reflexes involve P2X receptor-mediated transmission from interneurons to motor neurons in guinea-pig ileum. J Physiol. 2000; 528(Pt 3): 551-560.

116. Fahrenkrug J, Emson PC. Vasoactive intestinal polypeptide: functional aspects. Br Med Bull. 1982; 38(3): 265-270.

117. Costa M, Furness JB, Pompolo S, Brookes SJ, Bornstein JC, Bredt DS, Snyder SH. Projections and chemical coding of neurons with immunoreactivity for nitric oxide synthase in the guinea-pig small intestine. Neurosci Lett. 1992; 148(1-2): 121-125.

118. Furness JB, Bornstein JC, Murphy R, Pompolo S. Roles of peptides in transmission in the enteric nervous system. Trends Neurosci. 1992; 15(2): 66-71.

119. Aimi Y, Kimura H, Kinoshita T, Minami Y, Fujimura M, Vincent SR. Histochemical localization of nitric oxide synthase in rat enteric nervous system. Neuroscience. 1993; 53(2): 553-560.

120. Grider JR. Interplay of VIP and nitric oxide in regulation of the descending relaxation phase of peristalsis. Am J Physiol. 1993; 264(2 Pt 1): G334-340.

121. Barbiers M, Timmermans JP, Scheuermann DW, Adriaensen D, Mayer B, De Groodt-Lasseel MH. Nitric oxide synthase-containing neurons in the pig large intestine: topography, morphology, and viscerofugal projections. Microsc Res Tech. 1994; 29(2): 72-78.

122. Timmermans JP, Barbiers M, Scheuermann DW, Bogers JJ, Adriaensen D, Fekete E, Mayer B, Van Marck EA, De Groodt-Lasseel MH. Nitric oxide synthase immunoreactivity in the enteric nervous system of the developing human digestive tract. Cell Tissue Res. 1994; 275(2): 235-245.

123. Lomax AE, Zhang JY, Furness JB. Origins of cholinergic inputs to the cell bodies of intestinofugal neurons in the guinea pig distal colon. J Comp Neurol. 2000; 416(4): 451-460.

124. Münnich J, Gäbel G, Pfannkuche H. Intrinsic ruminal innervation in ruminants of different feeding types. J Anat. 2008; 213(4): 442-451.

125. Grider JR, Katsoulis S, Schmidt WE, Jin JG. Regulation of the descending relaxation phase of intestinal peristalsis by PACAP. J Auton Nerv Syst. 1994; 50(2): 151-159.

126. Ny L, Larsson B, Alm P, Ekström P, Fahrenkrug J, Hannibal J, Andersson KE. Distribution and effects of pituitary adenylate cyclase activating peptide in cat and human lower oesophageal sphincter. Br J Pharmacol. 1995; 116(7): 2873-280.

127. De Backer O, Lefebvre RA. Mechanisms of relaxation by carbon monoxide-releasing molecule-2 in murine gastric fundus and jejunum. Eur J Pharmacol. 2007; 572(2-3): 197-206.

128. Hasler WL, Kurosawa S, Chung OY. Regional cholinergic differences between distal and proximal colonic myenteric plexus. Am J Physiol. 1990; 258(3 Pt 1): G404-410.

129. Takahashi T, Owyang C. Regional differences in the nitrergic innervation between the proximal and the distal colon in rats. Gastroenterology. 1998; 115(6): 1504-1512.

130. Freytag C, Seeger J, Siegemund T, Grosche J, Grosche A, Freeman DE, Schusser GF, Härtig W. Immunohistochemical characterization and quantitative analysis of neurons in the myenteric plexus of the equine intestine. Brain Res. 2008; 1244: 53-64.

131. Wattchow D, Brookes S, Murphy E, Carbone S, de Fontgalland D, Costa M. Regional variation in the neurochemical coding of the myenteric plexus of the human colon and changes in patients with slow transit constipation. Neurogastroenterol Motil. 2008; 20(12): 1298-1305.

132. Brown DR, Timmermans JP. Lessons from the porcine enteric nervous system. Neurogastroenterol Motil. 2004; 16(S1): 50-54.

133. Song ZM, Brookes SJ, Costa M. Identification of myenteric neurons which project to the mucosa of the guinea-pig small intestine. Neurosci Lett. 1991; 129(2): 294-298.

134. Angel F, Go VL, Szurszewski JH. Innervation of the muscularis mucosae of canine proximal colon. J Physiol. 1984; 357: 93-108.

135. Steele PA, Costa M. Opioid-like immunoreactive neurons in secretomotor pathways of the guinea-pig ileum. Neuroscience. 1990; 38(3): 771-786.

136. Messenger JP. Immunohistochemical analysis of neurons and their projections in the proximal colon of the guinea-pig. Arch Histol Cytol. 1993; 56(5): 459-474.

137. Holzer P, Holzer-Petsche U. Tachykinins in the gut. Part I. Expression, release and motor function. Pharmacol Ther. 1997; 73(3): 173-217.

138. Costa M, Furness JB, Llewellyn-Smith IJ, Cuello AC. Projections of substance P-containing neurons within the guinea-pig small intestine. Neuroscience. 1981; 6(3): 411-424.

139. Brodin E, Sjölund K, Håkanson R, Sundler F. Substance P-containing nerve fibers are numerous in human but not in feline intestinal mucosa. Gastroenterology. 1983; 85(3): 557-564.

140. Domeneghini C, Radaelli G, Arrighi S, Bosi G, Dolera M. Cholinergic, nitrergic and peptidergic (Substance P- and CGRP-utilizing) innervation of the horse intestine. A histochemical and immunohistochemical study. Histol Histopathol. 2004; 19(2): 357-370.

141. Mitsui R. Immunohistochemical characteristics of submucosal Dogiel type II neurons in rat colon. Cell Tissue Res. 2010; 340(2): 257-265.

142. Brown DR, Miller RJ. Neurohormonal control of fluid and electrolyte transport in intestinal mucosa. Handbook of Physiology. 1991. S. G. Schultze. Baltimore, Waverly Press. The gastrointestinal system IV: 527-589.

143. Wood JD. Cellular neurophysiology of enteric neurons. Physiology of the gastrointestinal tract, 4 edition. 2006. L. R. Johnson, Academic Press. 1: 629-664.

144. Keast JR, Furness JB, Costa M. Somatostatin in human enteric nerves. Distribution and characterization. Cell Tissue Res. 1984; 237(2): 299-308.

145. Dharmsathaphorn K, Sherwin RS, Dobbins JW. Somatostatin inhibits fluid secretion in the rat jejunum. Gastroenterology. 1980; 78(6): 1554-1558.

146. Sandle GI, Warhurst G, Butterfield I, Higgs NB, Lomax RB. Somatostatin peptides inhibit basolateral potassium channels in human colonic crypts. Am J Physiol. 1999; 277(5 Pt 1): G967-975.

147. Dharmsathaphorn K, Mandel KG, Masui H, McRoberts JA. Vasoactive intestinal polypeptide-induced chloride secretion by a colonic epithelial cell line. Direct participation of a basolaterally localized Na+,K+,Cl- cotransport system. J Clin Invest. 1985; 75(2): 462-471.

148. Cooke HJ. Neurobiology of the intestinal mucosa. Gastroenterology. 1986; 90(4): 1057-1081.

149. Weber M, Cole T, Conlon JM. Specific binding and degradation of somatostatin by membrane vesicles from pig gut. Am J Physiol. 1986; 250(5 Pt 1): G679-685.

150. Beyer J, Jabari S, Rau TT, Neuhuber W, Brehmer A. Substance P- and choline acetyltransferase immunoreactivities in somatostatin-containing, human submucosal neurons. Histochem Cell Biol. 2013; 140(2): 157-167.

151. Cummings JF, Sellers AF, Lowe JE. Distribution of substance P-like immunoreactivity in the enteric neurons of the large colon of normal and amitraz-treated ponies: an immunocytochemical study. Equine Vet J. 1985; 17(1): 23-29.

152. Burns GA, Cummings JF. Neuropeptide distributions in the colon, cecum, and jejunum of the horse. Anat Rec. 1993; 236(2): 341-350.

153. Chiocchetti R, Bombardi C, Mongardi-Fantaguzzi C, Venturelli E, Russo D, Spadari A, Montoneri C, Romagnoli N, Grandis A. Intrinsic innervation of the horse ileum. Res Vet Sci. 2009; 87(2): 177-185.

154. Pompolo S, Furness JB. Origins of synaptic inputs to calretinin immunoreactive neurons in the guinea-pig small intestine. J Neurocytol. 1993; 22(7): 531-546.

155. Stebbing MJ, Bornstein JC. Electrophysiological mapping of fast excitatory synaptic inputs to morphologically and chemically characterized myenteric neurons of guinea-pig small intestine. Neuroscience. 1996; 73(4): 1017-1028.

156. Spencer NJ, Dickson EJ, Hennig GW, Smith TK. Sensory elements within the circular muscle are essential for mechanotransduction of ongoing peristaltic reflex activity in guinea-pig distal colon. J Physiol. 2006 Oct; 576(Pt 2): 519-531.

157. Dickson EJ, Spencer NJ, Hennig GW, Bayguinov PO, Ren J, Heredia DJ, Smith TK. An enteric occult reflex underlies accommodation and slow transit in the distal large bowel. Gastroenterology. 2007; 132(5): 1912-1924.

158. Smith TK, Spencer NJ, Hennig GW, Dickson EJ. Recent advances in enteric neurobiology: mechanosensitive interneurons. Neurogastroenterol Motil. 2007; 19(11): 869-878.

159. Portbury AL, Pompolo S, Furness JB, Stebbing MJ, Kunze WA, Bornstein JC, Hughes S. Cholinergic, somatostatin-immunoreactive interneurons in the guinea pig intestine: morphology, ultrastructure, connections and projections. J Anat. 1995; 187 (Pt 2): 303-321.

160. Uemura S, Pompolo S, Furness JB. Colocalization of neuropeptide Y with other neurochemical markers in the guinea-pig small intestine. Arch Histol Cytol. 1995; 58(5): 523-536.

161. Song ZM, Brookes SJ, Costa M. Characterization of alkaline phosphatase-reactive neurons in the guinea-pig small intestine. Neuroscience. 1994; 63(4): 1153-1167.

162. Neal KB, Bornstein JC. Mapping 5-HT inputs to enteric neurons of the guinea-pig small intestine. Neuroscience. 2007; 145(2): 556-567.

163. Porter AJ, Wattchow DA, Brookes SJ, Costa M. Cholinergic and nitrergic interneurones in the myenteric plexus of the human colon. Gut. 2002; 51(1): 70-75.

164. Furness JB, Jones C, Nurgali K, Clerc N. Intrinsic primary afferent neurons and nerve circuits within the intestine. Prog Neurobiol. 2004; 72(2): 143-164.

165. Bornstein JC, Furness JB, Costa M. An electrophysiological comparison of substance P-immunoreactive neurons with other neurons in the guinea-pig submucous plexus. J Auton Nerv Syst. 1989; 26(2): 113-120.

166. Kirchgessner AL, Tamir H, Gershon MD. Identification and stimulation by serotonin of intrinsic sensory neurons of the submucosal plexus of the guinea pig gut: activity-induced expression of Fos immunoreactivity. J Neurosci. 1992; 12(1): 235-248.

167. Song ZM, Brookes SJ, Steele PA, Costa M. Projections and pathways of submucous neurons to the mucosa of the guinea-pig small intestine. Cell Tissue Res. 1992; 269(1): 87-98.

168. Evans RJ, Jiang MM, Surprenant A. Morphological properties and projections of electrophysiologically characterized neurons in the guinea-pig submucosal plexus. Neuroscience. 1994; 59(4): 1093-1110.

169. Furness JB, Callaghan BP, Rivera LR, Cho HJ. The enteric nervous system and gastrointestinal innervation: integrated local and central control. Adv Exp Med Biol. 2014; 817: 39-371.

170. Kunze WA, Bornstein JC, Furness JB. Identification of sensory nerve cells in a peripheral organ (the intestine) of a mammal. Neuroscience. 1995; 66(1): 1-4.

171. Bertrand PP, Kunze WA, Bornstein JC, Furness JB, Smith ML. Analysis of the responses of myenteric neurons in the small intestine to chemical stimulation of the mucosa. Am J Physiol. 1997; 273(2 Pt 1): G422-435.

172. Kunze WA, Furness JB, Bertrand PP, Bornstein JC. Intracellular recording from myenteric neurons of the guinea-pig ileum that respond to stretch. J Physiol. 1998; 506(Pt 3): 827-842.

173. Kunze WA, Clerc N, Bertrand PP, Furness JB. Contractile activity in intestinal muscle evokes action potential discharge in guinea-pig myenteric neurons. J Physiol. 1999; 517(Pt 2): 547-561.

174. Furness JB, Keast JR, Pompolo S, Bornstein JC, Costa M, Emson PC, Lawson DE. Immunohistochemical evidence for the presence of calcium-binding proteins in enteric neurons. Cell Tissue Res. 1988; 252(1): 79-87.

175. Iyer V, Bornstein JC, Costa M, Furness JB, Takahashi Y, Iwanaga T. Electrophysiology of guinea-pig myenteric neurons correlated with immunoreactivity for calcium binding proteins. J Auton Nerv Syst. 1988; 22(2): 141-150.

176. Chiocchetti R, Poole DP, Kimura H, Aimi Y, Robbins HL, Castelucci P, Furness JB. Evidence that two forms of choline acetyltransferase are differentially expressed in subclasses of enteric neurons. Cell Tissue Res. 2003; 311(1): 11-22.

177. Van Nassauw L, Wu M, De Jonge F, Adriaensen D, Timmermans JP. Cytoplasmic, but not nuclear, expression of the neuronal nuclei (NeuN) antibody is an exclusive feature of Dogiel type II neurons in the guinea-pig gastrointestinal tract. Histochem Cell Biol. 2005; 124(5): 369-377.

178. Steele PA, Brookes SJ, Costa M. Immunohistochemical identification of cholinergic neurons in the myenteric plexus of guinea-pig small intestine. Neuroscience. 1991; 45(1): 227-239.

179. Li ZS, Furness JB. Immunohistochemical localisation of cholinergic markers in putative intrinsic primary afferent neurons of the guinea-pig small intestine. Cell Tissue Res. 1998; 294(1): 35-43.

180. Quinson N, Robbins HL, Clark MJ, Furness JB. Calbindin immunoreactivity of enteric neurons in the guinea-pig ileum. Cell Tissue Res. 2001; 305(1): 3-9.

181. Brehmer A, Croner R, Dimmler A, Papadopoulos T, Schrödl F, Neuhuber W. Immunohistochemical characterization of putative primary afferent (sensory) myenteric neurons in human small intestine. Auton Neurosci. 2004; 112(1-2): 49-59.

182. Dénes V, Gábriel R. Calbindin-immunopositive cells are cholinergic interneurons in the myenteric plexus of rabbit ileum. Cell Tissue Res. 2004; 318(2): 465-472.

183. Scheuermann DW, Krammer HJ, Timmermans JP, Stach W, Adriaensen D, De Groodt-Lasseel MH. Fine structure of morphologically well-defined type II neurons in the

enteric nervous system of the porcine small intestine revealed by immunoreactivity for calcitonin gene-related peptide. Acta Anat (Basel). 1991; 142(3): 236-241.

184. Wolf M, Schrödl F, Neuhuber W, Brehmer A. Calcitonin gene-related peptide: a marker for putative primary afferent neurons in the pig small intestinal myenteric plexus? Anat Rec (Hoboken). 2007; 290(10): 1273-1279.

185. Krammer HJ, Kühnel W. Immunohistochemistry for intermediate filaments in the enteric nervous system of the porcine small intestine. Ann Anat. 1992; 174(4): 275-278.

186. Szurszewski JH, Ermilov LG, Miller SM. Prevertebral ganglia and intestinofugal afferent neurones. Gut. 2002; 51(S1): i6-10.

187. Ermilov LG, Miller SM, Schmalz PF, Hanani M, Lennon VA, Szurszewski JH. Morphological characteristics and immunohistochemical detection of nicotinic acetylcholine receptors on intestinofugal afferent neurones in guinea-pig colon. Neurogastroenterol Motil. 2003; 15(3): 289-298.

188. Crowcroft PJ, Holman ME, Szurszewski JH. Excitatory input from the distal colon to the inferior mesenteric ganglion in the guinea-pig. J Physiol. 1971; 219(2): 443-461.

189. Szurszewski JH, Weems WA. A study of peripheral input to and its control by post-ganglionic neurones of the inferior mesenteric ganglion. J Physiol. 1976; 256(3): 541-556.

190. Kreulen DL, Szurszewski JH. Reflex pathways in the abdominal prevertebral ganglia: evidence for a colo-colonic inhibitory reflex. J Physiol. 1979; 295: 21-32.

191. Szurszewski JH, Miller SM. Physiology of prevertebral ganglia. Physiology of the Gastrointestinal Tract. 1994. J. LR. New York, Raven Press: 795-877.

192. Parkman HP, Ma RC, Stapelfeldt WH, Szurszewski JH. Direct and indirect mechanosensory pathways from the colon to the inferior mesenteric ganglion. Am J Physiol. 1993; 265(3 Pt 1): G499-505.

193. Ma RC, Szurszewski JH. Modulation by opioid peptides of mechanosensory pathways supplying the guinea-pig inferior mesenteric ganglion. J Physiol. 1996; 491(Pt 2): 435-445.

194. Walter GC, Phillips RJ, McAdams JL, Powley TL. Individual sympathetic postganglionic neurons coinnervate myenteric ganglia and smooth muscle layers in the gastrointestinal tract of the rat. J Comp Neurol. 2016; 524(13): 2577-2603.

195. Gabella G. Glial cells in the myenteric plexus. Z Naturforsch B. 1971; 26(3): 244-245.

196. Gabella G. Fine structure of the myenteric plexus in the guinea-pig ileum. J Anat. 1972; 111(Pt 1): 69-97.

197. Gabella G. Ultrastructure of the nerve plexuses of the mammalian intestine: the enteric glial cells. Neuroscience. 1981; 6(3): 425-436.

198. Jessen KR, Mirsky R. Astrocyte-like glia in the peripheral nervous system: an immunohistochemical study of enteric glia. J Neurosci. 1983; 3(11): 2206-2218.

199. Jessen KR, Mirsky R. Glial cells in the enteric nervous system contain glial fibrillary acidic protein. Nature. 1980; 286(5774): 736-737.

200. Ferri GL, Probert L, Cocchia D, Michetti F, Marangos PJ, Polak JM. Evidence for the presence of S-100 protein in the glial component of the human enteric nervous system. Nature. 1982; 297(5865): 409-410.

201. Cook RD, Burnstock G. The altrastructure of Auerbach's plexus in the guinea-pig. I. Neuronal elements. J Neurocytol. 1976; 5(2): 171-194.

202. Cook RD, Burnstock G. The ultrastructure of Auerbach's plexus in the guinea-pig. II. Non-neuronal elements. J Neurocytol. 1976; 5(2): 195-206.

203. Komuro T, Bałuk P, Burnstock G. An ultrastructural study of neurons and non-neuronal cells in the myenteric plexus of the rabbit colon. Neuroscience. 1982; 7(7): 1797-1806.

204. Baumgarten HG, Holstein AF, Owman C. Auerbach's plexus of mammals and man: electron microscopic identification of three different types of neuronal processes in myenteric ganglia of the large intestine from rhesus monkeys, guinea-pigs and man. Z Zellforsch Mikrosk Anat. 1970; 106(3): 376-397.

205. Gabella G, Trigg P. Size of neurons and glial cells in the enteric ganglia of mice, guinea-pigs, rabbits and sheep. J Neurocytol. 1984;13(1): 49-71.

206. Rühl A. Glial cells in the gut. Neurogastroenterol Motil. 2005; 17(6): 777-790.

207. Hoff S, Zeller F, von Weyhern CW, Wegner M, Schemann M, Michel K, Rühl A. Quantitative assessment of glial cells in the human and guinea pig enteric nervous system with an anti-Sox8/9/10 antibody. J Comp Neurol. 2008; 509(4): 356-371.

208. Hanani M, Zamir O, Baluk P. Glial cells in the guinea pig myenteric plexus are dye coupled. Brain Res. 1989; 497(2): 245-249.

209. Bannerman PG, Mirsky R, Jessen KR, Timpl R, Duance VC. Light microscopic immunolocalization of laminin, type IV collagen, nidogen, heparan sulphate proteoglycan and fibronectin in the enteric nervous system of rat and guinea pig. J Neurocytol. 1986; 15(6): 733-743.

210. De Giorgio R, Giancola F, Boschetti E, Abdo H, Lardeux B, Neunlist M. Enteric glia and neuroprotection: basic and clinical aspects. Am J Physiol Gastrointest Liver Physiol. 2012; 303(8): G887-893.

211. Boesmans W1, Lasrado R, Vanden Berghe P, Pachnis V. Heterogeneity and phenotypic plasticity of glial cells in the mammalian enteric nervous system. Glia. 2015; 63(2): 229-241.

212. Neunlist M, Van Landeghem L, Mahé MM, Derkinderen P, des Varannes SB, Rolli-Derkinderen M. The digestive neuronal-glial-epithelial unit: a new actor in gut health and disease. Nat Rev Gastroenterol Hepatol. 2013; 10(2): 90-100.

213. Gulbransen BD, Sharkey KA. Novel functional roles for enteric glia in the gastrointestinal tract. Nat Rev Gastroenterol Hepatol. 2012; 9(11): 625-632.

214. Neunlist M, Rolli-Derkinderen M, Latorre R, Van Landeghem L, Coron E, Derkinderen P, De Giorgio R. Enteric glial cells: recent developments and future directions. Gastroenterology. 2014; 147(6): 1230-1237.

215. Neunlist M, Van Landeghem L, Bourreille A, Savidge T. Neuro-glial crosstalk in inflammatory bowel disease. J Intern Med. 2008; 263(6): 577-583.

216. da Silveira AB1, de Oliveira EC, Neto SG, Luquetti AO, Fujiwara RT, Oliveira RC, Brehmer A. Enteroglial cells act as antigen-presenting cells in chagasic megacolon. Hum Pathol. 2011; 42(4): 522-532.

217. Steinkamp M, Geerling I, Seufferlein T, von Boyen G, Egger B, Grossmann J, Ludwig L, Adler G, Reinshagen M. Glial-derived neurotrophic factor regulates apoptosis in colonic epithelial cells. Gastroenterology. 2003; 124(7): 1748-1757.

218. Neunlist M, Aubert P, Bonnaud S, Van Landeghem L, Coron E, Wedel T, Naveilhan P, Ruhl A, Lardeux B, Savidge T, Paris F, Galmiche JP. Enteric glia inhibit intestinal epithelial cell proliferation partly through a TGF-beta1-dependent pathway. Am J Physiol Gastrointest Liver Physiol. 2007; 292(1): G231-241.

219. Savidge TC, Newman P, Pothoulakis C, Ruhl A, Neunlist M, Bourreille A, Hurst R, Sofroniew MV. Enteric glia regulate intestinal barrier function and inflammation via release of S-nitrosoglutathione. Gastroenterology. 2007; 132(4): 1344-1358.

220. Savidge TC, Sofroniew MV, Neunlist M. Starring roles for astroglia in barrier pathologies of gut and brain. Lab Invest. 2007; 87(8): 731-736.

221. Bouchard NC, Forde J, Hoffman RS. Carvedilol overdose with quantitative confirmation. Basic Clin Pharmacol Toxicol. 2008; 103(1): 102-103.

222. Van Landeghem L, Chevalier J, Mahé MM, Wedel T, Urvil P, Derkinderen P, Savidge T, Neunlist M. Enteric glia promote intestinal mucosal healing via activation of focal adhesion kinase and release of proEGF. Am J Physiol Gastrointest Liver Physiol. 2011; 300(6): G976-987.

223. Joseph NM, He S, Quintana E, Kim YG, Núñez G, Morrison SJ. Enteric glia are multipotent in culture but primarily form glia in the adult rodent gut. J Clin Invest. 2011; 121(9): 3398-3411.

224. Laranjeira C, Sandgren K, Kessaris N, Richardson W, Potocnik A, Vanden Berghe P, Pachnis V. Glial cells in the mouse enteric nervous system can undergo neurogenesis in response to injury. J Clin Invest. 2011; 121(9): 3412-3424.

225. Gomes P, Chevalier J, Boesmans W, Roosen L, van den Abbeel V, Neunlist M, Tack J, Vanden Berghe P. ATP-dependent paracrine communication between enteric neurons and glia in a primary cell culture derived from embryonic mice. Neurogastroenterol Motil. 2009; 21(8): 870-e62.

226. Gulbransen BD, Bains JS, Sharkey KA. Enteric glia are targets of the sympathetic innervation of the myenteric plexus in the guinea pig distal colon. J Neurosci. 2010; 30(19): 6801-6809.

227. Boesmans W, Cirillo C, Van den Abbeel V, Van den Haute C, Depoortere I, Tack J, Vanden Berghe P. Neurotransmitters involved in fast excitatory neurotransmission directly activate enteric glial cells. Neurogastroenterol Motil. 2013; 25(2): e151-60.

228. Otterson MF, Sarna SK. Neural control of small intestinal giant migrating contractions. Am J Physiol. 1994; 266(4 Pt 1): G576-584.

229. Bayliss WM, Starling EH. The movements and innervation of the small intestine. J Physiol. 1899; 24(2): 99-143.

230. Bayliss WM, Starling EH. The movements and the innervation of the large intestine. J Physiol. 1900; 26(1-2): 107-118.

231. Trendelenburg P. Physiologische und pharmakologische Versuche über die Dünndarmperistaltik. Arch. Exp. Pathol. Pharmakol. 1917; 81: 55-129.

232. Robinette ML, Colonna M. GI motility: microbiota and macrophages join forces. Cell. 2014; 158(2): 239-240.

233. Camilleri M, Hasler WL, Parkman HP, Quigley EM, Soffer E. Measurement of gastrointestinal motility in the GI laboratory. Gastroenterology. 1998; 115(3): 747-762.

234. Hansen MB. Small intestinal manometry. Physiol Res. 2002; 51(6): 541-556.

235. Kahrilas PJ, Sifrim D. High-resolution manometry and impedance-pH/manometry: Valuable tools in clinical and investigational esophagology. Gastroenterology. 2008; 135(3): 756-769.

236. Jones MP, Post J, Crowell MD. High-resolution manometry in the evaluation of anorectal disorders: a simultaneous comparison with water-perfused manometry. Am J Gastroenterol. 2007; 102(4): 850-855.

237. Dinning PG, Wiklendt L, Maslen L, Patton V, Lewis H, Arkwright JW, Wattchow DA, Lubowski DZ, Costa M, Bampton PA. Colonic motor abnormalities in slow transit constipation defined by high resolution, fibre-optic manometry. Neurogastroenterol Motil. 2015; 27(3): 379-388.

238. Castedal M, Abrahamsson H. High-resolution analysis of the duodenal interdigestive phase III in humans. Neurogastroenterol Motil. 2001; 13(5): 473-481.

239. Feinle C, Kunz P, Boesiger P, Fried M, Schwizer W. Scintigraphic validation of a magnetic resonance imaging method to study gastric emptying of a solid meal in humans. Gut 1999; 44(1): 106-111.

240. Bilecen D, Scheffler K, Seifritz E, Bongartz G, Steinbrich W. Hydro-MRI for the visualization of gastric wall motility using RARE magnetic resonance imaging sequences. Abdom Imaging. 2000; 25(1): 30-34.

241. Ohkubo H, Kessoku T, Fuyuki A, Iida H, Inamori M, Fujii T, Kawamura H, Hata Y, Manabe N, Chiba T, Kwee TC, Haruma K, Matsuhashi N, Nakajima A, Takahara T. Assessment of small bowel motility in patients with chronic intestinal pseudo-obstruction using cine-MRI. Am J Gastroenterol. 2013; 108(7): 1130-1139.

242. Fuyuki A, Ohkubo H, Higurashi T, Iida H, Inoh Y, Inamori M, Nakajima A. Clinical importance of cine-MRI assessment of small bowel motility in patients with chronic intestinal pseudo-obstruction: a retrospective study of 33 patients. J Gastroenterol. 2017; 52(5): 577-584.

243. Menys A, Butt S, Emmanuel A, Plumb AA, Fikree A, Knowles C, Atkinson D, Zarate N, Halligan S, Taylor SA. Comparative quantitative assessment of global small bowel motility using magnetic resonance imaging in chronic intestinal pseudo-obstruction and healthy controls. Neurogastroenterol Motil. 2016; 28(3): 376-383.

244. Malagelada C, De Iorio F, Azpiroz F, Accarino A, Segui S, Radeva P, Malagelada JR. New insight into intestinal motor function via noninvasive endoluminal image analysis. Gastroenterology. 2008; 135(4): 1155-1562.

245. Malagelada C, De Lorio F, Seguí S, Mendez S, Drozdzal M, Vitria J, Radeva P, Santos J, Accarino A, Malagelada JR, Azpiroz F. Functional gut disorders or disordered gut function? Small bowel dysmotility evidenced by an original technique. Neurogastroenterol Motil. 2012; 24(3): 223-228.

246. Seguí S, Drozdzal M, Pascual G, Radeva P, Malagelada C, Azpiroz F, Vitrià J. Generic feature learning for wireless capsule endoscopy analysis. Comput Biol Med. 2016; 79: 163-172.

247. Malagelada JR, Stanghellini V. Manometric evaluation of functional upper gut symptoms. Gastroenterology. 1985; 88: 1223-1231.

248. Stanghellini V, Camilleri M, Malagelada JR. Chronic idiopathic intestinal pseudo-obstruction: clinical and intestinal manometric findings. Gut. 1987; 28(1): 5-12.

249. Stanghellini V, Cogliandro R, Cogliandro L, De Giorgio R, Barbara G, Salvioli B, Corinaldesi R. Clinical use of manometry for the diagnosis of intestinal motor abnormalities. Dig Liver Dis. 2000; 32(6): 532-541.

250. Smout AJ. Manometry of the gastrointestinal tract: toy or tool? Scand J Gastroenterol Suppl. 2001; (234): 22-228.

251. Kellow JE, Delvaux M, Azpiroz F, Camilleri M, Quigley EM, Thompson DG. Principles of applied neurogastroenterology: physiology/motility-sensation. Gut. 1999; 45(S2): 17-24.

252. Bharucha AE, Camilleri M, Low PA, Zinsmeister AR. Autonomic dysfunction in gastrointestinal motility disorders. Gut. 1993; 34(3): 397-401.

253. Verhagen MA, Samsom M, Jebbink RJ, Smout AJ. Clinical relevance of antroduodenal manometry. Eur J Gastroenterol Hepatol. 1999; 11(5): 523-528.

254. Stanghellini V, Corinaldesi R, Barbara L. Pseudo-obstruction syndromes. Baillieres Clin Gastroenterol. 1988; 2(1): 225-254.

255. Knowles CH, Lindberg G, Panza E, De Giorgio R. New perspectives in the diagnosis and management of enteric neuropathies. Nat. Rev. Gastroenterol. Hepatol. 2013; 10: 206-18

256. Sanders KM, Ward SM, Koh SD. Interstitial cells: regulators of smooth muscle function. Physiol. Rev. 2014; 94: 859-907

257. Huizinga JD, Zarate N, Farrugia G. Physiology, injury, and recovery of interstitial cells of Cajal: basic and clinical science. Gastroenterology. 2009; 137: 1548-56

258. Panza E, Knowles CH, Graziano C, Thapar N, Burns AJ, Seri M, Stanghellini V, De Giorgio R. Genetics of human enteric neuropathies. Prog. Neurobiol. 2012; 96: 176-89.

259. Hirschsprung, H. StuhltragheitNeugeborener in Folge von Dilatation und Hypertrophie des Colons. Jahrb. Kinderheilk. 27: 1-7, 1888

260. Kapur RP. Hirschprung disease and other enteric dysganglionoses. Crit. Rev. Clin. Lab. Sci. 1999; 36: 225-73.

261. Goldstein AM, Hofstra RM, Burns AJ., Building a brain in the gut: development of the enteric nervous system. Clin. Genet. 2013; 83: 307-316

262. Pingault V, Bondurand N, Kuhlbrodt K, Goerich DE, Préhu MO, Puliti A, Herbarth B, Hermans-Borgmeyer I, Legius E, Matthijs G, Amiel J, Lyonnet S, Ceccherini I, Romeo G, Smith JC, Read AP, Wegner M, Goossens M. SOX10 mutations in patients with Waardenburg-Hirschsprung disease. Nat Genet. 1998;18:171-173.

263. Amiel J, Laudier B, Attié-Bitach T, Trang H, de Pontual L, Gener B, Trochet D, Etchevers H, Ray P, Simonneau M, Vekemans M, Munnich A, Gaultier C, Lyonnet S. Polyalanine expansion and frameshift mutations of the paired-like homeobox gene PHOX2B in congenital central hypoventilation syndrome. Nat Genet. 2003;33:459-61.

264. Cacheux V, Dastot-Le Moal F, Kääriäinen H, Bondurand N, Rintala R, Boissier B, Wilson M, Mowat D, Goossens M. Loss-of-function mutations in SIP1 Smad interacting protein 1 result in a syndromic Hirschsprung disease. Hum Mol Genet. 2001;10:1503-10.

265. Gabriel SB, Salomon R, Pelet A, Angrist M, Amiel J, Fornage M, Attié-Bitach T, Olson JM, Hofstra R, Buys C, Steffann J, Munnich A, Lyonnet S, Chakravarti A. Segregation at three loci explains familial and population risk in Hirschsprung disease. Nat. Genet. 2002; 31: 89-93.

266. Emison ES, McCallion AS, Kashuk CS, Bush RT, Grice E, Lin S, Portnoy ME, Cutler DJ, Green ED, Chakravarti A., A common sex-dependent mutation in a RET enhancer underlies Hirschsprung disease risk, Nature. 2005; 434: 857-863

267. Griseri P, Lantieri F, Puppo F, Bachetti T, Di Duca M, Ravazzolo R, Ceccherini I., A common variant located in the 3'UTR of the RET gene is associated with protection from Hirschsprung disease, Hum. Mutat. 2007; 28: 168-176

268. Wallace AS, Anderson RB. Genetic interactions and modifier genes in Hirschsprung's disease. World J Gastroenterol. 2011;17:4937-44.

269. Carrasquillo MM, McCallion AS, Puffenberger EG, Kashuk CS, Nouri N, Chakravarti A. Genome-wide association study and mouse model identify interaction between RET and EDNRB pathways in Hirschsprung disease. Nat Genet. 2002; 32: 237-44.

270. Cantrell VA, Owens SE, Chandler RL, Airey DC, Bradley KM, Smith JR, Southard-Smith EM. Interactions between Sox10 and EdnrB modulate penetrance and severity of aganglionosis in the Sox10Dom mouse model of Hirschsprung disease. Hum Mol Genet. 2004; 13: 2289-301.

271. Qualman SJ, Pysher T, Schauer G. Hirschprung disease: differential diagnosis and sequelae. Perspect. Pediatr. Pathol. 1997; 20: 111–26

272. Kapur RP, Reed RC, Finn LS, Patterson K, Johanson J, Rutledge JC. Calretinin immunohistochemistry versus acetylcholinesterase histochemistry in the evaluation of suction rectal biopsies for Hirschsprung Disease. Pediatr. Dev. Pathol. 2009; 12: 6-15

273. Weinberg AG. Hirschprung's disease. A pathologist's view. Perspect. Pediatr. Pathol. 1975; 2: 207–39

274. Ballard ET. Ultrashort segment Hirschprung's disease: a case report. Pediatr. Pathol. Lab. Med. 1996; 16: 319–25

275. Langer JC, Durrant AC, de la Torre L, Teitelbaum DH, Minkes RK, Caty MG, Wildhaber BE, Ortega SJ, Hirose S, Albanese CT. One-stage transanal Soave pull through for Hirschsprung disease: a multicenter experience with 141 children. Ann. Surg. 2003; 238: 569-83

276. Laughlin DM, Friedmacher F, Puri P. Total colonic aganglionosis: a systematic review and meta-analysis of long-term clinical outcome. Pediatr. Surg. Int. 2012; 28: 773-9.

277. Catto-Smith AG, Trajanovska M, Taylor RG. Long-term continence after surgery for Hirschsprung's disease. J. Gastroenterol. Hepatol. 2007; 22: 2273-82.

278. Jarvi K, Laitakari EM, Koivusalo A, Rintala RJ, Pakarinen MP. Bowel function and gastrointestinal quality of life among adults operated for Hirschsprung disease during childhood: a population-based study. Ann. Surg. 2010; 252: 977-81.

279. Niramis R, Watanatittan S, Anuntkosol M, Buranakijcharoen V, Rattanasuwan T, Tongsin A, Petlek W, Mahatharadol V. Quality of life of patients with Hirschsprung's disease at 5 - 20 years post pull-through operations. Eur. J. Pediatr. Surg. 2008; 18: 38-43

280. Gosain A, Brinkman AS. Hirschsprung's associated enterocolitis. Curr. Opin. Pediatr. 2015; 27: 364-9

281. Veiga-Fernandes H, Coles MC, Foster KE, Patel A, Williams A, Natarajan D, Barlow A, Pachnis V, Kioussis D. Tyrosine kinase receptor RET is a key regulator of Peyer's patch organogenesis. Nature. 2007; 446: 547-51.

282. Gosain A, Barlow-Anacker AJ, Erickson CS, Pierre JF, Heneghan AF, Epstein ML, Kudsk KA. Impaired Cellular Immunity in the Murine Neural Crest Conditional Deletion of Endothelin Receptor-B Model of Hirschsprung's Disease. PLoS One. 2015; 10: e0128822.

283. Yan Z, Poroyko V, Gu S, Zhang Z, Pan L, Wang J, Bao N, Hong L. Characterization of the intestinal microbiome of Hirschsprung's disease with and without enterocolitis. Biochem. Biophys. Res. Commun. 2014; 445: 269-74

284. Frykman PK, Nordenskjöld A, Kawaguchi A, Hui TT, Granström AL, Cheng Z, Tang J, Underhill DM, Iliev I, Funari VA, Wester T; HAEC Collaborative Research Group (HCRG). Characterization of Bacterial and Fungal Microbiome in Children with Hirschsprung Disease with and without a History of Enterocolitis: A Multicenter Study. PLoS One. 2015; 10: e0124172

285. Wang X, Li Z, Xu Z, Wang Z, Feng J. Probiotics prevent Hirschsprung's disease-associated enterocolitis: a prospective multicenter randomized controlled trial. Int. J. Colorectal Dis. 2015; 30: 105-10

286. Hoshino M, Omura N, Yano F, Tsuboi K, Kashiwagi H, Yanaga K..Immunohistochemical study of the muscularisexterna of the esophagus in achalasia patients. Dis. Esophagus 2013; 26: 14-21

287. Goldblum JR, Whyte RI, Orringer MB, Appelman HD. Achalasia. A morphologic study of 42 resected specimens. Am. J. Surg. Pathol. 1996a; 111: 648-54

288. Goldblum JR, Rice TW, Richter JE. Histopathologic features in esophagomyotomy specimens from patients with achalasia. Gastroenterology. 1996b; 111: 648–54

289. Clark SB, Rice TW, Tubbs RR, Richter JE, Goldblum JR. The nature of the myenteric infiltrate in achalasia: an immunohistochemical analysis. Am. J. Surg. Pathol. 2000; 24: 1153–8.

290. Raymond L, Lach B, Shamji FM. Inflammatory aetiology of primary oesophageal achalasia: an immunohistochemical and ultrastructural study of Auerbach’s plexus. Histopathology. 1999; 35: 445–53

291. Robertson CS, Martin BA, Atkinson M. Varicella-zoster virus DNA in the oesophageal myenteric plexus in achalasia. Gut. 1993; 34: 299–302

292. Birgisson S, Galinski MS, Goldblum JR, Rice TW, Richter JE. Achalasia is not associated with measles or known herpes and human papilloma viruses. Dig. Dis. Sci. 1997; 42: 300–6

293. Castagliuolo I, Brun P, Costantini M, et al. Esophageal achalasia: is the herpes simplex virus really innocent? J. Gastrointest. Surg. 2004; 8: 24–30.

294. Verne GN, Sallustio JE, Eaker EY. Antimyenteric neuronal antibodies in patients with achalasia. A prospective study. Dig Dis Sci 1997; 42: 307–13. Eaker EY. Antimyenteric neuronal antibodies and motility. Gastroenterology. 1998; 114: 421–2

295. Latiano A, De Giorgio R, Volta U, et al. HLA and enteric antineuronal antibodies in patients with achalasia. Neurogastroenterol. Motil. 2006; 18: 520-5.

296. Moses PL, Ellis LM, Anees MR, et al. Antineuronal antibodies in idiopathic achalasia and gastro-oesophageal reflux disease. Gut. 2003; 52: 629-36

297. Bruley des Varannes S, Chevalier J, Pimont S, et al. Serum from achalasia patients alters neurochemical coding in the myenteric plexus and nitric oxide mediated motor response in normal human fundus. Gut. 2006; 55: 319-26

298. De Giorgio R, Di Simone M, Stanghellini V, et al. Esophageal and gastric nitric oxide synthesizing innervation in primary achalasia. Am. J. Gastroenterol. 1999; 94: 2357-62

299. Di Nardo G, Tullio-Pelet A, Annese V, et al. Idiopathic achalasia is not allelic to alacrima achalasia adrenal insufficiency syndrome at the ALADIN locus. Dig. Liver Dis, 2005; 37: 312-5

300. Bredenoord AJ,Fox M,Kahrilas PJ, Pandolfino JE, Schwizer W, Smout AJPM, and International High Resolution Manometry Working Group. Chicago Classification Criteria of Esophageal Motility Disorders Defined in High Resolution Esophageal Pressure Topography (EPT). Neurogastroenterol. Motil. 2012; 24(Suppl 1): 57-65.

301. Scherer JR, Kwiatek MA, Soper NJ, Pandolfino JE, Kahrilas PJ. Functional esophagogastric junction obstruction with intact peristalsis: a heterogeneous syndrome sometimes akin to achalasia. J. Gastrointest. Surg. 2009; 13: 2219-25.

302. Pandolfino JE, Kwiatek MA, Nealis T, Bulsiewicz W, Post J, Kahrilas PJ. Achalasia: a new clinically relevant classification by high-resolution manometry. Gastroenterology. 2008; 135: 1526-33

303. Di Nardo G, Blandizzi C, Volta U, et al. Review article: molecular, pathological and therapeutic features of human enteric neuropathies. Aliment. Pharmacol. Ther. 2008; 28: 25-42.

304. Boeckxstaens GE, Annese V, des Varannes SB, Chaussade S, Costantini M, Cuttitta A, Elizalde JI, Fumagalli U, Gaudric M, Rohof WO, Smout AJ, Tack J, Zwinderman AH, Zaninotto G, Busch OR; European Achalasia Trial Investigators. Pneumatic dilation versus laparoscopic Heller's myotomy for idiopathic achalasia. N. Engl. J. Med. 2011; 364: 1807-16

305. Sharp NE, St Peter SD. Treatment of Idiopathic Achalasia in the Pediatric Population: A Systematic Review. Eur. J. Pediatr. Surg. 2015;

306. Meneghelli UG. Chagas' disease: a model of denervation in the study of digestive tract motility. Braz J Med Biol Res 1985; 18: 255–264

307. Clayton J. Chagas disease 101. Nature 2010; 465, S4-5.

308. Andersson J, Orn A, Sunnemark D. Chronic murine Chagas' disease: the impact of host and parasite genotypes. Immunol Lett 2003; 86: 207–212

309. Goin JC, Sterin-Borda L, Bilder CR, Varrica LM, Iantorno G, Rios MC, Borda E. Functional implications of circulating muscarinic cholinergic receptor autoantibodies in chagasic patients with achalasia. Gastroenterology 1999;117:798-805

310. Teixeira, A. R., Nitz, N., Guimaro, M. C., Gomes, C. & Santos-Buch, C. A. Chagas disease. Postgrad. Med. J. 2006; 82:788-798

311. Horowitz M, Fraser RJ. Gastroparesis: diagnosis and management. Scand. J. Gastroenterol. 1995; 213(Suppl.): 7-16.

312. Patrick A, Epstein O. Review article: gastroparesis. Aliment. Pharmacol. Ther. 2008; 27: 724-40

313. Waseem S, Islam S, Kahn G, Moshiree B, Talley NJ. Spectrum of gastroparesis in children. J. Pediatr. Gastroenterol. Nutr. 2012; 55: 166-72.

314. Sigurdsson L, Flores A, Putnam PE, Hyman PE, Di Lorenzo C. Postviral Gastroparesis: presentation, treatment and outcome. J. Pediatr. 1997; 131: 751-4.

315. You CH, Chey WY, Lee KY, Menguy R, Bortoff A. Gastric and small intestinal myoelectric dysrhythmia associated with chronic intractable nausea and vomiting. Ann. Int. Med. 1981; 95: 449-51.

316. Shellito PC, Warshaw AL. Idiopathic intermittent gastroparesis and its surgical alleviation. Am. J. Surg. 1984; 148: 408-12

317. Grover M, Bernard CE, Pasricha PJ, Lurken MS, Faussone-Pellegrini MS, Smyrk TC, Parkman HP, Abell TL, Snape WJ, Hasler WL, McCallum RW, Nguyen L, Koch KL, Calles J, Lee L, Tonascia J, Ünalp-Arida A, Hamilton FA, Farrugia G; NIDDK Gastroparesis Clinical Research Consortium (GpCRC). Clinical-histological associations in gastroparesis: results from the Gastroparesis Clinical Research Consortium. Neurogastroenterol. Motil. 2012; 24: 531-9

318. Chinn JS, Schuffler MD. Paraneoplastic visceral neuropathy as a cause of severe gastrointestinal motor dysfunction. Gastroenterology. 1988; 95: 1279–86

319. De Giorgio R, Barbara G, Stanghellini V, et al. Idiopathic myenteric ganglionitis underlying intractable vomiting in a young adult. Eur. J. Gastroenterol. Hepatol. 2000; 12: 613–6.

320. Rodriguez L, Irani K, Jiang H, Goldstein AM. Clinical presentation, response to therapy, and outcome of gastroparesis in children. J. Pediatr. Gastroenterol. Nutr. 2012; 55: 185-190.

321. Pasricha PJ, Parkman HP. Gastroparesis: definitions and diagnosis. Gastroenterol. Clin. North Am. 2015; 44: 1-7

322. Acosta A, Camilleri M. Prokinetics in gastroparesis. Gastroenterol. Clin. North Am. 2015; 44: 97-111

323. Stein B, Everhart KK, Lacy BE. Gastroparesis: A Review of Current Diagnosis and Treatment Options. J. Clin. Gastroenterol. 2015; 49: 550-8

324. Maranki J, Parkman HP. Gastric electric stimulation for the treatment of gastroparesis. Curr. Gastroenterol. Rep. 2007; 9: 286-94

325. Vanderwinden JM, Mailleux P, Schiffmann SN, Vanderhaegen JJ, De Laet MH. Nitric oxide synthase activity in infantile hypertrophic pyloric stenosis. New Engl. J. Med. 1992; 327: 511–5

326. Mashimo H, Kjellin A, Goyal RK. Gastric stasis in neuronal nitric oxide synthase-deficient knockout mice. Gastroenterology. 2000; 119: 766–73

327. Goyal RK, Hirano I. The enteric nervous system. N Engl J Med 1996;25:1106-5

328. Kimber, T.E., Blumbergs, P.C., Rice, J.P., Hallpike, J.F., Edis, R., Thompson, P.D., Suthers, G. Familial neuronal intranuclear inclusion disease with ubiquitin positive inclusions. J. Neurol. Sci. 1998; 160: 33–40.

329. Zannolli, R., Gilman, S., Rossi, S., Volpi, N., Bernini, A., Galluzzi, P., Galimberti, D., Pucci, L., D'Ambrosio, A., Morgese, G., Giannini, F. Hereditary neuronal intranuclear inclusion disease with autonomic failure and cerebellar degeneration. Arch. Neurol. 2002; 59: 1319–1326.

330. Sone, J., Hishikawa, N., Koike, H., Hattori, N., Hirayama, M., Nagamatsu, M., Yamamoto, M., Tanaka, F., Yoshida, M., Hashizume, Y., Imamura, H., Yamada, E., Sobue, G. Neuronal intranuclear hyaline inclusion disease showing motor-sensory and autonomic neuropathy. Neurology 2005; 65: 1538–1543.

331. Raue, F., Frank-Raue, K. Update multiple endocrine neoplasia type 2. Fam. Cancer 2010; 9: 449–457.

332. Wray, C.J., Rich, T.A., Waguespack, S.G., Lee, J.E., Perrier, N.D., Evans, D.B. Failure to recognize multiple endocrine neoplasia 2B: more common than we think? Ann. Surg. Oncol. 2008; 15: 293–301.

333. Eng, C., Clayton, D., Schuffenecker, I., Lenoir, G., Cote, G., Gagel, R.F., van Amstel, H.K., Lips, C.J., Nishisho, I., Takai, S.I., Marsh, D.J., Robinson, B.G., Frank-Raue, K., Raue, F., Xue, F., Noll, W.W., Romei, C., Pacini, F., Fink, M., Niederle, B., Zedenius, J., Nordenskjold, M., Komminoth, P., Hendy, G.N., Mulligan, L.M. The relationship between specific RET proto-oncogene mutations and disease phenotype in multiple endocrine neoplasia type 2. International RET mutation consortium analysis. JAMA 1996; 276: 1575–1579.

334. Smith, D.P., Houghton, C., Ponder, B.A. Germline mutation of RET codon 883 in two cases of de novo MEN 2B. Oncogene 1997; 15: 1213–1217.

335. Nguyen, A.T., Zacharin, M.R., Smith, M., Hardikar, W. Isolated intestinal ganglioneuromatosis with a new mutation of RET proto-oncogene. Eur. J. Gastroenterol. Hepatol. 2006; 18: 803–805.

336. Smith VV, Eng C, Milla PJ. Intestinal ganglioneuromatosis and multiple endocrine neoplasia type 2B: implications for treatment. Gut 1999; 45: 143–6

337. Kapur. Neuronal dysplasia: a controversial pathological correlate of intestinal pseudo-obstruction. Am J Med Genet A, 2003; 122: 287-293.

338. Koletzko S, Jesch I, Faus-Kebetaler T, Briner J, Meier-Ruge W, Müntefering H, Coerdt W, Wessel L, Keller KM, Nützenadel W, Schmittenbecher P, Holschneider A, Sacher P. Rectal biopsy for diagnosis of intestinal neuronal dysplasia in children: a prospective multicentre study on interobserver variation and clinical outcome. Gut 1999; 44: 853-861.

339. Kobayashi H, Hirakawa H, Surana R, O'Briain DS, Puri P. Intestinal neuronal dysplasia is a possible cause of persistent bowel symptoms after pull-through operation for Hirschsprung's disease. J Pediatr Surg 1995; 30: 253-259.

340. Meier-Ruge WA, Bruder E, Kapur RP. Intestinal neuronal dysplasia type B: one giant ganglion is not good enough. Pediatr Dev Pathol. 2006; 9:444-52.

341. Meier-Ruge WA, Brönnimann PB, Gambazzi F, Schmid PC, Schmidt CP, Stoss F. Histopathological criteria for intestinal neuronal dysplasia of the submucosal plexus (type B). Virchows Arch 1995; 426: 549-556.

342. Kapur RP: Neuropathology of paediatric chronic intestinal pseudo-obstruction and related animal models. J Pathol 2001; 194: 277-288.

343. von Boyen GB, Krammer HJ, Süss A, Dembowski C, Ehrenreich H, Wedel T. Abnormalities of the enteric nervous system in heterozygous endothelin B receptor deficient (spotting lethal) rats resembling intestinal neuronal dysplasia. Gut 2002; 51: 414-419.

344. Nagy N, Barad C, Graham HK, Hotta R, Cheng LS, Fejszak N, Goldstein AM. Sonic hedgehog controls enteric nervous system development by patterning the extracellular matrix. Development, 2016; 143: 264-275.

345. Gath R, Goessling A, Keller KM, Koletzko S, Coerdt W, Müntefering H, Wirth S, Hofstra RM, Mulligan L, Eng C, von Deimling A. Analysis of the RET, GDNF, EDN3, and

EDNRB genes in patients with intestinal neuronal dysplasia and Hirschsprung disease. Gut 2001; 48: 671-675.

346. Ford AC, Brenner DM, Schoenfeld PS. Efficacy of pharmacological therapies for the treatment of opioid-induced constipation: systematic review and meta-analysis. Am. J. Gastroenterol. 2013; 108: 1566-74.

347. van den Berg MM, Benninga MA, Di Lorenzo C. Epidemiology of childhood constipation: a systematic review. Am. J. Gastroenterol. 2006; 101: 2401-9.

348. Brandt LJ, Prather CM, Quigley EM, Schiller LR, Schoenfeld P, Talley NJ. Systematic review on the management of chronic constipation in North America. Am. J. Gastroenterol. 2005; 100 Suppl 1: S5-S2.

349. Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. Gastroenterology. 2006; 130: 1480-91.

350. Wald A. Constipation: pathophysiology and management. Curr. Opin. Gastroenterol. 2015; 31: 45-9.

351. Giorgio V, Borrelli O, Smith VV, Rampling D, Köglmeier J, Shah N, Thapar N, Curry J, Lindley KJ. High-resolution colonic manometry accurately predicts colonic neuromuscular pathological phenotype in pediatric slow transit constipation. Neurogastroenterol. Motil. 2013; 25: 70-78.

352. Bassotti G, Villanacci V. World J. Gastroenterol. 2006; 12: 4609–13

353. Bassotti G, Stanghellini V, Chiarioni G, Germani U, De Giorgio R, Vantini I, Morelli A, Corinaldesi R. Upper gastrointestinal motor activity in patients with slow-transit constipation. Further evidence for an enteric neuropathy. Dig. Dis. Sci. 1996; 41: 1999-2005.

354. Smith B. Myenteric plexus in Hirschprung's disease. Gut. 1967; 8: 308–12

355. Krishnamurthy S, Schuffler MD, Rohrmann CA, Pope CE. Severe idiopathic constipation is associated with a distinctive abnormality of the colonic myenteric plexus. Gastroenterology. 1985; 88: 26–34.

356. Porter AJ, Wattchow DA, Hunter A, Costa M. Abnormalities of nerve fibers in the circular muscle of patients with slow transit constipation. Int. J. Colorectal Dis. 1998; 13: 208-16.

357. Faussone-Pellegrini MS, Infantino A, Matini P, Masin A, Mayer B, Lise M. Neuronal anomalies and normal muscle morphology at the hypomotile ileocecocolonic region of patients affected by idiopathic chronic constipation. Histol. Histopathol. 1999; 14: 1119-34.

358. Wedel T, Roblick UJ, Ott V, et al. Oligoneuronal hypoganglionosis in patients with idiopathic slow-transit constipation. Dis. Colon Rectum 2002; 45: 54-62.

359. Lyford GL, He CL, Soffer E, et al. Pancolonic decrease in interstitial cells of Cajal in patients with slow transit constipation. Gut. 2002; 51: 496-501.

360. Wedel T, Spiegler J, Soellner S, et al. Enteric nerves and interstitial cells of Cajal are altered in patients with slow transit constipation and megacolon. Gastroenterology. 2002; 123: 1459-67.

361. Bassotti G, Villanacci V, Maurer CA, et al. The role of glial cells and apoptosis of enteric neurones in the neuropathology of intractable slow transit constipation. Gut. 2006a; 55: 41-6.

362. Bassotti G, Villanacci V, Cathomas G, et al. Enteric neuropathology of the terminal ileum in patients with intractable slow-transit constipation. Hum. Pathol. 2006b; 37: 1252-8.

363. Tomita R, Fujisaki S, Ikeda T, Fukuzawa M. Role of nitric oxide in the colon of patients with slow-transit constipation. Dis. Colon Rectum 2002; 45: 593-600

364. Koch TR, Carney JA, Go L, Go VL. Idiopathic chronic constipation is associated with decreased colonic vasoactive intestinal peptide. Gastroenterology. 1988; 94: 300-10

365. Lincoln J, Crowe R, Kamm MA, Burnstock G, Lennard-Jones JE. Serotonin and 5-hydroxyindolacetic acid are increased in the sigmoid colon in severe idiopathic constipation. Gastroenterology. 1990; 98: 1219-25.

366. Tzavella K, Riepl RL, Klauser AG, Voderholzer WA, Schindlbeck NE, Muller-Lissner SA. Decreased substance P levels in rectal biopsies from patients with slow transit constipation. Eur. J. Gastroenterol. Hepatol. 1996; 8: 1207-11.

367. Sjolund K, Fasth S, Ekman R, et al. Neuropeptides in idiopathic chronic constipation (slow transit constipation), Neurogastroenterol. Motil. 1997; 9: 143-50.

368. Knowles CH & Farrugia G. Gastrointestinal neuromuscular pathology in chronic constipation. Best Pract. Res. Clin. Gastroenterol. 2011; 25: 43-57.

369. Camilleri M. Novel therapeutic agents in neurogastroenterology: advances in the past year. Neurogastroenterol. Motil. 2014; 26: 1070-1078.

370. Mugie SM, Korczowski B, Bodi P, Green A, Kerstens R, Ausma J, Ruth M, Levine A, Benninga MA. Prucalopride is no more effective than placebo for children with functional constipation. Gastroenterology. 2014; 147: 1285-95.

371. Hyman PE, Di Lorenzo C, Prestridge LL, Youssef NN, Ueno R. Lubiprostone for the treatment of functional constipation in children. J. Pediatr. Gastroenterol. Nutr. 2014; 58: 283-91.

372. Gladman MA, Scott SM, Lunniss PJ, Williams NS. Systematic review of surgical options for idiopathic megarectum and megacolon. Ann. Surg. 2005; 241: 562-74.

373. Randall J, Coyne P, Jaffray B. Follow up of children undergoing antegrade continent enema: experience of over two hundred cases. J. Pediatr. Surg. 2014; 49: 1405-8

374. Patel AS, Saratzis A, Arasaradnam R, Harmston C. Use of Antegrade Continence Enema for the Treatment of Fecal Incontinence and Functional Constipation in Adults: A Systematic Review. Dis. Colon Rectum. 2015; 58: 999-1013.

375. Christison-Lagay ER, Rodriguez L, Kurtz M, St Pierre K, Doody DP, Goldstein AM. Antegrade colonic enemas and intestinal diversion are highly effective in the management of children with intractable constipation. J. Pediatr. Surg. 2010; 45: 213-9.

376. Metzger M, Bareiss PM, Danker T, Wagner S, Hennenlotter J, Guenther E, Obermayr F, Stenzl A, Koenigsrainer A, Skutella T, Just L,. Expansion and differentiation of neural progenitors derived from the human adult enteric nervous system. Gastroenterology. 2009; 137: 2063-2073

377. Kapur RP. Colonization of the murine hindgut by sacral crest-derived neural precursors: experimental support for an evolutionarily conserved model. Dev. Biol. 2000; 227: 146-155.

378. Metzger M, Caldwell C, Barlow AJ, Burns AJ, Thapar N. Enteric nervous system stem cells derived from human gut mucosa for the treatment of aganglionic gut disorders. Gastroenterology. 2009b; 136: 2214-2225.

379. Hotta R, Cheng LS, Graham HK, Pan W, Nagy N, Belkind-Gerson J, Goldstein AM. Isogenic enteric neural progenitor cells can replace missing neurons and glia in mice with Hirschsprung disease. Neurogastroenterol Motil. 2016 Apr;28(4):498-512.

380. Hotta R, Natarajan D, Thapar N. Potential of cell therapy to treat pediatric motility disorders. Semin. Pediatr. Surg. 2009; 18: 263-273.

381. Heanue TA, Pachnis V. Enteric nervous system development and Hirschsprung's disease: advances in genetic and stem cell studies. Nat. Rev. Neurosci. 2007; 8: 466-479.

382. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006; 126: 663-676

383. Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD. Genome editing with engineered zinc finger nucleases. Nat. Rev. Genet. 2010; 11: 636-646.

384. Fattahi F, Steinbeck JA, Kriks S, Tchieu J, Zimmer B, Kishinevsky S, Zeltner N, Mica Y, El-Nachef W, Zhao H, de Stanchina E, Gershon MD, Grikscheit TC, Chen S, Studer L. Deriving human ENS lineages for cell therapy and drug discovery in Hirschsprung disease. Nature. 2016; 531:105-9.

385. Xue H, Wu J, Li S, Rao MS, Liu Y. Genetic Modification in Human Pluripotent Stem Cells by Homologous Recombination and CRISPR/Cas9 System. Methods Mol Biol. 2016; 1307: 173-90.

386. Zou J, Mali P, Huang X, Dowey SN, Cheng L. Site-specific gene correction of a point mutation in human iPS cells derived from an adult patient with sickle cell disease. Blood. 2011; 118: 4599-608.

387. Song B, Fan Y, He W, Zhu D, Niu X, Wang D, Ou Z, Luo M, Sun X. Improved hematopoietic differentiation efficiency of gene-corrected beta-thalassemia induced pluripotent stem cells by CRISPR/Cas9 system. Stem Cells Dev. 2015; 24: 1053-65.

388. Corti S, Nizzardo M, Simone C, Falcone M, Nardini M, Ronchi D, Donadoni C, Salani S, Riboldi G, Magri F, Menozzi G, Bonaglia C, Rizzo F, Bresolin N, Comi GP. Genetic correction of human induced pluripotent stem cells from patients with spinal muscular atrophy. Sci Transl Med. 2012; 4: 165ra162.

389. Gabbard SL, Lacy BE. Chronic intestinal pseudo-obstruction. Nutr Clin Pract 2013;28:307-316.

390. Yeung AK, Di Lorenzo C. Primary gastrointestinal motility disorders in childhood. Minerva Pediatr 2012;64:567-584.

391. Connor FL, Di Lorenzo C. Chronic intestinal pseudo-obstruction: assessment and management. Gastroenterology 2006;130:29-36.

392. Stanghellini V, Cogliandro RF, De Giorgio R et al. Natural history of chronic idiopathic intestinal pseudo-obstruction in adults: a single center study. Clin Gastroenterol Hepatol 2005;3:449-458.

393. Stanghellini V, Cogliandro RF, De Giorgio R et al. Chronic intestinal pseudo-obstruction: manifestations, natural history and management. Neurogastroenterol Motil 2007;19:440-452.

394. Heneyke S, Smith VV, Spitz L, Milla PJ. Chronic intestinal pseudo-obstruction: treatment and long term follow-up of 44 patients. Arch Dis Child 1999;81:21-27.

395. Vargas JH, Sachs P, Ament ME. Chronic intestinal pseudo-obstruction syndrome in pediatrics. Results of a national survey by members of the North American Society of Pediatric Gastroenterology and Nutrition. J Pediatr Gastroenterol Nutr 1988;7:323-332.

396. Di Lorenzo C. Pseudo-obstruction: current approaches. Gastroenterology 1999;116:980-987.

397. Muto, M Matsufuji H, Tomomasa T et al. Pediatric chronic intestinal pseudo-obstruction is a rare, serious, and intractable disease: a report of a nationwide survey in Japan. J Pediatr Surg 2014;49:1799-803.

398. Iida H, Ohkudo H, Inamori M, Nakajiama A, Sato H. Epidemiology and clinical experience on chronic intestinal pseudo-obstruction in Japan: a nationwide epidemiologic survey. J Epidemiol 2013;23:288-294.

399. Mousa H, Hyman PE, Cocjin J, Flores AF, Di Lorenzo C. Long-term outcome of congenital intestinal pseudoobstruction. Dig Dis Sci 2002;47:2298-2305.

400. Faure C, Goulet O, Ategbo S et al. Chronic intestinal pseudo-obstruction syndrome: clinical analysis, outcome, and prognosis in 105 children. French-Speaking Group of Pediatric Gastroenterology. Dig Dis Sci 1999;44:953-9.

401. Shen O, Schimmel MS, Eitan R, Granovsky-Grisaru S, Rabinowitz RR. Prenatal diagnosis of intestinal pseudo-obstruction. Ultrasound Obstet Gynecol 2007;29:229-231.

402. De Giorgio R, Guerrini S, Barbara G et al. Inflammatory neuropathies of the enteric nervous system. Gastroenterology 2004;126:1872-1883.

403. Schäppi MG, Smith VV, Milla PJ, Lindley KJ. Eosinophilic myenteric ganglionitis is associated with functional intestinal obstruction. Gut 2003;52:752–755.

404. Ooms AH, VerheiJ J, Hulst JM et al. Eosinophilic myenteric ganglionitis as a cause of chronic intestinal pseudo-obstruction. Virchows Arch 2012;460:123-127.

405. Accarino A. Mast cell neuromuscular involvement in patients with severe gastrointestinal dysmotility (SGID). Gut 2007;56(Suppl III):A18.

406. Mann SD, Debinski HS, Kamm MA. Clinical characteristics of chronic idiopathic intestinal pseudo-obstruction in adults. Gut 1997;41:675-681.

407. Oton E, Moreira V, Redondo C et al. Chronic intestinal pseudoobstruction due to lymphocytic leiomyositis: is there a place for immunomodulatory therapy? Gut 2005;54:1343-1344.

408. Dewit S, de Hertogh G, Geboes K, Tack J et al. Chronic intestinal pseudo-obstruction caused by an intestinal inflammatory myopathy: case report and review of the literature. Neurogastroenterol Motil 2008;20:343-348.

409. Weiner P, Shlum H, Ganam R, Plavnick L. Colonic pseudo-obstruction: a late complication of jejunoileal bypass. Isr J Med Sci 1984;20:405-406.

410. Amiot A, Joly F, Alves A et al. Long-term outcome of chronic intestinal pseudoobstruction adult patients requiring home parenteral nutrition. Am J Gastroenterol 2009;104:1262-1270.

411. Lindberg G, Iwarzon M, Tornblom H. Clinical features and long-term survival in chronic intestinal pseudo-obstruction and enteric dysmotility. Scand J Gastroenterol 2009;44:692-699.

412. De Giorgio R, Stanghellini V, Barbara G et al. Primary enteric neuropathies underlying gastrointestinal motor dysfunctions. Scand J Gastroenterol 2000;35:114-122.

413. Cogliandro RF, Antonucci A, De Giorgio R et al. Patient-reported outcomes and gut dysmotility in functional gastrointestinal disorders. Neurogastroenterol Motil 2011;23:1084-1091.

414. Lyford G, Foxx-Orenstein A. Chronic intestinal pseudo-obstruction. Curr Treat Options Gastroenterol 2004;7:317-325.

415. Pini Prato A, Rossi V, Fiore M et al. Megacystis, megacolon, and malrotation: a new syndromic association? Am J Med Genet 2011;155A:1798-1802.

416. de Betue CT, Boersma D, Oomen MW, Benninga MA, de Jong JR. Volvulus as a complication of chronic intestinal pseudo-obstruction syndrome. Eur J Pediatr 2011;170:1591-1595.

417. Di Nardo G, Stanghellini V, Cucchiara S et al. Enteric neuropathology of congenital intestinal obstruction: a case report. World J Gastroenterol 2006;12:5229-5233.

418. Schuffler MD, Pagon RA, Schwartz R, Bill AH. Visceral myopathy of the gastrointestinal and genitourinary tract in infants. Gastroenterology 1988;94:892-898.

419. Anuras S, Mitros FA, Soper RT et al. Chronic intestinal pseudo-obstruction in young children. Gastroenterology 1986;91:62-70.

420. Glassman M, Spivak W, Mininberg D, Madara J. Chronic intestinal pseudo-obstruction: a commonly misdiagnosed disease in infants and children. Pediatrics 1989;83:603-608.

421. Lapointe SP, Rivet C, Goulet O, Fekete CN, Lortat-Jacob S. Urological manifestation associated with chronic intestinal pseudo-obstruction in children. J Urol 2002;168:1768-1770.

422. Bardosi A, Creutzfeldt W, Di Mauro S, et al. Myo-, neuro-, gastrointestinal encephalopathy (MNGIE syndrome) due to partial deficiency of cytochrome-c-oxidase. A new mitochondrial multisystem disorder. Acta Neuropathol 1987;74:248-258.

423. Giordano C, Sebastiani M, De Giorgio R et al. Gastrointestinal dysmotility in mitochondrial neurogastrointestinal encephalomyopathy is caused by mitochondrial DNA depletion. Am J Pathol 2008;173: 1120-1128.

424. Lara MC, Valentino ML, Torres-Torronteras J, Hirano M, Martí R. Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE): biochemical features and therapeutic approaches. Biosci Rep 2007;27:151-163.

425. Amiot A, Tchikviladzé M, Joly F et al. Frequency of mitochondrial defects in patients with chronic intestinal pseudo-obstruction. Gastroenterology 2009;137:101-9.

426. De Giorgio R, Bovara M, Barbara G et al. Anti-HuD-induced neuronal apoptosis underlying paraneoplastic gut dysmotility. Gastroenterology 2003;25:70-79.

427. Hubball A, Martin JE, Lang B, De Giorgio R, Knowles CH. The role of humoral autoimmunity in gastrointestinal neuromuscular diseases. Prog Neurobiol 2009;87:10-20.

428. Merlin A, Soyer P, Boudiaf M, Hamzi L, Rymer R et al. Chronic intestinal pseudo-obstruction in adult patients: multidetector row helical CT features. Eur Radiol 2008;18:1587-1595.

429. Attar A, Kuoch V, Ducreux M, Benamouzig R, Malka D. Simultaneous decompression colonoscopy and radiologic G-tube insertion in a patient with megacolon because of chronic colonic pseudo-obstruction. Gastrointest Endosc 2005;62:975-6.

430. Maqbool S, Parkman HP, Friedenberg FK. Wireless capsule motility: comparison of the SmartPill GI monitoring system with scintigraphy for measuring whole gut transit. Dig Dis Sci 2009;54:2167-2174.

431. Fell JME, Smith VV, Milla PJ. Infantile chronic idiopathic intestinal pseudo-obstruction: the role of small intestinal manometry as a diagnostic tool and prognostic indicator. Gut 1996;39:306-11.

432. Hyman PE, Di Lorenzo C, McAdams L et al. Predicting the clinical response to cisapride in children with chronic intestinal pseudo-obstruction. Am J Gastroenterol 1993;88:832-836.

433. Di Lorenzo C, Flores AF, Buie T, Hyams PE. Intestinal motility and jejunal feeding in children with chronic intestinal pseudo-obstruction. Gastroenterology 1995;108:1379-85.

434. Cucchiara S, Annese V, Minella R et al. Antroduodenojejunal manometry in the diagnosis of chronic idiopathic intestinal pseudoobstruction in children. J Pediatr Gastroenterol Nutr 1994;18:294-305.

435. Cucchiara S, Borrelli O, Salvia G et al. A normal gastrointestinal motility exclude chronic intestinal pseudo-obstruction in children. Dig Dis Sci 2000;45:258-264.

436. Amiot A, Joly F, Cazals-Hatem D et al. Prognostic yield of esophageal manometry in chronic intestinal pseudo-obstruction: a retrospective cohort of 116 adult patients. Neurogastroenterol Motil 2012;24:1008-e542.

437. Waseem SH, Idrees MT, Croffie JM. Neuroenteric Staining as a Tool in the Evaluation of Pediatric Motility Disorders. Curr Gastroenterol Rep 2015;17:30.

438. Angkathunyakul N, Treepongkaruna S, Molagool S, Ruangwattanapaisarn N. Abnormal layering of muscularis propria as a cause of chronic intestinal pseudo-obstruction: A case report and literature review. World J Gastroenterol 2015;21:7059-64.

439. Knowles CH, Veress B, Tornblom H et al. Safety and diagnostic yield of laparoscopically assisted full-thickness bowel biopsy. Neurogastroenterol Motil 2008;20:774-779.

440. Knowles CH, De Giorgio R, Kapur RP et al. Gastrointestinal neuromuscular pathology: guidelines for histological techniques and reporting on behalf of the Gastro 2009 International Working Group. Acta Neuropathol 2009;118:271-301.

441. Pironi L, Joly F, Forbes A et al. Long-term follow-up of patients on home parenteral nutrition in Europe: Implication for intestinal transplantation. Gut 2011;60:17-25.

442. Meyer R, Foong RX, Thapar N, Kritas S, Shah N. Systematic review of the impact of feed protein type and degree of hydrolysis on gastric emptying in children. BMC Gastroenterol 2015;15:137.

443. Pironi L, Arends J, Bozzetti F, et al; Home Artificial Nutrition & Chronic Intestinal Failure Special Interest Group of ESPEN. ESPEN guidelines on chronic intestinal failure in adults. Clin Nutr 2016;35:247-307.

444. Scolapio JS, Ukleia A, Bouras EP, Romano M. Nutritional management of chronic intestinal pseudo-obstruction. J Clin Gastroenterol 1999;28:306-312.

445. Joly F, Amiot A, Messing B. Nutritional support in the severely compromised motility patient: when and how? Gastroenterol Clin North Am 2011;40:845-851.

446. Emmanuel AV, Shand AG, Kamm MA. Erythromycin for the treatment of chronic intestinal pseudo-obstruction: description of six cases with a positive response. Aliment Pharmacol Ther 2004;19:687-694.

447. Rao AS, Camilleri M. Review article. Metoclopramide and tardive dyskinesia. Aliment Pharmacol Ther 2010;31:11-19.

448. Soudah HC, Hasler WL, Owyang C. Effect of octreotide on intestinal motility and bacterial overgrowth in scleroderma. N Engl J Med 1991;325:1461-1467.

449. Verne GN, Eaker EY, Hardy E, Sninsky CA et al. Effect of octreotide and erythromycin on idiopathic and scleroderma-associated intestinal pseudoobstruction. Dig Dis Sci 1995;40:1892-1901.

450. Gmora S, Poenaru D, Tsai E. Neostigmine for the treatment of pediatric acute colonic pseudo-obstruction. J Pediatr Surg 2002;37:E28.

451. Calvet X, Martinez JM, Martinez M. Repeated neostigmine dosage as palliative treatment for chronic colonic pseudo-obstruction in a patient with autonomic paraneoplastic neuropathy. Am J Gastroenterol 2003;98:708-709.

452. O'Dea CJ, Brookes JH, Wattchow DA. The efficacy of treatment of patients with severe constipation or recurrent pseudo-obstruction with pyridostigmine. Colorectal Dis 2010;12:540-8.

453. Sanger GJ. Translating 5-HT receptor pharmacology. Neurogastroenterol Motil 2009;21:1235-1238.

454. Camilleri M, Deiteren A. Prucalopride for constipation. Expert Opin Pharmacother 2010;11:451-461.

455. Emmanuel AV, Kamm MA, Roy AJ, Kerstens R, Vandeplassche L. Randomised clinical trial: the efficacy of prucalopride in patients with chronic intestinal pseudoobstruction- a double-blind, placebo-controlled, cross-over, multiple n = 1 study. Aliment Pharmacol Ther 2012;35:48-55.

456. Husebye E. Gastrointestinal motility disorders and bacterial overgrowth. J Intern Med 1995;237:419-427.

457. Cuoco L, Montalto M, Jorizzo RA et al. Eradication of small intestinal bacterial overgrowth and oro-cecal transit in diabetics. Hepatogastroenterology 2002;49:1582-1586.

458. Madrid AM, Hurtado C, Venegas M, Cumsille F, Defilippi C. Longterm treatment with cisapride and antibiotics in liver cirrhosis: effect on small intestinal motility, bacterial overgrowth, and liver function. Am J Gastroenterol 2001;96:1251-1255.

459. Singh VV, Toskes PP. Small bowel bacterial overgrowth: presentation, diagnosis, and treatment. Curr Gastroenterol Rep 2003;5:365-372.

460. Pimentel M. Review of rifaximin as treatment for SIBO and IBS. Expert Opin Investig Drugs 2009;18:349-358.

461. Gomez R, Fernandez S, Aspirot A et al. Effect of amoxicillin/clavulanate on gastrointestinal motility in children. J Pediatr Gastroenterol Nutr 2012;54:780-784.

462. Prapaitrakool S, Hollmann MW, Wartenberg HC, Preckel B, Brugger S. Use of buprenorphine in children with chronic pseudoobstruction syndrome: case series and review of literature. Clin J Pain 2012;28:722-725.

463. Geller A, Petersen BT, Gostout CJ. Endoscopic decompression for acute colonic pseudo-obstruction. Gastrointest Endosc 1996;44:144-150.

464. Chun C, Aulakh S, Komlos F, Triadafilopoulos G. Tube to freedom: use of a venting jejunostomy in a patient with chronic intestinal pseudo-obstruction. Dig Dis Sci 2012;57:3076-3079.

465. Kim JS, Lee BI, Kim BW. Repetitive Colonoscopic Decompression as a Bridge Therapy before Surgery in a Pregnant Patient with Chronic Intestinal Pseudo-Obstruction. Clin Endosc 2013;46:591-594.

466. Küllmer A, Schmidt A, Caca K. Percutaneous endoscopic cecostomy (introducer method) in chronic intestinal pseudo-obstruction: Report of two cases and literature review. Dig Endosc 2016;28:210-5.

467. Murr MM, Sarr MG, Camilleri M. The surgeon's role in the treatment of chronic intestinal pseudo-obstruction. Am J Gastroenterol 1995;90:2147-51.

468. Sabbagh C, Amiot A, Maggiori L et al. Non-transplantation surgical approach for chronic intestinal pseudo-obstruction: analysis of 63 adult consecutive cases. Neurogastroenterol Motil 2013;25:680-686.

469. Pakarinen MP, Kurvinen A, Koivusalo AI et al. Surgical treatment and outcomes of severe pediatric intestinal motility disorders requiring parenteral nutrition. J Ped Surg 2013;48:333-338.

470. Irtan S. Bellaïche M, Brasher C et al. Stomal prolapse in children with chronic intestinal pseudo-obstruction: a frequent complication ? J Ped Surg 2010;45:2234-2237.

471. Pironi L, Spinucci G, Paganelli F et al. Italian guidelines for intestinal transplantation: potential candidates among the adult patients managed by a medical referral center for chronic intestinal failure. Transplant Proc 2004;36:659-661.

472. Millar AJ, Gupte G, Sharif K. Intestinal transplantation for motility disorders. Semin Pediatr Surg 2009;18:258-262.

473. Lao OB, Healey PJ, Perkins JD et al. Outcomes in children after intestinal transplant. Pediatrics 2010;125, 550-558.

474. Masetti M, Di Benedetto F, Cautero N et al. Intestinal transplantation for chronic intestinal pseudo-obstruction in adult patients. Am J Transplant 2004;4:826-9.

475. Tzakis AG, Kato T, Levi DM et al. 100 multivisceral transplants at a single center. Ann Surg 2005;242:480-93.

476. Sudan DL, Chinnakotla S, Horslen S et al. Basiliximab decreases the incidence of acute rejection after intestinal transplantation. Transplant Proc 2002;34:940-1.

477. Lauro A, Zanfi C, Pellegrini S et al. Isolated intestinal transplant for chronic intestinal pseudo-obstruction in adults: long-term outcome. Transplant Proc 2013;45:3351-5.

478. Lauro A, Zanfi C, Dazzi A et al. Disease-related intestinal transplant in adults: results from a single center. Transplant Proc 2014;46:245-8.

479. Ceulemans LJ, Monbaliu D, De Roover A et al. Belgian multicenter experience with intestinal transplantation. Transpl Int 2015;28:1362-70.

480. Colledan M, Stroppa P, Bravi M et al. Intestinal transplantation in children: the first successful Italian series. Transplant Proc 2010;42:1251-1252.

481. Halter JP, Michael W, Schüpbach M et al. Allogeneic haematopoietic stem cell transplantation for mitochondrial neurogastrointestinal encephalomyopathy. Brain 2015;138:2847-58.

482. De Giorgio R, Pironi L, Rinaldi R, Boschetti E, Caporali L, Capristo M, Casali C, Cenacchi G, Contin M, D'Angelo R, D'Errico A, Gramegna LL, Lodi R, Maresca A, Mohamed S, Morelli MC, Papa V, Tonon C, Tugnoli V, Carelli V, D'Alessandro R, Pinna AD. Liver transplantation for mitochondrial neurogastrointestinal encephalomyopathy. Ann Neurol. 2016; 80(3): 448-455.

483. Ohkubo H, Fuyuki A, Arimoto J, Higurashi T, Nonaka T, Inoh Y, Iida H, Inamori M, Kaneda T, Nakajima A. Efficacy of percutaneous endoscopic gastro-jejunostomy (PEG-J) decompression therapy for patients with chronic intestinal pseudo-obstruction (CIPO). Neurogastroenterol Motil. 2017 Dec;29(12).

484. Lebouvier T, Coron E, Chaumette T et al. Routine colonic biopsies as a new tool to study the enteric nervous system in living patients. Neurogastroenterol Motil 2010;22:e11-14

485. Lindberg G, Törnblom H, Iwarzon M, Nyberg B, Martin JE, Veress B. Full-thickness biopsy findings in chronic intestinal pseudo-obstruction and enteric dysmotility. Gut 2009;58:1084-1090.

486. Valli PV, Pohl D, Fried M, Caduff R, Bauerfeind P. Diagnostic use of endoscopic full-thickness wall resection (eFTR)-a novel minimally invasive technique for colonic tissue

sampling in patients with severe gastrointestinal motility disorders. Neurogastroenterol Motil in press;doi: 10.1111/nmo.13153.

487. Ponec RJ, Saunders MD, Kimmey MB. Neostigmine for the treatment of acute colonic pseudo-obstruction. N Engl J Med 1999;341:137-141.

488. Wong Toh Yoon E, Yoneda K, Nakamura S. Percutaneous endoscopic gastrostomy (PEG) using a novel large-caliber introducer technique kit: a retrospective analysis. Endosc Int Open 2016;4:E990-E996.

489. Ławiński M, Gradowski Ł, Bzikowska A, Goszczyńska A, Jachnis A, Forysiński K. Gastrojejunostomy inserted through PEG (PEG-J) in prevention of aspiration pneumonia. Clinical nutrition complication in dysphagic patients. Pol Przegl Chir 2014;86:223-229.

490. Alyami J, Spiller RC, Marciani L. Magnetic resonance imaging to evaluate gastrointestinal function. Neurogastroenterol Motil 2015;27:1687-92.

491. Rao SS, Camilleri M, Hasler WL, et al. Evaluation of gastrointestinal transit in clinical practice: position paper of the American and European Neurogastroenterology and Motility Societies. Neurogastroenterol Motil 2011;23:8-23.

492. Malagelada C, Drozdzal M, Seguí S, et al. Classification of functional bowel disorders by objective physiological criteria based on endoluminal image analysis. Am J Physiol Gastrointest Liver Physiol 2015;309:G413-9.

493. Camilleri M, Bharucha AE, di Lorenzo C, et al. American Neurogastroenterology and Motility Society consensus statement on intraluminal measurement of gastrointestinal and colonic motility in clinical practice. Neurogastroenterol Motil 2008;20:1269-82.

494. Veress B, Nyberg B, Törnblom H, et al. Intestinal lymphocytic epithelioganglionitis: a unique combination of inflammation in bowel dysmotility: a histopathological and immunohistochemical analysis of 28 cases. Histopathology 2009;54:539-49.

495. De Giorgio R, Barbara G, Stanghellini V, et al. Clinical and morphofunctional features of idiopathic myenteric ganglionitis underlying severe intestinal motor dysfunction: a study of three cases. Am J Gastroenterol 2002;97:2454-9.

496. Camilleri M. Jejunal manometry in distal subacute mechanical obstruction: significance of prolonged simultaneous contractions. Gut 1989;30:468-75.

497. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science 1998;281:1322-6.

498. Kellow JE, Phillips SF, Miller LJ, et al. Dysmotility of the small intestine in irritable bowel syndrome. Gut 1988;29:1236-1243.

499. Evans PR, Bak YT, Shuter B, et al. Gastroparesis and small bowel dysmotility in irritable bowel syndrome. Dig.Dis.Sci. 1997;42:2087-2093.

500. Bassotti G, Villanacci V, Nascimbeni R, et al. Colonic mast cells in controls and slow transit constipation patients. Aliment Pharmacol Ther 2011;34:92-9.

501. Malagelada C, Karunaratne TB, Accarino A, et al., Comparison between small bowel manometric patterns and full-thickness biopsy histopathology in severe intestinal dysmotility. Neurogastroenterol Motil. 2017 Sep 22.

502. Murphy EM, Defontgalland D, Costa M, et al. Quantification of subclasses of human colonic myenteric neurons by immunoreactivity to Hu, choline acetyltransferase and nitric oxide synthase. Neurogastroenterol Motil 2007;19:126-34.

503. Ippolito C, Segnani C, De Giorgio R, et al. Quantitative evaluation of myenteric ganglion cells in normal human left colon: implications for histopathological analysis. Cell Tissue Res 2009;336:191-201.

504. Doleshal M, Magotra AA, Choudhury B, et al. Evaluation and validation of total RNA extraction methods for microRNA expression analyses in formalin-fixed, paraffin-embedded tissues. J Mol Diagn 2008;10:203-11.

ACKNOWLEDEGMENTS

I would like to thank my supervisors, professor Vincenzo Stanghellini and professor Roberto De Giorgio for their kindness and support through out my studies at University of Bologna. I would especially like to thank professor Roberto De Giorgio, who I look up to as father figure, for his inspiration and immense influence on strengthening and moulding my career and life. I wish to thank professor Giovanni Barbara for his support and guidance during this time. I am very grateful to doctor Rosanna Cogliandro for her input in my work and teaching me how to perform and interpret manometry. I especially would like to thank Professor Umberto Volta for his cordiality and involving me in his exciting work. I would also like to thank doctor Ilaria Carrino for supporting me with the enormous paperwork required by Italian system. I would like to thank doctor Ana Accarino, doctor Carolina Malagelada, professor Juan Ramon Malagelada and professor Fernando Azpiroz for welcoming me at Vall D'Hebron hospital in Barcelona, Spain during my secondment and their excellent collaboration with the studies I did during my PhD studies. Also, I like to thank professor Paul Enck, doctor Sigrid Diether and doctor Thomas Wheeler-Schilling for putting together and managing the NeuroGUT project which provided me with a platform to carry out my studies at the University of Bologna. I like to thank my fellow colleagues, Elisa, Raffaella, Francesca, Fiorella, Elena, Alessandra and Giacomo in Bologna and Annika, In-Seon, Jasper, Eulisa, Friederike, Teodoa, Jacob, Thomas, Huiying (Vera), Maria, Ahmed, Natasja, Nazar and Michal of NeuroGUT project for all the support and good times we had together. There are many people I wish to thank who have helped and supported me during various times in my life. But I like to acknowledge and thank people below who are more important to me than anything. Frist of all, with all the respect I thank and pay tribute to my parents, Sisira Karunaratne and Geethangani Opatha who gave me this beautiful life, nurtured me with unconditional love and instilling great values in me. Above all else I thank my father, whose

tireless dedication and commitment allowed me to achieve these great things in my life. Secondly, I like to thank my great friend and brother, doctor Wayne Chou who always believes in me, stand by me through thick and thin and greatly influences my life to become a better person. Last but most importantly, I take pride in thanking my dearest friend doctor Silvia Cola to whom I would like to dedicate this thesis to. I am able to present this thesis due to her magnanimity in standing by me, caring about and supporting me at a most crucial moment in this journey.

THANK YOU ALL FOR EVERYTHING!!!!