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CICLO XXXI

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The study of the enteric nervous system in the distal colon: the GLP-2 protective effects against cisplatin-induced neuropathy and the neo-neurogenesis in colon-rectal cancer

Settore Scientifico Disciplinare BIO/17 - Istologia

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To the most important people of my life
Marisa, Silvia, Riccardo, Carlo

Abbreviations

15-deoxi $\lambda^{12\text{-}14}$-prostaglandin J2	15d-PGJ2
2, 4, 6-trinitrobenzene sulfonic acid	TNBS
5- fluorouracil	5-FU
5-hydroxytryptamine	5-HT
Acetylcholine	ACh
Adenomatous polyposis coli	APC
Autonomic nervous system	ANS
Brain-derived neurotrophic factor	BDNF
Calcitonin Gene Related Peptide	CGRP
Central nervous system	CNS
Chemotherapy induced constipation	CIC
Chemotherapy induced diarrhea	CID
Choline acetyltransferase	ChAT
Colon rectal cancer	CCR
Cyclossigenase-2	COX-2
Dipeptidyl peptidase-IV	DPP-4
Dorsal root ganglion	DRG
Enteric glia cells	EGCs
Enteric nervous system	ENS
G protein-coupled receptor	GPCR
Glial fibrillary acidic protein	GFAP
Glucagon-like peptide 2	GLP-2
Glucose transporter	SGLT1
Glutathione	GSH
Human intestinal organoids	HIOs
Inducible nitric oxide synthase	iNOS
Inflammatory bowel diseases	IBD
Insulin-like growth factor 1	IGF-1
Interstitial cells of Cajal	ICC
Intrinsic primary afferent neurons	IPANs
L1 cell adhesion molecule	L1CAM
Microtubule-Associated Protein 2	MAP2
Multidrug resistance protein-2	MRP2
N-myc downstream regulated gene 4	NDRG4
Nerve growth factor	NGF
NeuroFilament-200	NF-200
Neuronal nitric oxide synthases	nNOS
Neuronal Specific Enolase	NSE
Neuropeptide Y	NPY
Neurotrophic factor	GDNF
Neurotrophin-3	NT-3
Nitric oxide	NO
Non-histone chromosomal high-mobility group proteins 1	HMG1
Non-histone chromosomal high-mobility group proteins 2	HMG2
Nuclear factor-κB	NF-κB
Nucleotide excision repair	NER
Perineural invasion	PNI

Phosphatidylinositol-3-kinase/Protein kinase B	PI3K-AKT
Pituitary adenylyl cyclase activating peptide	PACAP
Pressure sequences	PSs
Primary cultures of enteric nervous system	pcENS
Proglucagon gene	*Gcg*
Prohormone convertase	PC
Prostaglandin E2	PGE2
Protein Gene Product 9.5	PGP 9.5
Proteins and mismatch repair	MMR
Quality of life	QoL
Receptor for advanced glycation end products	RAGE
Short bowel syndrome	SBS
Smooth muscle cells	SMC
Substance P	SP
Thromboxane A2	TXA2
Tumor epithelial cells	TEC
Tumor microenvironment	TME
Vasoactive intestinal polypeptide	VIP

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1. Summary

The enteric nervous system (ENS) is defined as the essential intrinsic microcircuit embedded within the gut wall able to orchestrate the gastrointestinal functions independently of central nervous system (CNS) inputs. The ENS regulates the gut functions and intestinal homeostasis via the control of the gastrointestinal (GI) motility, the regulation of the fluids' movement, nutrients absorption across the epithelium, the maintenance of intestinal barrier integrity and the management of the local blood flow [1]. Abnormalities that impact the ENS seriously compromise gut functions and can lead to severe gastrointestinal (GI) diseases [2]. Given that, over the last twenty years, the ENS has become an increasingly important research topic for the study of a wide and heterogeneous range of not only digestive but also extra intestinal diseases. Although the ENS has been extensively studied in the context of developmental, inflammatory and functional diseases [2], its role in chemotherapy-induced neuropathies and in colon rectal cancer (CRC) is understudied and remains largely elusive.

This present doctoral thesis addresses in part these two particular aspects (topics), which have been developed along two different projects and, here, they will be presented as two different research investigations.

The first research regarded the study of the cisplatin gut damages and the protective effects of the hormone Glucagon-like peptide-2 (GLP-2) studied in mouse distal colon, with a greater focus on the ENS, such as a novel target of cisplatin-induced toxicity. Indeed, it is well known that platinum-based (such as cisplatin) treatments cause distressing GI side effects during their administration, mainly due to the development of an acute inflammatory condition called mucositis. However, the causes of the persistence of vomiting, constipation and diarrhea, even after the end of the chemotherapeutic treatment, are still unclear. Recent studies have highlighted the association between chemotherapy-induced damage to the ENS and GI symptomatology, showing the ENS as a potential target of chemotherapy and conversely also as a putative therapeutic target to alleviate chemotherapy-induced toxicity [3–9]. Since strategies are needed to overcome these side effects, which seriously affect the patience's quality of life and therapy outcome, the aim of the present work of thesis is providing insight into the putative protective role of the analogue of the hormone GLP-2 against the cisplatin-induced toxicity. The GLP-2 is an endogenous hormone well known for its trophic role within the intestinal mucosa exerted both in physiological and experimental-induced pathological conditions [10]. Furthermore, it has been described as a neuroendocrine molecule able to

communicate directly with the ENS leading to neuronal activation and thereby to the regulation of the GI motility and also to neuronal protection. GLP-2 has also been described as a hormone able to mediate anti-inflammatory effects [11–15]. Therefore, the long-term co-administration of GLP-2 analogue and cisplatin, at two different dosages, was investigated using the mouse as experimental model. Then, morphological and molecular techniques were performed to characterize alterations both on the mucosal layer such as the target of chemotherapy-induced mucositis and on the ENS as target of cisplatin-induced cytotoxicity and putative responsible for the persistence of the chemotherapy-induced side effects.

On one hand the results carried out through confocal and fluorescence microscopy, showed that GLP-2 is able to protect the ENS in the myenteric plexus, reverting the loss of glial and neuronal cells due to the higher dosage cisplatin treatment. More specifically, the nitrergic component, rather than the cholinergic and the vipergic ones, has been shown as the component more involved in the GLP-2 protection. On the other hand, the cisplatin-induced gut remodelling, characterized by changes in morphological parameters such as the mucosal area and mucins expression, was not reverted by the GLP-2 co-administration. Finally, anti-inflammatory properties of the GLP-2 upon gut response to cisplatin were highlighted using ELISA techniques to measure Interleukin-1ß and Interleukin-10 levels. The co-administration of cisplatin and GLP-2 has been able to normalize the levels of both two cytokines compared to control group. In conclusion, these studies have revealed the GLP-2 as a molecule able to exert neuronal and glial protection on the myenteric plexus of the mouse distal colon and as a molecule having anti-inflammatory effects against mucosal inflammation due to cisplatin chronic treatment. Hence, this analogue could represent an effective strategy to overcome the intestinal symptoms induced by cisplatin therapy.

The second project developed in this thesis was to further explore the putative role of the ENS in colorectal carcinogenesis. It is increasingly recognized that the tumour microenvironment (TEM) could play a central role in the development of colorectal cancer (CCR). Notably, the ENS has been proposed as a novel cellular component of TEM, able to provide physical and functional support to the tumour development and progression in CCR [16]. TEM is defined like the overall cellular and molecular environment surrounding the tumour mass, able to establish a bidirectional cross-talk within the cells that it is composed of and the tumour epithelial cells (TEC) [17]. This communication is carried out by direct cell-cell interaction or via paracrine way signalling in which many cellular and molecules types, such as several growth factors, are involved.

Next to evidences that have showed immune cells and fibroblast as cells belonging to TEM and contributors to the tumour development, the existence of a direct interaction between tumour cells and peripheral nerve fibers, defined as perineural invasion (PNI), has been described in many cancers[18,19], including CCR [20]. In addition, PNI has been frequently associated to aggressive diseases and poor prognosis. In PNI, the invasion of the tumour cells around and within nerve sheaths has been described as a process that leads to the tumour migration along nerve pathways contributing, in this way, to the tumour development and sprouting [19,21]. Moreover, recent studies have demonstrated the correlation between the tumour development and autonomic neurogenesis in prostatic cancer [22], and furthermore, similar data have shown that neurogenesis could occur even in the ENS in CCR [23]. According to these findings, recent and preliminary evidences carried out by the French hosting laboratory have shown using immunohistochemistry an increased enteric neuronal density detected in human colon tissue surrounding the tumour areas as compared to 'healthy' tissues distant from the tumour. In addition, *in-vitro* experiments performed using primary culture of enteric ENS treated with tumour supernatants obtained from human tissue (within tumour and distant area) confirmed the evidences observed in situ. However, the factors involved in these neurotrophic effects remained unknown. In this context, the present work of thesis, aimed at further gaining insights into the mechanism through which factors released by TEM or TEC were able to induce the enteric neurogenetic process. At first my work contributed to confirm the ability of tumour supernatants to increase neuronal cells number in primary culture of ESN derived from embryonic rats. Then, the same experiments were performed using Caco-2 obtained-supernatants in order to confirm the hypothesis that TEC could directly account for these neurogenetic effects trough the release of specific factors. After having identified the Glial-derived neurotrophic factor (GDNF) as a putative mediator present in TEM and involved in tumour induced neurogenesis, we performed *in-vitro* experiments in presence of GDNF blocking antibody, showing that the neuronal number increase induced by tumour supernatants of TEM or TEC was significantly reverted. Finally, in order to validate the GDNF neurotrophic effect in an adult nervous system model, we developed organotypic cultures of rat adult colonic explants. Preliminary results showed that GDNF is able to induce a slight but not yet significant recovery of the myenteric number neurons compared to the untreated tissues. Up to date, these results, to be confirmed with additional studies, support our hypothesis according to which GDNF might be a molecule released by the tumour and thus being part of the TEM which we supposed to be directly involved in the tumour development process through neurogenic

properties exerted on the ENS. In conclusion, the work presented in this thesis contributes to the emerging concept that defines the ENS, on one hand, as an important contributor to tumour development in CCR, on the other hand, as a target of chemotherapy-induced neurotoxicity that likely contributes to the long-lasting GI side effects. The modulation of ESN functions and phenotype might represent a novel target for the treatment and/or prevention of CCR and conversely, for the protection against the post chemotherapy-induced GI complications.

2. Introduction

2.1. Large intestine

2.1.1. Anatomical and histological organization of the large intestine

The large intestine is the last part of the gastrointestinal tract. In humans, the large intestine is about 1.5 metres long, which is about one-fifth of the whole intestine. Anatomically it extends from the ileocecal junction to the anus and it is composed of mainly 4 parts: cecum, colon, rectum and anus [24]. The colon is divided further into four parts called ascending, transverse, descending and sigmoid colon. The ascending colon continues from the cecum to the right upper abdomen where it turns at the hepatic flexure to become transverse colon. It crosses anteriorly the whole width of the abdominal cavity to reach the splenic flexure, which marks the beginning of the la descending part. Since the present research is focused on the distal colon, this and next chapters will address this issue.

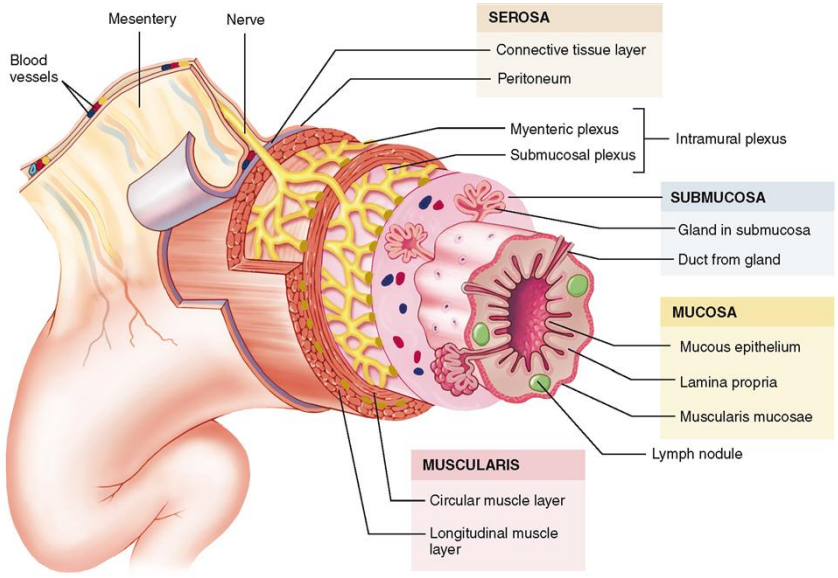

Figure 1; Macroscopic overview of the gastrointestinal wall. *The colonic wall, as the rest of the digestive tube is composed of the following layers, from inside to outside: Mucosal layer, composed of ephitelium, lamina propria and muscolaris mucosae; Submucosal layer; Muscolaris externa, composed of the circular and longitudinal muscel layers; Serosa composed of connective tissue layer and peritoneum.*

Like the rest of the digestive tube, the colon is made of four tissue layers (*Figure 1*): The innermost layer is the mucosa, in turn consists of the epithelium and associated glands, the lamina propria and the muscularis mucosa; the submucosal layer; the muscularis externa and the sierosa. The luminal part of the mucosal layer is lined with simple and

smooth columnar epithelium. It gives rise to highly repetitive invaginations, also called colonic crypts or crypts of Lieberkühn, which represent the basic functional units of the large intestine. These crypts are simple (unbranched) glands displayed parallel to each other and placed into the whole thickness of the mucosal layer. They appear such as tightly packed tubules shaped such as microscopic thick tubes with a central canal opening on the surface epithelium. Mucus-secreting goblet cells largely line both the epithelium and the crypts surface acting an important role in maintaining the mucosal barrier. At the base of the crypts, actively proliferating colonic stem cells are present and able to fuel the self-renewal of the epithelium giving mainly rise to four terminally differentiated epithelial cell types: the absorptive cells, the mucus-secreting goblet cells, peptide hormone-secreting endocrine cells and Paneth cells (*Figure 2*).

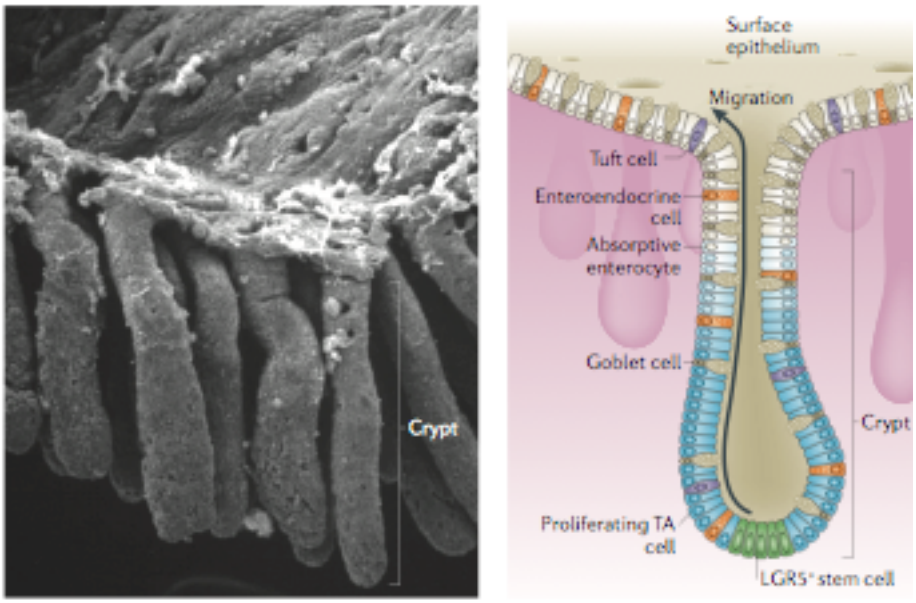

Figure 2; Colonic glands. *The structural organization is shown in the scanning electron micrograph in left panel. Within the colon, the luminal side of the mucosa is arranged in multiple invaginations, forming the intestinal glands (or crypts of Lieberkühn), that are lined with columnar epithelial cells. The crypts are displaced close each other, alternated with portions of a flat luminal surface. The adult epithelium undergoes rapid renewal to maintain optimal function and to recover from tissue damages. Epithelial turnover occurs every 5–7 days. Regeneration resides at the crypt base. Here a small population of adult stem cells (LGR5$^+$) regularly divides to produce highly proliferative progenitors known as transit amplifying (TA) cells. These cells undergone to several divisions while they move towards the crypt surface. Once reached the crypt luminal side, these cells complete their differentiation process to origin absorptive or secretory cells.* *(Image source: Barker et al. [25])*

The lamina propria is a loose connective tissue that contains lymphatic and blood vessels, nerves and smooth muscle cells. The muscularis mucosae is well developed and a bit more prominent in the large bowel compared to small intestine. It consists of distinct inner circular and outer longitudinal layers, which together contributes to the formation of the numerous mucosal folds. Surrounding the mucosa there is the submucosal layer, a loose connective tissue rich of blood vessels and nerves supporting the mucosal layer. The muscularis externa surrounds the submucosa and contains two muscle layers that

contract and move the large intestine. It is composed of a thick inner circular layer and a thinner outer longitudinal smooth muscle layer. Finally, the serosa forms the outermost layer. The serosa is composed of a thin layer of simple squamous epithelial tissue that secretes watery serous fluid to lubricate the surface of the large intestine, protecting it from friction between abdominal organs and the surrounding muscles.

2.1.2. Functions of the large bowel

The large bowel is involved in a wide range of functions, including the absorption of water, electrolytes and unabsorbed nutrients and the formation, storage and transport of feces [26,27]. The water and electrolyte absorption carried out by the epithelial cells is important for maintaining an appropriate hydration and electrolyte balance, but it is also important for the stool desiccation. The water present in the semi digested fluid material coming from the small intestine is absorbed more and more the chymo passes through the transverse and ascending colon, where the greatest absorptive capacity is present. The water absorption is strictly connected to the sodium transport, indeed, the lower sodium concentration in luminal part than the cytoplasm of enterocytes, allow the passage of the water towards mucosal direction [28]. Moreover, under normal conditions the colon is able to absorb sodium, chloride and to secrete bicarbonate and potassium [27,28]. The presence of a double mucus layer mainly secreted by goblet cells [29], provide not only the epithelial protection against the frictional and chemical trauma caused by the passage of chymo; but it also provides an optimal microenvironment to host over 400 species of bacteria responsible for the absorption of any remaining absorbable nutrients. Indeed, these microorganisms are able to break down complex carbohydrates into short- chain fatty acids, which are in turn absorbed by the enterocytes placed into the colonic epithelium. At lesser extent these bacteria are also able to metabolize proteins partly in amines, short- and branched-chain fatty acids [30]. Moreover, to the microorganisms metabolize vitamins such as vitamin K, thiamine and riboflavin which are resistant to digestion in the small intestine. Furthermore, through fermentative processes they are able to convert urea into ammonia, contributing in this way to the urea metabolism [27].

2.1.3. General overview of the colonic motility

All of functions stated above are strictly related to and regulated by a complicate interaction between muscle, secretory and nervous systems. Indeed, a precise coordination of all these systems is needed to achieve the mixing and propulsive colonic movements necessary for digestion, absorption and transit of intraluminal contents. Altogether these movements are usually referred as colonic motility. Although altered motility has been described playing a great role in various GI diseases, little is known regarding the colonic motility patterns. A variety of gut motility patterns has been described, but the most robust and commonly used classification system, which encompasses other previous observations, was created by Bassotti and colleagues [31]. They described two main forms of colonic contractile activities: propagating pressure sequences (PSs) and segmental contractions. PSs are reported to be an important determinant of luminal propulsion and defecation [27]. Whereas segmental contractions give rise to localised mixing contractions which facilitate the contact of colonic contents with colonic mucosa for absorption of water and other contents. These contractions may occur as single events or as a group of waves presenting singly or in rhythmic or arrhythmic bursts and accounting for the majority of colonic activity, particularly at rest [26,27,32]. Colonic motility is regulated by several neuronal and hormonal interactions, which are extremely complicated. Briefly, the main neural control is intrinsically orchestrated by the enteric nervous system (further explained in chapter 2.2.3), even thought, the extrinsic control, due to hormonal and extrinsic neuronal pathways, is a key to normal colonic physiology [27]. Several hormones released directly by the gut, such as gastrin, cholecystokinin, insulin, glucagon etc., have been demonstrated to exert a chemical control of the colonic motility [26]. These factors are released in relation to the nature of nutrients to adapt the gut to the digestive status. Regarding to the extrinsic neuronal control, sympathetic nerves exert an excitatory effect upon colonic motility by means the release of acetylcholine and tachykinins (ACh an SP), whilst parasympathetic nerves exert a tonic inhibition of colonic peristalsis, but it will be not address in this work [33].

2.2. Enteric nervous system

The enteric nervous system (ENS) is a complex neural network distributed along the whole length of the gut wall, from the oesophagus to the rectum, and in associated glands (pancreas, gallbladder and biliary tree). The ENS is composed of a large number of intrinsic neural circuits placed in close apposition to the effector systems that controls many functions even when it is completely separated from the central nervous system (CNS). In the gut, the effector systems include the musculature, the epithelium, the immune system and the blood vessels, through which the ENS controls gut motility, local blood flow, mucous transport and secretion, immune and endocrine functions [34]. Nonetheless, the innervation of the gut is not completely autonomous. Indeed, the neuronal control of all these functions is due to an integrated system involving local enteric reflexes, reflexes that pass through the ganglia of the autonomic nervous system (ANS) and reflexes that pass from the gut and back through the CNS [35]. The ENS is often referred to as the "little brain" because it resembles in complexity and autonomy the CNS more than other components of the ANS. Enteric neurons and glial cells are organized along the gut in aggregated structure unities (clusters) called ganglia. Thousands of small ganglia are interconnected each other's by inter-ganglionic strands, that, together with nerve fibers reaching the effector tissues, make up the complete enteric reflex circuit able to process and integrate many sensory and motor information [36]. The structure and functions of the enteric ganglia, however, diverge considerably from ganglia found in the ANS that merely act as relay-distribution centres. Histologically, the enteric ganglia are composed of neuronal bodies, glial cells and fibers bundles consisting of the axons of enteric neurons, axons of extrinsic neurons (arising from the parasympathetic and sympathetic parts of the ANS) that project to the gut wall, and glial cells' prolongment [1,36]. Therefore, as mentioned before, there is a rich histological and functional interaction, in both directions, between the enteric nervous system, sympathetic prevertebral ganglia and the CNS.

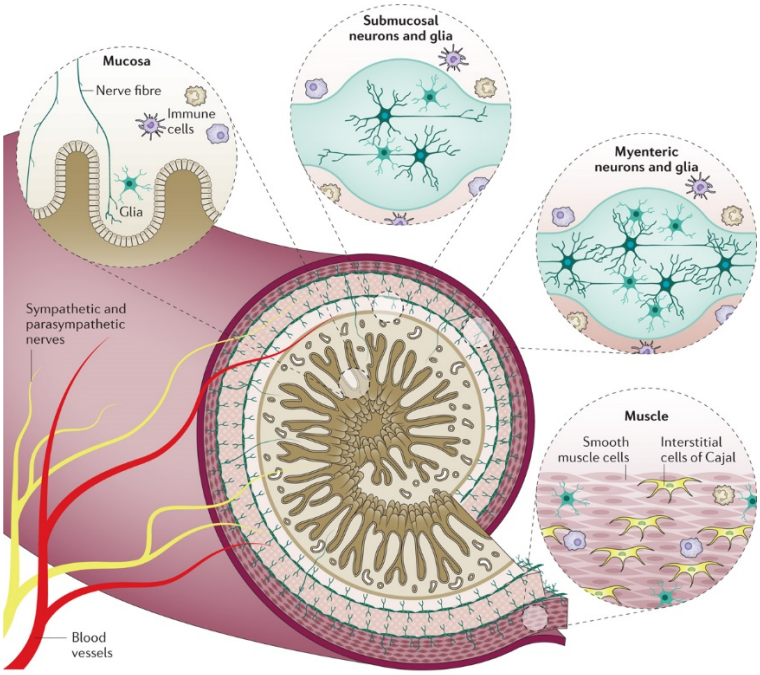

Figure 3; Microanatomical organization of the ENS within the large bowel wall. *The figure illustrates the general organization of the ENS within the gut wall (from the left to the right). Within the mucosal layer there are several nerve fibers mainly arising from submucosal neurons, together with glial cell; The submucosal plexus, also known as Meissner's plexus, is located within the submucosal layer and composed of submucosal neurons surrounded by glial cells; The myenteric plexus, also called Auerbach's plexus, is located between the circular and longitudinal muscle layers. It is composed of neurons mainly innervating the muscle wall. In the lower close up, the interstitial cells of Cajal are represented as cells intercalated between the smooth muscle cells of the circular muscle layer.* *(Image source: Boesmans et al. [37])*

The ENS is organized in ganglionated and aganglionated plexuses whose architecture varies among species and shows differences within the diverse gut regions [38]. Globally, among the ganglionated component, two are the submucosal and the myenteric plexus, showed in *Figure 3* . The myenteric plexus, also known as Auerbach's plexus, is located between the longitudinal and circular muscle layers and it extends from the upper esophagus to the internal anal sphincter. Most of the motor neuron' bodies that innervate both muscle layers originate from this plexus even if they can extend their prolongments to neurons placed in the submucosal plexus [36]. According to this anatomical disposition, the main role of the myenteric plexus is to direct the smooth muscle functionality. The submucosal plexus, also known as Meissner's plexus, is situated in the submucosal region between the circular muscle and the mucosa. In larger mammals (e.g., humans), the intestinal submucosal plexus consists of more than one layer. Although during the past decades many authors attempted to give a clearer morphological overview concerning the number of these layers, a unique definition has not been found. Brehmer and colleagues in 2010, described the human submucosal plexus composed of two continuous networks: an innermost monolayered plexus placed in close apposition to the circular muscle layer, and a multilayered one place in the inner part of the submucosal plexus [39]. The

submucosal plexus forms ganglia smaller and more irregular in shape than those present in the myenteric one. Furthermore, these ganglia are less dense respect to the myenteric plexus and relatively numerous in the small and large intestines, while are extremely rare in the stomach and esophagus [36]. From this plexus arises the majority of the motor innervation that reach the mucosal epithelium and intestinal secretory glands, as well as submucosal arterioles, in order to control and maintain water and electrolyte balance, secretion and vascular tone [1,40]. Concerning the aganglionated component, six plexuses have been described in literature [38]: The *sub-serous plexus* [38]; The *longitudinal muscle plexus* [41]; The *circular muscle plexus* and *deep muscular plexuses* [42]; The *muscularis mucosae plexus* and *mucosal plexus* [43].

2.2.1. Enteric neurons

The human ENS contains about 100 million neurones and comprises many different types of neurones, which are differently distributed depending on gut segments and species [34,44]. Combinations of several features as morphology, neurochemical properties and cell physiology have helped to define approximately 20 types of enteric neurons [45]. Dogiel was the first author who described the morphology of the enteric neurons, classifying them into three main groups: Dogiel types I, II, III [46]. Following studies based on new developed techniques, confirmed the existence of these neuronal types, pointing out Dogiel type I and II as the most common in many species of mammal, and the type III less common [36,38,47]. The classification of enteric neurons by shape has been further developed by several authors, most notably Stach and Brehmer [38,46]. These authors have identified neurons that are designated types IV to VII and mini-neurons. In this work, only the Dogiel types I and II will be briefly described as enteric neuronal types more represented in humans.

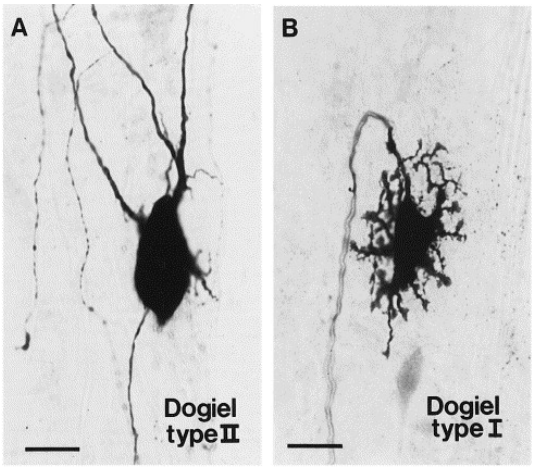

Figure 4; Dogiel type II and Dogiel type I neurons (B and A). *(A) Dogiel type I neurons are characterized by many short and a single long procesess, whereas Dogiel type II presents multipolar conformation with many long, smooth processes.* *(Image source: adapted from Clerk et al. [48])*

The Dogiel type I shows stellar-shaped cell body surrounded by many short dendrites and a single long axon that extends along the whole gut wall circumference passing through the inter-ganglionic fiber tracts. Usually, axons arise from the cell body, but sometimes emerge from a dendrite giving rise to short spines in their initial parts [47]. The majority of this neuronal type are motor neurons that make up synapsis with the musculature and secretory epithelium [38,47] and they are more numerous within the myenteric plexus than in the submucosal one. Depending on the projecting direction (aboral or oral), neurons belonging to this group are able to release specific neurotransmitters such as: nitric oxide (NO), vasoactive intestinal polypeptide (VIP) that are inhibitory neurotransmitters; substance P (SP) and acetylcoline (ACh) that are excitatory ones [47]. The type II neurons are angular, stellar or spindle shaped with a large, smooth, round or oval cell body. They have one axon and few dendrites (from 3 to 10) which can be long or short and displayed in a variety of configurations. Many of them have long processes that may extend through inter-ganglionic fibers tracts in either circumferential, oral or aboral direction. Unlikely, they may have short processes that project only into their own ganglia. Several of type II neurons project processes to the mucosa/submucosa and express the SP and ACh as neurotransmitters [36,38,47]. Dogiel type II neurons (*Figure 4)* are the most prominent neurons in myenteric and submucosal ganglia of the small intestine and colon but are very rare in the stomach.

2.2.2. Functional classification of enteric neurons

Besides the morphological classification, enteric neurons can be functionally classified into three main classes: intrinsic primary afferent neurons (IPANs, also referred to as intrinsic sensory neurons), interneurons and motor neurons (*Figure 6*). IPANs have the Dogiel type II shape and are present in both myenteric and submucosal ganglia representing respectively the 30% and 14% of total neurons [44]. These, which have been described as sensory neurons, detect the physical state of the gut such as the presence of tension on the gut wall or chemical features in the luminal contents, acting as chemo-sensor, mechanoreceptors and stretch responsive neurons [34–36,44,49]. From electrophysiological point of view they are characterized by delayed and prolonged after hyperpolarizing potential, and so that, they are defined as AH neurons [47]. IPANs are connected each other but also directly with motor neurons and interneurons, composing a wide number of enteric neural reflex able to initiate appropriate control of motility, secretion and blood flow [1].

Interneurons are usually Dogiel type I and are classified in two main classes: ascending and descending interneurons [38]. At least, one type of ascending and three types of descending interneurons have been characterised, although the greater part found in mammals belongs to the descending type [34,36,44]. The ascending neurons are mainly cholinergic, whereas the descending ones have a complex chemical coding including neurotransmitters as ACh, NO, VIP, 5-HT, and somatostatin [50]. They are able to receive, and then process and integrate the sensory information incoming from IPANs and therefore, they can be involved in the modulation of peristaltic motility reflexes or in local secretomotor reflexes depending on what neuronal types establish connection with. Indeed, the combination among different neurotransmitters gives rise to different functions (see the chapter 2.2.2). For instance, ACh/NO/VIP/somatostatin neurones are involved in gut motility, while ACh/5-HT neurones are involved gut secretion [50].

Motor neurons represent the final motor output of the enteric reflex circuit that act to a large number of effector cells. Depending on the effector system, they are designed using different names. Muscle motor neurons, secretomotor neurons, vasodilator neurons, motor neurons innervating enteroendocrine cells, motor neurons effecting on nutrient uptake, and neurons innervating lymphatic tissue have been identified in the gut [35]. The ENS contains two types of muscle motor neurons: excitatory neurons that mainly release ACh and tachykinins as transmitters and inhibitory neurons that release VIP, NO, ATP, pituitary adenylyl cyclase activating peptide (PACAP) [44]. Both of these two types have a

Dogiel type I shape and innervate the smooth muscle of the muscularis externa and muscularis mucosa, controlling in this way, the mixing and propulsive movements of the whole gut. Neurons innervating the circular musculature have their soma mainly placed into the myenteric ganglia whether part of those innervating the longitudinal one have the cell body even in the outer submucosal plexus [36,51]. Moreover, the submucosal plexus has been proposed also as the main source of innervation of the muscularis mucosae in humans [51]. Secretomotor and vasodilator neurons present their soma predominantly into the submucosal ganglia but some of them are also placed in myenteric one. They innervate the intestinal mucosa of the small and large intestines promoting respectively the fluid passage from body compartments into the gut lumen and controlling the mucosal blood flow [52]. Both of these two classes utilize VIP as the main neurotransmitter, but other transmitters, notably acetylcholine, also participate [1,51].

Concerning the innervation of enteroendocrine cells, little is known. Nevertheless we do know that twelve classes of endocrine cells lie on the mucosal layer and that numerous nerves bundles are placed in close apposition to these cells, until recently, it was not clear yet whether their functions were neuronally regulated [1]. Indeed, in the past the enteroendocrine cells have been considered as sensory epithelial cells able to communicate by means the production and release of several hormones. Recently they have been better characterized as cells having features reminiscent of excitable cells such as small synaptic vesicles, voltage-gated channels and further, specific structures called, neuropods [53,54]. The discovery of these latter structures as a communication route with nerves, let them to be considered as specialized innervated epithelial sensors taking part to a neuro-endocrine network recently called “connectome” [55]. Other characters involved within this network are the enteric neurons and especially, glial cells, that have been recently described as cells able to physically interact with the enteroendocrines’ pseudopods for modulating the transmission of sensory gut information [53]. Nonetheless, the composition of all networks involved in the connectome are not well known. The best documented motor neurons innervating enteric endocrine cells are those controlling release of gastrin from G cells, which has been demonstrated being under the influence on both vagal and intrinsic gastric pathways [36]. The release of hormone as Peptide YY from L cell or motilin from M cells has been supposed to be regulated by the extrinsic innervation neural control [56]. Finally, recent evidences have suggested that the increase of glucose transport is nerve mediated [57]. Indeed, after the nutrient’s stimulation, the glucose transporter SGLT1 are functionally activate by the hormone glucagon-like

peptide 2 (GLP-2) which presents its receptors not on mucosal epithelial cells but on submucosal neurons [58] (see the chapter 2.4.5).

2.2.1. Neuronal markers

Pan-neuronal markers predominantly used for studying enteric neurons, are: HuC/D [59], Neuronal Specific Enolase (NSE), Protein Gene Product (PGP) 9.5, NeuroFilament (NF) 200, β-tubuline III, Microtubule-Associated Protein (MAP)2 and NeuN. They are in common with those utilized for studies on CNS because a specific enteric neuronal marker is not present yet. HuC/D belongs to the family of Hu proteins, which is reported to be able to regulate the stability of specific mRNA and thereby modulate the precise temporal expression of certain proteins [60,61]. Because of these roles, HuC/D normally resides in the body cytosol and not in the fibers, making it a useful neuronal marker for analysis focused on the number neurons quantitation. However, some authors described an expression heterogeneity in different subcellular compartments showing that it can also be present in the nucleus [61,62]. Indeed, even though specific transporter proteins able to shuttle Hu proteins through the nuclear envelope have been described, its role in the nucleus is still elusive [61]. This heterogeneity has been addressed only in some intestinal ischemia-reperfusion studies, where nuclear expression of Hu proteins, was reported and associate with condition of neuronal stress and damage such as elevated nitrosylation levels [63]. Finally, low oxygen conditions has been showed representing an important trigger which lead to increased nuclear HuC/D immunoreactivity [62]. NSE is a glycolytic enzyme that catalyses the conversion of 2-phosphoglycerate to phosphoenolpyruvate whereas PGP 9.5 is a member of the ubiquitin hydrolase family. These two markers are expressed in both soma and fibers by allowing studies focused not only on the number's neurons, but also on their tissue distribution. NeuN protein is localized in nuclei and perinuclear cytoplasm of most of the neurons in the central nervous system of mammals. The accumulated experimental data, mainly obtained from studies performed on CNS, provide evidence that the intensity of the immunocytochemical reactions for NeuN in the nucleus and even in the cytoplasm may vary both within the same type of neurons and between different types of neurons [64]. Even though NeuN immunoreactivity has been found in ENS, it is poorly utilized for studying the enteric neurons [65]. Finally, NF-200, β-tubuline III, MAP2, are neuronal marker mainly expressed in the neuronal extensions. Their expression differs from one species to another and the expression profile is not overlapping.

2.2.2. Chemical coding

Another way to differently classify the enteric neurons is considering their neurochemical phenotype consisting of the combinations of different neurotransmitters, neuropeptides, enzymes, and neuroendocrine markers expressed by the enteric neurons. This concept is the basis for the chemical coding hypothesis which states that each neuron expresses a unique neurochemical combination composed of 1 to 11 different mediators [36]. Several studies have highlighted the correlation between this chemical coding hypothesis and the neuronal functions in physiological and pathological conditions [18,66]. For instance, chemical codes of neurons in the mouse small intestine are showed in Figure 5.

Neuron subtype	Neurochemical coding	Neuron function	Proportion
Myenteric neurons			
Intrinsic sensory neuron, or intrinsic primary afferent neuron	• CGRP (also known as CALCA) • ACh • Neurofilament (145 kDa) • Calbindin ± calretinin	Main sensory neurons of the ENS, detect chemical and mechanical changes in gut lumen	26%
Descending interneuron	• ACh • NOS	Descending inhibition	3%
Descending interneuron	• ACh • 5-HT	Descending excitation	1%
Descending interneuron	• ACh • Somatostatin • Calretinin	Propagation of migrating motor complexes	4%
Ascending interneuron	• ACh • Calretinin • Tachykinin	Ascending excitation	4%
Excitatory circular muscle motor neuron	• ACh • Tachykinin ± calretinin	Elicits contraction of circular muscle	21%
Inhibitory circular muscle motor neuron	• NOS1 • VIP • NPY	Inhibits contraction of circular muscle	23%
Excitatory longitudinal muscle motor neuron	• ACh • Calretinin ± tachykinins	Elicits contraction of longitudinal muscle	13%
Inhibitory longitudinal muscle motor neuron	• NOS1 • VIP	Inhibits contraction of longitudinal muscle	3%
Submucosal neurons			
Vasodilator neuron	• VIP • NPY • Calretinin	Control of submucous arterioles	30%
Cholinergic secretomotor neuron	• ACh • CGRP • Somatostatin • Calretinin	Control of secretion from mucosa	30%
Non-cholinergic secretomotor neuron	• VIP • NPY • Calretinin • TH	Control of secretion from mucosa	22%
Intrinsic primary afferent neuron?	Has not been identified in mouse submucosal plexus	NA	–
Cholinergic	ACh but no other marker	Function unknown	10%
Unidentifiable markers	Unknown	Function unknown	8%

Figure 5; Table describing the neuronal chemical coding in myenteric and submucosal neurons correlated with their specific functions. *(Image source: Boesmann et al. [67])*

2.2.3. Motility enteric reflex

The intestinal motility is defined as the alternation of contraction and relaxation cycles performed by the smooth muscle cells (SMC) placed within the muscularis externa. Peristalsis and segmentation are the two principles colonic movements [1,36]. These motor functions are primarily controlled by complex and integrated circuitry composed of SMC, connected each other's trough gap junction to form an electrical syncytium, myenteric neurons, that innervate SMC, and interstitial cells of Cajal (ICC) able to trigger the electrical stimulus and synchronize, in this way, the musculature contraction [68,69]. More precisely, in peristaltic reflex *(Figure 6)*, mechanical and/or chemical stimuli cause the release of serotonin from the enteroendocrine cells. In turn, serotonin will active IPANs neurons which in turn release several neuromodulators as ACh, SP, Calcitonin Gene Related Peptide (CGRP) in order to activate descending or ascending interneurons. Descending nerves are connected with motor inhibitory enteric neurons that synapsis with the SMC upstream to the stimulation point, whilst ascending interneurons make synapsis with excitatory motor neurons that innerve the distal side of a stimulus. This leads to a relaxation and contraction respectively of the proximal and distal stimulation point. These cycles are coordinated in time and space resulting in an anally directed transport of gut contents by the activity of ICC. These cells are placed between the circular and longitudinal muscle layers and represents the intestinal pacemaker able to generate rhythmic and slow electric waves which pass through neighbouring ICC or SMC by gap-junction.

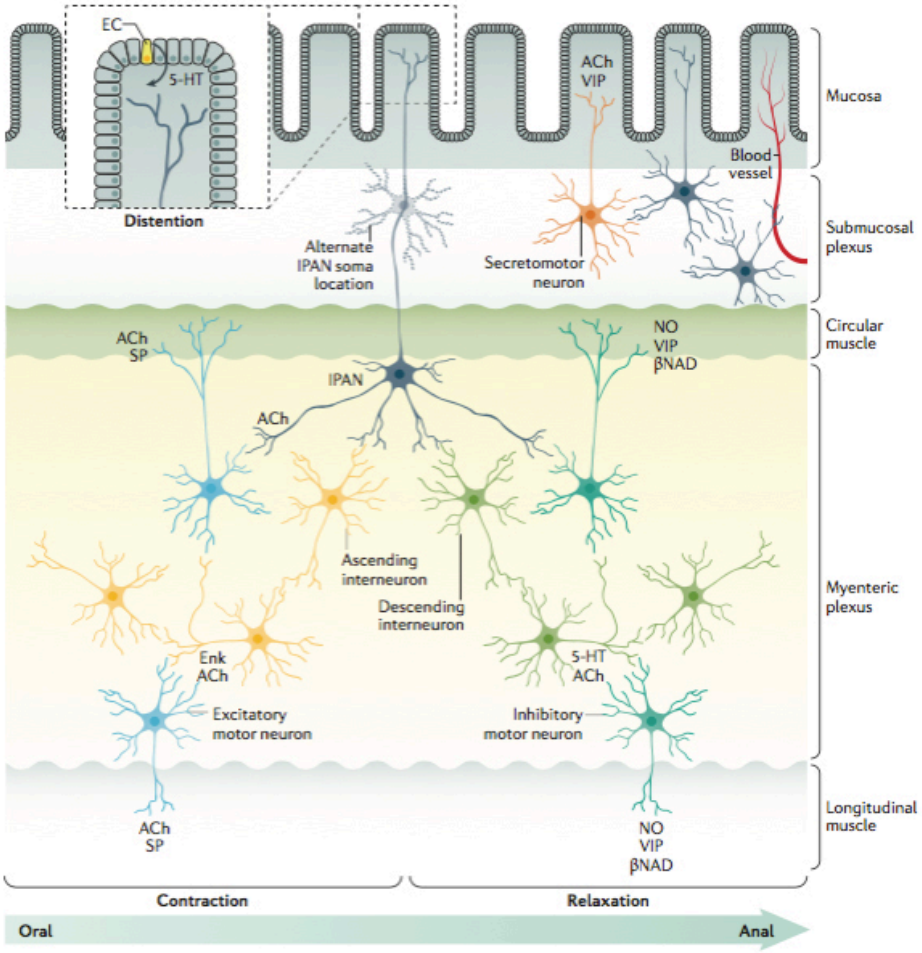

Figure 6; Model of intestinal peristaltic reflex. *The image sums up the anatomical interactions existing among the different neuronal types during a generic intestinal peristaltic reflex. IPANs neurons are colored in blue; Ascending interneurons in yellow, the descending ones in green; Excitatory motor neurons in light blue whilst the inhibitory one in turquoise. The reflex starts with the chemical/mechanical stimulation of the mucosal layer that induces the release of 5-HT from the enteroendocrine cells. Therefore, the information passes thought ascending or descending interneurons to reach excitatory or inhibitory neurons placed within the myenteric plexus. The result of this stimulation is the contraction and relaxation of the SMC placed, respectively, downstream and upstream of the stimulation point. (Image source Rao et al.* [70] *).*

2.2.4. Secretor- vasodilator enteric reflex

Similar to the motility reflexes, secretor-vasodilator enteric reflexes are present within the gut to modulate and control the secretion of water and electrolytes, as well as the blood flow.

As summarized in *Figure 7* these reflexes are generally triggered by mechanical or chemical stimuli that occur in mucosal layer. IPANs neurons can be activated by serotonin, released by enterochromaffin cells in response to the stimulation. Serotonin actives IPANs neurons which, in turn, can transmit the information through three ways. The reflex can occur through only one intrinsic neuron making up an axon reflex; alternatively, it can make synapsis with secretomotor and/or secretomotor vasodilator submucosal neurons or motor neurons placed in myenteric ganglia.

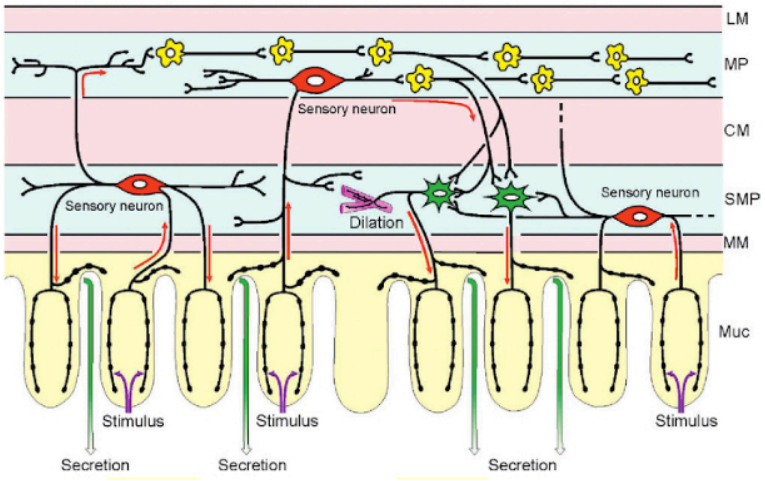

Figure 7; Schematic representation of a secretor- vasodilator enteric reflex. *These reflexes are mainly mediated by neurons placed in the submucosal plexus release ACh, VIP, NO neurotransmitters that mediate secretions and blood flow.(Image source Furness et al. [1])*

The integrated signal can, at this point, get back to secretomotor vasodilator place in the submucosal plexus [71]. Among these three ways, the secretor-motor reflex involved in a greater extent motor neuron placed in the submucosal plexus, even thought, it has been showed that cholinergic and vipergic myenteric neurons can project their extensions towards the mucosal layer [72]. In addition, it has been demonstrated in perfused rat colon that the ENS is able to control the mucus secretion by means the release of VIP and SP as neuromodulators, which activate in turn the secretion of TreFoil factor-3, which is a molecule described being involved in protection against intestinal mucosal damage and in epithelial repair [73].

2.2.5. Glial cells: Morphology, distribution and organization

Enteric glia cells (EGCs) are non-excitable (non-myelinated) and irregularly-shaped cells described for the first time by Dogiel in the 1899 as Schwann cells surrounding neurons placed into enteric ganglia. EGCs show an irregular stellate morphology characterized by an extensive array of highly branched processes, called gliofilaments, and a small cell body that is mostly filled by the nucleus, leaving very little cytoplasmic volume.

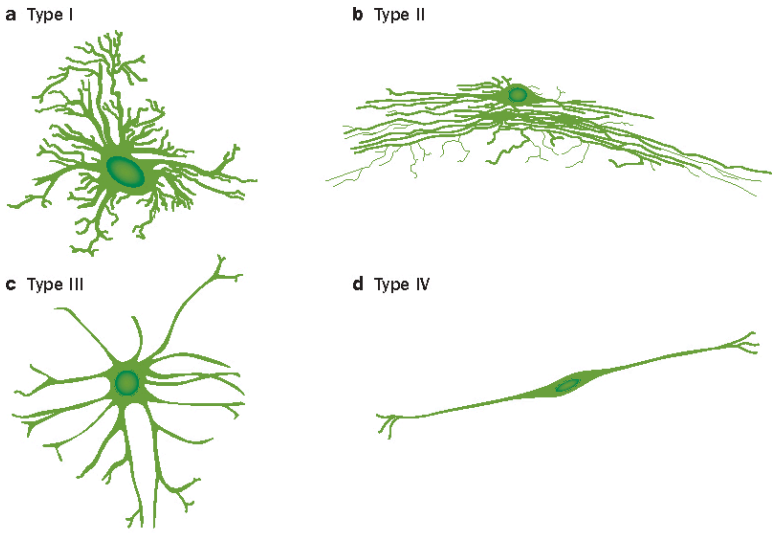

Figure 8; Types of enteric glial cells. *Schematic illustration of four different glial subtypes. Type I: Star shaped glial cells, characterized by short and numerous processes that lie within the ganglia. Type II: "fibrous" glial cells, characterized by irregular and numerous branches placed in the inter-ganglionic structures. Type III: "mucosal glia" placed underneath the epithelial barrier and characterized by several long and branched processes. Type IV: intramuscular glia with elongated shape, lining along nerve and muscular fibers.* *(Image source: Sharkey et al. [74])*

For almost a century EGCs had been considered as Schwann cell until when, in the 1970s Giorgio Gabella redefined them as unique glial lineage that shared many morphological, molecular and functional resemblances to astrocytes of the CNS [75]. Indeed, similar to astrocytes, EGCs showed a particular morphology associated with prominent and branched gliofilaments, and the expression of markers as Glial fibrillary acidic protein (GFAP) and S-100ß [76]. EGCs are arranged in close contact to the neurons throughout all layers of the gastrointestinal tract. They are organized in multiple diverse subpopulations resident within the different nervous structures. Major subpopulations of enteric glial cells reside within the myenteric and submucosal ganglia (intra-ganglionic ECGs), but other subpopulations are also placed within inter-ganglionic nerve fiber tracts, below the mucosal epithelial cells (subepithelial or mucosal ECGs) and associated with nerve fibers interspersed between SMC (intramuscular EGCs) [74,77]. Within the plexus glial cell bodies are placed between neurons and glial processes extend over the surface of neurons and between neuronal processes (*Figure 9).*

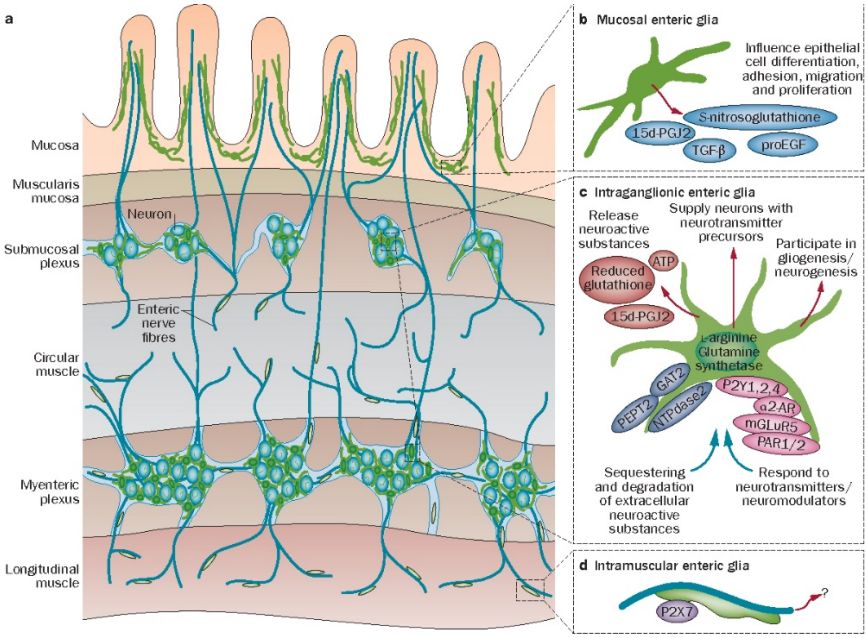

Figure 9; General distribution of different glial subpopulations within the gut wall and their related functions. *Mucosal enteric glia, placed in close apposition to the mucosal epithelium, regulates epithelial cell differentiation, adhesion, migration and proliferation. The intra-ganglionic glia located in both submucosal and myenteric plexuses, participates in gliogenesis/neurogenesis; communicates with enteric neurons responding to neurotransmitters/neuromodulators, as well as, sequestering/degrading the extracellular neuroactive substances; supplies neurons with neurotransmitters precursors. Intra-muscular glia.* *(Image source: Sharkey et al. [74])*

Although intensive studies were performed with the aim to better understand and characterize the diversity of enteric neurons subtypes, the origin, physiological and morphological diversification of EGCs are, nowadays, still unclear. Pioneering studies carried out by Hanani and Reichenbach proposed a first classification scheme for EGCs based on their morphology and location within the plexuses [78]. Nevertheless, despite this, EGCs were generally thought as homogeneous multitasking population showing differences in the expression of receptors, channels and enzymes. Along with the development of genetic and biomolecular tools able to better identify their physiological properties, their classification has been reexamined considering not only morphology and gut distribution, but also molecular and physiological features. Indeed, recently, Boesmans and co-workers, using a high-resolution genetic cell lineage tracing method, have defined four glial subtypes, each of them characterized by unique locations within the ENS structures and unique combinations of glial markers expression [37]. According to this work, Type I (also called protoplasmic glia) corresponds to star-shaped glial cells, placed into enteric ganglia and largely co-expressing the three glial markers: GFAP, SOX-10 and S-100ß. Type II, or fibrous glia, is a subtype composed of elongated glial cells situated into inter-ganglionic space. The extra-ganglionic glial group is represented by the Type III and IV, that are respectively composed of subepithelial glia, characterized by several long and branched processes, and intramuscular glia that run along nerve fibers

in muscle layers by means its elongated conformation (*Figure 8*) [37]. The minority cells belonging to the type II and III express the GFAP marker, meanwhile the majority one co-express S-100ß and SOX-10. Whether this phenotypic heterogeneity would be accompanied by functional differences and whether this heterogeneity could be developed during or after embryogenesis, are questions still unsolved. Concerning this issue, the same authors have proposed evidences based on physiological approach, showing that different glial subpopulations exhibit different activity when stimulated through ATP [37]. Other evidences provided by few *in vivo* studies have further reinforced the existence of an intrinsic functional glial heterogeneity. Moreover, given the fact that the gut is one of the most dynamic organs of the body, able to adapt itself to variable microenvironment (exposition to unpredictable availability of nutrients or exposure to pathogenic bacteria), the presence of potential plasticity of the different EGCs subtypes must be considered.

2.2.6. Markers of enteric glial cells

Three are the markers currently used in the enteric glial studies: GFAP, S-100ß and SOX-10 (*Figure 10*). GFAP is a protein belonging to cytoscheletric intermediate filaments, thus expressed within the whole cytoplasm [79]. The GFAP expression is not straightly specific for enteric glia, indeed, other cell types of peripheric and central glia such as astrocytes and Schwann cells share this marker [76]. Furthermore, a recent work has highlighted that some colonic epithelial cells found in the mouse intestine, might express this fibrillary protein [80]. GFAP cellular levels has been correlated with the functional state of EGC and it has been reported that GFAP expression in mature EGCs is modulated by cell inflammation [81].

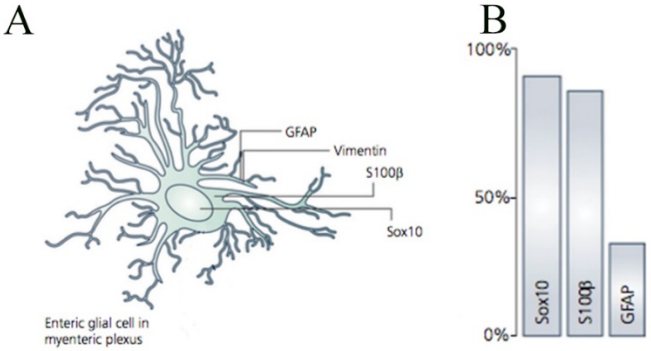

***Figure 10;The expression of the glial markers within myenteric plexus (A)**. GFAP is expressed in glial processes, SOX-10 in the nucleus, S-100ß in the cytoplasm. Percentage of the expression of SOX-10, S-100ß, GFAP glial markers (B). (Image source: adapted from Grubišić et al. [82]).*

SOX-10 is a transcription factor belonging to the SOX family. Although it has important roles during the development of the enteric [83], specifically during the glial differentiation, its expression has been found also in mature EGCs, becoming a very well-suited glial marker used for quantification purposes given its selective nuclear localization and expression [84]. The diffusible S-100ß protein belongs to the S100 family that includes more than 20 EF-hand Ca^{2+}-Zn^{2+}–binding proteins expressed by astrocytes and by several nervous and non-nervous cellular types, such as Schwann cells, chondrocyte, oligodendrocytes and adipocytes [85]. Despite of this, S-100ß is specifically and physiologically expressed and released by EGCs in the gut, making it a specific enteric glial marker [76]. Both in gut and brain, S-100ß can be considered a "Janus-faced" protein, because in nanomolar concentrations, it regulates microenvironmental homeostasis, whereas in micromolar amounts it is correlated with a pathologic and inflammatory status. Especially in the latter case, S-100ß exerts its actions by binding to the receptor for advanced glycation end products (RAGE), with the downstream phosphorylation of several kinases which lead to the consequent activation of the nuclear factor-κB (NF-κB), which in turn leads to the transcription of different cytokines and inducible nitric oxide synthase (iNOS) protein [86].

2.2.7. Functions of enteric glial cells in the ENS

Although over the past 40 years a great deal of progress has been achieved in understanding the role of enteric neurons in the control of gut functions in health and disease conditions, it is only recently that EGCs have started to be investigated as central actor of gut and ENS homeostasis. Indeed, the EGCs have long been considered as mere supportive cells for enteric neurons but so far, several reviews elucidated the emerging concept according to which the EGCs are able to actively regulate several gut activities by means neuromodulation, neuroprotection, and neurogenetic properties carried out through a bidirectional communication with enteric neurons (*Figure 11*) [82,87].

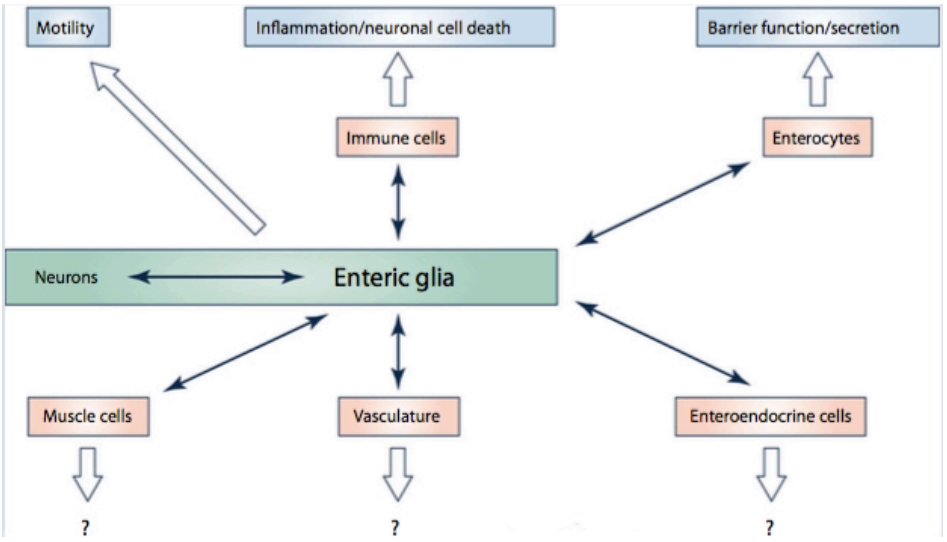

Figure 11; Schematic representations of functions exerted by the neuro-glial interactions. *(Image source: Grubišić et al.* [82]*)*

EGCs are known as excitable cells able to answer to neuronal stimulation with transient elevation of intracellular calcium concentration $[Ca^{2+}]_i$ [87]. A number of studies have shown that glial activity is recruited by neurotransmitters/neuromodulators released during synaptic communication and recently it has been demonstrated that these glial $[Ca^{2+}]_i$ transients are involved in gut motility [88,89]. Lately, McClain et al. have showed that impairing the activity of glial cells *in vivo* disrupts the neural control of gut motility, pointing out that the mechanisms enacted by $[Ca^{2+}]_i$ responses in enteric glia regulate the activity of enteric neural networks [82,90]. These evidences are not surprising, considering that changes affecting glial integration such as changes in glia numbers or their cellular Ca^{2+} handling have been highlighted to play roles in GI dysmotility disorders such as chronic constipation [88,91]. Further to this, EGCs have shown able to exert neuroprotective effects in oxidative stress-induced conditions. Indeed, *in vitro* co-culture experiments highlighted that the release of eicosanoids, such as15-deoxi $\Lambda^{12\text{-}14}$-prostaglandin J2 (15d-PGJ2) by EGCs enhanced neuronal survival in cultures [92–94]. Enteric glia are probably also involved in the metabolism of the neurotransmitters through their degradation or sequestration once released at the synapses, and via the production of neurotransmitter precursors such as L- arginine or glutamine [76,77] . Besides neurons, however, growing number of studies strongly support the notion that EGCs also engage functional crosstalk with all the different cell types present in the gut wall as immune cells, enterocytes, enteroendocrine and muscle cells, making them as important regulators of physiological processes in the gut mucosa. Neunlist et al. has recently coined the term 'neuronal–glial–epithelial unit' to correlate the anatomical proximity to functional interaction between

enteric glia and the intestinal epithelium, highlighting the role of EGCs in the homeostasis and maintenance of epithelium barrier integrity and functionality [95]. More precisely, *in vivo* experiments of EGCs ablation, carried out through different severity degrees (severe and moderate), resulted respectively in the disruption of epithelial barrier integrity and changes in paracellular permeability [96]. *In vitro* experiments looked further inside the molecular mechanisms through which the EGCs can modulate these functions. Savidge et al., proposed the S-nitrosoglutatione as a modulator of mucosal barrier secreted by EGCs and associated with high expression of epithelial tight-junctions proteins [97]. Furthermore, ECG can also enhance epithelial barrier repair after mechanical and inflammatory injury and actively participate to secretory and absorptive processes [98]. Interestingly, recent evidences carried out through electron scanning and confocal microscopy showed contacts between EGCs and enteroendocrine cells trough specific axons-like structures called "neuropods" [53] . Finally, EGCs have been reported to be able to block the epithelial barrier proliferation and to induce the differentiation process by means the release of 15d-PGJ2 [99]. Another exciting glial role within the intestinal mucosa is represented by the bi-directional communication with the gut microbiota. Up to now, the nature of this interaction is poorly understood, but it has been recently proposed that microbiome is likely involved in the homeostasis and development of mucosal glia [100].

2.3. The plasticity of the ENS in diseases and in response to environmental challenges

As outlined in the chapters above, the ENS is able to orchestrate many GI functions and therefore alterations charged to one or more enteric components can lead to a wide range of GI dysfunctions. Among these, the most clinically evident are those in which intestinal motility or sensory functions are affected. Alterations in the ENS can arise from congenital abnormalities as the best studied Hirschsprung's disease, or from acquired alterations that are generally believed to be caused by neuronal degeneration, immune-mediated inflammation, or infection [101]. In certain cases, the ENS represents the main contributor to a disease, in others, it is encompassed in the development of the enteric disturbance. The ENS plasticity is defined as the overall range of morphological and functional changes that can occur during physiological conditions as a morphological adaptation to environmental-induced changes [102], but also during pathological conditions considered as the capacity of the ENS to undergo adaptive changes and/or reparative events [103]. In inflammatory bowel diseases (IBD) changes in neurophysiological,

neurochemical and morphological properties of enteric nerves have been well described. The body of the literature concerning these diseases is derived from multiple studies performed on humans and various experimental models, that highlighted a wide range of gut remodeling during intestinal inflammatory conditions [103,104]. For instance, nerve rearrangement (hypertrophy and hyperplasia) have been shown in Crohn's disease and ulcerative colitis [105]. Moreover, neuronal loss and changes in neurochemical coding have been observed in neurons and nerve fibers in tissue specimens of patients with IBD [106]. Notably, SP and VIP have been the most widely studied among all gut neuropeptides in both IBD and animal models of gastrointestinal inflammation [66,107,108]. Electrophysiological studies have demonstrated an hyperexcitability of IPANs neurons suggesting that a perturbation of the afferent component of intrinsic motor reflexes may occur during inflammation and may contribute to gut dysmotility observed in patients affected by inflammatory dysfunctions [109]. Furthermore, next to alteration in enteric neurons, glial reactivity has been also underlined as an upregulated neural component involved in these inflammatory-induced remodeling [110]. Interestingly, the glial response to stress is probably in part aimed at restoring normal ENS homeostasis, in particular via the ability of EGC (ie Sox-10-IR cells) to generate de novo neurons under pathological conditions such as inflammation or BAC induced neuronal cell death.

Despite to this, more recently, the ENS has also been proposed as novel contributor to other major GI disease as digestive cancer and their response to chemotherapy treatment. However, so far, few studies addressed these issues and therefore they will be the two main topics at the center of my thesis and further described in the following paragraph of the introduction.

2.4. Research study 1; "The GLP-2 protective effects against cisplatin-induced neuropathy."

2.4.1. Chemotherapy and side effects

In the last 50 years, the development of several new antineoplastic agents and new protocols in oncology have revolutionized the treatment of cancer diseases leading to curative or palliative chemotherapeutic intervention to several human cancers [111]. The use of combinate chemotherapy regimens based on the use of antineoplastic drugs with different mechanisms of action, and the early prevention have improved patient survival

and declined the mortality rates [111]. Nevertheless, a longer life expectancy of the patients has been accompanied by a growth in the incidence of several side effects responsible for a worsening of patients' quality of life (QoL) [112]. Poor QoL can influence adversely the possibility for patients to continue and successfully complete the treatment. For this reason, the need to better understand and identify the mechanisms at the basis of the wide spectrum of unwanted effects have become increasingly important. Indeed, several attempts are ongoing to look for new and more specific antitumoral regimens, and for the development of adjuvant strategies focused on counteracting the chemotherapy-induced side-effects. Among the main chemotherapy classes, platinum-based drugs are treatments whose mechanism of action acts interfering with cells cycle, inducing, predominantly, the apoptosis process in high proliferating cells as cancer cells. Next to this antitumor efficacy, there is the general nonspecific cytotoxicity exerted on healthy cells that induce the side effects just mentioned above. Cells with an high proliferating rate as those placed in the bone marrow, digestive tract or hair follicles are the worst affected [111].

Clinically these unwanted effects are classified as early and late side effects depending on the time of occurrence and persistence. The early side effects can appear during the treatment as consequence of early toxicity exerted on tissues with a high proliferating index, whereas the late side effects appear after the treatment is ceased, and can even persist for long periods starting from 6 months to years [113]. This persistence has been indicated as result of damages on tissues with a low proliferating index as cardiac cells, skeletal muscle, cells in the organ of Corti and neurons causing fibrosis, atrophy, vascular and neural damage [114].To date, although the late toxicity represents a major health problem for the long-term cancer survivors [115], it represents a field still poorly explored.

2.4.2. Cisplatin

Cisplatin, cis-diamminedichloroplatinum (II), discovered in the 1961 by Rosemberg, has been the progenitor of the platinum-based drugs family and to this day, one of the most effectiveness chemotherapy drugs. Indeed, it is still used for treatment of a wide spectrum of solid neoplasms, including ovarian, testicular, bladder, colon-rectal, lung and head and neck cancers [116,117]. Despite of its antitumor efficacy, however, cisplatin therapy is accompanied by intrinsic and acquired resistance as well as severe side effects, including dose-limiting nephrotoxicity, cumulative peripheral sensory neuropathy, ototoxicity, and GI complications such as nausea and vomiting, diarrhea and constipation [118]. Over the years, various platinum complexes, some of which are shown in *Figure 12*, have been

studied in an attempt to overcome these problems. Although some of them were tested in clinical trials, only a few (e.g., carboplatin and oxaliplatin) received worldwide approval for clinical practice. For instance, Carboplatin has reduced toxicity but is cross-resistant with cisplatin, whereas oxaliplatin displays a lack of cross-resistance and has been used to treat colorectal cancer [117].

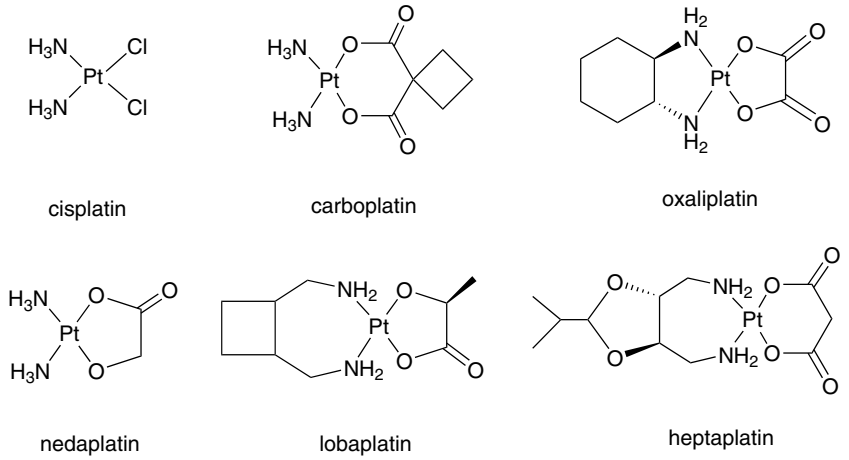

Figure 12; Chemical structures of the clinically used platinum drugs.

Although cisplatin has been successfully used in the chemotherapy of cancer for more than 25 years, its precise mechanism of action is still unclear. The current accepted paradigm states that DNA platination is the crucial step for triggering the cell death processes [119,120]. However, multiple cellular events appear to contribute to its cytotoxicity. From the chemical point of view, cisplatin is an inorganic and water-soluble platinum complex that has a square planar structure composed of metal platinum Pt linked to 2 chloride and 2 ammonia (NH_3) ligands [121]. These substituent atoms can assume a -*cis* or a *trans* conformations, but only the *cis* is showed as the most pharmacologically active. Moreover, the presence of two chloride atoms allow it to be reactive with H_2O and other intracellular nucleophilic molecules. Depending on the chloride concentration, cisplatin can react with H_2O giving rise to the hydrated configurations. Thus, since in the bloodstream the serum chloride concentration is lower than the cytoplasmic one, cisplatin remains a neutral molecule, whilst in cells cytoplasm, where the chloride is higher, the hydration occurs forming to two main complexes monoaqua $[Pt(NH_3)2Cl(H_2O)]^+$ and diaqua $[Pt(NH_3)2(H_2O)2]^{2+}$ [122]. It had been long assumed that the passive diffusion was the main way by means cisplatin was able to enter into cells. However, some facilitate, or active transport mechanisms have been highlighted as contributor to cisplatin uptake and intracellular accumulation. For instance, CTR1 or MDR1 receptors play an important role in the uptake of cisplatin as well the development of cisplatin resistance [123,124]. The

cisplatin hydrolyzed species present electrophiles characteristics which make these compounds more reactive then the native one and thus able to bind nucleophiles species present into the nucleus and cytoplasm. DNA and RNA are the most important cisplatin biological target described in literature even if, as cited above, many other cytoplasmic targets are ongoing to be characterized as responsible of the cytotoxic cisplatin activity. Due to their higher nucleophilicity, N^7 atoms of guanosine and adenosine residues are the most two DNA reactive sites which cisplatin is able to form cross link on. To be more specific, depending on which residues are involved, intra-strand or inter-strand cross-links can be formed. The most frequent cross-links are 1,2-d(GpG) and 1,2-d(GpA), which are formed between two adjacent guanine or adjacent guanine and adenine [119].

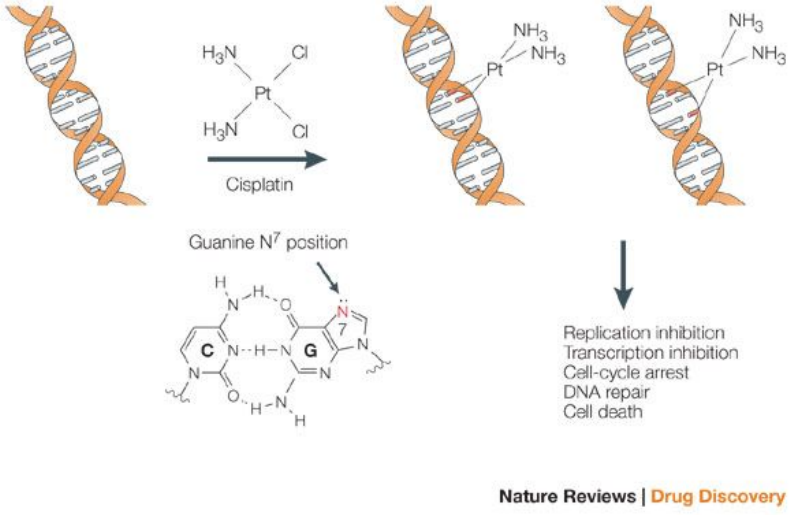

Figure 13; Cisplatin mechanism of action. *The guanine N^7 position is able to covalently bind the platinum atom of the cisplatin hydrated form. This process leads form DNA inter- or (1,2 or 1,3) intrastrand cross-links, also called cisplatin adducts, that can cause various cellular responses, such as the arrest of replication, transcription, DNA repair processes, that in turn can lead to trigger the apoptotic process. (Image source: Whang et al. [125])*

These cross links, also defined as platinum adducts, are able to induce a significant distortion in the DNA double helix, which, in turn, can lead to block DNA replication and gene transcription, triggering diverse signaling pathways. Regarding to this, AKT, c-ABL, p53, MAPK/JNK/ERK pathways have been related with the modulation of cisplatin toxicity [125]. Downstream of these intracellular pathways, cell death may occur by means apoptosis and/or necrosis processes [120,125], when the DNA repair mechanisms fail. Indeed, these adducts can be recognized by several cellular proteins leading to the repair, replication bypass or eventually initiation of apoptosis. Several protein families are involved in the recognition of Pt-DNA adducts including non-histone chromosomal high-mobility group proteins 1 and 2 (HMG1 and HMG2), nucleotide excision repair (NER) proteins and mismatch repair (MMR) proteins [125]. Among DNA repair mechanism, NER

has been recognized as the factor involved in cisplatin-adducts defense [120]. It is noteworthy that oxaliplatin adducts, for instance, are recognized preferentially by other repair mechanisms respect those involved in cisplatin protection, although the nature of their adducts is similar. This differential recognition of cisplatin and oxaliplatin adducts contribute to the differences in cytotoxicity and tumor range of cisplatin and oxaliplatin [126]. As previously stated, cisplatin can form adducts not only with nucleic acids but also with other intracellular molecules, such as glutathione (GSH), methionine, metallothioneins and other cysteine-rich proteins. Particularly, the formation of GSH-cisplatin adducts leads to decreased GSH cytoplasmic reserves which results in oxidative stress, but also in cisplatin resistance, given that the complex has been demonstrated to be readily excreted out of the cells by multidrug resistance protein-2 (MRP2), a member of the ABC family ATPases [127]. Furthermore, as most other metal compounds, intracellular cisplatin presents an high oxide-reductive potential though which can induce the formation of many reactive oxygen species (ROS) in cytoplasm environment depending on the drug concentration and/or duration of exposure [122]. This oxidative imbalance has been described also, as a result of cisplatin mitochondrial damage that through the disruption of the electron transport chain and the consequent release of ROS within the cytoplasm, contribute to the oxidative stress and the related apoptotic death [121,122]. Furthermore, alterations in mitochondrial respiration lead to a transient intracellular calcium increase, which plays a significant role in calcium homeostasis and hence cell functions [121].

Among all these molecular actions, those mainly responsible for the diverse set of toxicities have not been well established yet. However, some reports showed that, for instance, the oxidative stress cisplatin-induced is one of the mechanisms responsible for the severe nephrotoxicity and hepatotoxicity [128]. Although it has been supposed that the cisplatin-induced DNA damage mainly affect proliferating cells, interestingly, it has been proposed that the main contributor to the dorsal root ganglion (DRG) neurons damage, would be the formation of DNA adducts [114,126]. Indeed, even as DRG neurons are post-mitotic cells, it has been demonstrated trough *in vivo* studies that the chronic cisplatin administration resulted in an accumulation of DNA adducts which determined increase levels of neuronal death [129]. Furthermore, it has been highlighted that high DNA adducts can lead to inhibit the global transcription activities which are essential for neuronal functions, given the fact that neurons are characterized by a high metabolism rate [130]. Therefore, DNA damage can block transcription processes leading to a neuronal atrophy and disruption of neuronal connections [130]. Finally recent evidences have proposed that

cisplatin-induced mitochondrial dysfunctions might be the secondary mechanism able to contribute and aggravate the neuronal damage [131,132].

2.4.3. Delayed GI side effects chemotherapy-induced

As mentioned above, most other chemotherapy treatments, included platinum-based treatments, are associated with debilitating and dose limiting GI side effects. Nausea, vomiting, diarrhea and constipation affect more than 40% of patients receiving standard dose chemotherapy and 100% of patients receiving high dose chemotherapy [115,133,134]. Particularly, among the delayed GI side effects, chemotherapy induced constipation and diarrhea (CIC and CID) occur in about 50% of cancer survivors and can persist up to 10 years after the cessation of treatment [115]. Even though, cisplatin has been known as one of the most emetogenic chemotherapy drugs, CIC and CID have been reported as intestinal consequences of cisplatin long term treatment [135].The pathophysiology of CIC and CID have been overlooked until recently, but recent evidences have suggested these GI alterations as complex processes most likely involving multiple different and intersecting processes such as secretion, osmosis, inflammation. Nurgali and co-workers summarized all evidences present in literature in a review recently published [115]. They described CID as a by-product of GI inflammatory condition referred as mucositis most commonly described with fluoropyrimidines (particularly 5-fluorouracil (5-FU) and capecitabine) and irinotecan [136–138]. The initiation of mucositis is believed to result from direct or indirect cytotoxic effects exerted by chemotherapy treatment on rapidly diving cells as those present in the intestinal mucosa, which, in turn, can contribute to malabsorption and hypersecretion[136]. Furthermore, the upregulation of proinflammatory cytokines is described as a contributor to mucosal inflammation and ulceration. Several studies demonstrated how increased expression of cyclossigenase-2 (COX-2), prostaglandin E2 (PGE2), thromboxane A2 (TXA2) was able to stimulate colonic secretion and gut hyperistalsis, as well as, modulate the secretion of different electrolytes contributing to the onset of diarrhea [139]. Moreover, next to the mucosal damage and inflammation that could be responsible for alteration in absorption and GI secretion, changes in intestinal microflora have been recently highlighted playing a role in intestinal homeostatic dysfunctions, but also in the development of mucositis itself modulating the inflammatory response [140,141]. Concerning CIC, these authors reported that almost 80-90% of patients receiving cisplatin, thalidomide and others cytotoxic agents experience that symptomatology [115]. Severe constipation can result in a variety of symptoms

including abdominal distention, pain, hemorrhoids, bleed and rectal fissures, as well as, following bowel obstruction, perforation, ischemia and necrosis [115]. Little is known about the pathological processes associate to these symptoms, but it has been hypothesized that CIC which is predominantly motor in nature may result from direct damage chemotherapy-induced on gut innervation.

2.4.4. Impact of cisplatin on the ENS

The affinity of platinum compounds to the peripheral nervous system has been proven by several works focused on DRG. Recently, it has been hypothesized that this affinity could be exert even towards the ENS [142,143]. Hence, ENS is emerging as key player in chemotherapy-induced GI dysfunctions which may contribute to permanent gastrointestinal damages in cancer survivors [8].

Very recently attempts have been made to elucidate how long-term chemotherapy treatments could impact the ENS and GI functions. The use of distinct animal models, different cytotoxic agents at different administration timings and dosages showed differential alterations on ENS within the different GI regions. Vera and coworkers aimed to study the early and delayed effects of the long-term cisplatin administration using different dosages (1, 2 and 3 mg/kg) given for 5 weeks (1 time/week) to rats, chosen as experimental model. In several published works, these authors demonstrated that long-term cisplatin administration induced significant reduction of the upper intestinal transit and obliteration of colonic contractile activity, immediately after and one week later the treatment ceased. Conversely, they highlighted a significant gastric distention and delayed gastric emptying just at the end of the treatment, but not after one week [3,4,9]. These functional outcomes have been accompanied by morphological changes charged to the general tissue architecture and particularly to the myenteric neurons [4,9]. Within the distal colon and terminal ileum, they observed dose-dependent histopathological features especially referred to the mucosal architecture altogether with structural and neurochemical alterations in myenteric plexus. Cisplatin 3 mg/kg dose decreased the total number of neurons and was associated with decreased ganglionic area and an increased proportion of neuronal nitric oxide synthases (nNOS)-IR neurons in the distal colon [4]. In another study they demonstrated that the dosage of 2 mg cisplatin was able to induce increased mRNA expression of several marker neuronal markers such as nNOS, Choline acetyltransferase (ChAT) and of the c-kit as specific marker for ICC within the muscularis externa of the small bowel [9]. No changes in total number of myenteric neurons was

observed. Other observations have been reported by Pini and co-workers that showed as the cisplatin long-term administration (2mg/kg for 4 weeks) caused dilatation of the mice gastric fundus supported by enhanced neuronally induced contractile responses and loss of myenteric neurons. The authors demonstrated also a decrement in both number and proportion of nNOS-IR myenteric neurons [7].

Further studies performed by Nurgali's research team have highlighted the long-term GI effects elicited by another platinum compound as oxaliplatin. They have showed, similarly to cisplatin treatment, significant loss of myenteric and submucosal neurons in the ileum and distal colon after 21 days of treatment [5,6,144]. Concerning the studies on the distal colon, they showed in myenteric plexus increased proportion of nNOS-IR neurons just after 7 day-treatment both in ex-vivo and in vivo experiments, while decrements of total number neurons have been detected only after 21 days-treatment [5]. They further showed that after 14 days the frequency and propagation speed of colonic migrating motor complexes were significantly decreased in vitro. They looked further into the mechanism of these alterations, studying the oxidative stress as a putative molecular mechanism through which oxaliplatin could induce neuronal damages [144]. They demonstrated that the formation of reactive oxygen species (O_2^-), nitration of proteins and release of cytochrome c from mitochondrial membrane depolarization, resulted in the neuronal apoptosis in both the submucosal and myenteric plexuses of the colon. In addition, increased inducible nitric oxide synthases (iNOS) expression has been correlated to changes in intestinal smooth muscle tone and neuromuscular transmission underlying colonic dysmotility which could lead to chronic constipation associated with oxaliplatin treatment [144]. Regarding the mouse ileum, they showed no histological alterations charged to mucosal and muscle layers, but differences in neuronal density and expression of two different glial markers, were observed both in myenteric and submucosal plexuses during the chronic oxaliplatin administration. In particular, decrease in GFAP-IR and increase in S-100ß were observed in submucosal and myenteric plexus together with loss of enteric neurons [6].

2.4.5. GLP-2: general overview and biological functions

Glucagon-like peptide 2 (GLP-2) is a 33-aa peptide produced from a single gene, called proglucagon gene (*Gcg*), that is expressed in several organs including pancreas, intestine and CSN [145,146]. *Gcg* gene further encodes for glucagon and for a large number of others glucagon-like peptides in mammals [147]. This differential peptide profile is carried out through the action of the prohormone convertase (PC) enzymes which are differentially expressed in several tissues [148]. Indeed, enzymatic isoforms, such as PC2, PC1 and PC3, are present in pancreatic α cells, intestinal L cells and CNS, respectively [148,149]. Because of their alanine or proline residues, common to GLP-2 and other members of the PACAP/glucagon superfamily, GLP-2 is highly susceptible to the degradation protease-mediated, exerted specifically by dipeptidyl peptidase-IV (DPP-4), which is a serine peptidase widely distributed along tissue but high concentrated in kidney, liver, at the brush border of enterocytes as even in the endothelial cells of the entire vascular bed [146,150].

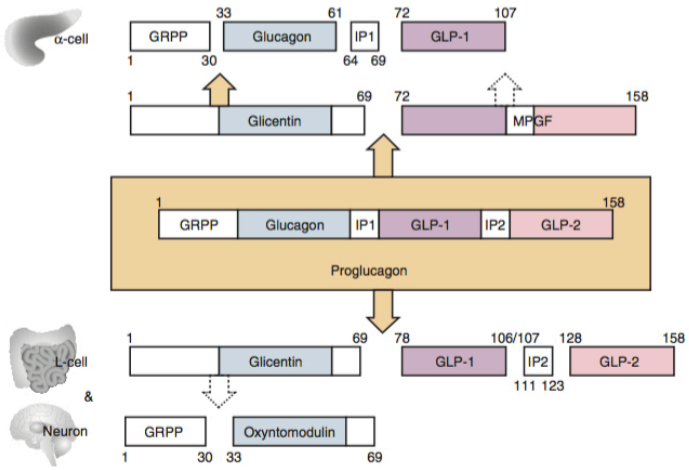

Figure 14; Schematic presentation of the several products arising from post-trascriptional processes occuring in pancreas, intestine and central nervous system. *In the intestine, L-cells product mainly glicentin, GLP-1, GLP-2 starting from proglucagon by means the proteolic clivage mediated by the pro-hormone convertasi 3 PC3, specifically expressed in the intestine.*

Within the intestine, the key stimulus for the induction of *Gcg* gene transcription and, then, for the GLP-2 synthesis and secretion is the food ingestion that leads rapidly to increased GLP-2 plasma levels through mechanisms requiring engagement of neural and endocrine circuits, as well as direct nutrient contact with L cells [151]. Once into the blood stream, GLP-2 is cleaved into its non-bioactive form in few minutes by DPP-4 [152]. Besides this physiological response, plasma levels of GLP-2 have been described increasing rapidly in response to intestinal injury or major intestinal resection as adaptive response,

even though the signals involved in the sensing of gut injury that lead to production and secretion of GLP-2 remain obscure [146]. GLP-2 is synthetized and secreted by the enteroendocrine "L" cells of ileum and colon [146,153]. L cells are specialized enteroendocrine cells with maximum density in the ileum and colon, although L cells are also present more proximally in the small intestine, including jejunum and even duodenum [154]. GLP-2 exerts several functions binding its specific receptor, GLP-2R, which is a G protein-coupled receptor (GPCR) belonging to the glucagon-secretin class B receptor family. The distribution of GLP-2R has been found to be largely restricted to intestine but limited mRNA transcript expression has been found in CNS, and lung [146,155,156]. Although the exact localization of the GLP-2R within the gut of different animal and human is still on-going debate, GLP-2R has been detected in the stomach and in both the small and large intestines [157]. Using different technical approaches, several authors have described the GLP-2R in human enteroendocrine cells, in murine enteric neurons and in subepithelial myofibroblasts in rodents and humans [12,146,158–160]. Nonetheless, despite this highly localized expression within the gut, the mechanism underlying the wide range of function exerted by GLP-2 has proven to be complex and up to date not well understood. Indeed, even though GLP-2 was firstly discovered as an intestinotrophic factor in 1996, today, it is recognized as a pleiotropic hormone that influences multiple physiological functions specifically in the GI tract. In literature it is mainly described as a molecule able to regulate the intestinal energy absorption and maintain the mucosal morphology and integrity through trophic effects (*Figure 16*). Several works have reported GLP-2 as a key beneficial effector on the gut by means the expansion of the mucosal epithelium owing to increment of the villus height and crypt depth. This effect has been described as a consequence of the GLP-2 ability to promote the crypt cell proliferation and inhibition of apoptosis [146,158,161]. However, the mechanisms through which GLP-2 acts on the mucosal epithelium have not been fully explained. Since the GLP-2R was not found to be expressed on epithelial cells but on subepithelial fibroblast, enteric neurons and enteroendocrine cells, it has been proposed that GLP-2 could exert its actions indirectly, perhaps through a paracrine mechanism, which involves second messengers deriving from GLP-2R expressing cells [158]. The *Figure 15* shows the model proposed to explain the mucosal trophic effect GLP-2-induced.

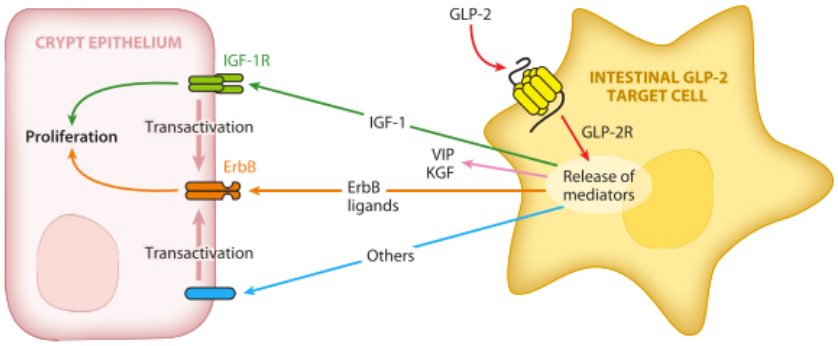

Figure 15; Key growth factor pathways transducing the intestinotrophic actions of GLP-2. IGF-1R and ErbB signaling mediates the intestinotrophic response to GLP-2. *Abbreviations: KGF, keratinocyte growth factor; IGF-1, insulin-like growth factor 1; VIP, vasoactive intestinal peptide .(Image source Drucker et al.* [146]*)*

As first, Dubé and Drucker provided evidences that the mitogenic factor IGF-1 produced by intestinal subepithelial myofibroblast cells plays a key role in the intestinotrophic effects of GLP-2 [162]. Moreover, further studies carried on murine intestinal subepithelial myofibroblasts suggested that the IGF-1 released by myofibroblast may involve ErbB ligands which is able in turn to activate the phosphatidylinositol-3-kinase/Protein kinase B (PI3K-AKT) and beta-catenin signaling [157,163]. Besides this, GLP-2 has been shown as a molecule able to exert other several actions within the GI tract related to the promotion of energy absorption. Indeed, it has been demonstrated that GLP-2 is able to enhance intestinal barrier function decreasing transcellular and paracellular epithelial permeability in murine diabetic and food allergy models [164–166]. Further, GLP-2 was able to modulate the uptake of luminal nutrients increasing the expression of digestive enzymes and nutrient transport channel [167,168]. Finally, GLP-2-dependent increase in mesenteric blood flow have been highlighted in studies on human and pig [159,169]. Recently, Mulè and co-workers [170] shed further insight into the role of GLP-2 within the gut by describing this hormone as a neuroendocrine molecule able to directly involve the ENS in the regulation of some GI functions such as motility and inflammatory response. Evidences showing the presence of GLP-2R in the ENS, together with changes in gut motor activity, especially exerted to gastric level, have led to consider the slowing motility GLP-2-induced as the result not only of the CNS-mediate mechanism but also of the involvement of ENS [12,159,171,172]. Indeed, it has been shown that GLP-2 in the mouse small intestine and colon is able to reduce the spontaneous smooth muscle activity by increasing nitric oxide release [160], while in the colon, where GLP-2R is expressed and colocalized with ACh-IR myenteric neurons, the peptide acts by slowing the motility through inhibition of ACh release from the neurons [12]. Lastly, in various rat and mouse model of inflammatory

bowel diseases, GLP-2 treatment decreased levels of inflammatory cytokines as tumor necrosis factor a and interleukin-1 and -6. Sigalet et al., proposed an indirect mechanism mediating the GLP-2-induced anti-inflammatory actions that would be exerted either through the maintenance of gut barrier integrity or through induction of anti-inflammatory mediators but involving the ENS [14,15]. Indeed, they reported that GLP-2-activated VIP-immunoreactive neurons leading to anti-inflammatory response. In particular, using 2,4,6-trinitrobenzene sulfonic acid (TNBS) mouse model, they first observed in mice treated with TNBS and GLP-2 an increase in the number and proportion of VIP expressing submucosal neurons. Second, they showed the activation of submucosal VIP neurons following the exogenous GLP-2 administration that was associated with a reduction in mucosal inflammation, suggesting, hence, anti-inflammatory and neuroprotective GLP-2-dependent roles [14,15].

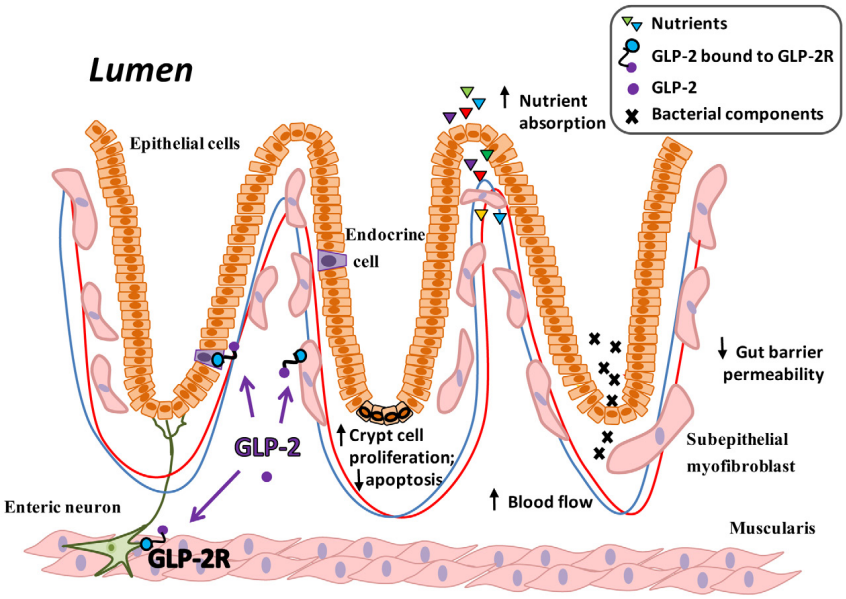

Figure 16; Physiological effects of GLP-2 in intestinal mucosa. *GLP-2 is able to increase the intestinal blood flow, enhance proliferation and decrease apoptosis in cryptic intestinal cell, modulate the barrier permeability and reduce the gut motility. All of these functions aim to increase the absorptive capacity. (Image source: Connor et al.* [173] *).*

Ekblad and collaborators reported, finally, the neuroprotective properties of GLP-2 against mast cell-induced cell death in *in vitro* experiments [13]. However, the ability of GLP2 to induce in vivo neuroprotective effects both in enteric neurons as well as in enteric glial cells remain currently unknown.

Collectively, these evidences have led to underline the emerging role of this peptide as a neuroendocrine signal molecule involved in the modulation of GI motility via neural/GLP-2R signaling.

2.4.6. GLP-2 in pathological conditions

A large number of pharmacological studies, carried out through exogenous administration of GLP-2 in rodents and humans, have helped to gain further insights into the biological role of GLP-2 during physiological but also pathological conditions. Activation of GLP-2R signaling was shown to protect the small and large bowels in a broad spectrum of experimental models of intestinal injury. Indeed, several studies demonstrated its therapeutic potential in the prevention or treatment of several intestinal diseases, including short bowel syndrome (SBS) [174,175], inflammatory bowel diseases[176] and chemotherapeutically-induced mucositis [146,151] (*Figure 17*). Given the short life half-life of endogenous GLP-2, different analogues have been synthetized in order to obtain a molecule more resistant with respect to the native form. Among these, DPP-4-resistant analogues such as human [Gly2]–GLP-2 (h[Gly2]-GLP-2, or teduglutide) has been proposed in several pre-clinical studies as treatment for short bowel syndrome (SBS), Chron's disease and chemotherapy-, indomethacin-induced and radiation-induced enteritis [151,177]. Concerning the SBS, the chronic administration of teduglutide to adult subject with this disease has been reported able to reduce the need of parental nutrition in almost the 63% of patients [174,178].

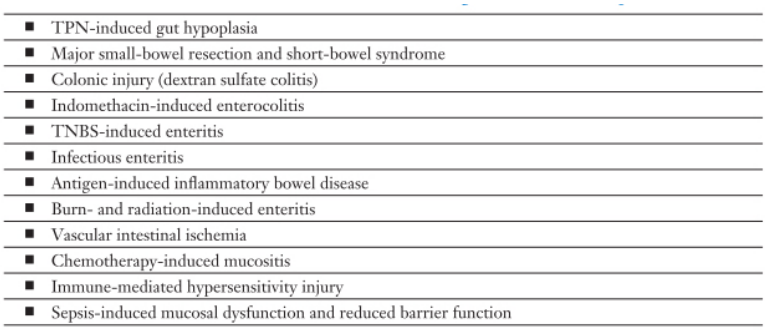

Figure 17; Preclinical models of intestinal disorders exhibiting amelioration in response to GLP-2. *(Image source: Drucker et al. [146])*

For this reason, Teduglutide has been recently approved by European Medicines Agency and the US Food and Drug Administration as an innovative adjuvant treatment in condition of intestinal injury such as the short bowel syndrome [179], and further clinical studies are developing for the treatment of Crohn's disease or SBS in children [151]. Conversely, the cytoproliferative and anti-apoptotic effects of GLP-2 have raised concerns about the potentiality for promoting or inducing the growth of localized polyps or malignancy. For these reasons, several studies have been performed in animals treated

with tumorigenic agents, in order to elucidate whether the treatment at different lengths of time might induce increment in number or size of polyps into the intestinal mucosa. Comparing the results obtained from the treatment with native GLP-2 or Teduglutide, these studies showed increased number of small polyps only during the long-term treatment with Teduglutide. Despite of this, there is no evidence to date concerning the association between GLP-2 treatment and development colonic polyps or cancers in humans later life. Nonetheless, since a predisposition could arise from the GLP-2 treatment, most of protocols recommend colonoscopy before and after the treatment [180].

2.5. Research study 2; "The enteric neo-neurogenesis in colon-rectal cancer."

2.5.1. Colon Rectal Cancer – Generality and epidemiology

Cancer is a leading cause of death worldwide, accounting for an estimated 9.6 million deaths in 2018. In particular, Colon rectal cancer (CCR) is defined as the third cause of cancer with 1.80 million cases per year and among these, 862 000 persons dye every year of this cancer [181]. The incidence rate varies within different world's regions, traditionally occurring with higher rates in developed and industrial countries, whereas the less-developed countries present lower rates. Nevertheless, CRC incidence is dramatically increasing in many developing countries [182]. Reports from Eastern Asian regions, such as Hong Kong, Taiwan, urban China, Singapore, and Thailand, and Eastern European part indicate a rapid rise in CRC incidence, close to the rates reported in Western populations [183,184]. About 75% of colorectal tumors refers to sporadic forms which do not result directly from inherited genetic mutations, but from the exposition to several risk factors and individual preposition [182,185]. Indeed, environment and lifestyle are associated with increased incidence of CCR. Obesity, sedentary behavior, and "western diet" characterized by high-meat consumption, high-calorie and fat-rich diet missing fibers have been linked to increased colorectal cancer risk [182,186,187]. In addition, alcohol consumption and tobacco smoking are also increasing CCR risks [188]. Next to the sporadic form, a small proportion of colorectal tumors have been linked to significant genetic basis. Mutations in oncogene adenomatous polyposis coli (APC) and aberrant DNA mismatch repair genes (MLH1, MSH2, PMS2 and MSH6) have been recognized as causative mutation involved in the development of CCR [189].

CCR refers to a slowly developing cancer that begins as a tumor or tissue growth on the inner lining of the rectum or colon over a period of 10 to 20 years. Colonic carcinogenesis is a complex, multistep process that, in over the 95% of the cases, originates from damages in the colonic epithelium, called adenomatous polyps, which through a stepwise series of pathologic neoplastic changes associated with accumulation of genetic and epigenetic molecular alterations develops into an advanced adenoma with high-grade dysplasia, also called adenocarcinoma, and then progress to invasive cancer [190]. Until when the tumor mass is confined within the colonic wall is consider curable (stage I and II), but if untreated, it can spread toward surrounding tissue reaching blood and lymph vessels (stage III) and metastasize to surrounding tissues (Stage IV) becoming invasive

adenocarcinoma [190]. Up to now, surgical excision, radiotherapy and combined chemotherapy are the strategies used in tumor stages I-III, but there a not an efficacy treatment for the metastatic form and it mostly remains incurable and associated with a poor prognosis [190].

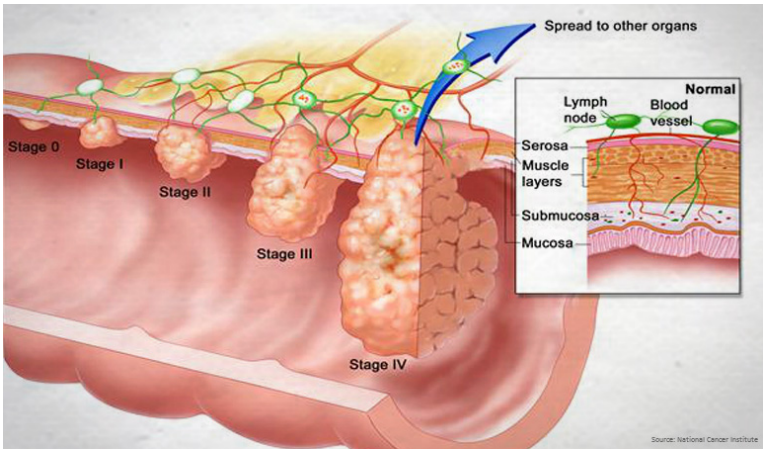

Figure 18; Schematic representation of the stages involved in the CRC development. *Stage 0: carcinoma in situ; Stage 1: Tumor invades submucosa; Stage 2: Tumor invades muscularis propria; Stage 3: Tumor invades through muscularis propria into pericolorectal tissues and spreads into regional lymph nodes; Stage 4: Tumor penetrates to the surface of the visceral peritoneum and directly invades adherent structures and spreads to other organs.*

Despite of the development in CRC treatments, in early diagnostic systems and changes in lifestyle that have led to decrease CCR specific mortality rates, up to date approximately 20% of patients with CRC already present metastases at diagnoses and thus, CRC still represents a major clinical and public health concern [191]. Recent progress in metastasis research has vastly expanded our understanding of metastasis on the cellular and molecular level but unfortunately, but the prognosis remains poor [191–194].

2.5.2. Nervous system and tumour microenvironment

Until the last decade, the study of metastatic molecular process had been focused on genetic abnormalities which promote cancer progression. Recently, however, the tumor microenvironment (TME) has attracted many attentions as a novel actor playing an important role in tumor cells invasion and metastasis [195,196]. Indeed, it was believed that the tumor surrounding tissues acted as by-stander, but new perspectives have highlighted their active participation in tumor progression and invasion. The TME consists of cellular and extracellular components, such as resident fibroblasts, endothelial cells, pericytes, leukocytes and extracellular matrix, that talk through a bidirectional communication with the tumor epithelial cells (TEC) [17]. This communication is carried out by direct cell-cell

interaction or via paracrine way signalling in which many cellular and molecules types, such as several growth factors, are involved. Accumulating evidences have confirmed that TME is able to recruit and reprogram surrounding normal cells, for instance, modulating adhesion and proliferating pathways [17]. Conversely TEM is able to release soluble factors contributing, in turn, to modulate tumor invasive properties. Recent *in-vivo* and *in-vitro* findings have proposed the nervous system as a novel factor involved in this bi-directional communication and thus in metastatic cascade. In this regard, Nurgali at al. [197] in a recent review resumed the knowledge concerning the ability of the nervous system to modulate several metastatic processes such as the clonal expansion, evasion from apoptosis, epithelial-mesenchymal transition and tissue invasion and colonization, angiogenesis, evasion of immune response and establishment of TME. Neurotrophic factors, neuropeptides, neurotransmitters have been described as neuronal-related factors, released by nerve endings that modulate the processes listed above, facilitate, in this way, the metastasis development [197]. Next to angiogenesis and lymphangiogenesis processes that have been already described such as examples of TME-MET interactions and also the main cancer route of spread, perineural invasion (PNI) has been considered as under-recognized route of metastatic spread associated with cancer-related pain and poor prognosis [19]. Indeed, although the existence of PNI was described since the 1800s, it has long been overlooked as inert by-standers in solid malignancies. To date PNI is gaining recognition as potentially important component of TME. Clinically PNI is defined as the presence of cancer cells in the perineurium spread along nerve sheaths. It was firstly reported in head and neck cancers but then it has emerged as a key pathologic feature of many other malignancies as colon-rectal [198], gastric [199], oral tongue squamous cell carcinoma [200] and pancreatic cancers [201]. The physiopathology of PNI is not still well understood. In prostatic and pancreatic cancers, several neurotrophic factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF) and neurotrophin-3 (NT-3), have been implicated in promoting tumour cell invasion and may be key mediators in the pathogenesis of PNI [18,201,202] along extrinsic nerves. Particularly, GDNF has been showed using in vitro and in vivo models, to induce cancer cell migration in human pancreatic cancer [202].

2.5.3. Role of enteric nervous system in colon rectal cancer

Besides the all evidences mentioned before, which have highlighted the importance of peripheral neurons in carcinogenesis, several papers have preliminarily showed the interaction between CRC and ENS (reviewed by Rademakers and co-workers) [16] proposing the ENS as a novel important factor involved in CCR carcinogenesis. Some established CRC molecular biomarkers have been found expressed in ENS. For instance, the netrine-1 pathway, normally expressed in developing ENS, has been reported also as an important contributor in CCR [203]. Moreover, N-myc downstream regulated gene 4 (NDRG4), an established early biomarker for CRC, has been found specifically expressed in enteric neurons. According to observation obtained from studies performed on PNS, whereby this protein is able to modulate the intracellular vesicular trafficking, it has been supposed that this factor could be involved in the modulation of the neurotransmitters release by enteric neurons during the colon-rectal carcinogenesis [204]. Recent work also pointed out the physical supporting role of the ENS in the invasion and spreading processes of CCR. Indeed, it has been demonstrated that TEC adhere preferentially to enteric neurons respect to mesenchymal cells, similarly to PNI in extrinsic myelinated neurons. Particularly, this adhesion has been shown mediated by two adhesion molecules, N-cadherin and L1 cell adhesion molecule (L1CAM) [205].

The role in of ENS in CRC is also indirectly suggested by data derived from clinical studies involving patients with megacolon, a pathology showing decreased enteric neuronal innervation, showed very low incidence of CCR. Indeed only 3 cases over 802 total patients were reported as affected by polyps formation [206].

Other evidences have highlighted the ability of cancer cells to modulate the expression of several neurotransmitters in regions adjacent to the tumour areas. Indeed, by using immunohistochemical analysis, decreased numbers of Neuropeptide Y (NPY), CGRP, SP expressing neurons was observed in myenteric and, to a lesser extent, in submucosal neurons of patients operated due to CCR. Only galanin-IR neurons results augmented and unlike, the VIP-IR ones didn't show any differences [207,208]. Conversely, changes in enteric neurochemical coding of enteric neurons can either promote or inhibit the colon rectal carcinogenesis. Additionally, tumour cells can express different NT receptors and in turn, response to this neuronal stimulation with the release of other NT [209]. For instance, the VIP has been the first NT to be associated with the CRC. Controversial findings have described its putative role in both the enhancement of cancer cells proliferation *in vivo* [210] and inhibitions of the migratory/invasive processes of 26-L5 adenocarcina cells [211]. ACh

is another NT described as factor involved in CRC carcinogenesis. Cheng et al., have reported that the ACh, released by human colon cancer cells, was able to act as growth factor with regards to tumour cells, inducing tumour proliferation in autocrine manner [212]. Others NT are mentioned in literature but they will not reviewed in this work of thesis [16]. In addition, the increased nerve density, called neurogenesis in their work, has been reported in tumour mass by Albo and co-workers. More precisely, they demonstrated increased PGP 9.5-IR in colonic adenocarcinoma human specimens, indicating that high degree of neurogenesis could represent an independent predictor of poor outcomes. Furthermore, by using *in vitro* model of DRG neurons co-cultivated with colon adenocarcinoma cells, they characterized the phenomenon that they called neurogenesis ,rather than neuritogenesis, as result of the active formation of new neurites [23]. Nevertheless, whether the neurogenesis is a process inducible directly by tumour or whether concomitant and cancer-related pathological conditions, as inflammation or other lesions, have a role in this process is currently unknown. Furthermore, up to date, whether changes in the number of enteric neurons might occurs during the colon-rectal carcinogenesis and whether the nature of mediators produced by the TM could be responsible for these effects are questions still unsolved.

3. Aim

As stated in the introduction, the ENS is involved in the genesis and regulation of many GI functions and therefore abnormalities affecting the ENS components seriously compromise the gut physiology, leading to severe GI diseases [2]. For this reason, the ENS has become an increasingly important research topic for studying a wide and heterogeneous range of not only digestive but also extra intestinal diseases. In this context, the ENS has been described as a factor involved in the development of GI dysfunction but also as the main contributor for the pathophysiology of the disease. Whether the role of ENS in developmental, inflammatory and functional diseases has been well characterized [2], the involvement of ENS in acquired enteric morbidities as the chemotherapy-induced neuropathies and the colon rectal cancer (CRC) is poorly addressed and understudied. This present doctoral thesis addresses in part these two particular topics, which have been developed along two different projects.

The aim of the Research study 1 was studying the putative protective properties of the GLP-2 analogue on the distal colon, by using a chemotherapy animal model characterized by the cisplatin chronic administration. Precisely, we focused our attention on:

- Colonic Mucosa, as common seat of chemotherapy-induced mucositis and target of GLP-2 intestinotrophic functions. We evaluated several histological parameters as mucosal area and mucus secretion, as well as the tissue inflammatory response. Further, the expression of vasoactive intestinal peptide (VIP) was assessed as molecule involved in neuronal plasticity during inflammation and as a putative downstream signal of the GLP-2 activity.
- Myenteric neurons and glial cells, as novel targets of cisplatin-induced neurotoxicity, whose alterations have been associated to the long-term chemotherapy-induced dysmotility. Thus, we investigated on the putative neuroprotective action of GLP-2 administration. Firstly, we studied the total number of myenteric neurons and glial cells, and then we extended the analysis to the nitrergic, cholinergic and vipergic neuronal subpopulation, as well as the expression of two different glial markers, GFAP and S-100ß.

The aim of the Research study 2 was further gain insight into the role of ENS, recently considered as novel component of TEM, in CRC carcinogenesis. As a continuation of previous studies, indicating the existence of enteric neoneurogenesis within the myenteric and submucosal plexuses of TME, we aimed to investigate, by using an *in vitro* system of enteric primary cultures:

- Whether soluble factors derived from TME were able to reproduce in vitro the neo-neurogenesis process observed *in situ*;
- Whether these factors could be released directly by TEC.
- Whether GDNF might be a factor released by TME responsible for the observed neurotrophic effects.

Furthermore, we aimed to confirm the neurotrophic capability of GDNF by using an alternative experimental model, as the *ex vivo* organotypic cultures obtained from rat explants of distal colon.

Finally, we aimed to quantify the expression of GDNF mRNA in human specimens obtained from the tumour area, tumour surrounding area and healthy area.

4. RESEARCH STUDY 1

4.1. Material and methods

4.1.1. Animals and experimental design

C57BL/6 female mice (18–22 g; n = 28) were obtained from Harlan laboratories (Correzzana, Italy). They were allowed to acclimatize to the climate- and light-controlled animal facility for one week and before starting the treatment the mice were randomly divided into seven groups: controls group, vehicle-treated group, [Gly2]GLP-2 treated group, Cisplatin 2mg-treated group, Cisplatin 2mg+[Gly2]GLP-2-treated group, Cisplatin 4mg-treated group and Cisplatin 4mg+[Gly2]GLP-2-treated group (4-5 per cage). The experimental protocol was designed in compliance with the recommendations of the European Community (D.M.116192; O.J. of E.C. L358/1 12/18/1986) for the care and use of laboratory animals and was approved by the animal care Committee of the University of Florence (Italy). The animals lived under standard conditions, as previously described [7]. The general condition of the mice was daily assessed, and the bodyweight was weekly registered. Specifically, the bodyweight was measured at the animal arrival and every Thursday before the cisplatin injection.

The mice were daily treated with [Gly2]GLP-2 (Caslo Laboratory, Lyngby, Denmark), a degradation-resistant GLP-2 analogue that has a longer half-life in vivo than the native peptide, dissolved in sterile saline solution and injected intraperitoneal (i.p.) at the concentration of 50 μg/kg. The [Gly2]GLP-2 dosage used correspond to that recommended for the treatment of SBS [1,2]. Cisplatin injectable solution (kindly provided by Dr T. Falai, SOD of Pharmacy, AOU Careggi, Florence, Italy) was. administered i.p, twice a week (on Monday and Thursday) at two different concentrations: [2 and 4 mg/kg]. To prevent cisplatin-induced nephrotoxicity, 1 mL of saline were injected subcutaneously just before each cisplatin injection and the mice, aside from untreated controls, received the same number of injections. The [Gly2]GLP-2 treatment was commenced on Monday of the first week of treatment, simultaneously to the first weekly cisplatin administration. The treatments were performed for 4 weeks. On Monday of the fifth week, 3 days after the last cisplatin injection, the animals were sacrificed. Distal colon specimens were collected for all the analyses.

4.1.2. Tissue sampling

After the sacrifice, the abdomen was immediately opened, and the distal colon was quickly removed. The content of the excised segments was washed with ice-cold physiological saline solution and segments of about 0.5 cm in length were cut in a full section way in order to maintain the whole tissue's architecture. Then, they were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) pH 7.4 and then embedded in paraffin for morphological analysis. Others colon specimens were frozen at -80°C for molecular biology experiments.

4.1.3. Morphological studies

Histology and Histochemistry analysis

Full-thickness cross sections (5µm thick) stained with haematoxylin/eosin (H&E) or with Periodic Acid Schiff (PAS) reaction were used for semi-quantitative score evaluation and quantitative morphometric analysis of mucosal area and mucus secretion. All sections were stained in a single session to minimize artefactual differences in the staining. Score analysis adapted from Vera et al. [4] was performed on two sections per animal by two blinded observers (PN, MGV) using the following four criteria: i) loss of crypt architecture (graded 0-3, normal, mild, moderate, severe), ii) extent of inflammatory cell infiltrate in the lamina propria (graded 0-3, normal, mild, moderate, severe), iii) loss of epithelial integrity (graded 0-1, presence-absence), iv) loss of muscularis mucosae continuity (graded 0-1, presence-absence). Thus, a numerical score of 0-8 was assigned to each animal.

The mucosal changes, expressed as total mucosal cross-sectional area and as mucus secretion alterations expressed as PAS-positive area, were performed using ImageJ software (NIH, Bethesda, ML, USA) on digitalized images acquired at 10X or 40X objectives, respectively, using a microscope fitted with a camera (Leica DFC310 FX 1.4-megapixel camera, Leica Microsystems, Mannheim, Germany). The mucosal area was measured in two sections per animal. Mucin content was quantified on 10 photomicrographs randomly taken for each section (2 section/animal) measuring the PAS-positive areas, exclusively selected using the photograph's threshold values.

Immunofluorescence analysis

Two different procedures were used since different experimental sets were developed and performed in different laboratories. All analyses were performed on paraffin embedded sections.

One experimental set was performed in the laboratories of the University of Florence.

The deparaffinization was carried out through two incubations in xylol reagent (8 min each), while the rehydration was performed using a decreasing alcohols scale, ranging from absolute ethanol to 50° ethanol and the incubations were 2 minutes for each step. The antigen retrieval was performed using Tris buffer (10 mM) with EDTA (1 mM, pH 9.0) for 20 min at 90–92 °C, followed by cooling to room temperature (RT) for 30 minutes. The sections were then washed three times in PBS (each of them 2 min long) and blocked with 1.5% or 5% bovine serum albumin (BSA; Applichem, Darmastad, Germany) in PBS for 20 min at RT to minimize non-specific binding.

Antigen	Species	Source	Concentration
Primary antisera			
nNOS	Rabbit	Abcam	1:2000
NeuN	Mouse	Millipore	1:200
ChAT	Goat	Millipore	1:200
Vip	Mouse	Santa Cruz	1:200
Secondary antisera			
Alexa Fluor 488	Goat	Invitrogen (Carlsbad, CA, USA)	1:333
Alexa Fluor 594	Mouse	Jackson ImmunoResearch (West grove, PA, USA)	1:333
Alexa Fluor 488	Rabbit	Jackson ImmunoResearch	1:333

Table 1; Primary and secondary antisera used

The primary antibodies (listed in *Table 1*) diluted in BSA 1,5% PBS (5% BSA PBS was used only for ChAT labelling) were incubated overnight at 4 °C in humid chamber. The omission of the primary antibodies was used as negative control. The next day, after three washes with PBS, the sections were incubated for 2 h, at RT in the dark with appropriate fluorochrome-conjugated secondary antibodies diluted in BSA 1,5% PBS or 5% BSA PBS for ChAT labelling. Subsequently, the specimens were washed three times with PBS (two minutes each) and then mounted in an aqueous medium (Mountant Permafluor, Thermo scientific, Rockford, IL, USA). The double labelling was performed in some sections as follows: nNOS/NeuN, ChAT/NeuN. In these cases, two mixed antibodies

solutions were used, diluted in 1,5% or 5% BSA in PBS as appropriated. Fluorophores were visualized under an epifluorescence Zeiss Axioskop microscope (Zeiss, Oberkochen, Germany), using excitation filters for Alexa 594 red and Alexa 488 green. The fluorescence images were captured using a Leica DFC310 FX 1.4-megapixel digital camera, equipped with the Leica software application suite LAS V3.8 (Leica Microsystems, Mannheim, Germany).

The total number of the nitrergic and cholinergic-IR neurons was evaluated within the myenteric plexus, considering only the ganglia oriented parallel to the section axis. The labelled neurons were counted along the entire perimeter by two observers (PN, MGV) blindly to each other. The double labelling with the pan neuronal marker, NeuN was used as neuronal positive control. The results were expressed as number of neuronal cell bodies per sections ± S.E.M (two sections per animal; 4-6 animals/group).

The quantitative assessment of the VIP was performed analysing the VIP-IR area both in the mucosa and in external muscular layers of each sections (2 section/animal; 4-6 animals/group). The analysis was done on digitized images acquired with a 20x objective covering the entire cross-sectional area. Regarding the mucosal analysis, Squared regions of interest (ROI) were designed using ImageJ in order to cover all of the mucosal area, then, the labelling in each ROI was converted to a binary image and the photographs' threshold values were set to analyse the structures of interest exclusively. 40 ROI (each of them measured 600 μm^2) were randomly selected from each animal in order to analyse at least a tissue area of 24.000 μm^2. The results were expressed as mean VIP-IR area (μm^2) ± S.E.M. Regarding the analysis performed on the muscle layer, unlike, the VIP-IR area was analyses on the whole muscle wall. Using the free hand selection tool of ImageJ, ROI composed of only the external muscle layer were drawn. Therefore, after fixing a threshold value in order to analyse only the structures of interest, the VIP-IR area was quantified and then normalized for the total muscle layer considered in the analysis.

The second experimental set was carried out in the specimens collected in the Florence lab in the hosting laboratories of the UMR 1235, INSERM Nantes. Before starting with the deparaffinization step, the sample were incubated in heater at 60°C for 30 minutes. After that, samples were incubated for two times in xylene (8 min each) and in absolute ethanol, 90% and 70% ethanol (respectively for 7, 6, 5 minutes long) in order to remove the paraffin from the slices and rehydrate the samples. Tissue sections were incubated

with antigen retrieval solution (Dako, Santa Clara, CA) at 110° C for 90 seconds. After cooling in ice for 10 minutes, sections were incubated successively in blocking solution (Dako) for 1 hour, in humid chamber at RT, followed by primary and secondary antibodies incubation steps. The primary and secondary antibodies were diluted in antibody diluent solution (Dako) using dilutions listed in the *Table 2*.

Antigen	Species	Source	Concentration
Primary antisera			
SOX-10	Goat	Santa Cruz	1:500
HuCD	Rabbit	Santa Cruz	1:200
GFAP	Mouse		1:500
S-100ß	Rabbit	Dako	Ready to use
Secondary antisera			
Anti-mouse–Cy3	Donkey	Jackson ImmunoResearch,	1:500
Anti-rabbit-FP488	Donkey	Interchim	1:200

Table 2; Primary and secondary antisera used.

The omission of the primary antibodies was used as negative controls. The next day, after three washes with PBS (each of them was five minutes long), the sections were incubated for 2 h, at RT in the dark with appropriate fluorochrome-conjugated secondary antibodies (see the *Table 2*). Subsequently, the specimens were washed three times with PBS and then mounted in aqueous medium (ProLong gold antifade mountant, Molecular probes, Life technologies, Carlsbad, CA, USA). The double labellings were performed on sequential slices as follow: GFAP/ S-100ß and HuCD/SOX-10 using two different mixing antibodies solutions. Fluorophores were visualized using a Nikon (Tokyo, Japan) A1R confocal microscope, and appropriate laser wavelength and filters, with 60 /1.4 objective and full-size images were recorded with NIS (Nikon) software. The acquisition was performed in collaboration with the Confocal Cellular and Tissutal Imaging Core Facility of Nantes University (MicroPICell). Two slices for each staining were analyzed and the morphometric analysis was focused on well-oriented ganglia of the myenteric plexus. Indeed, the smaller ganglia were excluded from the analysis since enteric neurons and glial cells might be not clearly visualized and thus the quantification might not be very accurate. At the outset, the number of well-oriented myenteric ganglia per section was analyzed in order to assess whether variations in their number were present and thus whether they were due to treatments or physiological variations. Since no differences were detected, we expressed all of quantitation as absolute value referred to the whole

section. Secondly, for each section, areas of each myenteric ganglion considered in analysis were quantified using the free-hand selection tool of ImageJ software. Therefore, in the two sections co-stained with HuCD and SOX-10 markers, the total number of myenteric neurons and glial cells was quantified, whereas in other two sequential sections stained with GFAP and S-100ß, the IR area was considered in the analysis. The results were expressed as total number of HuCD- or SOX-10-IR cells per section separately and also as a ratio SOX-10/HuCD cells. Moreover, given the cytoplasmic localization of both GFAP- and S-100ß markers, their IR areas were quantified selecting the labelling by means the photograph's threshold values and the results were expressed as total myenteric IR area per section.

4.1.4. Molecular biology study

Cytokine enzyme-linked immunoabsorbent assay

Distal colon samples previously stored at -80° were thawed slowly at 0° C and then homogenized with a tissue homogenizer (Ing. Terzano, Milan, Italy) in a cold lysis buffer: 10 mmol/L Tris/HCl, pH 7.4, 10 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1% Triton X-100, 0.1% SDS, added with 106 Sigmafast Protease Inhibitor cocktail tablets (Sigma Aldrich Corp., St Louis, MO, USA). The homogenized tissue was centrifuged at 14 000 g for 20 min at 4 °C to obtain the supernatants which were subsequently collected. The total protein content was measured spectrophotometrically using micro-BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) for calibration. Proteins concentration of IL-1beta and IL-10 in the tissue supernatants were determined using enzyme-linked immunoabsorbent assay (ELISA) kits (BioLegend, San Diego, CA, USA) according to the manufacturer's instructions. This analysis has been performed in collaboration with Professor Amedei' research group.

4.1.5. Data Analysis and Statistical Test

Statistical analyses were performed using GraphPad Prism software (GraphPad, San Diego, CA, USA). Statistical differences between mean values were analysed by analysis of variance (ANOVA) followed by Newman-Keuls to compared more than two groups. Statistical significance was defined as a p-value less than 0.05. When the data were not representative of a normal distribution, non-parametric test Kruskal-Wallis one-way ANOVA followed by Dunn's test was used.

4.2. Results

4.2.1. Physiologic parameters

Body weight

The long-term cisplatin 4mg administration caused a significant decrease of the bodyweight in treated mice compared with controls (*Figure 19*). This decrease started 2 weeks after the first injection and proceeded significantly throughout the following experimental period. The co-administration of [Gly2]GLP-2 did not prevent the weight loss due to cisplatin. The cisplatin 2mg group didn't induce significant decreased even thought a tendency was evident starting from the second week of treatment, analogously to what observed with the higher dose. When [Gly2]GLP-2 was administered alone, it did not alter the bodyweight gain as compared with the control mice, suggesting that at the established dose the hormone did not alter the food intake.

4.2.2. Morphological and biomolecular analysis of colonic mucosa

Histological and histochemical analysis

To establish whether the GLP-2 co-administration might exert protective effects against mucosal alterations due to the well-known cytotoxic/anti-mitotic properties of cisplatin, we performed morphometrical analysis considering different histological parameters. Firstly, we evaluated the tissue organization and the presence of inflammation. Secondly, we quantified the mucosal area, as marker of mucosal damage and PAS-stained area, as marker of mucin production.

Score analysis and measurement of mucosal area

Score analysis *(Figure 20, D*) showed significant histological changes in mucosal and submucosal layers of mice treated with cisplatin 4mg compared to controls. Cisplatin 4mg treatment (*Figure 20 B, B^{I}*) caused the loss of epithelium and muscularis mucosae continuity, the mucosa appeared unfolded and flattened, the colonic crypts showed an irregular profile and were narrower. Moreover, the lamina propria was infiltrated with inflammatory cells. GLP2 analogue administration did not prevent these changes (*Figure 20, C-C^{I}*).

The quantitation of the total colonic mucosal area (*Figure 20*, E) showed a significant decrease in cisplatin 4mg and 4mg+[Gly2]GLP-2 groups. On the contrary, cisplatin 2mg and 2mg+[Gly2]GLP-2 treated mice did not show differences regarding this parameter. Score analysis *(Figure 20, D*) showed significant histological changes in mucosal and submucosal layers of mice treated with cisplatin 4mg compared to controls Although cisplatin 2mg treated group showed microanatomical changes likewise cisplatin 4mg treated group, these changes were less pronounced and devoid of statistical significance compared to the control group. [Gly2]GLP-2, given in co-administration to cisplatin 4mg, prevent the cisplatin-induced damage only partially.

Mucus secretion analysis

PAS staining was observed in epithelium, distributed on the glandular crypts as well as, most intensively, in the goblet cells. The loss of PAS positive area, shown in *Figure 21* (D), was mostly evident charged to glandular crypts.

The quantitative analysis of the PAS staining *(Figure 21, F)* revealed a significant decrease of mucin expression in cisplatin 4mg treated mice, predominantly evident in the glands. Cisplatin 2mg treated group also showed a reduction of the PAS positive area, however, the difference with controls did not reach the significance. The co-treatment with [Gly2]GLP-2 never exert protective effect.

Evaluation of pro- and anti-inflammatory cytokines profile

IL-1β and IL-10 levels (*Figure 22* A and B) were quantified in tissue homogenates to verify whether these two cytokines were modulated by cisplatin treatment and whether the [Gly2]GLP-2 given in co-administration was able to reduce the tissue's inflammatory response. The results were expressed as pg of IL-1ß and IL-10 normalized for the total tissue proteins. The quantitation of IL-1ß and IL-10 levels did not show significant differences in cisplatin 2mg and cisplatin 2mg+[Gly2]GLP-2 groups when compared to controls. Conversely, the highest dose of cisplatin induced a significant increase of both IL-1ß and IL-10 levels. Interestingly, the levels of IL-1ß and IL-10 in cisplatin 2mg+[Gly2]GLP-2 treated mice returned toward baseline.

VIP-immunoreactivity (IR) in the colonic mucosa

VIP has been reported as a factor involved in inflammatory mucosal responses and neuroplasticity induced by several pathological conditions [107]. Additionally, it has been described as one of GLP-2's second messengers involved in inflammatory mucosal responses [157]. VIP-IR were distributed in numerous fibers along the villi's axis. Several IR varicosities lined the epithelium (*Figure 23,* A-D). In the mice treated with Cisplatin or GLP2 analogue alone or in co-administration, the labelled structures appeared increased.

The morphometry (*Figure 23, E)* revealed a significant increase of VIP expression in cisplatin 4mg and cisplatin 4mg+[Gly2]GLP-2 groups compared to controls. Moreover, significant increase of VIP-IR structures were observed in [Gly2]GLP-2 group in comparison to control group. The cisplatin treatment at the lower dose, in single or in co-administration with the GLP-2 analogue, didn't show differences compare to controls.

4.2.3. Analysis of myenteric neurons and glial cells in the muscle wall

In order to study the effects of cisplatin long-term treatment on the ENS and thus the putative neuro-glial protective actions of GLP-2 co-administration, we performed several morphological analyses focused on the myenteric plexus. At first, we analysed the ganglionic area and then the nervous cells (neurons and glia) hosted within the same ganglia. To fulfil these goals, we performed a co-staining using HuCD, as pan-neuronal marker located in the cytoplasm, and SOX-10, as glial one labelling the nuclei.

The number of neurons and glial cells and the ratio between these two cell types were quantified. Secondly, we passed to analyse three different neuronal subpopulations (nitrergic, cholinergic and vipergic) identified using antibodies against the following chemical codes: ChAT, VIP and nNOS. Regarding the nitregic and cholinergic one, we quantified the cells number, given the cytoplasmic localization of the enzymes (ChAT and nNOS). Conversely, concerning the vipergic subpopulation we quantified the VIP-IR structures distributed along the whole muscle wall because of the VIP-IR distribution in dispersed intracellular vesicles both in soma and fibers.

Area of myenteric ganglia

The myenteric ganglia (*Figure 24*) area was comparable among controls, cisplatin 2mg, cisplatin 2mg+[Gly2]GLP-2 and cisplatin 4mg+[Gly2]GLP-2 treated mice. A low but not significant decrease was observed in cisplatin 4mg group.

Total number of myenteric neurons and glial cells. Glial cells/neurons ratio.

HuCD-IR neurons appeared bigger in surface and number in *Figure 25* (A) than (B and C). The SOX-10-IR cells characterized by a nuclear labelling were observed in close apposition to HuCD-IR neurons within the myenteric ganglia. The results displayed in *Figure 25* showed a significant loss of the HuCD-IR neurons (B, D) and SOX-10-IR glial cells (B, E) in cisplatin 4mg treated mice when compared to controls. The lower dose of cisplatin, unlike, didn't induce any significant differences in both of these two markers (D, E) neither during the single or the co-administration with the GLP-2 analogue (C, D and E). It is worth noting that co-administration of Gly2]GLP-2 completely prevented the cisplatin 4mg effects.

Finally, *(Figure 25, F)* the glial cells/ neurons ratio revealed, a significant decrease in cisplatin 4mg group respect to controls suggesting a greater loss of glial cells compared to neurons. Cisplatin 4mg+[Gly2]GLP-2 treated mice, showed, likewise to previous results, a ratio comparable to controls.

Analysis of the Nitrergic and cholinergic subpopulations

The results (*Figure 26, G and H*) showed no significative differences for both markers in the groups treated and co-treated with the lower dose of cisplatin when compared to controls. Otherwise, the number of nNOS- and ChAT-IR neurons was significantly decreased in cisplatin 4mg treated group compared to controls (*Figure 26*; B, E, G, and H). Co-treatment with the GLP-2 analogue prevented the loss of the nitrergic neurons but did not affect the loss of the cholinergic ones (*Figure 26*; C, G, F and H).

Vipergic subpopulation

The VIP-IR was evaluated in the ganglia and in the two muscle layers (*Figure 27*). Because of the difficult to identify with certainty the neuronal positive somata all the measurements were pull together. Contrary to what observed for the cholinergic and nitrergic subpopulations, the vipergic positive structures showed no changes among the groups.

Glial markers analysis

After assessing the number of SOX-10-IR cells, we passed to analyse the expression of others two different glial markers: GFAP and S100ß. Although the literature reports that the majority of myenteric glial cells of the mouse colon co-express SOX-10, GFAP and S100ß [76], the expression of GFAP and S100ß has been demonstrated being modulated in several pathological conditions. Since both GFAP and S100ß have a cytoplasmic expression, we quantified the labelled area of both these markers separately, within the same well-oriented myenteric ganglia, following the same criteria used for the quantification of HuCD and SOX-10-IR cells.

GFAP- and S-100ß -IR areas

The expression of the GFAP protein in myenteric ganglia showed a significant decrement in cisplatin 4mg group compared to controls (*Figure 28, A-C, A^2-C^2, D*). It is worth noting that, although cisplatin 4mg+[Gly2]GLP-2 group showed values not significantly different from the controls, the difference between this co-treated group and the cisplatin 4mg one was not statistically significant. A similar condition was obtained for the S-100ß expression (*Figure 28, A^1-C^1 A^2-C^2, E*).

4.2.4. Images

Physiologic parameters

Body weight

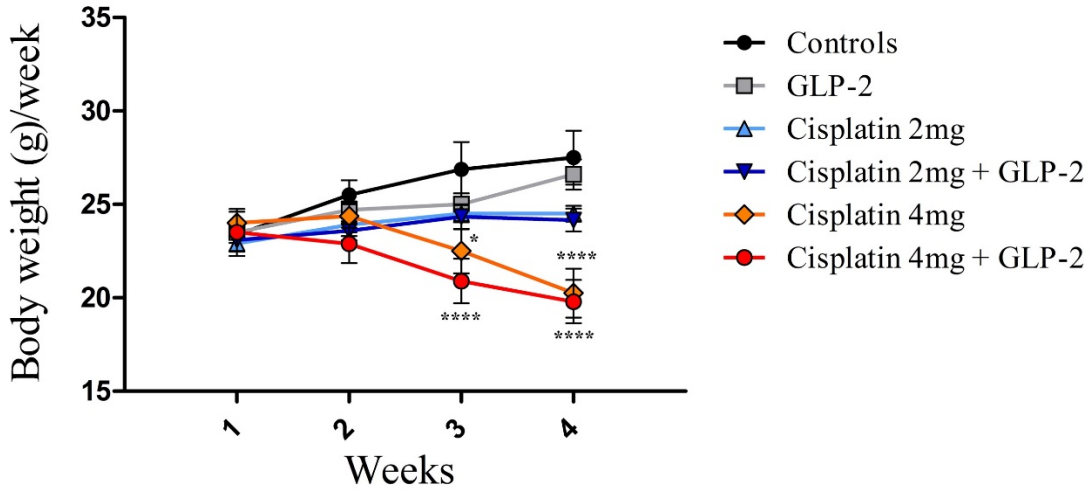

Figure 19; Mouse bodyweight during 4 weeks of treatment. *The mice were weighted every Thursday before the cisplatin injection. Data are expressed as mean ± SEM. Two-way ANOVA test - Post hoc Bonferroni's test. *($p < 0.05$), **** ($p<0.0001$) vs controls. N = 4-6.*

Morphological and biomolecular analysis

Histological and histochemical analysis of colonic mucosa

Score analysis and measurement of mucosal area

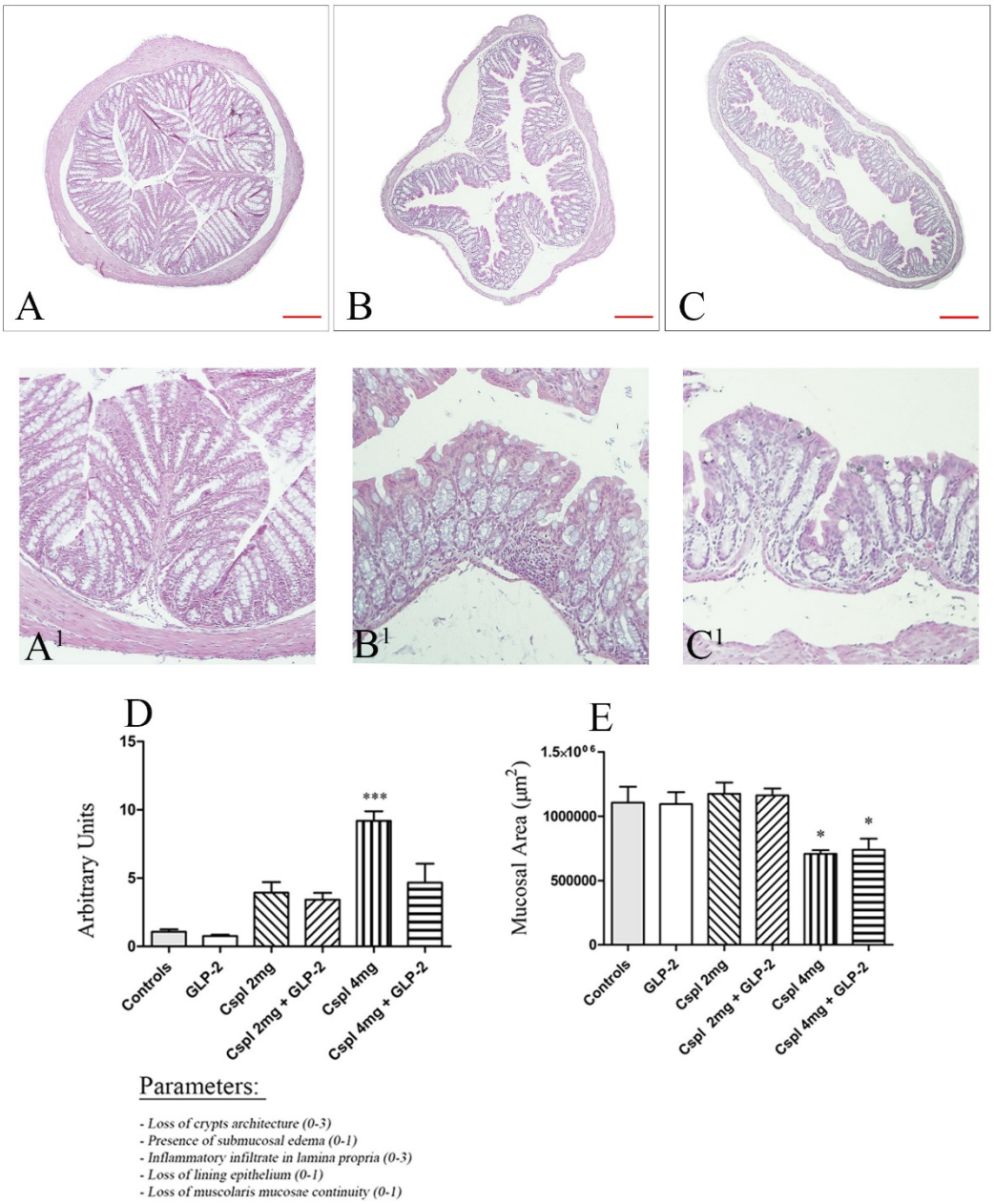

Figure 20; H&E staining of the mouse distal colon cross-section. *Control group (A), cisplatin 4mg group (B), cisplatin 4mg+[Gly2]GLP-2 group (C). A[1], B[1] and C[1] represent the magnification of tissue portions obtained from (A, B and C), respectively, whereby mucosal infiltration, glands and epithelial alterations are shown. Scale bar = 200µm.* ***Score analysis of mucosal damage (D) and quantitative analysis of the mucosal area (E).*** *Score analysis (D) was performed assigning values to the different parameters listed below the graph. Values are the median ± SEM. Non-parametric Anova Kruskal–Wallis - Post hoc Dunn's test. ***(P < 0.0001) vs controls and [Gly2]GLP-2 group. The quantitation of mucosal area (E) was done on the entire transversal section of the mouse colon. Data are expressed as mean ± SEM. One-way ANOVA test - Post hoc Dunn's test. *(p < 0.05) vs controls and [Gly2]GLP-2 group. N = 4-6.*

Mucus secretion analysis

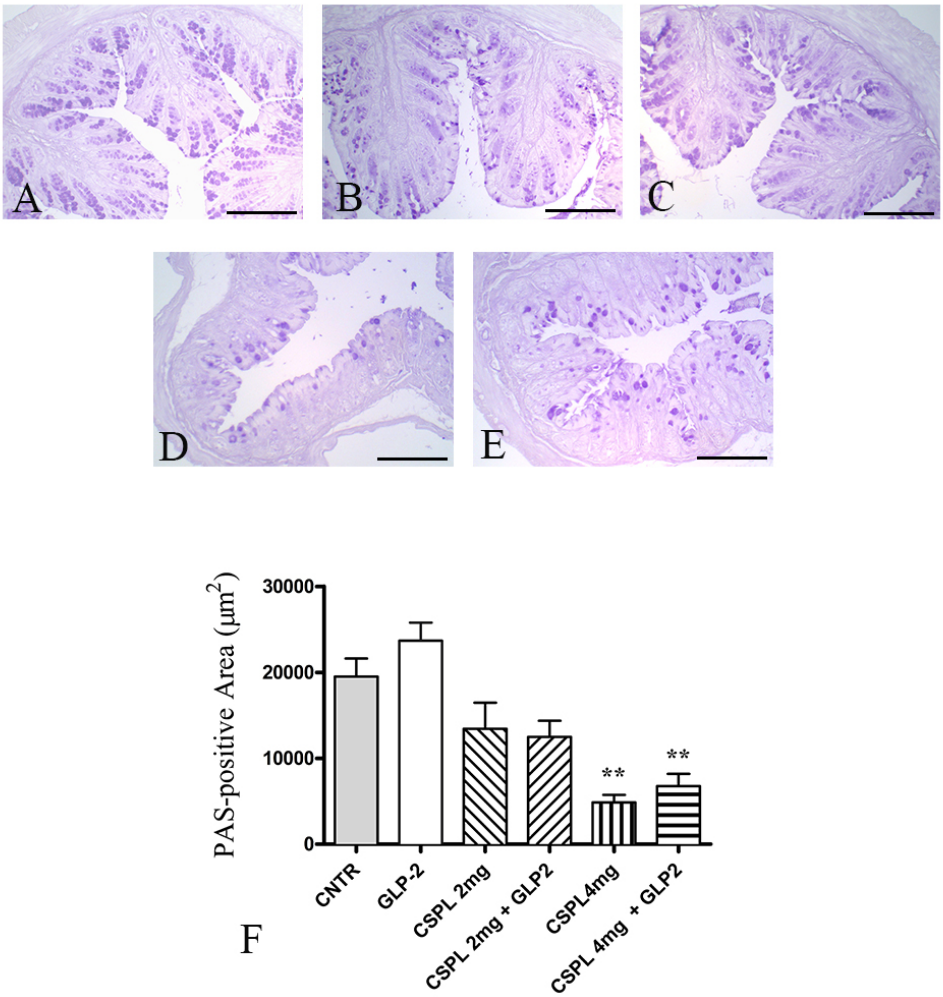

Figure 21; PAS staining of mouse distal colon (A-E). *Control group (A), cisplatin 2mg group (B), cisplatin 2mg+[Gly2]GLP-2 group (C), cisplatin 4mg group (D), cisplatin 4mg+[Gly2]GLP-2 group (E). Scale bar = 100µm.* ***Quantitation of PAS staining (F)****. The measurement was done on 10 ROI randomly chosen from the total mucosal area. Data are expressed as mean ± SEM. One-way ANOVA test - Post hoc Newman Keuls' test. **($p < 0.001$) vs controls and [Gly2]GLP-2 group. N = 4-6.*

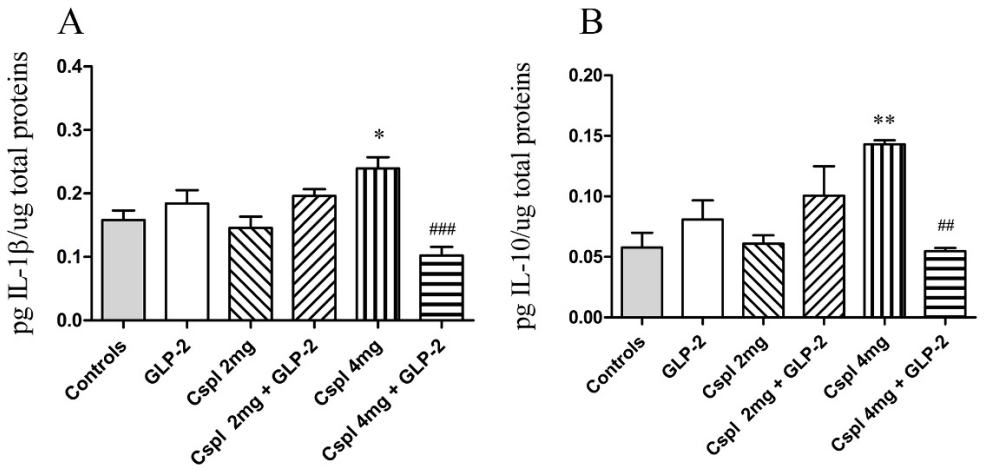

Figure 22; Evaluation of tissue pro- and anti-inflammatory cytokines. *The measurement performed throught ELISA assay were expressed mean ± SEM of cytokines pg normalized for the total tissue proteins. One-way ANOVA test - Post hoc Newman Keuls'. * ($p < 0.05$), ** ($p<0.001$) vs controls; ## ($p<0.001$), ### ($p<0.0001$) vs cisplatin 4mg. N = 4-6.*

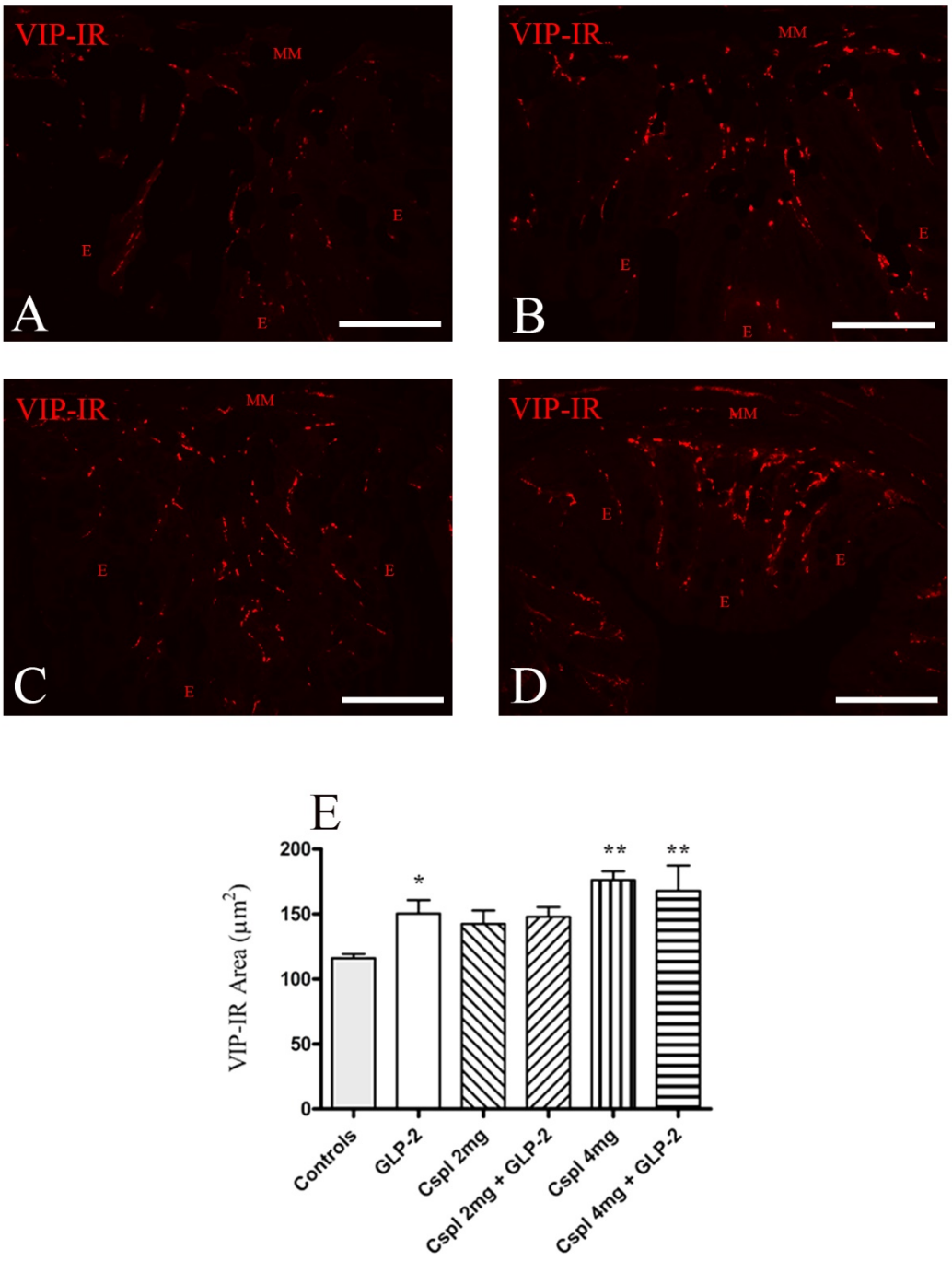

Figure 23; VIP labelling in colonic mucosa (A-D). *Control group (A), [Gly2]GLP-2 group (B), cisplatin 4mg group (C), cisplatin 4mg+[Gly2]GLP-2 group (D). Scale bar = 100µm.* ***Quantitation of VIP expression in colonic mucosa (E).*** *40 ROI were randomly chosen in order to analyze at least 24000 µm² of tissue. Results are expressed as mean ± SEM. One-way ANOVA test - Post hoc Newman Keuls'. * ($p < 0.05$), ** ($p<0.001$) vs controls; N = 4-6 for each group.*

Area of myenteric ganglia

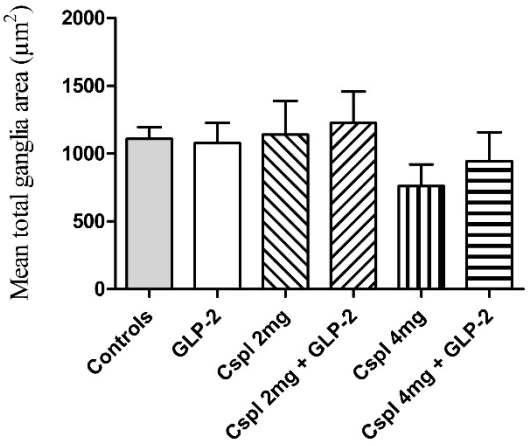

Figure 24; Myenteric ganglionic area. *The area was evaluated considering the well-oriented ganlia placed in whole cross-sections. Data are expressed as mean ± SEM. One-way ANOVA test - Post hoc Newman Keuls'. No significance. N = 4-6.*

Total number of myenteric neurons, glial cells, myenteric glial cells / neurons ratio.

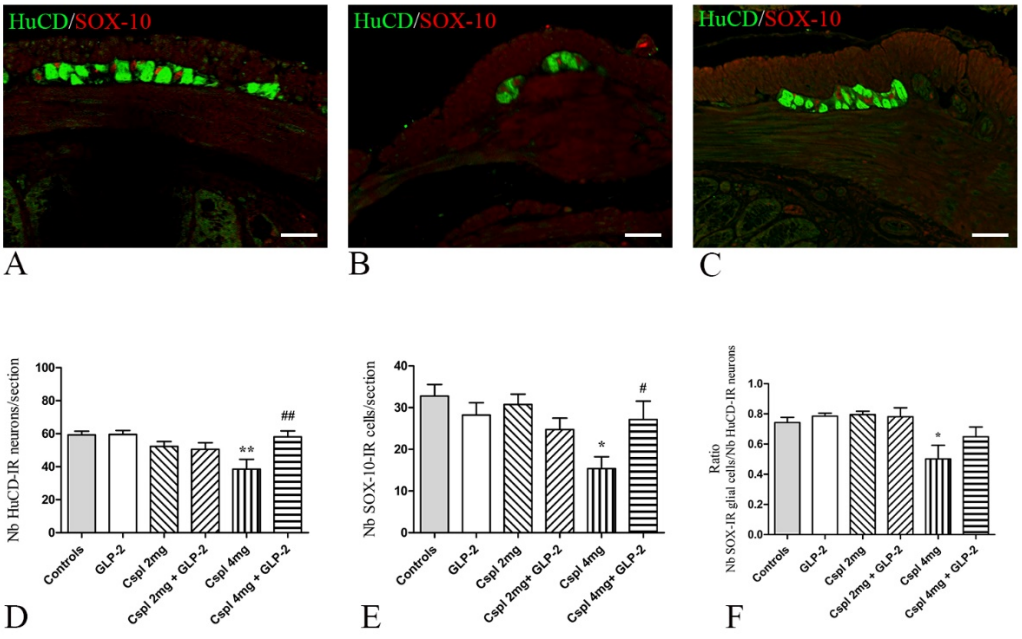

Figure 25; HuCD and SOX-10 co-labelling in myenteric ganglia (A-C). *Control group (A), cisplatin 4mg group (B), cisplatin 4mg+[Gly2]GLP-2 group (C). Scale bar = 50µm.* ***Quantitation of the total neuron and glial cells number (D, E); and myenteric glial cells / neurons ratio (F).*** *Measurements were performed considering the well-oriented ganglia placed within the cross section. Data are expressed as mean ± SEM. One-way ANOVA test - Post hoc Newman Keuls'. * (p<0.05), ** (p < 0.001) vs controls, # (p<0.05), ## (p < 0.001) vs cisplatin 4mg. N = 4-6.*

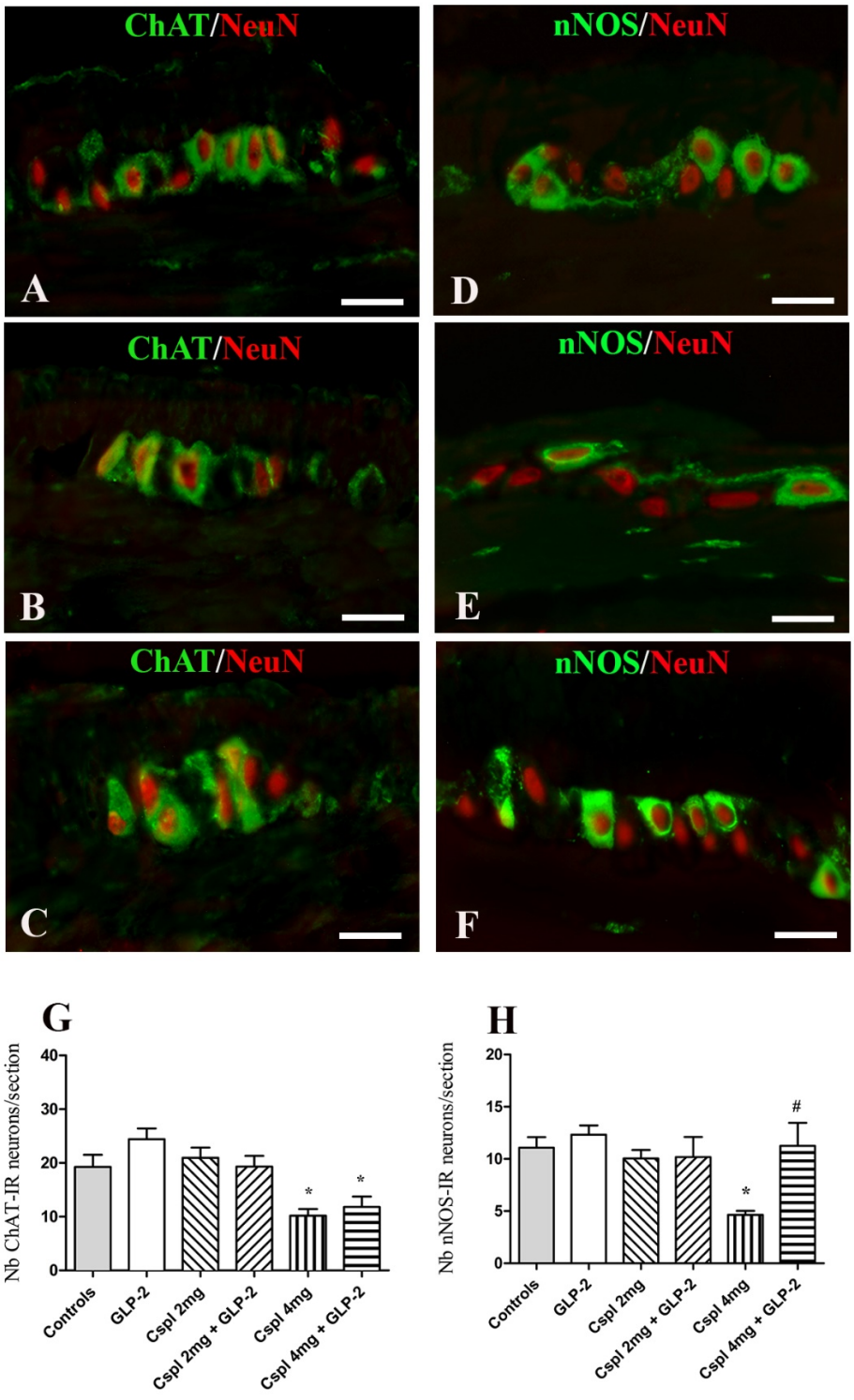

Figure 26; ChAT and NeuN co-labelling in myenteric plexus (A-C) and nNOS and NeuN co-labelling in myenteric plexus (D-F). *Control group (A, D), Cisplatin 4mg (B, E), cisplatin 4mg+[Gly2]GLP-2 group (C, F). ChAT- and nNOS-IR was observed in neuronal cytoplasm, whereas NeuN-IR was present in the nucleus of enteric neurons placed in myenteric ganglia. Scale bar = 25 µm.* ***Analysis of nitrergic and cholinergic neuronal subpopulations in myenteric ganglia (H and G).*** *Data are expressed as mean ± SEM. One-way ANOVA test - Post hoc Newman Keuls'. * ($p < 0.05$) vs controls, # ($p < 0.05$) vs cisplatin 4mg. N = 4-6.*

Vipergic subpopulation

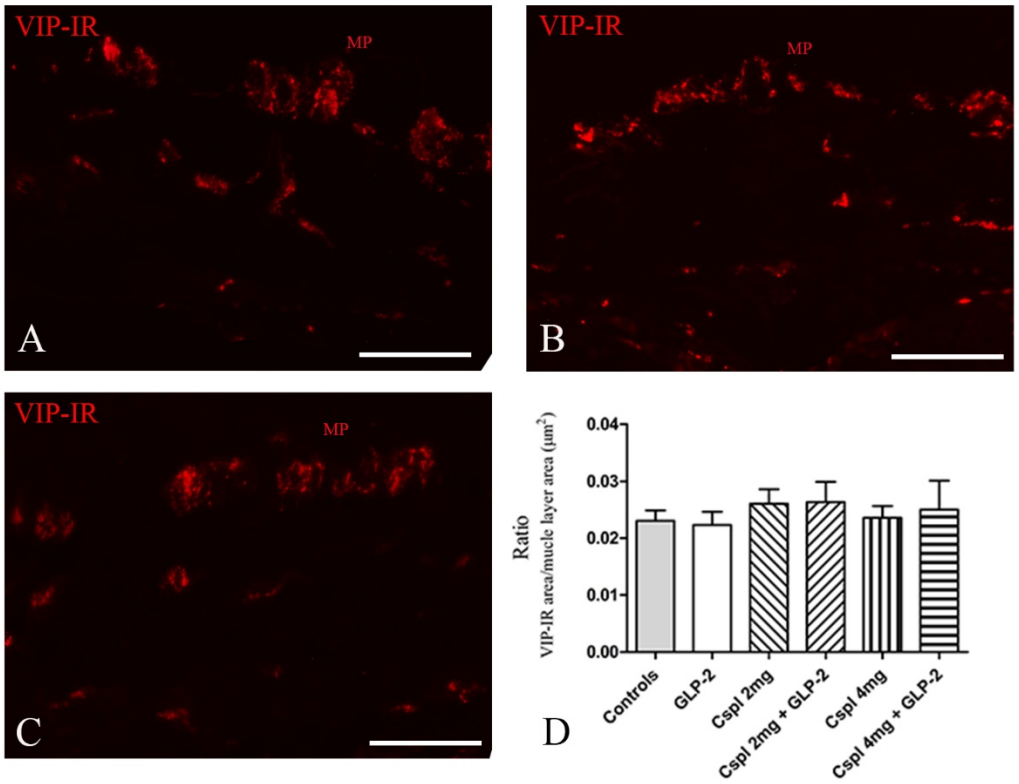

Figure 27; VIP labelling in muscle wall (A-C). *Control group (A), [Gly2]GLP-2 (B), cisplatin 4mg+[Gly2]GLP-2 group (C). VIP-IR was detected within the ganglia and in both muscle layers. Scale bar = 100µm.* ***Quantitation of VIP-IR (E).*** *The assessment of VIP expression was performed considering the whole cross-section and the results were expressed as VIP-IR over the total area. Data are expressed as mean ± SEM. One-way ANOVA test - Post hoc Newman Keuls'. No significance. N = 4-6.*

Glial markers analysis

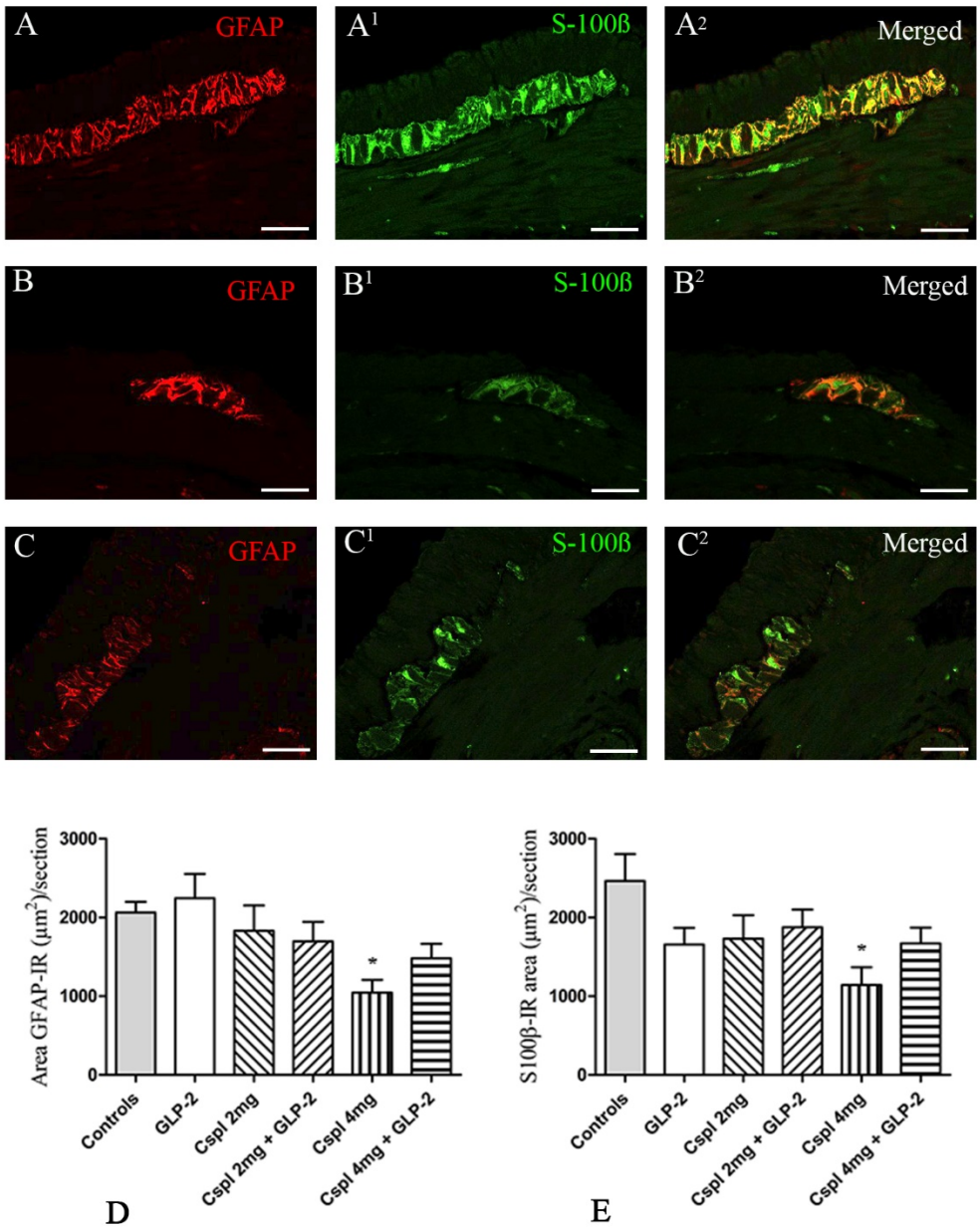

Figure 28; GFAP and S100β co-labelling in myenteric ganglia (A-C, A[1]-C[1]). *Control group (A, A[1], A[2]), cisplatin 4mg (B, B[1], B[2]), cisplatin 4mg+[Gly2]GLP-2 group (C, C[1], C[2]).* ***Evaluation of GFAP (D) and S100β-IR in the myenteric ganglia (E).*** *The areas of GFAP (D) and s100β (E) were measured in well oriented myenteric ganglia, following the same criteria used for the quantification of SOX-10 and HuCD-IR cells. Scale bar =30µm. Data are expressed as mean ± SEM. One-way ANOVA test - Post hoc Newman Keuls'. * ($p < 0.05$) vs controls. N = 4-6.*

4.3. Discussion

4.3.1. Physiologic parameters

Our findings show that the chronic cisplatin treatment cause a loss of body weight starting from the second week, evident at the dose of 2mg and statistically significant at the highest dose. The co-administration of the GLP-2 analogue does not revert this loss. The cause of this loss is likely due to the damages on the GI tract, both in the enteric innervation which regulates the transit of the ingested food and the absorption, and in the absorptive apparatus which lie on the mucosa. In a previous work published by our research group, it has been showed that the cisplatin chronical administration caused mouse gastric distention and damages on gastric epithelium and enteric neurons with significant changes in neurotransmission [7]. This was in line with other studies that correlated the impact of cisplatin treatment with the occurrence of nausea (evaluated as pica behaviour in rodents), food intake and gastric distention [3]. Since cisplatin is defined as one of the most emetogenic anticancer drug, we could suppose that the loss of weight is due to alterations affecting the stomach and, in turn the food intake, rather than damages on the distal colon. However, the observed damages on this latter district can contribute to a general GI dysregulation.

Whether the co-administration of GLP-2 has been shown to revert the gastric damage, here we show that it is not able to revert either the loss of body weight or the alteration observed in the distal colon mucosa, that we address in the next chapter.

4.3.2. Morphological and biomolecular analysis of colonic mucosa

Cisplatin induces damages on healthy GI mucosa, called mucositis. This condition is characterized by several features as tissue inflammation and mucosal barrier damages[213], whose physiopathology is still unclear. Although GLP-2 has been demonstrated a useful strategy for recovering several intestinal injuries, stimulating the proliferation and inhibiting the apoptosis of cryptic cells [214], our data showed that the GLP-2 analogue, when given together with the highest dose of cisplatin, did not protect the mucosal damage of the distal colon. Indeed, neither the loss of mucosal area or mucus secretion reduction were prevented by GLP-2 treatment.

These results could be explained considering the cumulative properties of platinum-based drugs that emerged namely during a chronic administration at elevate dosage. So that,

along the 4 weeks of treatment the impact of cisplatin cytotoxicity on intestinal epithelium could result too elevate to be counteracted by the co-administration with GLP-2 analogue at the established dose ad timing of administration. Although other studies demonstrated the protective properties of the GLP-2 exogenous administration against chemotherapy-induced mucositis, it should be pointed out that, differently from our experimental model, diverse anticancer drugs were used and they were administrated for shorter times [215–217]. Furthermore, differences in the kind of GLP-2 analogues used and in the methods of administration could also be play an important role. Indeed, different outcomes arising from the diverse timing, methods of administration, dosages and, further, the use of GLP-2 analogues or the native form [218] have been reported. For instance, Kaji et al., used the rat model of mucosal atrophy for studying the GLP-2 trophic effects during the high continuous or a low intermittent administration. They showed that the crypt cell proliferation was induced predominantly by the first way highlighting that the influence on cell proliferation is more affected by the peak rather than the constant exposure [218]. Moreover, Keefe et al., proposed a theory according to which the endogenous GLP-2 could exert the tropic effect mainly by inhibiting apoptosis, unlike, the exogenous one by increasing the proliferation [215]. Finally, certain authors emphasized the importance of GLP-2 pre-administration [215,217] whilst others, highlighted the GLP-2 post-treatment is more beneficial to patients suffering from chemotherapy-induced enteritis, rather than the GLP-2 pre-treatment [219].

Another aspect to be considered is a possible modulation of the GLP-2R expression during pathological conditions. Indeed, it has been highlighted that during inflammatory conditions in different mouse colitis models, the expression of the GLP-2R mRNA was decreased respect to the healthy condition [220]. Since we observed a significant tissue inflammation during the cisplatin 4mg administration, characterized by increased level of IL-1ß and IL-10, it is attempting to speculate that this inflammatory environment might induce variation in the expression of GLP-2R, leading to decreased response to exogenous GLP-2 administration.

Concerning the tissue inflammatory conditions, our findings, interestingly, show that the co-administration of GLP-2 prevents the increase of both IL-1ß and IL-10 caused by the highest dose of cisplatin, in association to increased levels of mucosal VIP-IR. Furthermore, the single GLP-2 administration reveals the same VIP-IR mucosal increments. Confirming the GLP-2 anti-inflammatory properties already demonstrated by other works, our results further point out that this anti-inflammatory role might be mediated by an increase in VIP within the mucosal layer. These observations are

consistent to evidences existing in literature that demonstrated the paracrine GLP-2 anti-inflammatory properties via VIP. More precisely, Sigalet and co-workers, by using mouse colitis models, demonstrated that the native GLP-2 was able to increase the number of activated VIP-IR submucosal neurons associated to decreased levels of inflammatory cytokines [14,15,58].

Considering the GLP-2 anti-inflammatory effect, however, we could address another point. In literature there are controversial theories that attempt to elucidate the action of the GLP-2 in several inflammatory conditions. Indeed, certain authors, studying the effect of GLP-2 treatment in animal model of necrotizing enterocolitis, pointed out the possibility that the GLP-2 trophic functions could decrease the levels of inflammatory cytokines by recovering the mucosal barrier [221]. Our results provide, indirectly, that the GLP-2 anti-inflammatory properties are not correlated with mucosal recovery.

Besides the effects of the GLP-2 co-administration, our experimental model of chemotherapy-induced GI damage contributes to the knowledge of cisplatin-induced mucositis. The present data shows that only the higher dose of cisplatin treatment, among the two used (2mg and 4mg), causes loss of mucosal area and mucin expression. These results are not consistent with our previous published work where we demonstrated a gastric fundus epithelial damage starting from the cisplatin 2mg dose and highlight that a different tissue susceptibility to cisplatin toxicity could occur among the different GI regions. In support of this statement, several evidences revealed discrepancies in mucositis occurrence. Indeed it has been shown that mucositis can differently emerged along the various GI districts and, further, the frequency and intensity have been correlated to the type and duration of the chemotherapy regimen [213]. We do know that certain drugs, as 5-fluorouracil (5-FU) and irinotecan, cause worse GI mucositis than others, mainly affecting the small bowel mucosa. However, when moderate mucosal lesions have been reported in stomach and large bowel [222], they have been shown mostly involving dysfunctions in quantity and composition of mucus secretion as well as in crypts' architectures [136]. Furthermore changes in mucosal secretion has been demonstrated as a feature associated to cisplatin intestinal mucositis [141], consistent to our results that show a great chemotherapy-induced damages charged on the mucus apparatus. Few papers addressed this topic. Yamamoto and co-workers, for instance, showed decreased mucus content in the ileum of mice treated with one high dose of cisplatin [223]. Conversely, Vera et al., showed increased number of goblet cells in the small intestine of mice treated with long-term cisplatin administration [9]. Although our evidences show differences to this latter study, it should be underlined that our

experimental model aimed to study the effect of cisplatin during the chronical administration; unlikely, Vera et al., performed their studies after one week after the treatment ceased. Hence, it is likely assuming that a mucosal renewal was occurred, leading to an increase of goblet cells necessary to restore the previous loss of mucosal barrier.

Finally, we show increased VIP mucosal expression during the cisplatin 4mg treatment together with chronic tissue inflammation, characterized by increased levels of IL-1ß and IL-10. VIP has been described as an important neuropeptide involved in the enteric neuronal plasticity in response of several intestinal injuries [107] and, recently, multiple evidences, summed by Torphe et al., have underlined the correlation between increased expression of VIP and increased mucus secretion in chemotherapy-induced mucositis [224,225]. Based on these literature evidences, we could speculate that the increased VIP expression represents an intrinsic protective response to inflammatory microenvironment and that, in turn, it might stimulate the release of mucins as a mechanism aimed to increase the mucus protective barrier.

4.3.3. Analysis of myenteric neurons and glial cells in the muscle wall

Over the last years, the knowledge concerning the neurotoxic effects induced by platinum-based treatments have been greatly enhanced. Notably, it has been outlined the ENS as a novel target of chemotherapy-induced neurotoxicity and alterations at this level have been associated with GI dysmotility in animal models and correlated with the long-lasting and distressing GI side effects experienced by patients undergoing antineoplastic treatments. Recently, Nurgali et al. investigated the functional and morphological properties of myenteric neurons in humans' specimens during chemotherapy. Indeed, in a pilot study they showed that the patients treated with typical regiments used in colon rectal cancer (CRC) presented neuronal hyperexcitability associated with morphological changes involving the translocation of HuCD from the cytoplasm to the nucleus (as feature of neuronal damage) and increase in the soma size of nNOS-IR neurons [226].

The objective of the second part of our project was studying the impact of cisplatin chronic treatment on myenteric neurons and glial cells. In parallel, we investigate the putative neuroprotective effects of the GLP-2, in order to elucidate its potential therapeutic role in chemotherapy-induced ENS alterations. As stated in the introduction, GLP-2 is a pleiotropic hormone proposed as molecule able to interact with the ENS and able to control many aspects of GI motility.

Our findings show that the repeated cisplatin 4mg administration causes reduction of both neuronal and glial cell number. Interestingly the measurement of ECG/neurons ratio in cisplatin treated mice shows a greater loss of ECGs respect to neurons. Previous works have already demonstrated myenteric neuropathies induced by cisplatin or oxaliplatin in rat and mouse ileum, colon and small intestine [4–6,9,144]. Nevertheless, here, for the first time we demonstrate the presence of myenteric cisplatin-induced gliopathy. Nurgali and co-workers addressed the effect of oxaliplatin on glial cells showing that the treatment was able to modulate the expression of two glial markers, GFAP and S100ß, in mouse ileum, increasing the levels of S100ß and decreasing those of GFAP [6]. Conversely, in our experimental model, besides the loss of glial cells (assessed by the reduced number of Sox-10-IR cells), we showed decreased expression of both GFAP and S100ß within the myenteric plexus. We could assume that the changes of these two markers are likely due to the loss of total number glial cells rather than to the direct cisplatin modulation of their expression. The involvement of EGC in enteric pathologies has been addressed by several authors and a differential modulation of glial markers have been associated with various pathological conditions [94,227]. Notably, a loss of ECG with the concomitant loss of enteric neurons has been described in patients affected by idiopathic constipation [228]. This is consistent with the presence of cisplatin-induced constipation in patients undergoing to cisplatin regimen [228].

As stated in the introduction, EGC communicate with enteric neurons modulating the GI motility and exerting neuroprotective actions [87,94]. The loss of EGC within the myenteric plexus might correspond to the lack of glial neuroprotection, that in turn could lead to the observed neuronal loss. Alternatively, we could speculate that the elevate cisplatin concentration might induce an unspecific toxicity upon the ENS, thus involving both enteric neurons and glial cells. In literature, there is a lack of information concerning the mechanism by means cisplatin and other platinum-based drugs induce damages to the ENS. Several evidences are referred to the dorsal root ganglion (DRG) neurons, which however, differ to the enteric ones in morphology and location [126,142,143,229]. Firstly, it has been supposed that a tissue chronic inflammation might induce the consequent neuronal damage. Nowadays, Nurgali et al., have proposed a model of oxaliplatin neurotoxicity whereby the oxidative stress is a key player in colonic neuropathies [144]. Despite of this, however, no works in literature have described the cellular mechanism of the chemotherapy-induced gliopathy yet.

In the present studies we showed also that the cisplatin 4mg administration induces imbalances in myenteric neurochemical code characterized by the decrease of the nNOS

and ChAT myenteric neurons. Impairments in the neuronal subpopulations within the myenteric plexus, have been associated to GI dysmotility. Several chemotherapy treatments as 5FU, irinotecan, cisplatin and oxaliplatin have been tested with chronical and long-term administration. The expression of neurotransmitters as ChAT, nNOS, VIP, has been analysed in different experimental models, showing diverse variations associated to different outcome depending on the treatment used [4,6,9,137,138]. For instance, the repeated administration of 5FU showed a loss of myenteric neurons associate with decreased number of ChAT-IR and nNOS-IR neurons that resulted in delayed and long-lasting colonic contractility [137]. The administration of irinotecan resulted in loss of myenteric neurons but associate to increased proportion of ChAT that lead to diarrhea [138]. Chronic treatments with cisplatin or oxaliplatin have been described inducing delayed colonic contractility and upper intestinal transit likely as a consequence of the loss of the nNOS-IR neurons. Regarding the VIP-IR myenteric neurons, Vera et al. reported no alteration during chronic cisplatin treatment in rats that it is in line with our observations showing no alterations in VIP-IR structures along the muscle wall. Regardless, it would be interesting to further gain inside into the reason why this myenteric subpopulation appears less susceptible to the chemotherapy-induced damages. Altogether these findings let us to notice that, although the chemotherapy treatment lead to a general neuronal loss within the myenteric plexus, the different anticancer agents are able to modulate differentially the diverse subpopulations, leading, in turn, to different outcomes. Besides, these data suggest that each drug might have its own mechanism of action able to target specific neuronal subpopulation, or it might be possible that each drug enter into the enteric neurons through specific membrane transporters, differentially distributed among the neuronal subpopulation. In this regards, the involvement of the membrane transporters has been reported as a component involved in platinum-based neurotoxicity [230].

Concerning the role of the GLP-2 co-administration, our findings show that it is able to protect both the enteric neurons and glial cells preventing the loss of both cell types. The GLP-2 neuroprotection it has been addressed by few studies. Ekblad et al., showed thought *in vitro* model, that the addition of GLP-2 in ENS primary cultures prevented the neuronal loss caused by mast cells [13]. Moreover, Pini et al. demonstrated that the co-administration of GLP-2 in mice chronically treated with cisplatin resulted in the protection of both the total number of myenteric neurons and of the nitrergic subpopulation in the gastric fundus [7]. This latter finding is in line to other observations within the distal colon, confirming that GLP-2 is able to play a specific neuronal protection upon the nitrergic component independently to the GI region involved.

The relationship between the GLP-2 and nNOS has been demonstrated in a recent work whereby the administration of GLP-2 in *in vitro* gastric strips modulates the nitrergic neurotransmission and expression [231]. Based on our data, it is tempting to hypothesize that the GLP-2 might exert the observed neuroprotection inducing an increase of NO production by means the modulation of the nNOS enzyme. The idea of NO as a neuroprotective agent has been described by Sandgren et al. showing that the NO added to myenteric neurons primary culture resulted in the increment of cell survival. However, the action of NO within the ENS is still controversial. Basically, it is known that NO can enhance neuronal survival in some circumstances, while it promotes neuronal death in others [232]. Nevertheless, the exact mechanism by means the GLP-2 might exert neuroprotection cannot be determined based on of the present experiment, but it represents an interesting research filed worthy to be enhanced.

Our results, for the first time to our knowledge, demonstrate that GLP-2 is able to protect the myenteric EGC, maintaining the total number of SOX-10-IR cells compared to controls. Regarding the existence of the GLP-2R in enteric glial cells and consequently the effect of GLP-2 on them is a question still matter of debate. Only Sigalet and co-worker reported *in vitro* evidences concerning the existence of GLP-2R on enteric glia [15]. However, whether the GLP-2R is expressed on enteric glial in animal model and what mechanism is involved in the GLP-2 induced glial protection is still obscure.

In addition, we show that GLP-2 is able to prevent the loss of the myenteric GFAP and S100ß expression only partially, unlikely to what observe using the measurement of SOX-10-IR cells. These could be explained considering the possibility of a putative selective protective action exerted by GLP-2 on all glial cells expressing SOX-10 but only on part of those co-expressing GFAP and S100ß. This statement might be in line to the hypothesis concerning the existence of several glial cells subpopulation laying within the myenteric plexus (*chapter 2.2.5*), however, considering our analysis, it is only a speculation because of the lack of evidences providing the colocalization of the three markers. Certainly, it is an interesting aspect that deserves to be enhanced.

4.4. Conclusions

Altogether, our results show, for the first time, that the GLP-2 analogue is able to counteract most of the cytotoxic effects induced by chronic cisplatin treatment in the mouse distal colon. Although we show that the GLP-2 co-administration does not prevent the cisplatin induced mucosal alterations, we demonstrate that it exerts anti-inflammatory activities decreasing the levels of pro- and anti-inflammatory cytokines likely through the enhancement of the mucosal VIP expression. Furthermore, we demonstrate that GLP-2 can protect both enteric neurons and glial cells from neurotoxicity, exerting a specific neuroprotective action on the nitrergic component within the myenteric plexus.
In addition, we provide further gain insight the mechanisms of GI toxicity cisplatin-induced. We show that the distal colon is a GI region relatively resilient to damages induced by cisplatin repeated administration. Indeed, only the highest dose of the drug is able to induce mucositis and alterations on the ENS. From our data, the chronic colonic mucositis emerges as a condition characterized by increased levels of IL-1ß and IL-10 associated to mucosal loss and reduced mucus secretion. Moreover, we interpret the increased VIP-IR in the colonic mucosa as a possible consequence of the tissue inflammation aimed to stimulate the mucus secretion as tissue defensive response to inflammatory-induced damages. Finally, we show that cisplatin induces enteric neuropathy and gliopathy within the myenteric plexus, likely associated to intestinal motor dysfunction. More precisely, besides the loss of total myenteric neurons, the nitrergic and cholinergic components appear susceptible to this treatment, whilst the vipergic ones seem not to be affected.

In conclusion, presently we provide evidences that the administration of the GLP-2 analogue during the cisplatin regimen might represent an effective strategy to overcome the cisplatin intestinal toxicity.

5. RESEARCH STUDY 2

5.1. Material and methods

5.1.1. In vitro experiments

ENS primary cultures

The rat ENS primary cultures (pcENS) were obtained using the current protocol used and developed in the laboratories of the UMR 1235 INSERM, Nantes [233]. Pregnant Sprague–Dawley rats were purchased each week (Janvier Laboratoires S.A., Le Genest-St-Isle, France) and manipulated in compliance with the French law and every effort was made to minimize animal suffering and the number of animals used. The rats were anaesthetised using isoflurane (3.5%) and killed by cervical dislocation at gestational day 15. The embryos (E15; 35–45 per isolation from 3 pregnant rats) were removed from the whole uterus which was in turn removed from the abdomen thought laparotomy. The embryos were pinned in rostro-caudal position into sylgard-coated dishes (Sylgard, 184 Silicone Elastometer, World Precision Instrument, Sarasota, FL, US) filled with a solution composed of HBSS (Gibco, Thermo Fisher Scientific, San José, CA, US) and 0.5% Penicilline-Streptomycine (Invitrogen, Carlsbad, CA). Thus, they were killed by decapitation. After opening the abdomen, intestines were collected paying attention to remove the pancreas from the intestinal proximal part. Tissue fragments were collected in sterile microtubes containing the same solution previously used for the preparation. All of the dissociation steps were done under the flux laminar hood. At first, the intestines were mechanically dissociated using a scalpel. The fragments obtained were, then, collected in a 50mL Falcon filled with 10mL of complete medium Dulbecco's modified eagle medium (DMEM) + Ham F12 (1:1) (Gibco, Thermo Fisher Scientific, San José, CA, US) supplemented with Penicilline (50 U/mL), Streptomycine (50µg/mL) and Glutamine (2mM) (Invitrogen, Carlsbad, CA) and then, dissociated using 2.5% trypsin (Sigma Aldrich Corp., St. Louis, MO) at 37◦C for 15 min for the chemical digestion. The trypsin reaction was stopped in 5 minutes at 37°C by adding 20 ml of complete medium (based on glucose 3g/L and supplemented with 10% foetal calf serum; Invitrogen, Carlsbad, CA). We added 300 µL of DNAse I (1% in sterile HBBS; Sigma Aldrich Corp., St. Louis, MO) and incubated it for 10 min at 37◦C in agitation. After triturating with a 10 ml pipette, the preparation was left to sediment for 5 minutes and the supernatant was

collected and centrifuged at 750 r.p.m. for 10 min. Finally, cells were resuspended in a mixture (composed of eosin 0.3% and medium supplemented with fetal calf serum (40 µL)) and then counted and seeded at a density of 2.4×10^5 cells cm^2 on 24-well plates previously coated for 6 h with a solution of 0.5% gelatine (Sigma Aldrich Corp., St. Louis, MO) in PBS. After 20 h, the medium was totally replaced with a serum-free medium DMEM (Glucose 0.9g/L) + F12 (1:1) (Gibco, Thermo Fisher Scientific, San José, CA, US) and containing 1% of N_2 supplement (Life Technologies, Cergy Pontoise, France). Cells were maintained in culture at 37°C with 5% of CO_2 for 13 days. Half of the medium was replaced every three days.

Human tumour supernatant

The human tumour/healthy supernatants were prepared from colorectal cancer surgical specimens (Authorization no. DC-2008-402). Informed consent was obtained for all included patients. The tumour supernatants were collected in a full thickness way tumour mass, whereas, the healthy ones were obtained from macroscopically healthy colon segments taken from a distance of at least 10 cm from the tumour. The healthy and tumour samples were cultures in Krebs Hepes solution (1mL/30mg) at 37°C for 1h. All the supernatants obtained were filtered in centrifuge tubes (Spin-X®, Sigma Aldrich Corp., St. Louis, MO). Finally, aliquots of supernatant 300 µL were stored at -80°C until assays.

Caco-2 cell line and Caco-2 supernatants

The Caco-2 is a cell line of heterogeneous human epithelial colorectal adenocarcinoma cells. They were originally provided by the society of the biologic resources ATTC. This cell line was cultured in medium DMEM (Gibco, Thermo Fisher Scientific, San José, CA, US) supplemented with 10% foetal bovine serum (Eurobio, Les Ulis, FR), 2mM of Glutamine, 50 µg/mL Streptomycin and 50U/mL Penicillin (Invitrogen, Carlsbad, CA) and incubated at 37°C with 5% of CO_2. The medium was changed every three days and the cells were passed when they reached the 90% confluence. The supernatant was collected when the cells reached the 70% confluence and was stored at -80°C.

Cultures treatments

In order to:

- Confirm the neurotrophic properties exerted by tumour supernatants, previously observed throughout preliminary experiments carried out by pcENS;
- Test GDNF effects on pcENS as a putative molecule present in human tumour supernatant able to induce those neurotrophic effects;
- Test the neurotrophic properties of supernatants obtained from Caco-2 cells line in order to verify the hypothesis which states that supernatants contained neurotrophic factors released by TEC;

Three experimental sets were performed in order to make up several experimental conditions, as follow:

1) pcENS were treated with human tumour/healthy supernatants;
2) pcENS were treated with human tumour/healthy supernatants and with or without anti-GDNF antibody;
3) pcENS were treated with human Caco-2 supernatants and with or without anti-GDNF antibody;

All the procedures were performed in sterile conditions using the laminar flux hood and supernatants, drugs and mediums were prepared and left at 37°C before adding them to the cultures. A specific medium was prepared for these experiments as follow: DMEM (0.9 g/L glucose) + F12 (1:1) was supplemented with 1% N_2 + 0.1% fetal calf serum, and then with supernatants, anti GNDF or specific isotype diluted 1:4 in order to set the different culture conditions. The treatments were performed at 11^{th} and 12^{th} days of culture as follow. CRC and TEC supernatants were added in mature pcENS medium at a 1:4 during 48h at 37°C. pcENS were treated daily with anti-GDNF blocking antibody (1.8 µg/ml, R&D System, Minneapolis). At the 13^{th} supernatants were collected, and cells were fixed in 4% paraformaldehyde (PAF) for 30 minutes at RT. Then, cells were washed with PBS for three times, each of them was 10 minutes long. Finally, they were stored at 4°C after adding to each well PBSNaN_3.

Immunohistochemistry

Cells were incubated permeabilized for 30min in PBS/NaN_3 containing 0.5% (v/v) Triton X-100 (Sigma) and 4% (v/v) horse serum before incubation overnight at 4◦C with mouse anti Human Neuronal Protein HuC/ HuD (HuC/D) as primary antibody. Then, cells were washed in PBS for three times (each of them ten minutes long) and incubated for 1h at RT with the anti-mouse-Cy3 (Jackson Immunoresearch) antibody as secondary antibody coupled to fluorophores (see table 3 for references and dilution).

Antigen	Species	Source	Concentration
Primary antisera			
HuC/D	mouse	Molecular probes, Invitrogen	1:200
Secondary antisera			
Anti-mouse-Cy3	Goat	Jackson Immunoresearch	1:500
Blocking antibody			
Anti GDNF	Mouse	R&D	1:250

Table 3; Primary, secondary and blocking Antibodies used for pcENS cultures.

Acquisition and morphometrical analysis

Fluorophores were visualized using an inverted microscope set up for fluorescence microscopy (Olympus IX50) with an excitation wavelength of 552 nm. Twenty ganglia for each well were considered for quantification. They were photographed by the camera using the software CellB and then the HuCD-IR neurons present in each ganglion were manually counted by two different and blinded researchers. The data were expressed as number neurons per ganglia (neuronal density).

5.1.2. Organotypic cultures

Cultures preparation

The colon used to perform the colonic organotypic cultures was explanted from the same pregnant Sprague–Dawley rats that were purchased each week (Janvier Laboratoires S.A., Le Genest-St-Isle, France) for preparing the pcENS. As usual the rats were manipulated in compliance with the French and every effort was made to minimize animal suffering and the number of animals used. After removing the uterus, the abdomen was opened, and the colon was rapidly excised and placed in the sterile, oxygenated and ice-cold Krebs solution of the following composition: NaCl: 117 mmol/2L; KCl: 4.7

mmol/2L; $MgCl_2$: 1.2 mmol/2L; NaH_2PO_4: 1.2 mmol/2L; $NaHCO_3$: 25 mmol/2L; $CaCl_2$: 2.5 mmol/2L; glucose: 11 mmol/2L; (pH 7.4). All dishes, glassware, surgical tools and solutions used were sterile. The tissue was placed in sylgard-covered petri dish, previously filled with oxygenated Krebs solution and then was pinned on the two extremity. After removing the proximal part, the explant was opened along the mesenteric border. The content was flushed away, and the tissue was pinned out and maximally stretched in order to make easier the peeling of the mucosal layer. During this procedure, the Krebs solution was changed every 10 min. Afterword, distal colon was cut into small full thickness pieces (about 1 cm long) and washed in agitation using sterile and cold Krebs solution for four times (each of them five minutes long). An 8 multi-well plate Sylgard-covered filled with sterile Krebs solution was used to let samples in culture and a special culture medium was prepared as follow: culture medium (Dulbecco's modified Eagle medium/F-12 Ham; Sigma Chemical, St. Louis, Mo., USA) was supplemented with $NaHCO_3$ 4M, 10% heat-inactivated fetal calf serum (CC pro; Karlsruhe, Germany), 10 ml Antibiotic antimycotic, 50mg/5ml amphotericin B, and 2mL gentamicin (Sigma Chemical, St. Louis, Mo., USA) and adjusted to pH 7.3. Except for one piece that was used as a positive control (T_0), all other pieces were placed into their own well using pins, trying to stretch it as long as possible and to leave an interface between the sample and the bottom of the well. These strategies favor the medium flow around and inside the tissue for avoiding the formation of central necrotic areas. After that, 1 mL of culture medium was added to each well and plates were maintained in a humidified-agitated chamber placed into an incubator set at 37° C with 5% of CO_2. Every day the chamber was equilibrated with 1 minutes of flowing carbogen and the culture medium was daily changed. Culture were stopped and fixed at two endpoints: 48 (Day 2) and 96 (Day 4) hours. The T_0 tissue after washes, was transferred in sylgard-covered petri dishes and pinned out one more time to be fixed in 4% PAF at RT for 1h and 30 min. After three washes in PBS (each of them 5 minutes long), it was ready for the microdissection step or the storage at 4°C adding $PBSNaN_3$.

Treatment

In order to study the effect of the GDNF on rat distal colon, some sample were treated with human GDNF (Eurobio). GDNF was used at the final concentration of 100 ng/ml and was added to the culture medium. It was daily prepared and added to the culture when the medium was changed.

Dissections and Immunohistochemistry

At day 2 and day 4, tissues were fixed as previously described for the T_0 tissue. Using a dissection microscope, each sample was micro dissected in order to obtain a longitudinal muscle-myenteric plexus preparation (LMMP). To reach this preparation, the submucosal layer and the muscle fibers of the circular layer were gently removed, paying attention not to disrupt the neuronal myenteric network. Immunohistochemical detection of pan-neuronal and apoptotic antigens was made using as primary antibodies: mouse anti-HuCD (1:200; Molecular probe) co-labelled with rabbit anti-caspase-3 antibodies (1:1000; Sigma). At first, tissues were permeabilized with solution composed of 0,1% Triton (v/v) 100x and 4% horse serum (v/v) in PBSNaN_3 incubated in agitation for 1h at RT. After this membrane permeabilization procedure, the tissues were incubated overnight at 4°C in agitation with the mixing solution composed of the two primary antibodies diluted in the same solution used for the permeabilization step. Therefore, samples were thoroughly rinsed with PBS (three times, each of them 5 minutes long) and incubated with a mixing solution composed of the two secondary antibodies: anti-mouse-AF488 (1:200; Interchim) and anti-rabbit CY3 (1:500; Jackson Immunoresearch) Finally, sample were washed three time with PBS (each of them 5 minutes long) and mounted on slices using the aqueous medium (ProLong gold antifade mountant, Molecular probes, Life technologies).

At first, a qualitative analysis was done in order to assess the effectiveness of the procedure. A general overview of the whole samples was obtained using the tilling procedure, that is an automatized function of the Zeiss Axio Zoom V16 stereomicroscope (Zeiss, Marly Le Roi, France) which allow us to obtain large field of view at high resolution.

Secondly, quantitative analysis was performed using Olympus BX-51microscope (Olympus) fitted with Leica DFC310 FX 1.4-megapixel digital camera and equipped with the Leica software application suite LAS V3.8 (Leica Microsystems, Mannheim, Germany). The number of HuCD-IR, Caspase-3-IR neurons and the intra-ganglionic area analysed were measured capturing photos at 20x on twenty ganglia per sample randomly chosen. Since no Caspase-3-IR neurons were observed, only the number of HuCD-IR neurons was measured and normalized for the intraganglionic area considered as ROI into the photos.

5.1.3. PCR analysis

PCR analysis was performed on human colonic biopsies collected from three different areas: healthy, taken (as previously described in the supernatant collection's chapter) from 10 cm after tumour mass; tumour, taken in a full thickness way from the tumour mass; and peripheral tumour area, taken from the tumour surrounding area. The gene analysed was the GDNF and its expression was quantified in 32 patients' biopsies. The primers were designed using primer-BLAST and the sequences are listed in the Table 4:

Gene		Sequences
hPanGDNF	Forward	5' AAAACCGGGGTTGTGTCTTA 3'
hPanGDNF	Reverse	3' TTGTCGTACGTTGTCTCAGC 5'

Table 4; GDNF primers

Primers are validated and controlled by sequencing at a specific platform (Genomique, SFR Bonamy, Nantes). The total RNAs were extracted from colonic biopsies using NucleoSpin®TriPrep (Macherey-Nagel EURL, Hoerdt, France) according to the manufacturer's instructions. For reverse transcription, 1 µg purified total RNA was denatured and subsequently the cDNA was synthetized using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, Courtaboeuf, France) according to the manufacturer's instructions. Real-time PCR was performed using the Fast Sybr Green Master Mix kit (Applied Biosystems, California, USA) in a total volume of 15 µL composed of 1.25 µL forward and reverse primer, 7.5 µL Light cycler 480 SYBR Green I Master (Roche, Mannheim, Germany) and 5 µL of cDNA at a concentration of 0.8 ng μL^{-1} RNA equivalent. Finally, the PCR reaction was run on a StepOnePlus thermocycler (Applied Biosystems, California, USA) and the same standard reaction condition was used for each primer pair, consisting of a denaturation at 95°C for 10 second, hybridization at 60°C for 15 second, and elongation at 72° for 15 second. This cycle was repeated 45 times. Samples were analysed in duplicate and a standard curve for each gene was prepared using serial dilutions from the cDNA extracted from colonic biopsies of each pool of patients. All the results obtained were expressed as a ratio of GDNF/S6 mRNA expression levels.

5.1.4. Data Analysis and Statistical Test

Statistical analyses were performed using GraphPad Prism software (GraphPad, San Diego, CA, USA). The Wilcoxon matched-pairs log-rank test was performed to compare continuous variations between 2 groups for the experiments on pcENS. Statistical differences between mean values were analysed by analysis of variance (ANOVA) followed by Bonferroni's post-hoc to compared more than two groups. Statistical significance was defined as a p-value less than 0.05.

5.2. Results

To explore the effect of tumour supernatants in ENS and to further gain insight into the study the mechanisms involved, pcENSs were chosen as in vitro assay to mimic the paracrine impact of TEC's released factors on ENS, as novel component of MET. In a first experimental set, pcENSs were treated with tumour and healthy supernatants (as described in *chapter 5.1*) in order to verify the neurogenic effect of tumour supernatants. Secondly, pcENSs were treated with TEC supernatants obtained from Caco-2 cells to verify whether the soluble factors present in human supernatants were released specifically by TEC. Finally, we passed to analyse whether the GDNF could be one factor involved in the enteric neoneurogenesis.

5.2.1. *In-vitro* studies:

Evaluation of the effect of tumour/healthy supernatant upon neuronal density

The analysis showed a significant an increase of 21% in the enteric neuronal density in cultures treated with tumour supernatants compared to cultures treated with supernatants of the healthy one *(Figure 29, C)*.

Evaluation of the role of GDNF as a putative factor involved in the effect of supernatant.

After assessing the potential of the tumour supernatants to increase the number of enteric neurons, we aimed to determine whether GDNF would have been a putative candidate able to exert neurotrophic properties previously observed. Based on the literature search, three factors were identified as putative candidates: artemin, NGF and GDNF. These factors belong to the GDNF family which has been suggested to be involved in PNI process [21]. In addition, in a previous study, we have shown that effects of high fat diet upon survival of in myenteric neurons were mediated by GDNF [234].

At the onset, in other experiments not presented in this thesis, GDNF levels were found to be significantly increased in the supernatants of tumour as compared to supernatants of healthy area. In order to test the GDNF as a putative factor involved in the effects of supernatants upon ENS, different experimental sets were performed using different experimental conditions (see *chapter 5.1.1*).

First, we showed that cultures treated with tumour supernatants presented a significantly greater neuronal density of 26% compared to the one treated with healthy supernatants (*Figure 29, C*). Second, the treatment with the antibody anti-GDNF and tumour supernatants reduced significantly the neuronal density as compared to the culture treated only with tumour supernatants (*Figure 30*, D). Moreover, the latter neuronal density was comparable to the one measured in culture treated with healthy supernatant.

Evaluation of the effects of TEC upon neuronal density and involvement of GDNF.

Subsequently, with the aim to confirm the fact that GDNF is effectively released by TEC, we measured the impact of supernatants obtained from the Caco-2 cell line upon neuronal density (*Figure 30, E*).

Similar to previous outcomes, in cultures treated only with TEC supernatants we observed a significant increase in the neuronal density compared to the culture treated with no supernatants and isotype, suggesting that TEC directly reproduce at least in part the effects of supernatants of the tumor. Furthermore, we showed that pcENS treated with TEC supernatants + anti-GDNF exhibited a significantly lower ganglionic neuronal density as compared to pcENS treated with TEC supernatants + isotype.

5.2.2. Ex-vivo studies:

With the aim to confirm the GDNF neurotrophic effect previously observed in embryonic-derived pcENS, organotypic cultures were developed as alternative and complemental experimental model. Indeed, organotypic cultures, prepared starting from colonic explants, are characterized by adult and well-developed ENS, surrounded by the whole physiological cellular and molecular microenvironment. This enable us to verify whether the GDNF exert neuroplastic changes properties directly on ENS, and more precisely on adult myenteric neurons, which is the enteric component studied in this work of thesis.

Development of organotypic cultures and evaluation of the neuronal survival

After setting the cultures conditions (medium, timing endpoints, best methodology for preparation the organotypic cultures from colonic explants), we analyzed the efficacy of the procedure, evaluating the neuronal survival.

Thus, we first quantified the number of HuCD and of caspase-3-IR cells to evaluate the potential loss of myenteric neurons during the cultures, and to characterize if loss of neurons would be associated to the neuronal death as result of apoptosis. *(Figure 31, A-C)*. The results showed myenteric neurons were present in all the three endpoints studied, T_0, T_1(48h), T_2(96h) and furthermore, no caspase 3-IR neurons were observed. However, a decreased number of myenteric ganglia in tissue cultures at both the two endpoints (48 and 96h) *(Figure 31, E-F and E^1-F^1)* was measured. More precisely, the second endpoint, T_2(96h), presented a more discontinuous myenteric network respect to T_1(48h) and T_0 together with significant neuronal loss *(Figure 31, G)*. It is worth noting that at T_2(96h) was observed in myenteric ganglia the presence of neuronal population composed of neurons with both nuclear HuCD and cytoplasmic staining. These latter one presented augmented dimensions compared to neurons in T_0 and T_1(48h).

Evaluation of neurotrophic effect GDNF-related.

In this part we tested the ability of GDNF to modify, the changes in neuronal density observed previously by adding GDNF (100 ng/ml) to organotypic culture medium.

The neuronal density in tissues treated with GDNF were not different as compared to control at the time point T_0, T_1(48h) and T_2(96h) (*Figure 32, G*). However, T_2(96h) tissues GDNF-treated showed a slightly, but not significant increases of neuronal density

compared to the T_2(96h) no-GDNF treated group. However, in all cultures condition, the treatment with GDNF did not prevent the neuronal loss observed over the time of organ culture.

5.2.3. Biomolecular analysis

Quantitative evaluation of GDNF mRNA expression in human biopsies.

To determine whether the GDNF mRNA expression was modulated in cancer compared to healthy specimens or to tumor surrounding area, quantitative PCR were performed on human samples, obtained from biopsies specimens. The results (*Figure 33*) showed no significant differences between the healthy group, tumor and tumor surrounding area.

5.2.4. Images

In-vitro studies:

Evaluation of the neurogenetic effect of tumor/healthy supernatant.

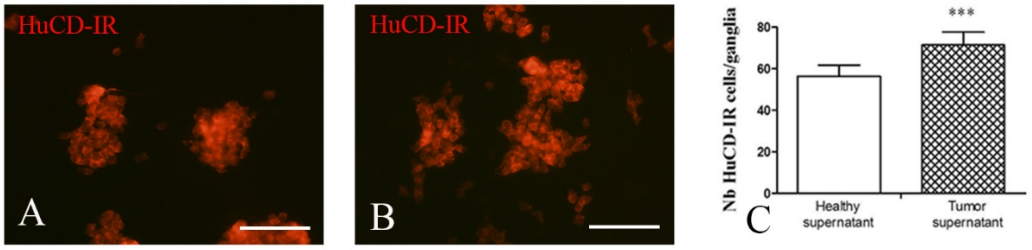

Figure 29; HuCD labelling in pcENSs (A and B). *Representative images of pcENS treated with Healthy supernatants (A), Tumor supernatants (B). HuCD-IR was observed in cytoplasm of enteric neurons, which in pcENSs treated with healthy supernatants (A) made up small and dispersed ganglia, whilst in pcENSs treated with tumor supernatants (B), aggregated to form bigger ganglia composed of higher neuronal number. Scale bar = 100 µm.* ***Evaluation of neuronal density in pcENSs treated with tumor supernatants (C).*** *Neuronal density was assessed quantifying the number of HuCD-IR cell per ganglia in primary ENS cultures. Data are expressed as mean ± SEM. Wilcoxon's test. *** ($p < 0.0001$) vs healthy supernatants. N = 15*

Evaluation of the neurogenetic effect due to GDNF, as a factor present in human tumor supernatant

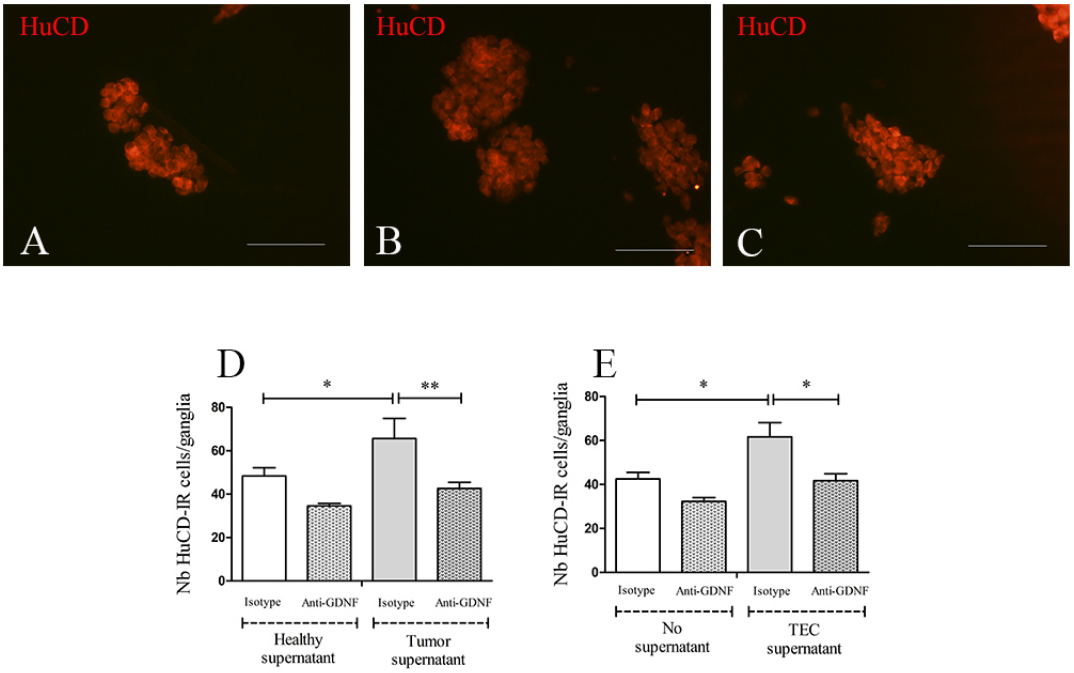

Figure 30; HuCD labelling in pcENSs (A-C). *Representative images of neuronal ganglia in pcENS treated with Healthy supernatants + isotype (A), Tumor supernatants + Isotype (B), Tumor supernatants + anti-GDNF antibody (C). Scale bar = 100 µm.* ***Evaluation of neuronal density in pcENSs treated with human healthy/tumor supernatants and isotype/anti-GDNF antibody (D) and pcENSs treated with no supernatants/TEC supernatants and isotype/anti-GDNF antibody (E).*** *Data are expressed as mean ± SEM. One-way ANOVA test - Post hoc Bonferroni's test. *($p<0.05$), **($p < 0.001$). N = 7.*

Ex-vivo studies:

Evaluation of the neuronal survival in organotypic cultures

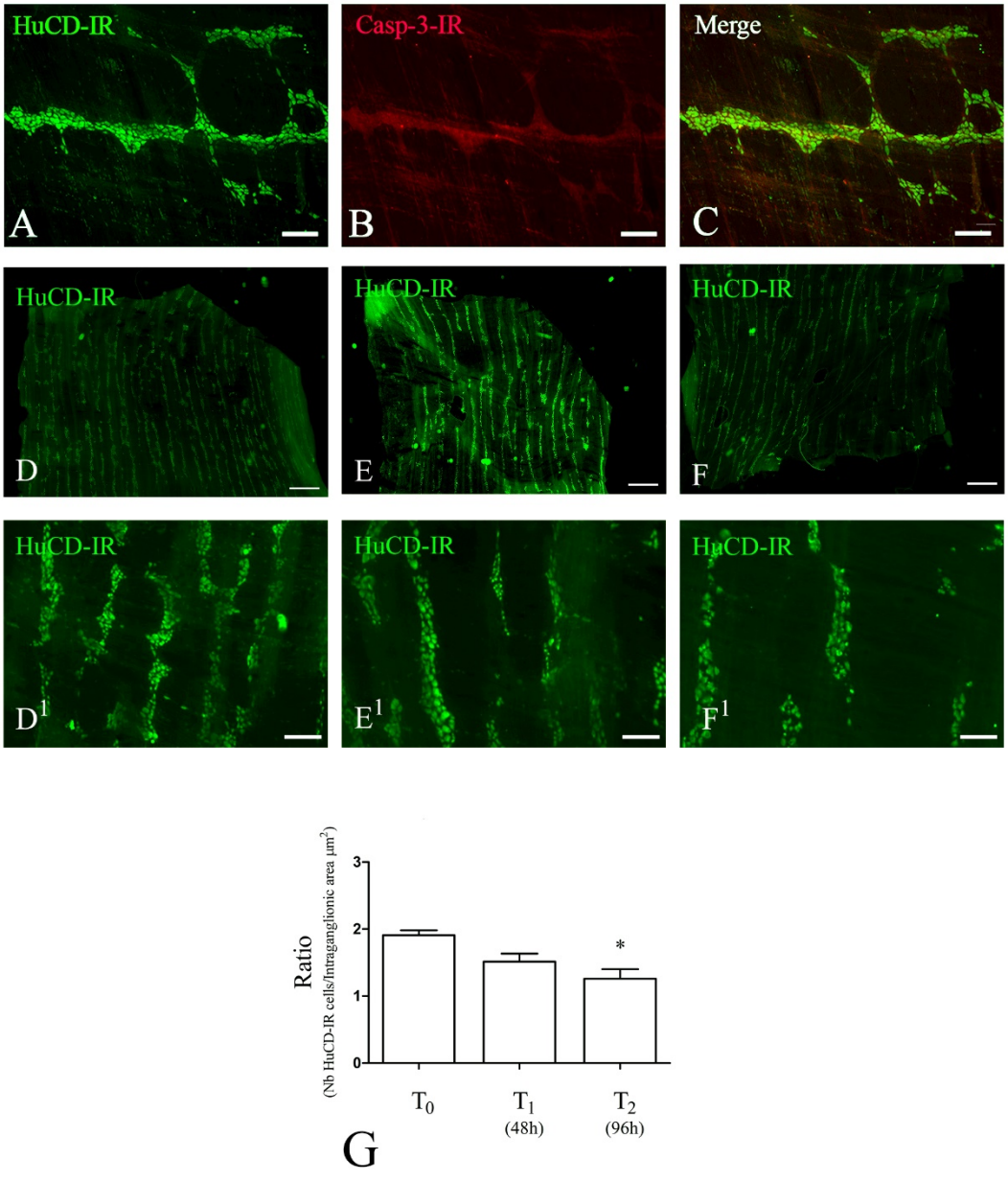

Figure 31; HuCD and caspase-3 labelling in colonic organotypic culture (A-C). *Representative images of HuCD-Caspase-3-IR in myenteric neuronal ganglia. Scale bar = (D-F: 1mm; D^1-F^1: 150µm).* ***Organotypic cultures obtained from adult rat distal colon (****D- F, D^1-F^1****).*** *Representative full-tissue images (D-F), and (D^1-F^1) are their respective magnifications. (D) shows the explant at T_0 which represents the control positive of the procedure. (E) and (F) are the two endpoints (T_1 and T_2) of the organotypic cultures derived from colonic explant (48 and 96 hours). We observed decreases in number of neurons and myenteric ganglia, especially, at 96h. However, no neurons HuCD-/caspase 3-IR were observed. In (F^1) we observed myenteric ganglia composed of a mixture of neurons with nuclear HuCD staining and neurons with HuCD cytoplasmic staining but bigger in shape respect to neurons observed in (A_1) and (B_1). Scale bar = 200 µm.* ***Evaluation of neuronal survival in organotypic cultures (G)****. The data were expressed as neuronal density and as mean ± SEM. One-way ANOVA test - Post hoc Newman Keuls'. * ($p<0.05$) vs T_0. N = 3.*

Evaluation of the neurogenetic effect due to GDNF, in adult experimental model:

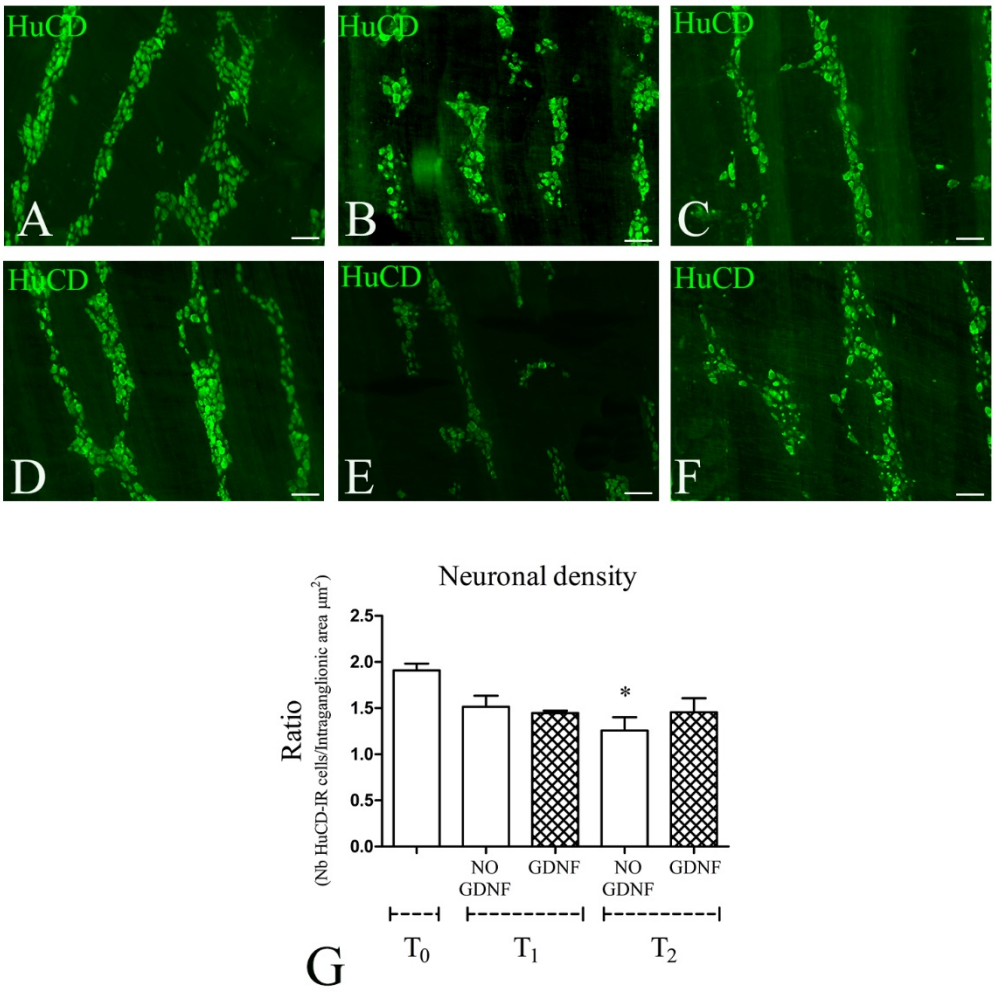

Figure 32; Organotypic cultures obtained from adult rat distal colon (A-F). *Representative images of T_0 (A, D), T_1 no GDNF (B), T_1 GDNF (C), T_2 no GDNF (E) and T_2 GDNF (F). T_1 and T_2 are the two endpoints utilized respectively of 48 and 96 hours. No neurons caspase-3-IR were observed, so that we didn't show those results.* ***Evaluation of neuronal density during GDNF treatment (G).*** *The data were expressed as neuronal density and as mean ± SEM. One-way ANOVA test - Post hoc Newman Keuls'. * ($p<0.05$) vs T_0. N = 3.*

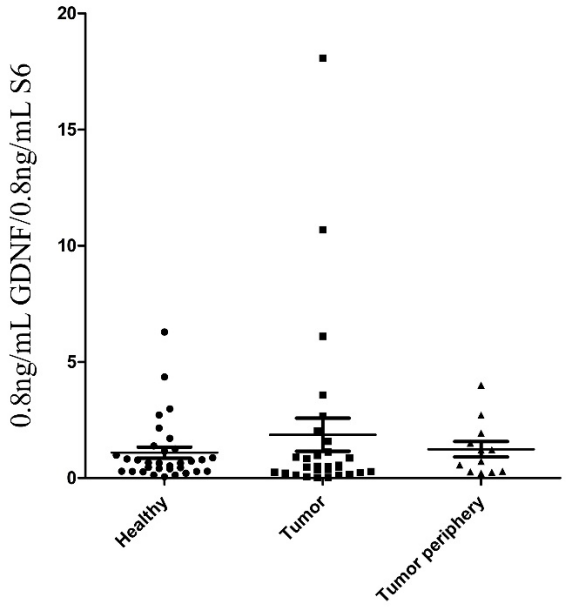

Figure 33; Quantitative PCR analysis in human biopsies. *GDNF mRNA expression in healthy, tumor, tumor peripheral area of specimens obtained from human biopsies. Q-PCR data are expressed as relative values (ng/mL GDNF/ng/mL S6). Data are expressed as mean ± SEM. Wilcoxon's test. No significance.* $N_{healthy} = 32$, $N_{tumor} = 29$, $N_{tumor\ periphery} = 12$

5.3. Discussion

5.3.1. In vitro studies

Several findings have established that the tumor microenvironment (TME) influence cancer growth, invasion and metastasis [196]. The nervous system has been described as a component of TME able to interact directly with cancer cells, providing physical supports for spreading, and indirectly, trough the release of soluble factors as neuropeptide and/or neurotransmitters [197]. Cancer cells can attract nerve fibers and stimulate nerve outgrowth within the tumor stroma by secreting neurotrophic factors [235,236]. Nerve fibers, unlike, can infiltrate TME and stimulate tumor growth and cancer cell dissemination [237]. In pancreatic and prostatic cancers, for instance, PNI has been presented as a preferential route of spread and dissemination [201]. Additionally, *in vivo* and *in vitro* models of tumor for the same cancers have showed that autonomic neuritogenesis occur within the tumor stroma inducing increments of novel neurites rather than the existing ones [238,239]. Moreover, the phenomenon of the autonomic neurogenesis has been recognized as a process common to several cancer types as prostatic, pancreatic and CRC often associate to a poor prognosis [23,239,240].

Recent evidences obtained at Inserm U1235 (host laboratory) have shown that interactions between tumor cells and intrinsic nerves as those belonging to the ENS can occur also in the case of CRC. Indeed, it has been shown that tumor cells can directly interact with enteric nerve fibers and preferentially migrate along them by means the L1CAM/N-Cadherin-dependent pathway. Conversely, preliminary data that have founded the scientific rational for my study, have highlighted the occurrence of ENS remodeling in CRC patients. This remodeling was characterized by an increased neuronal density within myenteric and submucosal ganglia. Based on this observation, we aimed to:

i) explore whether soluble factors derived from TME were able to reproduce in vitro the effects observed in situ;
ii) identify whether tumor epithelial cells (TEC) could contribute directly to these changes;
iii) identify the role of GDNF as a putative candidate released by the TME, responsible for the effects upon neuronal density.

Here, we demonstrated that soluble factors, released by CCR, induce *in vitro,* a cancer-related neo-neurogenesis, increasing the neuronal density within enteric ganglia. Further, we provide evidences indicating that these factors are directly released by TEC. These results, together with previous *in-situ* studies which observed increments in number neurons of both myenteric and submucosal plexuses within the tumour surrounding area, outline for the first time the existence of neo-neurogenesis within MET which involve the intrinsic neuronal component, the ENS. The origin of these newly generated enteric neurons is currently unknown. A possible speculation regards the direct neurotrophic effect exerted by tumor supernatants on existing neurons (neuronal neurogenesis). The second and more realistic hypothesis address the possibility for enteric progenitors to differentiate in neurons (stem cells neurogenesis). The current dogma refers to a little or no ongoing neo-neurogenesis in adult and well-develop ENS and pose a controversial question regarding the existence of true enteric neural precursors cells (ENPC) or regarding the neurogenic potential of the EGCs. Certain studies proven evidences concerning the presence of neurogenesis enteric progenitors'-derived proven through a murine model but not in humans [101,241]. Other works reported a limited neurogenic potential exerted by a small population of SOX-10-IR cells activated by certain in vitro and in vivo conditions (such as injury) [242]. Noteworthy, Kulkarni and co-workers recently showed the existence of Nestin^{+} cells placed in the myenteric plexus of adult and healthy mice. They provided evidences of their potential as precursors by means in vitro and in vivo experiments, highlighting not only their ability to differentiate in enteric neurons but also demonstrating that these cell have unique profile, not common to glial cells [243].

Our findings show also that the neurogenesis in pcENS was mediated by GDNF released by both the TME and also specifically by TEC, as demonstrated by the inhibitory effects of the anti-GDNF blocking antibody. In physiological conditions GDNF exerts central neurotrophic effects during ontogenesis of the ENS by regulating the proliferation of neuronal progenitors. Indeed, knock out of the GDNF or its receptors (GFRα1/RET) lead to enteric agangliosis [244]. Moreover, several studies have pointed out its role even during post-natal ENS development as promoter of proliferation, migration and differentiation of the neuro-glial precursors [245]. Finally, neuroprotection and neuromodulation in adulthood have been reported as processes GDNF-dependent [246]. Besides its overall physiological actions, GDNF has also been shown to directly modulate TEC functions. Consisted to our findings, GDNF has been shown to contribute to tumor progression and dissemination [247]. Certain authors have reported the GDNF as factor able to promote the

cancer adhesion and invasion through the enhancement of integrin expression [248] and VEGF-VEGF-R interaction [249]. However, what is its exact role within the enteric cancer-induced neoneurogenesis is still unknown and further studies are needed.

5.3.2. Ex-vivo studies

With the aim to establish whether GDNF could exert the observed neurotrophic function directly on adult myenteric neurons, we developed an *ex vivo* organotypic culture model as an alternative experimental method, characterized by of an adult and well-developed ENS. Firstly, this model was developed based on the assumption that the observed neurogenesis is induced during carcinogenesis (and not already present at the initiation of the diseases). Secondly, we aimed to overcome certain limitations inherent to the pcENS model itself. Indeed, pcENS are obtained from embryonic-derived cultures that, even differentiated, still contain a small residues of proliferating enteric progenitor cells (as assessed by Ki67 staining on previous studies). In order to determine whether GDNF could also promote neurogenesis in adult ENS, we tested its long term effect in a model of organotypic culture previously developed [250]. A major drawback of the development of this model is that we observed important neuronal cell loss over 96h of culture. This cell loss was probably not associated to apoptosis as not caspase 3-IR, but likely associated to a necrotic depend cell death. Although we aimed to reduce this cell death by adapting the experimental protocols (i.e. by modifying culture medium composition; increasing tissue O_2 exposure during culture) we did not achieve this goal. Alternatively, the use of novel techniques based on generation of gut derived from human intestinal organoids (HIOs) as currently being developed in the laboratory could allow us to answer these questions [251]. Nevertheless, although aware of the fragility of the model, we still decided to test the effects of GDNF in this organ culture of rat colonic tissue. Preliminary data obtained from the GDNF-treated cultures highlighted that GDNF slightly reduced the neuronal loss at 96h. Whether this reduction of cell loss is due either to stimulation of neurogenesis or neuroprotective effects counteracting neuronal cell death remains currently unknown. We rather feel that the effects observed could result from a neuroprotective GDNF-related role more than the neurotrophic one. Accordingly, Baudry et al., indeed, demonstrated that leptin exerted neuroprotective effects in myenteric neurons partly via GDNF in mouse treated with Western diet induced obesity [234]. Nevertheless, other works reported GDNF as molecule involved in diabetic neuronal loss

[246] and as stated above, involved in enhancement of neuronal survival in post-natal myenteric neurons [252].

Finally, from our findings, GNDF mRNA expression results not modulated by tumor, although previous experiments by using ELISA assay showed increased GDNF protein levels in tumor supernatant. The function of GDNF has been studied extensively, but little is known about the basic cell biology. Differently to brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), GDNF is synthesized as a pre-pro-protein that undergoes to post-transcriptional processing forming two GDNF isoforms, that, in turn, are differentially released [253,254]. So that, the discrepancies observed in our studies between GDNF mRNA and proteins levels might be likely due to tumor-induced regulation occurring during these processes. Further cell biology studies are required to investigate on this topic, as well as, analysis concerning the expression of its receptors, RET and GRF α-1.

5.4. Conclusions

Altogether, in this part of the study we have contributed to further gain insights into the mechanisms and actors involved in the remodeling of the ENS observed during CRC. Indeed, our results have identified GDNF as a putative candidate derived from the TME (and potentially produced in part by TEC) that could contribute to the increased neuronal density observed in CRC, in proximity of the tumor. This mainly in vitro study needs to be confirmed in vivo. In particular, the use of cancer induced mice models (AOM-DSS) in transgenic *Sox10*::*Cre*;R26ReYFP will allow us to determine:

1) whether neurogenesis occur in adult ENS in vivo and 2) whether the observed increased neurons originate from Sox10-IR cells. The use of this model could also allow us to see whether GDNF or blocking antibody against GNDF can directly interfere with tumor induced neurogenesis. In parallel, the development of tumors in intestinal organoids could allow us also to further validate in human the results obtained in our animal's models and gain also insights in the mechanisms involved in these effects.

Finally, the consequences of the remodeling of the ENS upon the development of the tumors remains to be determined. In order to address this question, it is first important to determine whether neurogenesis observed in CRC affects specific subpopulations of neurochemically defined populations. Indeed, neurotransmitters have been sown to

differentially affects TEC; for instance, ACh has been shown to exert pro proliferative effects while VIP has been shown to inhibit cells proliferation [210–212]. Therefore, whether ENS remodeling include by CRC ultimately favors or limits tumor development remains unknow. Better knowledge about these questions could therefore lead to the development of novel therapeutically approaches in the field of CRC by developing approaches aimed either at inhibiting specific neuromodulators or conversely activating the ENS (for instance via neuromodulation). Therefore, understanding the specific mechanisms of carcinoma/nerve interactions in neurogenesis is key to developing cancer-related therapies.

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7. Acknowledgments

"I always feel the movement is a sort of mosaic.
Each of us puts in one little stone,
and then you get a great mosaic at the end."

Alice Paul

I just think that we create our own fate. Day by day we build up our own mosaic, gathering thousands of experiences which make us, hopefully, better people.
At the end of my PhD, that was a scientific but also a deep and introspective journey, I picked up many little stones which are going to fill an important part of my own mosaic.
During this period, I learnt, of course, what the research is; an intense experience composed of conflicting feelings that give you excitement, happiness but also frustration and sometimes discouragement.
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PN