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Advanced studies on two animal models, murine and fish.

RadKI21^{A626T} mouse model: New Insights into Molecular Mechanisms of Enteric Neuro-Epithelial Pathology

European sea bass: Study of the effects of essential oils in different diets on the gastric system

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PREAMBLE

My PhD research period was focused on the anatomical, physiological and functional study of the gastrointestinal system on two different animal models. Therefore, a massive discussion of the two animals model gastro-enteric system will be illustrated in this work.

In two different contexts, the purpose of these two lines of research, conducted in parallel during my PhD period, was contribute to understand and analyze how a specific genetic mutation or the adoption of a particular dietary supplement can affect gastrointestinal function .

ABSTRACT

Background: Functional gastrointestinal disorders (FGID) are chronic conditions characterized by symptoms for which no overt organic cause can be found. They affect up to 20% of western populations with evidence suggesting a steadily increasing percentage also in less industrialized countries (Spiller 2005). Although symptoms are generally mild or moderate, a small subset of cases shows severe manifestations, i.e. nausea, vomiting, bloating, abdominal distension, intractable constipation and chronic pain, with such an intensity hampering normal feeding and compromising considerably patients' quality of life. In addition, this subset of patients may also have recurrent intestinal sub-occlusive episodes, which occur in the absence of demonstrable mechanical causes, leading to numerous hospitalizations as well as useless and potentially harmful surgical interventions. This condition is referred to as chronic intestinal pseudo-obstruction (CIPO), a rare and intractable chronic digestive disease with symptoms and signs of intestinal obstruction without demonstrable mechanical cause. CIPO can result from derangements affecting the integrity of a variety of regulatory cells/tissues, i.e. smooth muscle cells, the interstitial cells of Cajal (ICC) (pace-makers of gut motility), and neurons (either intrinsic – the enteric nervous system- or extrinsic nerve pathways). CIPO can have an idiopathic origin, or can be secondary to other diseases. From a genetic perspective, some mutations have been associated with CIPO in different genes (SOX10, ACGT2, SGOL1, TYMP, POLG, FLNA, L1CAM and RAD21). A novel causative RAD21 (Ala622Thr) missense mutation was identified in a large consanguineous family, segregating a recessive form of CIPO with a clinical phenotype characterized by prominent severe enteric dysmotility. RAD21 regulates different cellular mechanisms as part of the cohesin complex, including 3D genome organization and transcriptional regulation.

Aims and experimental studies: The present thesis was aimed to elucidate the mechanisms leading to neuropathy underlying CIPO via a recently developed conditional KI mouse carrying the RAD21 mutation. The experimental studies are

based on the characterization and functional analysis of the conditional KI Rad21^{A626T} mouse model.

Conclusion: My thesis focuses on detection of new genes in CIPO to a thorough characterization of a conditional KI^{A626T} mouse with a genetic profile derived from patients will better understand mechanisms leading to CIPO neuropathy.

LIST OF ABBREVIATIONS

APOB48 Apolipoprotein B48

CdLS Cornelia de Lange syndrome

ChAT choline acetyltransferase

CIPO Chronic intestinal pseudo-obstruction

CNS central nervous system

CSM circular smooth muscle

DAPI 4,6-diamidino-phenylindole, dihydrochloride

DMSO dimethyl sulfoxide

dpf days post fertilization

EC enterochromaffin cells

ECs endothelial cells

EMSA electromobility shift assays

ENS Enteric Nervous System

ENCCs enteric neuronal crest cells

FGID Functional gastrointestinal disorders

GFAP glial fibrillary acid protein

GI gastrointestinal

IBD inflammatory bowel disease

IBS Irritable Bowel Disease

IBS-C constipation predominant IBS

IBS-D diarrhea-predominant

IBS ICC interstitial cells of Cajal

IR Immunoreactivity

KI knock-in

KO knock-out

KHB Krebs-Henseleit Buffer

MNGIE Mitochondrial Neurogastrointestinal Encephalomyopathy

MO morpholino

MP myenteric plexus

nNOS neuronal nitric oxide synthase

NPY Neuropeptide Y

NT non-targeting

PBS phosphate buffered saline

POLG1 polymerase gamma

scRNA-seq Single-cell RNA sequencing

Syn synaptophysin

SMP submucous plexus

UES upper esophageal sphincter

WT wild type

WES Whole Exome Sequencing

MMIHS megacystis-microcolon-intestinal hypoperistalsis syndrome

INTRODUCTION

Chapter I

1. MORPHO-FUNCTIONAL ORGANIZATION OF THE ENTERIC NERVOUS SYSTEM

The enteric nervous system (ENS) is the third division of the autonomic nervous system (ANS). Conversely from any other ANS components, the ENS exhibits the peculiar ability to control many key physiological functions independently of the central nervous system (CNS). That is the reason why the ENS is also named the "second brain" or the "brain-in-the-gut" [1]. Over the years, the ENS has been thoroughly investigated. The first data derived from early purely morphological studies unravelling that enteric neurons are organized in a neural network, which finds its basic structure in two types of neuronal aggregates or ganglia or ganglionic plexuses embedded in the intestinal wall. Hence the myenteric plexus, bearing the name of the discoverer, Leopold Auerbach (who illustrated in his senior thesis in 1862 the myenteric ganglia of the small bowel of small mammals), is located within the two muscular coats (the outer – longitudinal - the inner - circular) of the gut. The submucosal plexus is located in the submucosa and has been studied by Meissner even earlier than the myenteric one. Although in the nineteenth century nobody knew the role of enteric ganglia and even less the identity of single neurons, *in vitro* studies on isolated gut segments carried out by legendary physiologists, such as Bayliss and Starling (1899), allowed to understand that the enteric innervation could act autonomously in controlling basic and fundamental functions. This was the case of 'the law of the intestine' later re-defined 'peristalsis' by Trendelenburg. Specifically, Bayliss and Starling clearly reported that local neural reflexes in isolated (or extrinsically denervated) intestinal segments of dogs and rabbits were activated by distension of the gut wall, which evoked a muscle contraction oral and a relaxation anal to the distended area thereby providing an oral-caudal pressure gradient capable of intraluminal propulsion [2]. "The law of the intestine" has been confirmed later with more sophisticated methods [3]. Since then, a vast

array of knowledge about the ENS has been collected. Nowadays, we know that the ENS consists of more than hundred million nerve cells, grouped in ganglia and with their cell bodies located in the gut wall (Figure 1). The myenteric plexus (MP) is present along the whole gut from the distal esophagus to the internal anal sphincter, whereas the submucosal plexus (SMP) is significantly present in the small and large intestine [4, 5, 6, 7, 8, 9]. Furthermore, an accurate evaluation discloses differences in the size of the ganglia: the SMP is generally smaller and the related interconnecting strands thinner than those of the MP [10]. Because of its strategic location, the MP mainly regulates motility, whereas the SMP regulates mainly secretion / absorption as well as the microvascularization [10]. Novel results indicate that the ENS acts in a remarkable number of physiological functions involving immunological aspects, entero-endocrine cells, blood flow, crosstalk with the immune system as well as playing a role in the maintenance of the GI epithelial barrier, body energy homeostasis, and major interplay (either direct or indirect) with the gut microbiota. An intact ENS is essential for body integrity and well-being, whereas abnormalities of ENS cause significant digestive disorders.

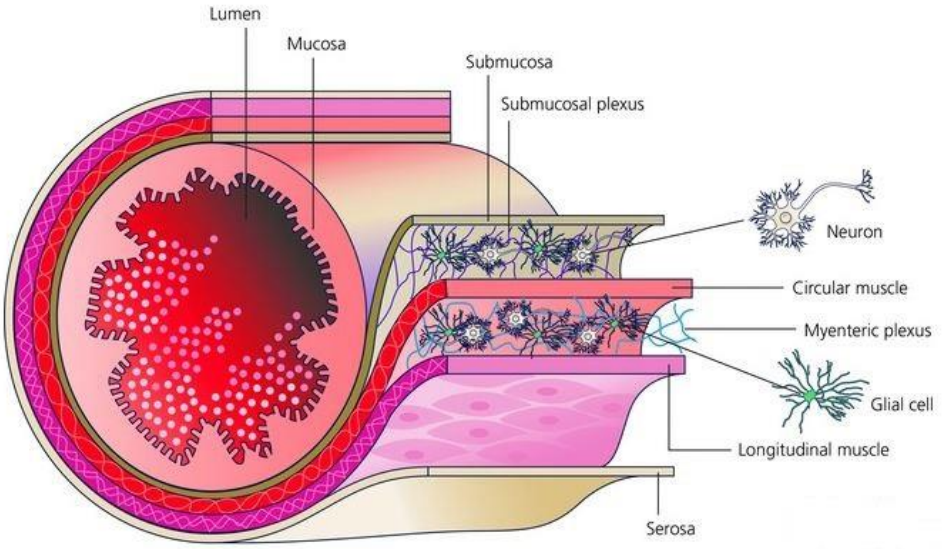

Figure 1. Organization of the ENS of human and medium–large mammals.
The ENS has ganglionated plexuses, the myenteric plexus between the longitudinal and circular layers of the external musculature and the SMP that has outer and inner components. Nerve fiber bundles connect the ganglia and form plexuses that innervate the longitudinal muscle, circular muscle, muscularis mucosae, intrinsic arteries and the mucosa. Abbreviations: ENS, enteric nervous system; SMP, submucosal plexus. Modified from Furness, J.B. The Enteric Nervous System (Blackwell Oxford, 2006).

The GI tract is a continuous tube that extends from the mouth to the anus. The alimentary canal includes the mouth, pharynx, esophagus, stomach, small intestine (consisting of duodenum, jejunum, and ileum) and large intestine (consisting of proximal and distal colon). It is within this complex system that a number of coordinated processes occur to allow for digestion and absorption of many different types of nutrients. Muscular sphincters compartmentalize the bowel, dividing it into functionally distinct regions with different luminal environments. The upper esophageal sphincter (UES) maintains the highest resting pressure of all sphincters, preventing air from entering the esophagus. It consists of striated muscle, is under control of the swallowing center in the medulla, and relaxes during swallowing to permit food to pass through the esophageal lumen. The lower esophageal sphincter (LES) separates the esophagus from the stomach and consists of smooth muscle that relaxes during swallowing. It functions to coordinate the passage of food into the stomach after swallowing and prevent the reflux of gastric contents, including acid, into the esophagus. A defective LES contributes to generate symptoms such as

heartburn. The pyloric sphincter separates the stomach from the duodenum, and its resting pressure contributes to regulate gastric emptying and prevent duodeno-gastric reflux. Reflux of bile acids and digestive enzymes can lead to gastritis, ulcer formation, and risk of perforation. The ileo-cecal sphincter is a valve-like structure that separates the ileum from cecum, preventing back flux of colonic contents into the ileum. Finally, the internal (smooth muscle) and external (skeletal muscle) anal sphincters control elimination of waste products.
A constant detection of luminal contents induces the transport of ingested material caudally at a physiological rate, allowing each region of the gut to perform their respective function. Skeletal (in the esophagus and anus) muscle contractions are thus coordinated into activity patterns, such as segmentation (small intestine) or haustration (colon) that grind, mix, and propel aborally the ingested food.
Secretory mechanisms exert a pivotal role in order to maintain an appropriate pH and a regulated concentration of electrolytes, enzymes, and mucous. The pH of the highly acidic chyme in the stomach reaches 1.5 to 3.5. At this pH, the hydrogen ion concentration is around 3 million times than that of the arterial blood. Secretion of bicarbonate ions into the lumen of the duodenum by the exocrine pancreas neutralizes the acidic chyme delivered from the stomach to duodenum. These processes are necessary to promote digestion, absorption, and detoxification of ingested materials. A continuously regenerated semipermeable epithelial barrier separates the lumen from the internal milieu of the body. This barrier promotes absorption, but also avoid the leakage of indispensable molecules into the intestinal lumen as well as the transit of digestive enzymes, toxins, and germs into the body from the lumen. Clearly, this highly organized structure of the GI tract and its related physiological functions require a sophisticate degree of regulation and coordination, provided by the ENS. Thus, since the ENS contributes significantly to body homeostasis, it is not surprising that any noxae perturbing ENS maintenance and integrity result in a

variety of disorders some of them so severe to be life threatening and / or hinder significantly the patients quality of life.

1.1 Developmental aspects

During ENS development, precursors emigrate to the gut from the vagal and sacral components of the neural crest. This cell population is pluripotent and diverges to give rise to enteric neurons and glia. Notably, there is little cell death within the bowel and at the end, neurons are generated in the appropriate number. Gliogenesis also occurs. In contrast to the developmental period, neuronal precursor cells of the mature bowel do not express SOX10, although they continue to express nestin and p75NTR (Figure 2) [11].

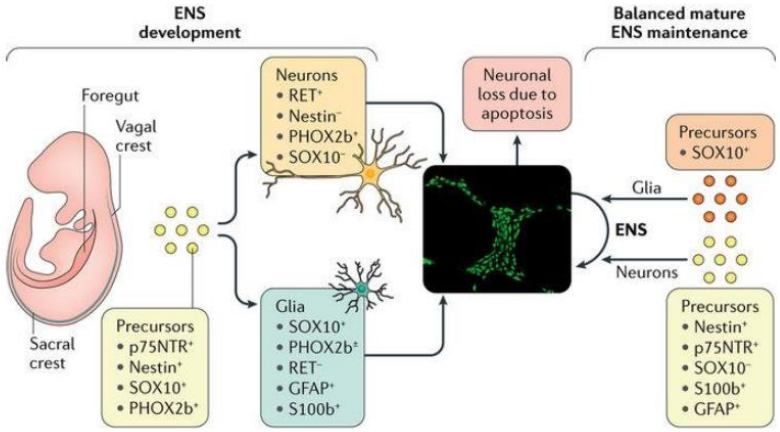

Figure 2. The dynamic life history of enteric neurons and glia. Modified from Rao M. et al 2017.

The development of the ENS has been investigated in a wide array of animal models, including mice and chicken [12], zebrafish [13] and invertebrates [14]. Consistent evidence acquired over many years of research in this field indicates that all the neurons and glia of the ENS arise from neural crest-derived cells that migrate into the developing GI tract during development. The majority of these arise from the vagal enteric neuronal crest cells (ENCCs), which originate in the caudal hindbrain region of the neural tube, opposite somites 1-7 [15, 16]. During development, these vagal ENCCs migrate into the foregut (developing esophagus

and stomach) and colonize the developing GI tract in a rostral to caudal wave [17]. In the developing mouse, ENCCs reach the foregut at E9.5, enter the midgut (developing small intestine) at E10.5, and reach the anal end of the colon at E14.5 [18] (Figure 3).

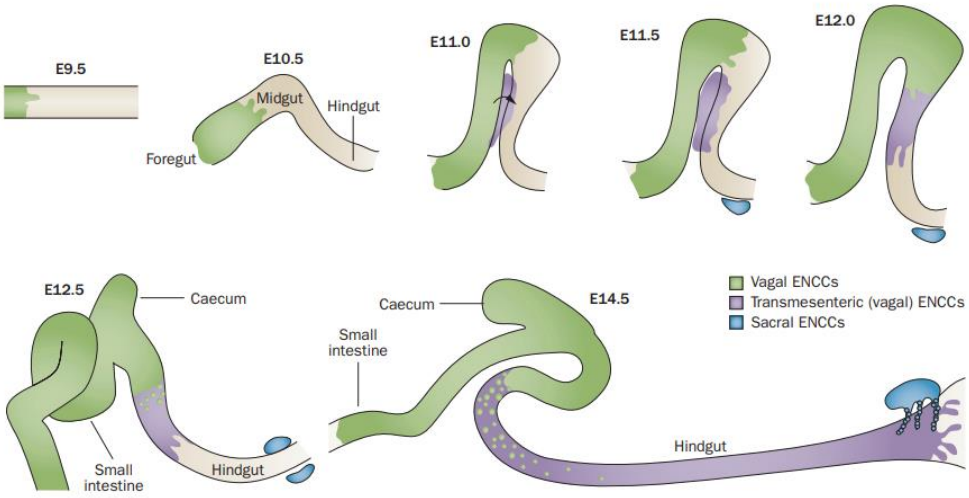

Figure 3. Migratory pathways of enteric neuronal cell crests (ENCCs) through the mouse gut during embryonic life. ENCCs enter the foregut around developmental stage E9 and migrate caudally within the gut mesenchyme. At E11.0–E11.5, there is a transient apposition of the midgut and postcaecal gut, and a subpopulation of vagal ENCCs (shown in purple) takes a short-cut across the mesentery to colonize the colon. The transmesenteric cells give rise to the ENS in a large part of the colon. Vagal ENCCs reach the anal end of the gut around E14.5. Cells derived from the sacral neural crest (shown in blue) emigrate to the vicinity of the hindgut around E11.5, but then undergo a waiting period and enter the distal hindgut around E13.5 along with nerve fibres arising from extrinsic neurons.7 As the gestation period for a mouse is ~19 days, colonization of the gut by ENCCs takes >25% of the gestation period. Abbreviations: ENCCs, enteric neural crestderived cells; ENS, enteric nervous system. From Obermayr et al. 2013

This is the longest migration of cells during embryonic development and takes place over approximately three weeks in human foetal development [19]. Sacral ENCCs also contribute to a small population of neurons and glia in the colon [20] [21] [16] [22]. Neuron differentiation takes place before ENCCs have reached the anal end of the gut. In the developing mouse embryo, around 10-15% of ENCCs in the midgut express pan-neuronal markers at E10.5 [23, 24]. These neurons are located along the entire length of the colonized gut, including at the migratory wavefront [24]. Upon entering the foregut, undifferentiated enteric neural crest cells initiate well-orchestrated programs of proliferation, migration and differentiation,

which culminate in the generation of the neuronal and glial lineages of the ENS [25]. Phenotypically distinct neurons are generated at different developmental windows, suggesting a temporal mode of neuronal diversification [26, 27]. Recent single cell transcriptomic analysis of adult mouse and human ENS has classified enteric neurons based on their gene expression profile [28]. Furthermore, transcriptional profiling of mouse ENS cells during development has provided evidence that the rich diversity of enteric neurons in adulthood is generated by the diversification the two cardinal neuronal subtype branches (cholinergic/excitatory and nitrergic/inhibitory) that are derived from postmitotic committed neuronal precursors [29].

Over the past five years, a series of single-cell sequencing experiments have been conducted to study the development of ENS, from NC to mature ENS circuit. Single-cell RNA sequencing (scRNA-seq) gives a global view of the cell heterogeneity and allows reconstruction of differentiation trajectories underlying the early embryonic development of the organisms [30]. Various single-cell sequencing tools, stage/cell-specific reporter mouse lines, isolation methods for cell enrichment/isolation and analytic strategies have been applied for the discovery of rare cells and delineating the molecular basis of neuron specification and diversification [31]. The early events of ENS development, such as migration and proliferation, have been extensively examined in several studies [1, 17, 32, 33, 34, 35, 36, 37, 38]. A failure of ENCCs to reach the anal end of the colon results in Hirschsprung disease (HSCR), a congenital disease characterized by the lack of enteric ganglia for a variable length throughout the colon. The aganglionic segment of the intestine causes a functional obstruction since the affected region cannot generate peristalsis and therefore propel its contents. Complete colonization of the GI tract requires complex coordination of ENCC proliferation, survival, migration, and differentiation. These events are regulated by a number of key factors including: 1) soluble factors released from the gut mesenchyme; 2) expression of extracellular matrix molecules; 3) expression of cell surface receptors by ENCC; and 4) expression of transcription factors by ENCCs that

regulate downstream events. The two main signalling pathways involved are the glial cell line derived neurotrophic factor (GDNF) – Ret / GDNF family receptor α1 (GFRα1) and the endothelin 3 (ET-3) – endothelin receptor B (EDNRB) pathway. GDNF and ET-3 are produced by the gut mesenchyme and ENCCs express Ret, and its GFRα1 co-receptor, as well as EDNRB [34]. There are also genetic interactions between many of these factors [39]. Mice that lack GDNF, Ret, or GFRα1 have complete aganglionosis of the small and large intestines [40, 41, 42, 43] whereas mice that lack ET-3 or EDNRB only have aganglionosis of the terminal colon [44, 45].

The next sections will detail the critical step occurring during development of the ENS, namely cell migration of vagal and sacral component; proliferation and survival; development of the two main ganglionated (myenteric and submucosal) plexuses; and, finally, development of enteric neuron subtypes.

5.1.1 Cell migration. Many factors are important for the control of vagal ENCC migration. The migratory behavior of these ENCCs has been examined in several time-lapse imaging studies using intact preparations of embryonic gut in the mouse [46, 47, 48]. ENCCs migrate in chains and cell-cell interactions are important for regulating this behaviour [49, 50, 51, 52]. In some cases, delays in the timing of ENCC migration can result in colonic aganglionosis. For example, the aneural hindgut of older embryos (E16.5) is less permissive to ENCC migration than that of younger embryos (E11.5) [53]. This can be due to several factors, such as changes in the expression of certain attractive and repulsive factors (e.g., increased laminin expression in the colon between E13.5 and E14.5) [54]. The same factors can exert different effects on ENCCs at different ages (e.g., GDNF has a proliferative effect on ENCCs from E12.5 gut, but this effect is reduced on ENCCs from older embryonic gut, at E14.5 - E16.5) [55]. The increased laminin expression is thought to prevent ENCC migration through the colon at E14.5. Delays in ENCC migration would result in colonic aganglionosis at birth, as observed in Ednrb-/-, β1 integrins conditional knockout, and Ret 9 hypomorphic mice [56, 47]. However,

more recent studies in L1-/y, Tcof1 +/- and Gdnf +/- mice have shown that although ENCC migration is delayed, the cells can still reach the anal end of the colon [57, 58, 59] and therefore no aganglionosis occurs at birth. In the case of Tcof1 +/- mice, there is increased proliferation of ENCCs at the wavefront of migration at E11.5, and decreased neuronal differentiation, thereby promoting the migration of ENCCs through the hindgut [59]. Therefore, finely tuned processes involving ENCC proliferation, survival, migration and differentiation, along with that of the microenvironment of the mesenchyme are required for complete colonization of the hindgut.

In addition to the most prominent component (i.e. vagal), another neural crest component contributes to form the ENS, i.e. the sacral NCCs. These precursor cells migrate from the neural tube at later stages, E9-9.5, than the vagal component, at least in the mouse [21]. They then pause at the nerve of Remak (which is an extension of the pelvic plexus) in chick embryos [22] and at the pelvic ganglia in mouse embryos [60] for several days, prior to migrating into the hindgut. In both chick and mouse embryos, the sacral NCCs enter the gut (hence ENCCs) after the entry of vagal NCCs into the hindgut [22, 61]. However, there is a small period when the two populations are distinct, as the sacral NCCs enter the caudal hindgut prior to its colonization by vagal ENCCs [50]. The presence of vagal NCCs is not necessary for the migration of sacral ENCC into the gut, as because sacral NCC derived neurons are found in the gut when vagal ENCCs are ablated [62, 63] and in transgenic animal models, where vagal-derived ENCCs are absent from the gut [64, 65, 32, 66]. Derivatives of the sacral ENCCs in the ENS account for up to 17% of neurons in the distal hindgut and cannot completely colonize the colon to compensate the absence of vagal NCC derivatives [22, 67, 66]. The developmental potential of sacral ENCCs is reduced in comparison to vagal ENCCs, as transplantation of vagal ENCCs to the level of pre-migratory sacral NCCs results in increased colonisation of the hindgut [22, 68]. The increased potential of vagal ENCCs appears to be due to increased expression of Ret in comparison to sacral ENCCs [69]. The migratory behaviour of sacral ENCCs has been observed in in vitro

co-cultures where a piece of pelvic ganglion was opposed to a segment of aneural hindgut [48]. The donor sacral ENCCS appear to migrate along nerve fibers that had extended into the gut from the pelvic ganglion donor tissue [63, 70]. Sacral NCCs contribute to the ENS of the colon, more in the myenteric, rather than submucosal, plexus [22]. Overall, the physiological effect of ablation of sacral ENCCs remains unknown.

1.1.2 Proliferation and survival. Proliferation of ENCCs is important to produce sufficient number of cells for gut colonization. ENCCs proliferate as they migrate towards [59] and throughout the gut [71, 72]. A reduction in the number of vagal NCCs results in incomplete colonization of the GI tract [15, 73, 67], and a minimum number of NCCs is required for full colonisation [59]. The density of ENCCs affects directly their migratory behaviour. For example, when the ENCC population at the wavefront is decreased, the distance covered during their migration decreases in direct proportion to the number of remaining cells [49, 46]. A model of "frontal expansion" has been proposed, where proliferation of ENCCs near the migratory wavefront drives the invasion of ENCCs into the uncolonized gut [74]. In these experiments, increased cell proliferation near the wavefront was detected in gut organ cultures in vitro [74]; however, this increased proliferation has not yet been detected in vivo [72, 59], except in the zebrafish gut [75]. In the mouse, the proportion of dividing ENCCs is not significantly different near the wavefront in comparison to the colonized regions of the gut [72]. Furthermore, there does not appear to be distinct proliferative zones within the GI tract, with dividing ENCCs and neurons located throughout the colonized gut [72, 76, 59]. It seems likely that, while high proliferation rates are required at the wavefront to drive migration into the uncolonized regions [74], high proliferation rates are also required behind the wavefront in vivo to maintain the density of cells while the gut is growing rapidly in length.

Regulation of cell death is also important during ENS development [77, 78]. Prior to entering the foregut, there is an apoptotic death of vagal ENCCs in route to the

gut, which is important for regulating the ENCC number [79]. Once they enter the gut, very little ENCC or neuronal cell death has been detected [71, 80, 81], which is, as indicated above, very different from many other parts of the developing nervous system. One study has found that conditional loss of GFRα1 signalling in mice at E12.5 – E15.5 in embryonic development results in neuronal cell death in the colon and subsequently, colonic aganglionosis at birth [82]. However, cell death in these mice did not involve common cell death executors, i.e. caspase-3 or caspase-7, or the proapoptotic protein Bax, nor did dying cells exhibit the morphological features of apoptosis such as chromatin compaction and mitochondrial pathology [82]. This has raised the possibility that this form of cell death has not been detected in previous experiments using traditional techniques of TUNEL or caspase staining. Instead, cell death was detected by abnormal nuclear shapes using electron microscopy [82]. The death of Sox10-expressing ENCCs has been detected in mice without defects in signalling pathways, using live imaging of ENCCs that express the fluorescent protein Venus in their nucleus [83]. In these cells, death was observed as fragmentation of the nucleus, and was identified in a small percentage of Sox10-expressing ENCCs at all different regions along the colonised gut [83]. A very recent study has also reported a decrease in the number of Hu-expressing neurons per ganglion in the rat antrum between 4-12 weeks after birth suggesting significant postnatal neuronal cell death in the antrum [84].

1.1.3 Development of the myenteric and submucosal plexuses

In the small intestine of both chickens and mice, ENCCs first congregate in the outer mesenchyme, forming the future MP, while SMP develops several days later, from a secondary migration of cells from the MP [85, 26, 18]. In mice, neurons can be detected in the presumptive SMP of the small intestine at E15.5 [31]. The secondary migration of ENCCs into the SMP is dependent on netrin-deleted in colo-rectal cancer (DCC) signaling. Netrins are expressed in the mucosa of the developing small intestine, while DCC, a netrin receptor, is expressed by ENCCs.

Netrin -/- mice lack submucosal ganglia, indicating that netrin acts as a chemoattractant to a sub-population of myenteric ENCCs [31]. Recently, GDNF was shown as ab important chemoattractant for the migration of ENCCs from the presumptive MP to the SMP [56]. In the embryonic mouse, the circular muscle does not differentiate until after the colonisation of the hindgut at E14.5 [86]. As the ENCCs migrate down the hindgut, they are dispersed through the presumptive circular muscle layer. As the circular muscle differentiates, the ENCCs condense into a single layer on the serosal surface of the circular muscle, thus forming the MP [86]. Neurons are present in the location of the presumptive SMP of the colon at E18.5, but are sparsely distributed, and colonic submucosal ganglia are not evident until post-natal day (PD)3 [86].

Vagal NCCs do not express any neuronal markers end route to the foregut [32], and differentiation takes place as ENCCs migrate through the GI tract. In the developing mouse, ENCCs that express neuronal markers are present in the foregut at E9.5, and in the midgut at E10.5 [23]. These cells express a variety of pan-neuronal markers such as Hu [87], neuronal class III β-tubulin (TUJ) [87; 88], neurofilament-M (NF-M) [89], protein gene product 9.5 (PGP9.5; [90] and microtubule associated protein 2 (MAP2) [91]. Enteric neurons at E10.5 and E11.5 are also known as the "transiently catecholaminergic" population of ENCCs as all ENCCs that express pan-neuronal markers also express tyrosine hydroxylase (TH) at these ages [23, 90]. By E12.5, TH expression is downregulated and is only present in a subpopulation of enteric neurons. The embryonic transiently catecholaminergic neurons are different from the TH positive dopaminergic neurons of the adult ENS [92]. It does not appear to be any distinct neurogenic zones in the ENS as these early enteric neurons are dispersed amongst the undifferentiated ENCCs, including at the migratory wave front of ENCCs [93]. The differentiation of ENCCs into neurons affects the colonization of the GI tract by ENCCs, which is vital to ENS development. ENCCs migrate through the gut as chains and lay down a network of ENCCs. Time-lapse imaging experiments have shown that the chains are maintained for many hours after their initial formation

[49]. Through development, cells aggregate together to form distinct ganglionic clumps with interconnecting neurite fiber strands. The formation of ganglia proceeds in a rostral-to-caudal progression [94]. In the mouse, enteric ganglia start to become distinct at E13, as revealed by an increased separation between groups of cells [93]. Expression of Ret-, p75NTR-, Phox2a-, Phox2b-, and tyrosine hydroxylase-immunoreactivity by undifferentiated neural crest-derived cells and different classes). An increase in the number of neurons per ganglion has been described during embryonic development [95]. Changes in the morphology of ganglia have been described in the early postnatal development of the rat [96]. In the duodenum the MP at birth appeared more densely packed with cells than that of older postnatal ages, whilst in the colon, distinct ganglia were not present until P7 [96].

The neuronal cell adhesion molecule (NCAM) and its polysialylated form, PSA-NCAM, are critical in ganglion formation. Immunohistochemical studies have shown that both NCAM and PSA-NCAM are present in developing enteric neurons, and PSA-NCAM is important for the clustering of enteric neurons in vitro [97]. Regulation of NCAM expression and polysialylation involves the bone morphogenic protein 4 (BMP4) pathway and the transcription factor Hand2 [97; 98]. Inhibition of BMP4 signalling results in smaller ganglia in chick embryos, and in a reduced neurite fasciculation in mouse embryos [12; 99]. The clustering of neurons in vitro is dependent on the presence of BMP4 signalling [97]. The formation of enteric ganglia is disrupted in a conditional Hand2 mutant, and the organized network structure of the ganglia is absent in these mice [98]. This effect is considered to be caused by the altered regulation of NCAM expression in the ENS of these mice [98]. In β1 integrin mutant mice, there is also abnormal ganglia formation. However, this does not appear to be as severe as that of the Hand2 mutant, as distinct ganglia are still present [51]. In the β1 integrin mutant, ENCCs aggregate together during migration, leaving large cell-free space, which leads to the formation of an abnormal ganglia network [51]. The expression of cell adhesion molecules, including NCAM, did not appear to be disrupted in the β1

integrin mutants [51]. Mathematical modelling of ganglia formation suggested that threshold criteria for ENCC proliferation, differentiation, and cell-cell adhesion are required for the normal formation of enteric ganglia [100].

Glial cells originate from the same neural crest progenitors, as the enteric neurons, and they migrate and colonize the GI tract. Upon colonization of the embryonic gut, neural-crest derived progenitors mature into neurons and glia via Hedgehog/Notch pathway [101]. In the mouse ENS, the development of enteric glia occurs later than that of enteric neurons [102]. Cells immunoreactive for specific glia markers S100b are present only in the duodenum at E14.5 [102], and cells expressing GFAP are present at E16.5 [77]. The terminal portion of the ls/ls mouse is congenitally aganglionic because the precursors of enteric neurons fail to enter this region. This animal was studied in order to gain insight into the origin of enteric glia and into the process by which the precursors of these cells colonize the gut. In control (CD-1) mice, immunoreactivity of the glial marker, glial fibrillary acidic protein, appeared for the first time in the fetal bowel at day E16 and, in adults, was much more intense within intraenteric neural elements than in nerves outside the bowel. Glial fibrillary acidic protein developed in tissue cultures of fetal intestine explanted before the protein appeared in situ, and before the bowel became innervated by extrinsic nerves; thus, the precursors of cells able to elaborate glial fibrillary acidic protein must have been present, but unrecognizable, in the original explants. This explant assay demonstrated that these glial precursors were present in all regions of the bowel of control mice, but not in the presumptive aganglionic bowel of ls/ls mice. The nerves (of extrinsic origin) in the aganglionic tissue of ls/ls mice showed a high level of immunoreactive glial fibrillary acidic protein; nevertheless, their ultrastructure was typical of peripheral nerve, not enteric plexus, and they contained Schwann cells, not enteric glia. These observations support the view that enteric glia are derived from the single wave of neural crest colonists that populates the enteric nervous system before the gut receives its extrinsic innervation. These glial

precursors, like neuronal precursors, tend to be excluded from the presumptive aganglionic ls/ls bowel. In contrast, Schwann cells grow into the abnormal ls/ls gut with the extrinsic innervation. The enteric microenvironment appears to promote the expression of glial fibrillary acidic protein in both enteric glia and Schwann cells; however, even within the bowel, Schwann cells retain their characteristic morphology. It is thus probable that the normal enteric nervous system contains supporting cells of separate lineages, enteric glia and Schwann cells [77]. Prior to this, the precursors of enteric glia maintain Sox10 expression, which is downregulated in neurons [102]. Expression of B-FABP, restricted to glial precursors and not common precursors of both neurons and glia, is present in the foregut and midgut at E11.5 [102]. The molecular pathways influencing the development of enteric glia have not yet been fully elucidated. Sox10 is essential for the generation of enteric glia [103, 104]. Notch and Foxd3, which indirectly regulate the expression of Sox10, are therefore also important for gliogenesis in the ENS [105, 106, 107]. One pathway has been identified whereby signalling through the ErbB3 receptor has been found to promote glial differentiation [108]. The ErbB3 receptor is expressed by the developing enteric glia and regulated by Sox10 [103]. Glial growth factor 2 (GGF2) is a ligand of ErbB3 expressed by the enteric mesenchyme and promotes glial differentiation [108]. BMPs, which promote neurogenesis, also promote gliogenesis via this pathway as they stimulate ErbB3 expression [55]. In the adult ENS, enteric glia has been shown to play many different roles in regulating GI function [10; 106]. One recent study has shown that they act as adult neural stem cells of the ENS and can differentiate to enteric neurons in response to injury [109].

1.1.4 Development of enteric neuron subtypes. The age at which a neuronal precursor exits the cell cycle is defined as the birthdate of that neuron. In the developing CNS, it is thought that neuronal precursors exit the cell cycle prior to expressing neuronal markers, hence, neurons are “postmitotic”. In contrast to this, neurons in sympathetic ganglia [110] and the developing ENS continue to divide after initiating their expression of pan-neuronal markers [111; 72]. However,

there does not appear to be any division of enteric neurons that express subtype specific markers [72], hence, enteric neurons appear to exit the cell cycle prior to their subtype differentiation.

5-hysroxytryptamine (5-HT) / serotoninergic and choline acetyltransferase (ChAT) / cholinergic neurons are "born" at E8, which is prior to the entry of vagal NCCs into the mouse gut [26]. Several other subtypes begin to be born at E10, including VIP, NYP, enkephalin and CGRP neurons [26]. A subsequent study showed that NOS, CALB and GABAergic neurons were born from E12.5 to P1, however, E12.5 was the earliest birthdate examined [108]. In both studies, the birthdates of neurons in the MP were found to be earlier than those of submucosal plexus neurons [112; 108]. In a third study, the birth of VIP neurons was examined in chick embryos [113]. So far, no correlation between the time of cell cycle exit and the first immunohistochemical detection of its neurochemical phenotype has been determined. This length of time appears to vary for different neuronal subtypes.

1.2 Structural features.

1.2.1 Myenteric plexus (MP). The MP shows numerous differences in its morphological organization, among GI segments of different species [114; 115,116]. Generally, in the MP, ganglia are bigger than the SMP in terms of number of neurons and are linked by interconnecting strands (or primary strands), which constitute the so-called primary plexus [117]. Neurons residing in the myenteric plexus are constantly deformed during muscle contraction and relaxation [118]. The MP ganglia are located in parallel to the circular muscle layer, although this feature can vary among species; primary interconnecting strands show longitudinal course, an organization that seems to be a distinctive feature of the MP in most small and large mammals [119, 120, 121, 122, 10, 123, 124]. The other two components of MP are the secondary and tertiary plexuses [117]. The secondary plexus is constituted by fine bundles of nerve fibers running parallel to the circular muscle layer and primary plexus [125, 10]. The tertiary plexus is made by thin

interconnecting strands (the smallest in size) supplying the longitudinal muscle layer [119, 125, 10].

1.2.2 Submucosal plexus (SMP)

The SMP is well developed in the small and large intestine, while only a few submucous ganglia can be found in the esophagus (third inferior part) and stomach, particularly in large mammals, including humans [126, 127, 9]. The interconnecting strands of the SMP are usually thin and SMP ganglia are small. The SMP is organized in a single layer in small mammals [128], whereas it shows a multilayered (two or three layers) organization in large mammals [129]. In these species two different ganglionated plexuses can be identified, namely the internal submucous plexus and the external submucous plexus [130, 131, 132, 134, 122]. External and internal SMP are separated by a thin connective tissue layer [130, 130, 125, 135] and by submucosal blood vessels (Balemba et al. 1998). The external and internal SMPs appear different among the investigated species. Generally, they can be distinguished on the basis of their location, architecture, meshwork density, size and form of the ganglia, and light microscopic appearance [136, 139]. The external SMP shows the most irregularly organized nerve meshwork, while the internal meshwork is smaller and more regular compared to the external SMP [140, 141, 142, 143, 144]. Additionally, the two compartments of SMP neurons show differences in the content of neurochemical messengers / transmitters, being the external SMP more similar to that of myenteric neurons [142]. In addition to the mucosa, some neurons of the external SMP also supply the muscular layer [145, 128, 146, 147]. The internal SMP neurons supply the mucosa and only a small number have projections to the muscle layers [128, 146]. Thus, in conclusion the external and internal SMP neurons overlap in terms of functional control of fluid movements, local blood flow and sensory functions, while the external SMP can also affect gut motility [143].

1.2.3 Neurochemistry of enteric neurons

Enteric neurons are able to synthesize and release different mediators / bioactive substances that may act as messengers, i.e. neurotransmitters, neuromodulators and neuropeptides (Table 1). Studies over the years have clearly shown that each subclass of enteric neurons can be characterized based on of the combination of messengers. This property is known as neurochemical coding and turned to be a feature of the ENS that is maintained through most animal species [10,]. Primary neurotrasmitters exert the same role in different species and along the GI tract. These substances include acetylcholine (ACh) and tachykinins in enteric excitatory motor neurons, and nitric oxide (NO), vasoactive intestinal polypeptide (VIP) and other messengers in inhibitory motor neurons. Secondary neurotransmitters or modulators include substances, which may vary among different groups of neurons depending on the GI tract and the species considered [148] (Table 1). Research performed in the last thirty years has shown that classification of the several classes of enteric neurons is the result of a combination of various technical approaches and criteria.

These features include: neuronal shape; histochemical and immunohistochemical staining; projections; and electrophysiological behaviour; pharmacological and functional /neurochemical properties.

Table 1. Multiple transmitters of functionally distinct enteric neurons that control digestive function. Abbreviations: 5-HT, 5-hydroxytryptamine; ACh, acetylcholine; CCK, cholecystokinin; ChAT, choline acetyltransferase; CGRP, calcitonin gene-related peptide; GRP, gastrin releasing peptide; ND, not determined; NPY, neuropeptide Y; NOS, nitric oxide synthase; PACAP, pituitary adenylyl-cyclase activating peptide; VIP vasoactive intestinal peptide. From Furness 2006.

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

1.2.4 Neuronal shape. A detailed description of different shapes of enteric neurons was done more than 100 years ago, but is still valuable until present [149]. This short review describes the conceptual development in the search for the enteric neural circuits with the initial identifications of the classes of enteric neurons on the bases of their morphology, neurochemistry, biophysical properties, projections and connectivity. The discovery of the presence of multiple neurochemicals in the same nerve cells in specific combinations led to the concept of “chemical coding” and of “plurichemical transmission”. The proposal that enteric reflexes are largely responsible for the propulsion of

contents led to investigations of polarised reflex pathways and how these may be activated to generate the coordinated propulsive behaviour of the intestine. The research over the past decades attempted to integrate information of chemical neuroanatomy with functional studies, with the development of methods combining anatomical, functional and pharmacological techniques. This multidisciplinary strategy led to a full accounting of all functional classes of enteric neurons in the guinea-pig, and advanced wiring diagrams of the enteric neural circuits have been proposed. In parallel, investigations of the actual behaviour of the intestine during physiological motor activity have advanced with the development of spatio-temporal analysis from video recordings. The relation between neural pathways, their activities and the generation of patterns of motor activity remain largely unexplained. The enteric neural circuits appear not set in rigid programs but respond to different physico-chemical contents in an adaptable way (neuromechanical hypothesis). The generation of the complex repertoire of motor patterns results from the interplay of myogenic and neuromechanical mechanisms with spontaneous generation of migratory motor activity by enteric circuits [149]. Generally, enteric neurons are morphologically divided into three types, referred to Dogiel type I, II and III. Dogiel type I neurons are flattened, slightly elongated neurons with stellate or angular outlines that have 4 – 20 lamellar dendrites and a long process which is likely the axon (Dogiel 1895). Their cell bodies have a large diameter of 13 – 35 µm and a small diameter of 9 – 22 µm. Dogiel type II neurons in contrast have large oval or round somata. They have a large diameter of 22 – 47 µm and a small diameter of 13 – 22 µm. Dogiel proposed that they have 3 – 10 dendrites and one axon, but nowadays it is believed that they are multiaxonal [150,151]. They are numerous and make up 10 – 25% of the MP population of the small and large intestine. 80 – 90% of the Dogiel type II neurons of guinea pig myenteric ganglia are immunoreactive for the calcium-binding protein calbindin [152, 153]. Furthermore, the majority of them is immunoreactive for choline acetyltransferase (ChAT) [154]. Dogiel type III neurons are filamentous neurons that have 2 – 10 dendrites. The dendrites are

short, became thinner and are branching. The axon can arise from a protrusion of the cell body or from a dendrite (Dogiel 1899). After Dogiel, many other authors proposed additional classifications based on individual neuronal morphology revealed by silver impregnation technique, immunoreactivity for neurofilaments and / or other markers [137, 150]. Taken together, these studies confirmed and extended the Dogiel classification from three up to seven types of neurons in addition to "mini-neurons". The morphological classifications of enteric neurons has been underestimated [129, 155], however, the possibility to correlate the morphological appearance of a given neuron to its neurochemical code, as well as bioelectrical / functional properties has also refuelled the importance of studying the morphology of the enteric nerve cells.

*1.2.5 Electrophysiological aspects***.** The electrophysiological classification in AH and S neurons was introduced in 1974 [156]. S neurons are characterized by high excitability and can exhibit fast excitatory postsynaptic potentials. On the contrary, AH neurons show large action potentials, followed by a long phase of hyperpolarization in which they are not excitable [10]. There are normally two phases of hyperpolarization, an early and late phase [156]. Part of their spike component is driven by calcium influx. They rarely receive fast synaptic input, but generate slow excitatory postsynaptic potentials (EPSPs). In the guinea-pig ileum, all AH neurons have Dogiel type II morphology. The action potentials of AH neurons show a tetradotoxin (TTX) sensitive Na+ current and a TTX insensitive Ca2+ current component [157]. In contrast S neurons exhibit brief action potentials that lack the slow hyperpolarization. They receive fast EPSPs and their action potentials are TTX sensitive. Typically, they show Dogiel type I morphology and have a single axon [156]. In the guinea-pig small intestine, they never have Dogiel type II morphology [10].

1.2.6 Functional properties

From a neurochemical / functional point of view, it is possible to identify motor neurons, interneurons, sensory neurons (or better-defined intrinsic primary sensory neurons, IPANs) [149, 158] and intestinofugal afferent neurons (IFANs) [159].

Motor neurons. They are uniaxonal neurons with S-type electrophysiology. This category of neurons includes excitatory and inhibitory neurons directed to the GI musculature; secretomotor / vasodilator neurons are able to regulate mucosal secreting cells and vasodilation / vasoconstriction of the intestinal vasculature. The excitatory motor neurons have Ach and tachykinins as primary transmitters. Inhibitory motor neurons have different kind of neurotransmitters such as nitric oxide (NO), vasoactive intestinal peptide (VIP) and adenosine triphosphate (ATP) [10,]. Furthermore, another subset is represented by secretomotor neurons and neurons innervating entero-endocrine cells [158]. The motor neurons innervating the smooth muscle of digestive tract are located within the MP of rodents [160] and within MP and external and internal SMPs of large mammals [MP > external SMP > internal SMP] and humans [MP > external SMP > internal SMP] [132, 145–147]. These neurons could be distinguished in circular muscle motor neurons, longitudinal muscle motor neurons, and motor neurons innervating the muscularis mucosae. The soma of excitatory motor neurons are localized aborally to the innervated muscle [161, 162], while the inhibitory motor neurons are generally localized orally to the innervated circular and longitudinal muscle [161–163].

Interneurons. Interneurons are involved in the formation of local circuits and exhibit descending and ascending projections.

These cells, which have been identified with certainty only in the guinea-pig [149], mouse, rat, and human are mainly localized in the MP. Interneurons form long functional chains of ascending and descending elements through which information may travel for short or long distances [164]. Interneuron projections extend up to 14 mm anally and up to 136 mm orally in guinea-pig small intestine [152]. So far, four types of interneurons have been identified in the guinea-pig small

intestine: one ascending and three types of descending interneurons. The ascending interneurons are Dogiel type I cholinergic neurons [159, 162] and may contain also calretinin, substance P (SP), neurofilament triplet protein (NFP), and enkephalin [161]. Descending interneurons (5% of all ENS cells in guinea pig) are phenotypically cholinergic neurons further distinct into three types based on their immunoreactivity for NOS/VIP, SOM and 5-hydroxytryptamine (5HT) [165; 166; 162]. nNOS/VIP/ChAT/immunoreactive Dogiel type I interneurons can contain also neuropeptide Y (NPY) [161], gastrin releasing peptide (GRP), bombesin (BN) [162] and the enzyme alkaline phosphatase [167]. SOM/ChAT immunoreactive Dogiel type III MP neurons [165]; [166] project to other MP ganglia and also to SMP guinea-pig ganglia [162]. Also, 5HT/ChAT immunoreactive Dogiel type I neurons send their projections to guinea pig MP and SMP and seem to have significant roles in excitatory pathways regulating both motility and secretion [168].

Intrinsic primary afferent neurons (IPANs). IPANs make up ~ 20% of all neurons in the ENS [10]. They are the first neurons of the reflex pathways in the intestine [169]. They encode the information about the chemical and mechanical environment of the tissue they innervate and respond in an adequate way. They are involved in the control of physiological functions as motility, blood flow and secretion, being responding to several stimuli, such as distention, luminal chemistry and mechanical stimulation of the mucosa [10]. IPANs have typical electrophysiological properties. In fact, they have broad action potentials that are carried by both sodium and calcium currents and are followed by early and, generally, by late afterhyperpolarization potentials (AHPs) [169]. Their targets are represented by mucosa and other MP and SMP neurons [170–172]. Cell bodies of multi-axonal IPANs form the 10-30% of neurons in SMP and MP ganglia of the small and large intestine; no IPANs are detected in the esophagus and in the stomach [173]. All the IPANs in the intestine of guinea-pig show Dogiel type II morphology (non-dendritic, multi-axonal type II neurons) and AH electrophysiology [174–180]. A large percentage (82-84%) of myenteric IPANs of the guinea-pig ileum expresses IR for the calcium-binding protein calbindin (CALB)

[148,181] and almost all MP and SMP IPANs express cytoplasmic NeuN-IR [182]. Furthermore, all of them show ChAT-IR [154,183] . Notably, only 30% of submucosal IPANs of the guinea-pig ileum appear to be IR for CALB [153,184], and that CALB is not confined to the IPANs since it is also localized in 50% of submucosal calretinin-IR secretomotor / vasodilator neurons [184]. Many researchers studied CALB-IR also in other species, with the aim to establish whether CALB could be considered an IPAN marker. In the pig small intestine, CALB cannot be considered markers of IPANs, being mainly localized in interneurones and intestinofugal neurons [185]. Dénes and Gábriel [186] described CALB-IR myenteric neurons in rabbit small intestine. These cells showed Dogiel type I morphology, ChAT-IR, and were identified as interneurons. Also in the mouse colon, CALB cannot be considered a good marker of IPANs, while the anti-CGRP antibody is considered the most specific marker of these cells [169]. Non-dendritic multiaxonal type II neurons involved in mucosal innervations have been demonstrated also in porcine [187] and human [188] small intestine. Unlikely guinea-pig, porcine and human (and mouse) IPANs express CGRP, which has been considered a specific marker of type II neurons in these species [119, 128, 142, 144, 187].

Intestinofugal primary afferent neurons (IFANs). Intestinofugal neurons represent a unique subset of enteric neurons with their cell body located in the myenteric ganglia and projections giving off the intestinal wall [189]. Most of them show a Dogiel type I morphology, whereas a minority have Dogiel type II features [190, 191]. IFANs act as mechanoceptors, being able to detect changes in volume and to respond to the stretch (but not to the tension) of the smooth muscle cells [192, 193]. Once activated, IFANs usually release Ach in the in the prevertebral ganglion (PVG) thereby evoking nicotinic fast EPSPs [194]. A subset of IFANs responds to colonic distention by releasing gamma-amino butyric acid (GABA), which facilitate Ach release from cholinergic IFANs in PVG. Because of IFANs activation, the response of the reflex entero-PVG circuitry is the release of noradrenaline by sympathetic neurons in GI wall. This effect modulates smooth muscle contraction or myenteric neuron activity [195, 196], [197]. The functional significance

of this reflex arc is to counteract large increases in tone and intraluminal pressure during filling [189].

1.2.7 Enteric glia

The existence of accompanying non-neuronal cells (hence the term glia) dates back to the beginning of the 19th century with the first glial cells were described by Robert Remak who detailed very elegantly "special cellular elements" while reporting in his thesis (published in 1838) on nerve fibers and their surrounding sheets, later on called Schwann cells. However, the term "glia" (in the ancient Greek "γλία" meaning "glue") was introduced by Virchow (1856). Virchow used for the first time the word glia describing the neuroglia in the CNS. He thought neuroglia was a kind of connective tissue and found that this tissue also contained cellular elements. We owe the first drawings of a star-shaped glial cell to Otto Deiters (1865).

Concerning the ENS, the first description of nucleated satellite cells around nerve cell bodies of the enteric ganglia was given by Dogiel (1899), and many other investigators have studied these cells in the ganglia and nerve strands of the GI plexuses and referred to them as "Schwann cells" (Stöhr 1952). The term "enteric glial cell" was used for the first time by Giorgio Gabella in his original and extremely accurate research made along 10 years (Gabella 1971 to 1981) [198,199]. Other studies, which have used immunohistochemical markers to locate enteric glia, revealed that these cells are common in the ganglia and nerve fiber bundles. Enteric glia express glial fibrillary acidic protein (GFAP) [200] and the S-100 Ca2+-binding protein [201], both of which are typical of CNS astrocytes. Glial cells in other autonomic ganglia do not contain GFAP [200]. Electron microscopy studies also showed that what it was intended as "enteric neuronal satellite cells", actually are glial cells closely reminiscent of astrocytes of the CNS and distinct from Schwann cells of other peripheral ganglia or nerve trunks [198,199,202,203]. Specifically, enteric glial cells partly surround nerve cell bodies and axons in the ganglia, leaving bare large areas of neuronal membrane at the surface of ganglia;

also, their processes contain bundles of gliofilaments and are surrounded by a single basement membrane (none of these aspects are detectable in Schwann cells). In small mammals, glial cell processes fail to penetrate in the interstitium between nerve cell bodies as well as axons in the neuropil [198, 203]. In fact, many nerve processes show direct membrane-to-membrane contacts with each other; the glial cells only separate them into groups and rarely form a sheath around an individual axon. In contrast, in enteric ganglia of human and monkey, axons are separated from one another by intervening glial cell processes [204].

Compared with other peripheral glial cells (e.g., Schwann cells), enteric glial cells do not form basal laminae and they ensheath nerve bundles and not individual axons [205]. In addition to the previously mentioned GFAP and S100b, other available immunohistochemical markers for glial cell labeling in the adult gut include Sox (SRY-box) 8/9/10, the first two being the most frequently used [206, 207]. Recently, marker expression analysis showed that the majority of glia in the MP co-express GFAP, S100b, and Sox10 [208]. However, a considerable fraction (up to 80%) of glia outside the myenteric ganglia, did not show labeling for these markers. The alternative combinations of markers reflect dynamic gene regulation rather than lineage restrictions, revealing an extensive heterogeneity and phenotypic plasticity of enteric glial cells [209] (Figure 4).

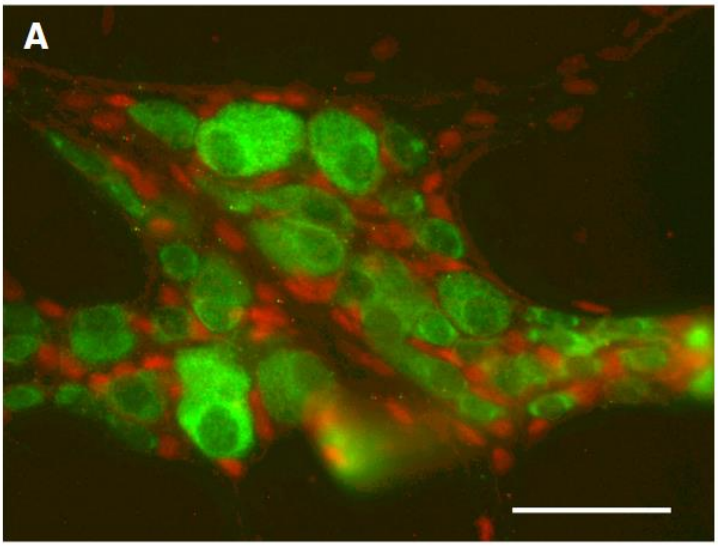

Figure 4. Differential expression of HuC/D and Sox10 in enteric glia of the myenteric plexus of the ileum. (a) Confocal photomicrograph showing double immunostaining for HuC/D (green fluorescence) a pan-neuronal marker and Sox10 (red) positive nuclei of glial cells. Note the presence of many Sox10-positive glial cells surrounding HuC/D labeled myenteric perikarya reflecting the structural and functional interplay of these two cell types in the ENS. Calibration bar= 25 μm. From De Giorgio et al. 2012.

Enteric glial cells have long been thought to exert a mere mechanical property by supporting neurons. Many evidences, however, indicate that these cells exhibit a number of functions, ranging from support to neurotransmission to enteric neuronal maintenance and survival [209–211]. In fact, glial cells are involved in many crucial tasks, such as synthesis of neurotransmitter precursors, uptake and degradation of neuroligands (i.e., detoxification of glutamate and g-aminobutyric acid), and expression of neurotransmitter receptors, thereby contributing to neuron-glia cross talk and neurotransmission [212]. Furthermore, glial cells exhibit immunological properties [206,213], participate in epithelial barrier functions [211,214–216] and are neuroprotective [211] (Figure 5). In addition, enteric glial cells have neurogenic potential being capable of generating enteric neurons in response to injury [217]. Recent works also demonstrated that enteric glial cells can respond to neurotransmitters by changes in intracellular Ca2+, such as purinergic (ATP) [218,219] serotonergic and cholinergic signaling mechanisms [209].

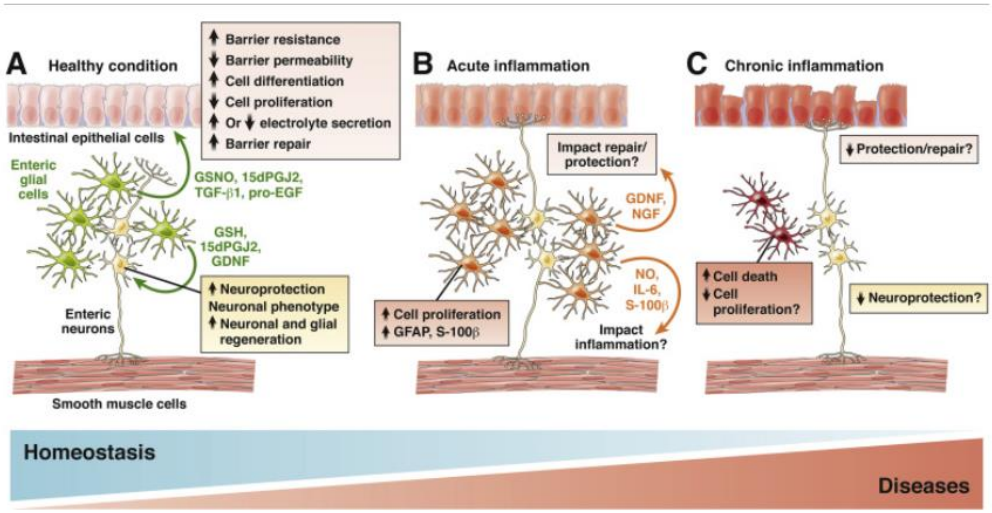

Figure 5. Enteric glial cells are central regulators of gut homeostasis and can play a role in gut diseases. (A) In normal conditions, glia control a number of neuronal aspects, i.e. neuroprotection, neuromediator expression, or neuronal renewal via liberation of different mediators. In addition, glia exhibit a key role in intestinal epithelial barrier integrity through the release of function-specific messengers (gliomediators). Taken together these features indicate that enteric glia possess protective and reparative properties in the gastrointestinal tract. (B) In pathological conditions, such as inflammation or bacterial stimulation, a phenomenon, known as reactive enteric gliosis (similar to astrogliosis in the brain) can occur. This contributes to the development of intestinal inflammation, but also participate in protection/repair of intestinal epithelial barrier/neuronal lesions evoked by these mechanisms. (C) Enteric glia death (induced by specific viruses or pathogens) or abnormal enteric gliosis could contribute to neuronal degeneration or barrier dysfunctions observed in some chronic intestinal or extra intestinal diseases. From Neunlist et al. 2014.

1.3 Mechanosensitivity in the gut

Muscle tone is important for the initiation and maintenance of peristalsis along longer segments of the gut [220] [221]. The “classic peristaltic reflex” should therefore be viewed as a pattern consisting of three components: an increased muscle tone at the site of distension, a contraction above and an relaxation of muscle activity below the distended region. To function independently and to generate muscle activity the ENS needs to possess neurons able to respond to mechanical stimuli and to initiate adequate motor responses. Moreover, the ENS needs to integrate signals that are generated by other nerves but, at the same time, must react to a constantly changing molecular composition of the micro milieu in the gut wall. Mechanosensitive mechanism is involved in these reflex activities [222].

Much progress has been made in identifying neurons that encode mechanical or chemical stimuli. Thus, muscle behaviors in the small and large intestines depend on mechanosensitive neurons which encode a variety of mechanical stimuli, ranging from brief deformation of the neurons soma or processes to sustained tissue stretch. [222] Mechanosensitivity has been recorded in a wide variety of neurons which behave like rapid or slowly adapting mechanosensors. Strikingly, mechanosensitive neurons do not appear to belong to a distinct class of highly specialised neurons but rather differ in their electrophysiology, neurochemistry and morphology. While some mechanosensitive neurons may respond to one stimulus type, others appear to be polymodal.

Mechanical forces are constantly present in the GI tract triggered by the muscle movements. Under physiological conditions, it has been shown that myenteric ganglia undergo deformation during muscle contraction and relaxation [223]. Neuronal cell bodies as well as interganglionic fibers are deformed [223]. The mechanism how the ENS is activated by mechanical stimuli is still under debate. Are the enteric neurons directly activated by mechanical stimuli or indirectly by the release of other transmitter substances? An example for an indirect activation is the release of 5-HT by enterochromaffin cells subsequent to mechanical stimulation of the mucosa [224]. Furthermore, in the gut there are other cell as smooth muscle cells and interstitial cells of Cajal (ICC) that can directly respond to mechanical stimuli [225]. The ICCs have pacemaker function whereas smooth muscle cells are the effector cells for contraction [225–227]. It is also challenging to detect the initiator of the peristaltic reflex. Partially contradictory results have been reported in the years. Early on a crucial role of the mucosa was postulated after the discovery that the peristaltic reflex was absent in preparations without mucosa [228]. On the contrary, other scientists could observe the peristaltic reflex without the presence of the mucosa [229,230]. It was also found that the absence of the MP inhibited the peristalsis [171]. These experiments and experiments in which normal peristalsis was detected in preparations without mucosa and SMP, proposed the MP as the neural site of

the initiation of the peristaltic reflex [3]. This suggests that neurotransmitters secreted by mucosal non-neuronal cells may have only modulatory action on the mechanotransduction pathway. Electrophysiological studies on the MP showed clear direct neuronal excitation by various mechanical stimuli [220,223,231,232]. Myenteric neurons receive from the muscle layers where they are embedded the crucial information and at the same time they control the muscle cell activity, forming a self-reinforcing network. The mechanosensititvity of IPANs has been shown by an ongoing action potential discharge in response to circumferential stretch and muscle contraction[177,178]. In addition, excitatory action of mechanical stimuli have been only shown for interganglionic nerve processes whereas compression of the neuronal soma in IPANs has an inhibitory effect [233]. The concept that there is only one specialized group of enteric neurons with sensory function is now in evolution. There are more and more evidences indeed that not only this specialized group of neurons is mechanosensitive, but also other myenteric neurons are activated by mechanical stimuli. For the first time it was shown in 2004 that also S interneurons are activated by circumferential stretch (Spencer and Smith 2004). Further studies showed that different enteric neurons like interneurons and motor neurons have multiple functions and give rise to a concept of multifunctional sensory neurons [223,232,234,235]. In addition, enteric neurons from different gut regions have to perform different functions in respect to their triggered muscle movement. Therefore, a difference in their electrophysiological and synaptic behavior is not surprising [236–240]. In guinea-pig small intestine there are S- as well as AH-neurons, whereas in the corpus region of the stomach AH-neurons are absent [156,241]. In addition, myenteric neurons in the stomach receive only fast synaptic input unlike small intestinal myenteric neurons. Fast synaptic input strongly suggests a modulatory activation. Recently it was found that all mechanosensitive neurons from guinea-pig small intestine receive fast synaptic input [232]. More recently, Beyder's group [242] identified that mechanosensory circuits built into the gastrointestinal wall allow for spatial and temporal integration of mechanical stimuli into a coordinated physiological

response (for example, peristaltic reflex), and connections to the extrinsic mechanosensory circuits are crucial for brain–gut communication (for example, sense of fullness). The gut is a mechanically active organ in which all cells must sense the forces emanating from the digestion of intraluminal contents and organ activity, such as motility [242]

Chapter II

2. ENTERIC NEUROPATHIES

2.1 General characteristics

Several GI disorders can result from enteric neuropathies, including both primary and secondary forms. The clinical phenotypes of the enteric neuropathies are the 'tip of the iceberg' of severe functional digestive diseases. Inflammatory and degenerative cellular mechanisms can contribute to neural changes in primary and secondary neuropathies. These neuropathies have been grouped as congenital or developmental neuropathies; sporadic and acquired neuropathies; neuropathies associated with other disease states and iatrogenic or drug-induced neuropathies [117]. Any damage, noxae or dysfunction involving the main effectors or the control system of the gut physiology may be responsible for the onset of a wide array of pathological conditions involving some lethal or seriously disabling manifestations [243,244]. Pathologies that involve the ENS include dysmotilities such as achalasia, CIPO and Hirschsprung's disease [245]. A view of the major milestones in ENS neuropathology over the years has been reported in Figure 6.

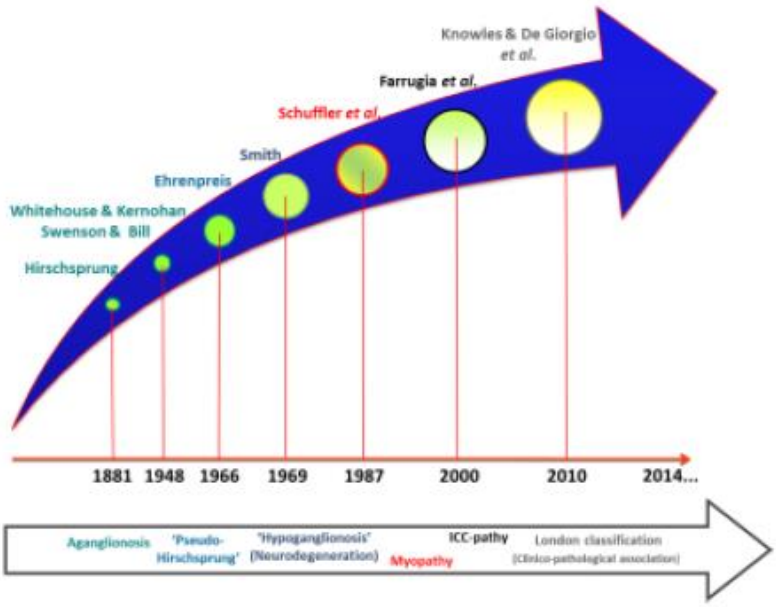

Figure 6. Synopsis summarizing some of the major milestones in ENS neuropathology over the years, i.e. from Hirschsprung's seminal description of congenital aganglionosis of the colon up to the London classification. Colors shown here couple the author(s) (above the curvilinear arrow) with the corresponding neuropathological acquisition (reported in the horizontal arrow). From De Giorgio et al., 2014).

With the rapid development of single-cell sequencing technologies, it has become a powerful strategy for the discovery of rare cells and delineating the molecular basis underlying various biological processes. Use of single-cell multimodal sequencing to explore the chromatin accessibility, gene expression and spatial transcriptome has propelled us to success in untangling the unknowns in the ENS and provided unprecedented resources for building new diagnostic framework for enteric neuropathies. Using scRNA-seq on ENS cells, ranged from the progenitors, neural crest (NC) cells, to the mature ENS circuit, in both human and mouse. These studies have highlighted the heterogeneity of ENS cells at various developmental stages and discovered numerous novel cell types [246].

2.2 Primary neuropathies

Enteric neuropathies are classified as primary in the case that the ENS is the major target of the disease process. Most are termed as "idiopathic" to denote the poor aetiological understanding of these conditions. Among primary neuropathies are: Hirschsprung's disease, idiopathic achalasia, idiopathic gastroparesis and CIPO.

Hirschsprung's disease is characterized by an absence of enteric neurons (aganglionosis) in terminal regions of the gut, leading to tonic contraction of the affected segment, intestinal obstruction and massive distension of the proximal bowel (megacolon). Constipation is the dominant presenting symptom in Hirschsprung's disease. The cardinal feature of Hirschsprung's disease is an absence of enteric nerve cell bodies in the distal part of the colon and in the rectum. The extent of the defect varies from patient to patient. The absence of enteric nerves results in the inability of the distal gut to pass meconium and later, feces. Various genetic mutations, including in the receptor tyrosine kinase RET gene [247] and in the endothelin receptor-B gene (ENDR-B) are associated with the familial Hirschsprung's disease [248]. Loss of interstitial cells of Cajal has also been reported in this disease but other studies report a normal complement of interstitial cells of Cajal [249]. A study by Chakravarti et al. observed that for most multigenic disorders, clinical manifestation (penetrance) and presentation (expressivity) are likely to be an outcome of genetic interaction between multiple susceptibility genes. As compared with wild-type mice intestinal gene expression, loss of Ret in null homozygotes led to differential expression of ~300 genes [203]. Recent studies identified 28 *de novo* mutations in 21 different genes. Eight of the *de novo* mutations were identified in RET, the main HSCR gene, but the remaining 20 mutations reside in genes not reported in the ENS. These genes are also expressed in human and mouse gut and/or ENS progenitors. Importantly, the encoded proteins are linked to neuronal processes shared by the central nervous system and the ENS [250].

CIPO indicates a condition in which affected subjects show failure of the propulsive forces of intestinal peristalsis to overcome the natural resistances to flow. Myogenic and neurogenic altered mechanisms contribute to CIPO [251]. Concerning CIPO-related neuropathies, there are evidence of neuronal degeneration and loss, with or without ganglionitis due to lymphocytic and eosinophilic infiltrate [252].

2.3 Secondary neuropathies

Secondary neuropathies are defined as pathological conditions in which the ENS is not the primary target of the disease. The classification of this neurophaties can be as degenerative and inflammatory, based on the mechanisms underlying the enteric neuronal pathology. Degenerative neuropathies include diabetes mellitus (DM), while inflammatory neuropathies include paraneoplastic enteric neuropathy and Chagas disease [243]. Symptoms depend on the type of nerves affected, more frequently there are described abdominal pain, heartburn, dysphagia, post prandial fullness, nausea, diarrhoea and constipation. In diabetic gastroparesis several key cell types are affected by diabetes. Etiology or risk factors have not been clearly identified. Failure to sustain elevated heme oxygenase-1 (HO1) expression is associated with delayed gastric emptying in diabetic mice and polymorphisms in the HO1 gene (HMOX1) are associated with worse outcomes in other diseases [253]. Recent study showed that longer poly-GT repeats in the HMOX1 gene are more common in African Americans with gastroparesis. Nausea symptoms are worse in subjects with longer alleles [253].

Chapter III

3. CHRONIC INTESTINAL PSEUDO-OBSTRUCTION (CIPO)

3.1 General features

CIPO is a rare and potentially life-threatening disorder with unknown prevalence and incidence, [254,255] is viewed typically as an insufficiency of the intestinal peristalsis that mimics a sub-occlusive disease in the absence of mechanical obstructions [256] [257]. The severity of the clinical presentation and the limited understanding of the disorder contribute to poor quality of life and increased mortality [258]. In addition, there are no specific biochemical or molecular biomarkers of CIPO, hindering further a correct diagnosis.

CIPO can result from derangement affecting the integrity of a variety of regulatory cells/tissues, i.e. smooth muscle cells (effectors of contractility / relaxation), the ICC, and neurons (either intrinsic – the enteric nervous system or extrinsic nerve pathways). In some cases, CIPO is associated with inflammation, predominantly of the enteric ganglia, which exhibit inflammatory neuropathy [251]. In the last years, several genes have been identified in different subsets of CIPO patients. The most recent update on enteric dysmotility related to CIPO are illustrated in this thesis, highlighting: (a) forms with a predominant underlying neuropathy; (b) forms with a predominant myopathy; and (c) mitochondrial disorders with a clear gut dysfunction as part of the clinical phenotype. Based on the type of cellular pathology, CIPO has been classified into three major categories: forms with a predominant underlying neuropathy; forms with a predominant myopathy; and mitochondrial disorders with a clear gut dysfunction as part of the clinical phenotype. Neuromuscular and ICC abnormalities observed in CIPO may be “secondary” to a number of recognized diseases (e.g, metabolic, paraneoplastic, or neurologic disorders), or “idiopathic” in nature [259]. Enteric neurodegenerative abnormalities and immune-mediated changes may occur in gut specimens of patients with neuropathic CIPO. Inflammatory neuropathies are characterized by a dense inflammatory infiltrate

characterized by CD3 positive (composed of both CD4 and CD8) lymphocytes almost invariably confined to the MP (hence the term of lymphocytic myenteric ganglionitis).

The close apposition of CD3 lymphocytes to myenteric neurons provides the basis to neuro-immune interactions targeting and affecting ganglion cell structure and survival [260]. Indeed, experimental evidence indicates that inflammation/immune activation in the GI tract can profoundly affect both morphology and function of the ENS.

A typical example of the high genetic heterogeneity underlying CIPO is given by mitochondrial encephalomyopathies that can be characterized by brain leukoencephalopathy and CIPO. Mitochondrial neurogastrointestinal encephalopathy (MNGIE), the 'tip of the ice-berg' of such rare diseases, is caused by mutations in *TYMP*, a gene encoding for thymidine phosphorylase (Filosto et al, 2011). Similar phenotypes are also caused by polymerase gamma (*POLG*) mutations, or mutations in the mtDNA as in mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) [261].

3.2 Genetic aspects

From a genetic perspective, X-linked, autosomal dominant and recessive forms have been identified with mutations in filamin A (FLNA) [262], actin G2 (ACTG2) [263], thymidine phosphorylase (TYMP) [264]/ polymerase gamma (POLG1) [265] SGOL1 [266]. Deglincerti et al. mapped a locus in a large consanguineous family segregating an autosomal recessive form of CIPO [267,268]. In the affected family members, the major clinical feature was represented by CIPO, in addition to megaduodenum, long-segment Barrett esophagus, and cardiac abnormalities of variable severity (OMIM 611376; Mungan syndrome, MGS).

Specifically, the main mutations known involve several genes:

- FLNA (Xq28 chromosome). The FLNA gene is involved in a form of X-linked CIPO; encoding for filamin A, an actin-binding protein that promotes the formation of branched networks of actin filaments the actin-binding

protein. The protein is involved in cytoskeletal remodelling allowing variations in cell shape and migratory processes. Studies of an Italian family have identified a homozygous mutation in in the FLNA gene in affected males. The deletion of two bases in exon 2 identified a premature stop codon in position 103. In the presence of this mutation, there is an excess in the formation of "constriction rings", structures important for the contractility of the cells. The shorter FLNA form may be responsible for cytoskeletal abnormalities by highlighting its role in the structure and function of enteric neurons [269].

- ACTG2A (chromosome 2p13.1). Actins are highly conserved proteins involved in various types of cell motility and cytoskeletal maintenance. Three types of actins have been identified: actin α, β and γ. Actin γ-2 is the protein encoded by the ACTG2A gene and represents an isoform expressed in the smooth muscle at the enteric level producing the SMA protein. The R148S mutation of ACTG2A was observed in Familial Visceral Myopathy (FVM), a form of myopathic pseudo-obstruction with autosomal dominant transmission [263,270] and appears to be associated with de novo sporadic CIPO cases. ACTG2AR148S variant is abnormally incorporated into actin filaments, with less actin-myosin II interaction and less stress fiber formation. This variant also appears to reduce the ability to interact with the actinic chaperonin CCT (Cytosolic Chaperonin Containing TCP-1), imposing correct actinic folding. In conclusion, R148S mutation interferes with the assembly and acto-myosin function of the intestinal musculature of FVM patients and the alteration in intestinal propulsion observed in FVM appears to be closely correlated with the decrease in the actin function at the enteric level. Through immunohistochemistry, accumulation of ACTG2A in the intestinal circular muscles, not found in the muscularis mucosae, can be observed in patients with FVM [263,270].

- MYH11 (chromosome 16p13.11). Autosomal recessive forms of megacystis-microcolon-intestinal hypoperistalsis syndrome (MMIHS) are caused by biallelic loss-of-function variants in MYH11 gene encoding for proteins involved in actin–myosin interactions. MMIHS is a rare congenital disorder characterized by impaired smooth muscle contraction isolated to the bladder and intestine. Only recently has the genetic aetiology of the disorder been identified with the majority of sporadic cases occurring secondary to ACTG2 mutations. Mutation in MYH11 have been identified by Gauthier et al [271]. Later Yetman et al [272] described the mutation in a premature infant with MMIHS and additional clinical features of congenital mydriasis, congenital sensorineural hearing loss, lung disease, pulmonary hypertension, and mild aortic dilation who lived to the age of 18 months. He had no substantial improvement in bladder or bowel function over time[272].
- LMOD1 (chromosome 1q32.1). Leiomodin 1, an actin-binding protein expressed primarily in vascular and visceral smooth muscle [273]. Diseases associated with LMOD1 include MMIHS and visceral myopathy. Several studies have highlighted the alterations in smooth muscle structural proteins and pathways related to smooth muscle function, providing mechanistic insights in the disease aetiology. As an example, loss of LMOD1 in vitro and in vivo results in a reduction of filamentous actin, with elongated cytoskeletal dense bodies and impaired intestinal smooth muscle contractility [273].
- TYMP (chromosome 22q13.32). The gene encodes Thymidine Phosphorylase (TP), a protein with enzymatic activity capable of converting thymidine nucleosides and deoxyuridine into thymine and uracil. Mutations in TYMP lead to a reduction of TP activity with consequent toxic accumulation of nucleosides in the plasma of patients with Mitochondrial Neurogastrointestinal Encephalomyopathy (MNGIE). At mitochondrial level there are alterations such as multiple deletions

and mitochondrial DNA depletion. MNGIE is characterized by peripheral neuropathy, chronic progressive external ophthalmoplegia, leukoencephalopathy and multiple mtDNA deletions in skeletal muscle. Thymidine phosphorylase also appears to play a role in the inhibition of glial cell proliferation (Hirano et al, 1999). Recent studies, conducted by our research group, have shown that in the liver there is a high expression of TP, suggesting that the transplant of this organ can be a valid therapeutic strategy [274,275].

- POLG1 (15q26.1). The gene encodes the catalytic subunit of the mitochondrial genome polymerase. The mitochondrial DNA polymerase is heterodimeric consisting of a homodimer of regulatory subunits plus a catalytic subunit. Mutations in this gene lead to multiple deletions in mtDNA with clinical features such as skeletal myopathy and visceral dysmotility, which are described in hypoperistalsis and CIPO. mtDNA depletion seems to be the likely cause of a visceral myopathy causing hypoperistalsis and intestinal pseudo-obstruction. Based on these findings, the external layer of muscularis propria is confirmed as the most susceptible point of the GI tract to develop mtDNA depletion, possibly because of the constitutive low abundance of mtDNA within smooth muscle cells at this site [265].
- SGOL1 (chromosome 3p24.3). The protein encoded by this gene is a component of the cohesin complex involved in the regulation of the cohesion of the sister chromatids which acts by protecting the centromere from the cleavage during the mitotic phase. Mutations in homozygosity of the SGOL1 gene cause the Chronic Atrial Intestinal Dysrhytmia (CAID), which is chronic intestinal atrial dysrhythmia. Dysrhythmias are part of disorders involving pacemaker activity, both at cardiac and intestinal levels. In 17 patients with CAID was observed the coexistence of a pathological phenotype both at intestinal and cardiac levels. Specifically, the heart level disorder manifested as Sick Sinus

Syndrome; instead, the intestinal level it manifested as CIPO for the involvement of ICC and ENS cells [266].

- RAD21 (chromosome 8q24.11). RAD21 is part of the cohesin complex, involved in the pairing and unpairing of sister chromatids during cell replication and division, and also regulating gene expression directly and independently of cell division [276]. The RAD21 subunit of the cohesin complex plays important structural and functional roles, in that it serves as the only physical link between the SMC1/SMC3 heterodimer and the STAG subunit and that its integrity regulates the association or disassociation of functional cohesin with chromatin. RAD21 has also a key role in double strand breaks DNA repair [277].

  Homozygous mutation was identified in a large consanguineous family segregating an autosomal recessive form of CIPO. In the affected family members, the major clinical feature was represented by CIPO, in addition to megaduodenum, long-segment Barrett esophagus, and cardiac abnormalities of variable severity (OMIM 611376; Mungan syndrome, MGS). The novel homozygous change c.1864 G>A in RAD21 (NM_006265.2) produce the damaging missense variant p.Ala622Thr [278]. Cohesin binds to many sites throughout the genome, in combination with the CCCTC-binding factor (CTCF) insulator protein, which is known to mediate chromatin loop formation. Cohesin co-localizes with CTCF along chromosome arms, cooperating with this protein in the regulation of gene expression and chromatin structure [279]. RAD21 is associated with other transcriptional regulators, such as estrogen receptor-α [97], and mediator (Kagey et al., 2010) in a cell type-specific manner. In combination with other factors, cohesin selectively binds genes with paused RNA polymerase II and can regulate transcription by determining the amount of elongating RNA polymerase II on genes [280]. Cohesin-mediated chromatin organization plays an important role in the formation/ stabilization of chromosome architecture and gene

transcription and repression. RAD21 interacts with CTCF and other cohesin-associated proteins to maintain and stabilize multidimensional organizations of topologically associating domains and chromatin loops [281].

In addition, human RAD21 is linked to the apoptotic pathways and RAD21cleavage can be induced by a broad spectrum of apoptotic stimuli at residue p.Asp279, which is different from the mitotic cleavage sites required for chromosomal segregation [282,283].

- ERBB2 (chromosome 17q12). Recent data demonstrated that dysregulation of ERBB3 or ERBB2 leads to a broad spectrum of developmental anomalies, including intestinal dysmotility [183]. The genetic variants resulted in loss of function with decreased expression or aberrant phosphorylation of the ERBB3/ERBB2 receptors. Experiments using mice revealed that Erbb3 and Erbb2 are expressed in enteric neuronal progenitor cells. This study is an example of descriptive observation that begs for mechanistic exploration to reveal precisely how the NRG1/ERBB3/ERBB2 pathway influences ENS development [284].

Since this thesis is focused on CIPO condition, a deep overview will be presented in the next chapter.

Chapter IV

4. NOVEL UNDERSTANDING ON GENETIC MECHANISMS OF ENTERIC NEUROPATHIES LEADING TO SEVERE GUT DYSMOTILITY

Modified from

"Novel understanding on genetic mechanisms of enteric neuropathies leading to severe gut dysmotility"

Francesca Bianco, Giulia Lattanzio, Luca Lorenzini, Chiara Diquigiovanni, Maurizio Mazzoni, Paolo Clavenzani, Laura Calzà, Luciana Giardino, Catia Sternini, Elena Bonora, Roberto De Giorgio

European Journal of Histochemistry 2021; volume 65(s1):3289

Abstract

The enteric nervous system (ENS) is the third division of the autonomic nervous system and the largest collection of neurons outside the central nervous system (CNS). The ENS has been referred to as "the brain-in-the gut" or "the second brain of the human body" because of its highly integrated neural circuits controlling a vast repertoire of gut functions, including absorption/secretion, splanchnic blood vessels, some immunological aspects, intestinal epithelial barrier, and gastrointestinal (GI) motility. The latter function is the result of the ENS fine-tuning over smooth musculature, along with the contribution of other key cells, such as enteric glia (astrocyte-like cells supporting and contributing to neuronal activity), interstitial cells of Cajal (the pacemaker cells of the GI tract involved in neuromuscular transmission), and enteroendocrine cells (releasing bioactivesubstances, which affect gut physiology). Any noxa insult perturbing the ENS complexity may determine a neuropathy with variable degree of neuro-muscular dysfunction. In this review we aim to cover the most recent update on genetic mechanisms leading to enteric neuropathies ranging from Hirschsprung's disease (characterized by lack of any enteric neurons in the gut

wall) up to more generalized form of dysmotility such as chronic intestinal pseudo-obstruction (CIPO) with a significant reduction of enteric neurons. In this line, we will discuss the role of the RAD21 mutation, which we have demonstrated in a family whose affected members exhibited severe GI dysmotility. Other genes contributing to gut motility abnormalities will also be presented. In conclusion, the knowledge on the molecular mechanisms involved in enteric neuropathy may unveil strategies to better manage patients with neurogenic gut dysmotility and pave the way to targeted therapies.

Key words: Chronic intestinal pseudo-obstruction; enteric neuropathies; genes; Hirschsprung's disease; neuroprotection; 5-HT4 receptors.

Introduction

The digestive system contributes significantly to the maintenance of body homeostasis[1] via a number of key physiological functions, including generating motor patterns to propel foods through the lumen of the gastrointestinal (GI) tract, mixing ingesta with different secretions, and absorbing nutrients. To serve this vast repertoire of coordinated activities, the GI tract requires finely tuned regulatory mechanisms, with the intrinsic innervation of the gut or enteric nervous system (ENS), being the most prominent one.[2] Because of its ability to operate independently from central nervous system (CNS) and the "brain-like" organization, the ENS is also referred to as '*the brain-in-the-gut'* or the *'second brain'*. Notably, the ENS is composed by as many neurons as those present in the mammalian spinal cord (*i.e.*, approximately 10^8), making it the largest division of the autonomic nervous system (the other being the parasympathetic and sympathetic components).[3]

Research performed over the past four decades by enteric neuroscientists has shown that in the two main ganglionated plexuses of the ENS, namely the myenteric (Auerbach's) one (embedded in the longitudinal and circular layers of the GI tract) and submucosal (Meissner's) one (localized throughout the

submucosa) there are functionally distinct classes of neurons.[4] These include motor neurons, interneurons, vasomotor neurons and secretomotor neurons synaptically linked in reflex circuits controlling “canonical” (*e.g.*, motility, absorption/secretion and splanchnic vasculature) and “non-canonical” functions (*e.g.*, neuro-immune interactions and epithelial barrier integrity) *via* the release of a cocktail of neuromodulators and neurotransmitters and related receptors.[5,6] The neurochemistry of the ENS prompted studies aimed at identifying novel pharmacological targets and therapeutic options, a typical example being 5-hydroxytryptamine (serotonin) and related receptors. [7] Furthermore, likewise the CNS, the ENS possesses a prominent glia component exhibiting morphological and functional features to some extent similar to the astrocytes of the CNS.[8-10]

Enteric neuropathies are challenging conditions characterized by a severe impairment of gut physiology, including motility. Overall, the clinical manifestations and their severity increase as the enteric neuron number decreases. A recent study from our group suggests that a 50% loss of neuronal cell bodies in the myenteric and submucosal ganglia (*vs* controls) may be considered a "critical threshold" to the occurrence of clinical manifestations, such as recurrent intestinal sub-occlusive episodes related to impaired motility and severe symptoms (*e.g.*, nausea, vomiting).[11]

Based on this background, the present review will focus on some aspects of genetically driven molecular abnormalities of enteric neuropathies ranging from Hirschsprung’s disease, a condition characterized by lack of any enteric ganglia in the gut wall, up to more generalized dysmotility, such as CIPO characterized by an overall reduced number of enteric neurons. Deciphering the molecular mechanisms involved in enteric neuropathies may unveil strategies to optimize the treatment of patients with severe gut dysmotility and related symptoms.

Hirschsprung's (HSCR) disease: a paradigm for complex diseases

An extreme form of ENS neuropathy is aganglionosis, *i.e*., the absence of any enteric neurons and ganglia in the GI tract. This can occur as an acquired condition in about 20-30% of patients with Chagas disease (which is due to the parasite *Trypanosoma cruzi* infecting humans *via* Triatominae, a subclass of insects also known as "kissing bugs" common in the tropical area) or as a congenital, genetically-driven, disorder in HSCR, which affects approximately 1:5000 newborn.[12,13] The classic clinical phenotype of HSCR is the lack of both myenteric and submucosal ganglia in the GI tract (usually the distal segments) as a result of an impaired enteric neuron migration and differentiation along the gut during prenatal life. The pathophysiological consequence of aganglionosis is a complete absence of peristalsis, the main motor behavior of the intestine leading to dilatation of the gut (a typical example being megacolon). Patients with HSCR require a rapid diagnosis and a surgical treatment aimed to remove the aganglionic segment.

HSCR is a disorder with genetic heterogeneity, but most cases present with mutations in the *RET* (rearranged during transfection), affecting either coding or non-coding parts of the gene.[14] Other mutated genes, such as *EDNRB* and *SOX10*, have an important impact on RET signaling thereby causing HSCR. In the last few years different genes and variants have been identified in association with this disease.[15] Rapid technological advancements in molecular genetics, including genome-wide association studies (GWAS), whole exome sequencing (WES) and next generation sequencing (NGS) contributed to detect new pathological variants in patients with HSCR.[15,16] In view of the considerable knowledge in this field, that described HSCR as a model of complex multigenic disorders, the new causative variants have been summarized in Table 2. The reader is referred to in-depth review on this topic that is beyond the scope of this work.

Table 2. Major genes with causative variants, which have been found in Hirschsprung disease.

Gene	Gene name	Map position
RET	Ret proto-oncogene	10q11.21
NRTN	Neurturin	19p13
GDNF	Glial Cell Derived Neurotrophic Factor	5p13
GFRa1	Glial cell line-derived neurotrophic factor family receptor 1	10q26
PSPN	Persephin	19p13
EDNRB	Endothelin Receptor Type B	13q22
ECE-1	Endothelin Converting Enzyme 1	1p36
EDN3	Endothelin 3	20q13
NTF3	Neurotrophin 3	12p13.31
NTRK3	Neurotrophic Receptor Tyrosine Kinase 3	15q25.3
SOX10	SRY-box containing gene 10	22q13
L1CAM	L1 cell adhesion molecule	Xq28
NRG1	Neuregulin 1	8p12
PHOX2B	Paired-like homeobox 2b	4p13
ZEB2	Zinc Finger E-Box Binding Homeobox 2	2q22.3
KIAA1279	Kinesin family binding protein; KIFBP	10q22.1
SEMA3A/ SEMA3C/ SEMA3D	Semaphorin 3A/3C/3D	7q21.11
DNMT3B	DNA Methyltransferase 3 Beta	20q11.21

Chronic intestinal pseudo-obstruction: understanding the molecular features of gut dysmotility

Chronic intestinal pseudo-obstruction (CIPO) is a very severe form of gut dysmotility, which manifests with recurrent sub-obstructive episodes, in the absence of any evidence of mechanical causes occluding the intestinal lumen.[17] CIPO can be either ‘‘secondary’’ to a wide array of recognized pathological conditions or ‘‘idiopathic’’ *i.e.*, forms related to unknown etiology.[18] So far, the management of CIPO patients remain largely unsatisfactory, thus leading to frustration for the patients, their families and physicians. Ideally, restoring GI motility may result in an improvement of the nutritional status and preserve the patient from a number of lifethreatening outcomes (septicemia being one of the most common complications).[19,20] Likewise aganglionosis in HSCR, we will address idiopathic CIPO and, specifically, focus on genetic abnormalities. Combined clinical, histopathological and genetic studies are eagerly awaited to identify new

perspectives in the understanding of CIPO. This is of utmost importance for classifying CIPO and establishing correlations between histopathological/clinical phenotypes and underlying genetic defects.

Likewise aganglionosis in HSCR, we will address idiopathic CIPO and, specifically, focus on genetic abnormalities. Combined clinical, histopathological and genetic studies are eagerly awaited to identify new perspectives in the understanding of CIPO. This is of utmost importance for classifying CIPO and establishing correlations between histopathological/clinical phenotypes and underlying genetic defects. A general view of the causative genetic variants associated to CIPO has been highlighted in Figure 7 showing the different locations in the gut of the gene products involved in CIPO. The reader should be aware that although CIPO can originate from smooth muscle abnormalities[21-,24] and changes to interstitial cells of Cajal (ICC - the pacemaker cells of the GI tract), herein, we will cover the genetically-driven forms of neuropathy-related (or "neurogenic") CIPO. Thorough *in vitro* and *in vivo* studies of gene variants are required to understand their impact in severe enteric dysmotility of CIPO patients. In this line, the discovery of mutated genes represents the first step for novel targeted therapeutic strategies aimed at overcoming downstream molecular impairments underlying severe gut dysmotility. The next paragraphs will deal with RAD21, GOL1, POLG, TYMP and other new gene-related neuropathies.

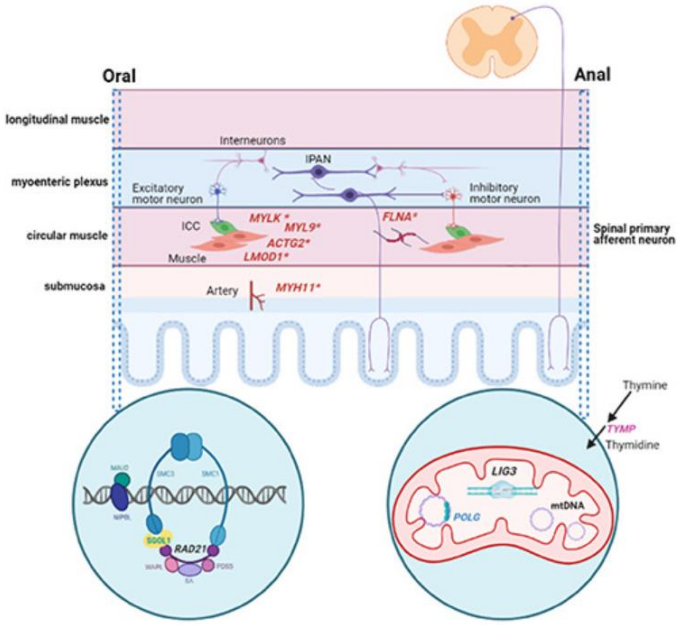

Figure 7. Possible topography of some genetic variants involved in gut neuro-muscular impairment and related dysmotility. MYLK, Myosin light chain kinase; MYL9, Myosin light chain 9; ACTG2, Enteric smooth muscle actin γ-2; LMOD1, leiomodin 1; MYH11, Myosin heavy chain 11; FLNA, filamin A; RAD21, Cohesin Complex Component; SGOL1, Shugoshin-like 1; TYMP, Thymidine phosphorylase gene; POLG, DNA polymerase gamma; LIG3, ligase III gene. IPAN, intrinsic primary afferent neuron; ICC, interstitial cells of Cajal; *genes associated to myopathic forms of CIPO. Created with BioRender.

SGOL1 and RAD21

Homozygous mutations in SGOL1 and RAD21, encoding for cohesin complex components, were identified in patients with CIPO. In 2014, Chetaille *et al*.[25] described a new syndrome affecting gut and heart rhythm caused by a S*GOL1* mutation. They defined this condition as chronic atrial and intestinal dysrhythmia (CAID) syndrome, *i.e*., a novel generalized dysrhythmia, indicative of a new role for SGOL1 in mediating the integrity of human cardiac and gut rhythmicity, the latter being generated by ICC. Since SGOL1 is part of the cohesin complex, its dysfunction could entail consequences for long-range transcriptional regulation, possibly interfering with the expression of gene products associated with CIPO.

In the same period, our group identified a homozygous causative variant in a Turkish large consanguineous family segregating an autosomal recessive form of

CIPO (Figure 8). In the affected family members, the major clinical feature was CIPO, in addition to megaduodenum, long-segment Barrett esophagus and cardiac abnormalities of variable severity (OMIM 611376; also referred to as 'Mungan syndrome', MGS). We performed WES analysis on the genomic DNA from two affected individuals and found the novel homozygous change c.1864G>A in RAD21 (NM_006265.2) producing the damaging missense variant p.Ala622Thr.2. [26]

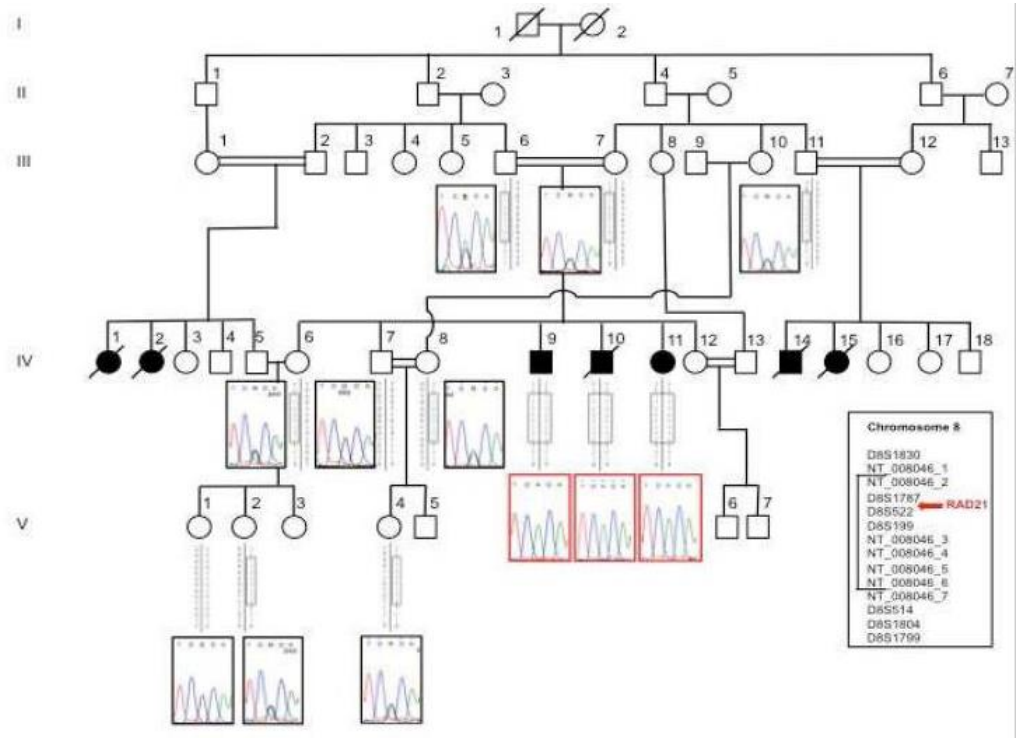

Figure 8. Pedigree of the Turkish consanguineous family showing the segregation of the RAD21 mutation in the available members. For the homozygous patients, electropherograms are boxed in red. Grey boxes represent the haplotypes derived from microsatellite analysis.

Likewise, SGOL1, also RAD21 (8q24.11 cromosome) is part of the cohesin complex. RAD21 is a transcription factor and a key central component of the multi-protein cohesin complex [27] (Figure 9), it provides the physical link between the SMC1/SMC3 heterodimer and the STAG subunit which functions to protect chromosome separation during the metaphase–anaphase transition of mitosis.

RAD21 is ubiquitously expressed in many tissues (Faure et al. 2012) and its critical role emerged from mouse knock-out, which showed a lethal early embryonic phenotype (Biswas et al. 2016).

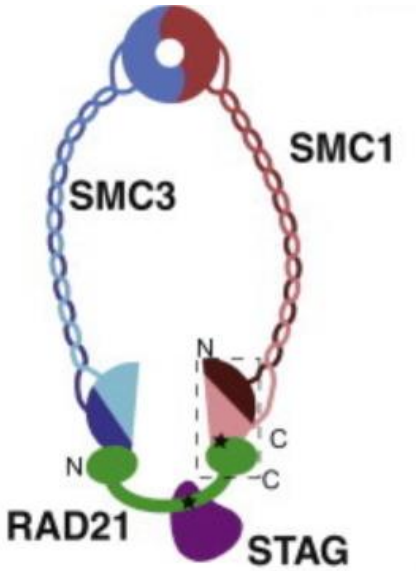

Figure 9. Cohesin complex Stucture: the structure include SMC1 and SMC3 subunits that linked proteins with ATP domain at one end. Togheter with RAD21 and STAG formed a large ring structure that can surround the sister chromatids. ATPase domains are needed to load cohesin to DNA. RAD21 is also able to link different sites within the genome, regulating the transcription of some genes; due to this characteristic it is involved in various processes linked to tumorigenesis (modified from Deardorff et al, 2012).

In our in vivo study we could recapitulate in the zebrafish gut the CIPO phenotype, i.e., marked hypoganglionosis and severe impairment of motility, both features observed in the patients with the homozygous RAD21 mutation. After injecting a morpholino (mo) specific for the functional ablation of rad21, zebrafish embryos were allowed to develop to 5 days post fertilization (dpf), when the digestive system developed (figure 10). Control and mo-treated embryos were fed fluorescent beads through microgavage, a technique that allows to assess the rate of intestinal motility as a function of time. After eight hours post bead injection, gut embryos were divided into four zones based on anatomical landmarks and the fluorescence detectable in each segment was

scored. Compared to controls, rad21 morphants showed delayed food transit along the gut. Furthermore, a significant depletion of enteric neurons, detected with antibodies to the neuronal marker HuC/D, was quantitatively shown in the gut of rad21 morphants vs controls. Taken together the marked impairment of gut motility along with the significant reduction of HuC/D-immunolabeled enteric neurons provided evidence of a neurogenic type of dysmotility reminiscent CIPO observed in patients with RAD21 mutations [26].

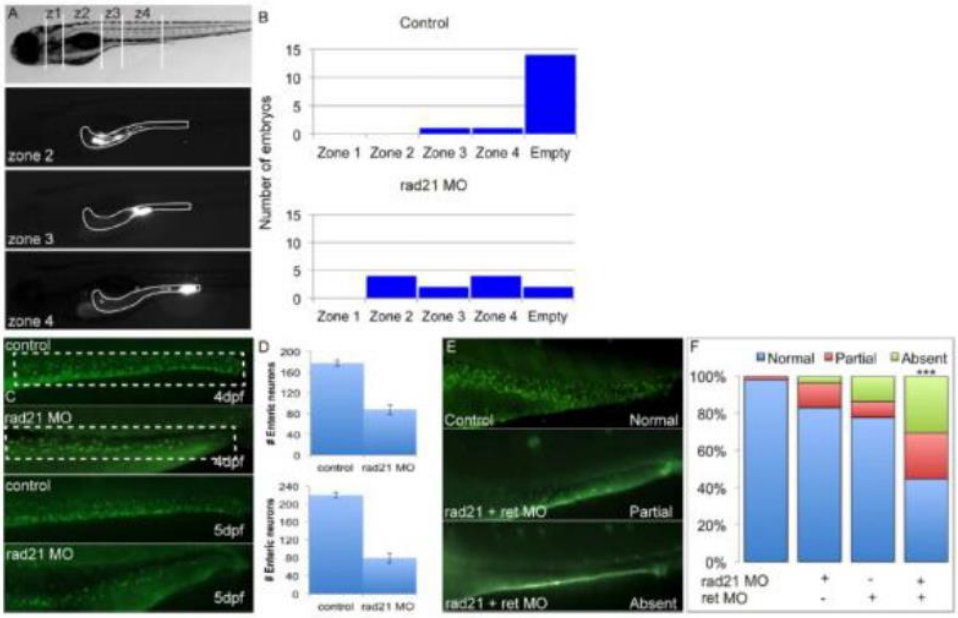

Figure 10. Gut dysmotility is caused by enteric neuronal loss in zebrafish embryos To assess gut motility in zebrafish larvae, we injected fluorescent beads into the mouth and recorded the rate of gut motility versus time. (A) Lateral view of 5dpf zebrafish larvae. Representative images of injected larvae show (after eight hours post injection, hpi) fluorescent beads in different gut compartments (zone 1–4). (B) In control embryos, most of the fluorescent beads have exited the gut by 8hpi, while rad21 MO injected embryos have reduced gut motility. (C-D) Compared to control larvae, rad21 morphants have a significant reduction of enteric HuC/D immunoreactive neurons at 4dpf (D; upper panel) and 5dpf (D; lower panel). (E) rad21 and ret interaction during the ENS development. Combination of suboptimal doses of rad21 and ret MOs causes a significant decrease in HuC/D enteric neurons, while embryos injected with suboptimal doses of rad21 MO or ret MO causes loss of HuC/D enteric neurons, not statistically significant (F).

The distribution of RAD21 in the mouse small intestine was another important step to better understand the RAD21-related functional implications in the ENS. High magnification revealed a predominantly cytoplasmic immunolabeling, although, as expected by its molecular nature (nuclear phospho-protein) and

function (cohesin complex / transcription factor), RAD21-IR was also detectable in nuclei[28]. No other cell types in the small intestinal muscularis propria displayed cytoplasmic RAD21-IR, including GFAP expressing glial cells, Kit+ ICC and PDGFRα-IR fibroblast-like cells. in order to understand whether RAD21 distribution in myenteric neurons was altered during development, we investigated the distribution and relative abundance of RAD21-IR neurons in neonatal mice. RAD21-IR neurons were quantitatively comparable in neonatal and adult tissues, a finding implying that there is no major age-dependent cellular redistribution of RAD21-IR in the cytoplasm of neuronal subsets. Moreover, in the mouse small intestine Rad21 immunoreactivity (IR) was detected in a subset of PGP9.5- and HuC/D-immunolabeled myenteric neurons, specifically in 42.5% of HuC/D-IR neurons/field, and same percentage of HuC/D-IR neurons/field in young mice. Based on this reliable immunolabeling, we investigated the intrinsic innervation of the human and mouse small intestine and identified RAD21 expression in myenteric ChAT-IR neurons, likely belonging to motor neurons / ascending interneurons. A subset of choline acetyl transferase (ChAT, a marker of cholinergic excitatory neurons)-positive neurons also expressed Rad21-IR. By contrast, nNOS expressing myenteric neurons, mainly inhibitory motor neurons / descending interneurons, did not show RAD21-IR in either species. Our data provided the first analysis of RAD21 localization in the mammalian enteric nervous system and clearly showed that this cohesin complex protein is expressed outside the nucleus of cholinergic neurons in a subset of ChAT-IR neurons.

RAD21 is known to exert a variety of regulatory functions critical to cellular homeostasis, thus our findings suggest a role for extra-nuclear RAD21 in maintenance and survival mechanisms of a subset of cholinergic neurons occurring in the healthy myenteric plexus. These data provided the basis to study how the RAD21 mutation affects the ENS by developing a genetically re-constructed Rad21 conditional knockin (Rad21KI) mouse carrying the Ala626Thr mutation found in affected family member. This model is expected to

recapitulate the main clinical and pathological features observed in the affected RAD21 variant family members.

TYMP, POLG, RRM2B: mitochondrial disorders and gut neuro(myo)pathy

The mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive disorder due to TYMP, POLG and RRM2B mutations[29]. In addition to a severe derangement of GI motility (i.e., gastroparesis and CIPO) resulting from an underlying enteric neuro-myopathy[11, 30], the clinical picture is characterized by peripheral neuropathy, ophthalmoplegia and brain leucoencephalopathy detectable at magnetic resonance imaging. TYMP encodes for the thymidine phosphorylase (TP) enzyme, which regulates the mitochondrial nucleotide pool. TP is a cytoplasmic enzyme expressed in most human tissues including the central and peripheral nervous systems, the GI tract, leukocytes, liver and platelets[31]. TP catalyzes the first step of mitochondrial dThd and dUrd catabolism by converting them to the nucleotides thymine and uridine, respectively, and 2-deoxy ribose 1-phosphate[32]. Thus, TYMP mutations evoke TP dysfunction leading to accumulation of both dThd and dUrd and a subsequent reduction of cytidine triphosphate (dCTP) in the plasma and tissues of MNGIE patients. This imbalance interferes with mtDNA replication, causing molecular abnormalities (mtDNA depletion, multiple deletions, and point mutations) and tissue damage (including the neuromuscular component of the GI tract) associated with the disease. Mutations in the gene coding for the catalytic subunit of the mitochondrial DNA (mtDNA) polymerase (POLG) are associated with a range of clinical syndromes characterized by secondary mtDNA defects, including mtDNA depletion and multiple deletions. The clinical manifestations are closely resembling those of MNGIE, although brain leukoencephalopathy is absent. Finally, compound heterozygous mutations in the gene encoding for the ribonucleotide reductase regulatory TP53 inducible subunit M2B (RRM2B) can cause a phenotype, characterized by ophthalmoplegia, ptosis, GI dysmotility,

cachexia, peripheral neuropathy, clinically indistinguishable from TYMP mutated MNGIE

What is new in mitochondrial disorders: the LIG3 mutation enteropathy

We recently characterized another mutant gene in seven patients with CIPO and neurological manifestations (reminiscent of MNGIE) from three unrelated families[33]. In addition to CIPO, the most prominent and consistent clinical signs were neurological abnormalities, including leukoencephalopathy, epilepsy, migraine, stroke-like episodes, and neurogenic bladder. DNA from these patients was examined via WES. Compound heterozygous variants were identified in the LIG3 gene in all patients. All variants were predicted to have a damaging effect on LIG3 protein. The LIG3 gene encodes the unique mitochondrial DNA (mtDNA) ligase that binds POLG and plays a pivotal role in mtDNA repair and replication. The study of the consequences of LIG3 mutations was performed in patient-derived primary skin fibroblasts and in transiently transfected cells expressing the different mutant vs WT proteins. All assays showed a markedly reduced amount of LIG3 protein in the mutant cells. In concordance, we demonstrated the lack of ligase activity in the mitochondrial extracts derived from patients' cells compared to control fibroblasts. The LIG3 gene defects altered the mitochondrial network and affected mtDNA maintenance leading to mtDNA depletion without the accumulation of multiple deletions observed in other mitochondrial disorders (e.g., MNGIE) and induced a severe imbalance in cell metabolism (i.e., impaired ATP production and increased mitochondrial reactive oxygen species generation). The resultant mitochondrial dysfunction was key in the causative effects leading to the clinical phenotype observed in these patients. In the gut, both traditional histopathological analysis and neuronal HuC/D immunoreactivity evaluation demonstrated a significant loss of the number of myenteric neuronal cell bodies in the gut (Figure 11). Furthermore, the lig3 disruption in the zebrafish model reproduced some key clinical features

observed in the patients, i.e., brain leukoencephalopathy and impaired gut transit, with altered mitochondrial networks. Thus, biallelic heterozygous loss-of-function variants in the LIG3 gene resulted in a novel mitochondrial disease characterized by predominant gut dysmotility due to enteric neuropathy, and encephalopathy.

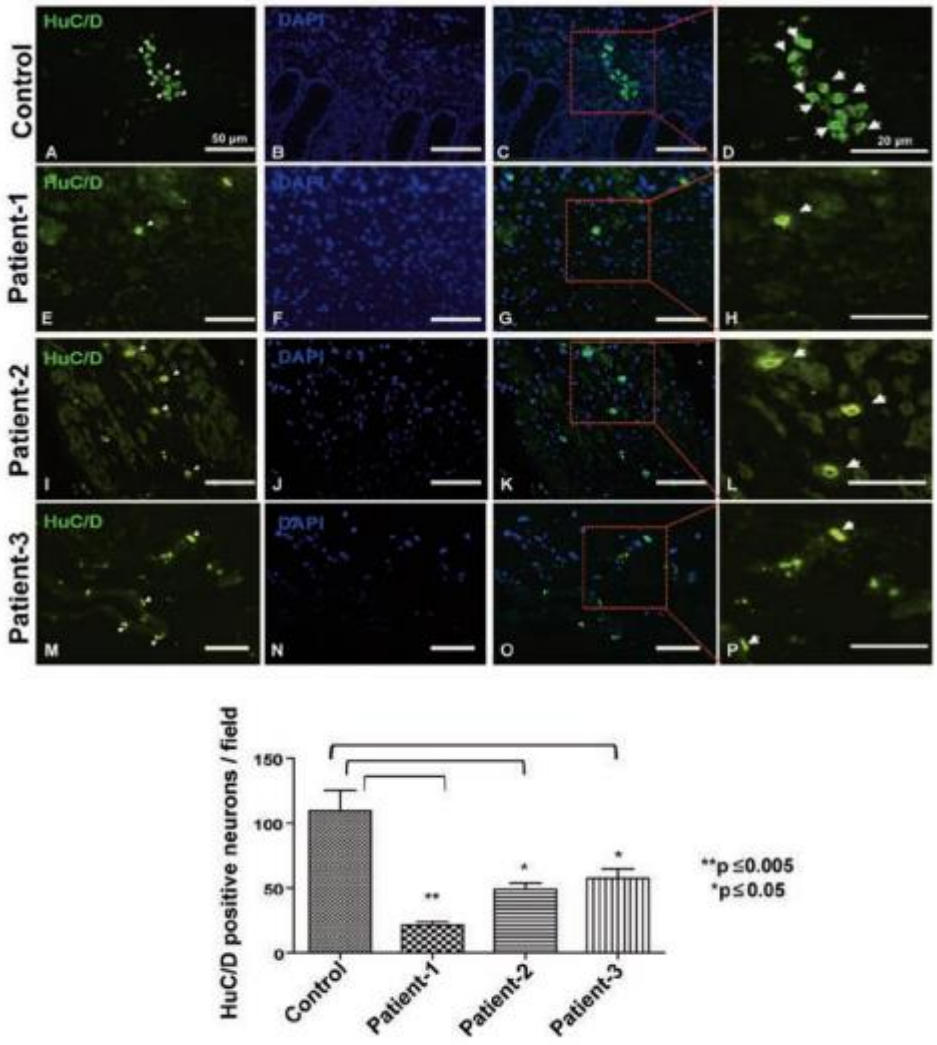

Figure 11. Mutant LIG3 mitochondrial-related entero-neuropathy. In the cryostat sections, few HuC/D immunolabeled (green) myenteric cell bodies (arrows) were observed in the gut (colon) biopsies of patients with LIG3 causative variants vs controls. Cell nuclei were stained with DAPI (blue). Quantitative analysis (graph below) confirmed that myenteric neurons were significantly reduced in patients carrying the causative variants in LIG3, compared to controls ($p \leq 0.005$ control vs patient-1 and $p \leq 0.05$ control vs patient-2 and 3; ANOVA). Scale bar: 50 µm. Photomicrographs in D, H, L and P illustrate higher magnification insets of the indicated region. Scale bar: 20 µm.

Future perspective

NGS has made available the simultaneous analysis of multiple genomic regions and reduced the time (and cost) of genetic tests. The discovery of new genes is of paramount importance for a better understanding of enteric neuropathies and related severe gut dysmotility. Also, this research is expected to develop novel therapeutic targets for affected patients who typically represent a challenge for their complex clinical picture. The lack of ENS models for studying the physio-pathological processes of enteric neuropathies detected in patients may account for the relatively slow progress in the diagnosis and minimally effective treatment options for these difficult cases. In recent years, however, new technological progresses cast hope for disentangling the complexity of enteric neuropathies. In fact, using the direct differentiation of human induced pluripotent stem cells (PSCs), it has been possible to obtain physiological three-dimension organ cultures, known as organoid [33]. A recent study applied a tissue-engineering approach with embryonic and PSC to generate a human intestinal tissue containing a functional ENS. The normal ENS development was recapitulated in vitro by combining human-PSC-derived neural crest cells and developing human intestinal organoids (HIOs), with migration to the mesenchyme, differentiation into neurons and glial cells, and generation of neuronal activity[34]. The ENS-containing HIOs in vivo formed neuro-glial structures similar to myenteric and submucosal plexuses, exhibited functional ICCs and generated waves of propagating contractions. The same approach was applied to study PHOX2B-/- related HSCR mouse model studied extensively by Workman et al [35]. The authors developed a functional intestinal tissue that could be transplanted in vivo and highlighted the potential of stem cell-based therapies for future treatment of severe gut dysmotility. Another frontier of ENS research concerns the molecular profiling, regional distribution, and relative conservation across species of various enteric neuron subsets (mainly the myenteric plexus). Emerging research has provided novel insights on marker genes specific for the different enteric neuron classes (ENCs). Recently, Morarach et al. [36] addressed

this extremely innovative area and provided molecular evidence of twelve ENCs within the myenteric plexus of the mouse small intestine. The authors identified inter-communication features and histochemical markers for discrete classes of neurons, including motor, sensory, and interneurons together with genetic tools for class-specific targeting[36]. A recent work from Ganz et al.[37] showed that genes controlling epigenetic modifications are important to coordinate intestinal tract development, providing the first demonstration that these genes influence ENS development. In this line, another study in human and mouse healthy tissues used laser-capture microdissection coupled to single-nucleus RNA-sequencing, to map out enteric neuron subtypes in the duodenum, ileum, and colon, which were overall conserved between humans and mice based on orthologous gene expression. Nevertheless, some enteric neuron subtype-specific genes in mice were expressed in completely distinct morphologically defined neuron subtypes in humans, thus suggesting that these species-specific differences should be taken into account when translating findings from mouse to human ENS[38]. Taken together these exciting results offer a conceptual and molecular resource for dissecting ENS circuits and predicting key regulators for the directed differentiation of distinct enteric neuron classes. Clearly, these data will change our approach and understanding of enteric neuropathy-related severe gut dysmotility.

Conclusions

In this review, we presented evidence that a thorough genetic analysis is a crucial approach to highlight the molecular pathways involved in ENS morpho-functional changes, hence enteric neuropathy, and severe gut dysmotility. Since current evidence suggests that the ENS may be viewed as a “dynamic system”, characterized by continuous turnover of neurons (i.e., those “dying” being replaced by resident precursors), clarifying the genetic abnormalities affecting neuronal subclasses of the ENS may be a step forward to develop effective targeted therapeutic options for patients. Likewise to CNS disorders, the

technological advances in genomics, molecular phenotyping and regenerative medicine (using pluripotent stem cells as possible treatment options) will represent the beginning of a new era in ENS disorders and related functional GI impairment.

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EXPERIMENTAL STUDIES

Chapter V

5. NOVEL FINDINGS ON GENETICALLY DRIVEN ENTERIC NEUROPATHY: THE RAD21 KNOCK-IN MOUSE

Abstract

Background and aims: RAD21 is a double-strand-break repair protein of the cohesin complex, which plays key roles in the maintenance and survival of various cell types including neurons. Studying a consanguineous family with a clinical phenotype of neurogenic chronic intestinal pseudo-obstruction (CIPO), we identified a novel causative RAD21 (Ala622Thr) missense mutation. CIPO is a very severe dysmotility disorder, which manifests with recurrent sub-obstructive episodes, in absence of any mechanical causes of gut occlusion. We developed a genetically re-constructed Rad21 conditional knock-in (Rad21KI) mouse carrying the mutation. In this study, we aimed to perform a qualitative, quantitative and functional characterization of the small and large intestine enteric nervous system of Rad21KI vs. wild type (WT) mice.

Methods: Gut transit time, stool production, *ex vivo* analysis of myogenic and nerve-mediated motility were investigated by organ bath experiment. Immunohistochemistry was performed in whole-mount myenteric plexus using the pan-neuronal marker HuC/D, choline acetyltransferase (ChAT), neuronal nitric oxide synthase (nNOS) and synaptophysin (Syn).

Results:

Motility index and mean active force to evaluate myoelectric activity of proximal colon smooth muscle in $Rad21^{A626T}$ *vs* WT showed a statistical reduction (3±0.23 *vs* 5.26±0.58.; P≤0.005) (0.45±0.05 *vs.* 0.94±0.1; P≤0.005). However, in the same portion neuro mediated muscolar activity after electrical stimulation in organ bath chamber did not display any differences.

The total number of HuC/D and HuC/D/nNOS-immunoreactive (IR) myenteric neurons did not significantly change in the small and large intestine of Rad21KI

vs. WT. However, in the small and large intestine of Rad21KI, we showed that HuC/D/ChAT-IR myenteric neurons/field were significantly lower *vs.* WT mice (18.9 ± 1.4 *vs.* 32.4 ±2; P≤0.005 and 19.8±2.1 in Rad21KI *vs.* 32.1±3.8; P≤0.005 respectevely). The synaptic vesicle analysis showed a significant reduction of Syn-IR in the Rad21KI large and small intestine compared to WT (P≤0.005). Carmine red dye study showed an increase of intestinal transit time in Rad21KI *vs.* WT (WT (260.5 ± 20.85 *vs.* 190 ±12.25; P≤0.005).

Conclusion: In Rad21KI mice there was a significant reduction of HuC/D/ChAT myenteric neurons along with a lower synaptic density (large/small intestine), a delayed intestinal transit. The reduction of specific neuronal populations suggests that a cholinergic deficit can contribute to the impairment of motility in CIPO patients with the RAD21 mutation.

This initial finding provides a basis to further investigate the full spectrum of abnormalities in this mouse model as a paradigm to CIPO in humans.
Keywords: Chronic intestinal pseudo-obstruction; mouse model; RAD21; enteric neuropathies; synaptophysin.

Introduction

Chronic intestinal pseudo-obstruction (CIPO) is a very severe form of gut dysmotility, which manifests with recurrent subobstructive episodes, in the absence of any evidence of mechanical causes occluding the intestinal lumen [251]. CIPO can be either ‘‘secondary’’ to a wide array of recognized pathological conditions or ‘‘idiopathic’’ i.e., forms related to unknown etiology [256]. So far, the management of CIPO patients remain largely unsatisfactory, thus leading to frustration for the patients, their families and physicians. Ideally, restoring gastrointestinal (GI) motility may result in an improvement of the nutritional status and preserve the patient from a number of life-threatening outcomes (septicemia being one of the most common complications).

RAD21 is expressed in mitotic and post-mitotic tissues, including neurons, and regulates different cell mechanisms as part of the cohesin complex, a ring-

shaped multiprotein complex crucial for 3D genome organization and transcriptional regulation during differentiation and development [276]. Also, RAD21 confers sister chromatid cohesion and facilitates DNA damage repair.

The discovery of new genes in patients with neuropathy-related CIPO is aimed at better understanding the mechanisms leading to severe gastrointestinal dysfunction, symptom generation and ultimately help developing novel therapeutic targets for this highly disabling and complex condition. The lack of models for studying the physio-pathological processes of enteric neuropathies may account for the large unmet need in effective treatment options for the management of CIPO patients. We aim to provide mechanistic insights into the pathogenesis of CIPO by characterizing a newly developed KI mouse, generated to reproduce the abnormalities identified in RAD21-mutated CIPO patients. The central hypothesis is based on the fact that RAD21 is necessary for the development and maintenance of the ENS, given that patients with mutated RAD21 have a significantly reduced number of enteric neurons [267,278], in addition to a severe impairment of intestinal motility.

Previous study showed that Rad21 immunoreactivity (IR) in the mouse small intestine, specifically, was detected in a subset of PGP9.5- and HuC/D-immunolabeled myenteric neurons. A subset of choline acetyl transferase (ChAT, marker of cholinergic excitatory neurons)-positive neurons also expressed Rad21-IR [285]. The generation and characterization of a mouse model ($Rad21^{A626T}$) carrying the CIPO-specific variant p.Ala626Thr (homologous of the p.Ala622Thr in humans), could led to identify whether the genetically determined model reproduces gut dysmotility in terms of GI transit and functional (i.e., electrophysiological) and structural alterations in RAD21 patients.

Materials and Methods

Animals

Animals were bred and maintained in community housing (≤5 mice/cage, 22˚ C) on a 12 h light/dark cycle with free access to water and standard pelleted food.

Care and use of mice for this study were in compliance with relevant animal welfare institutional guidelines in agreement with EU Directive 2010/63/EU for animals, the Italian Legislative Decree 4.03.2014, n. 26. The experimental protocols were approved by the Italian Ministry of Health (Animal protocol n. 382/2018-PR). Research staff received appropriate training in animal care. Mice were euthanized by CO_2 inhalation. For genetically modified animals, the evaluation of the severity was based on daily monitoring of the mice behavior, including evaluation of mobility and body weight.

Rad21^{A622T} conditional KI mouse generation

The Rad21KI mice were generated by Cyagen (Cyagen Biosciences Inc. Santa Clara, CA-USA).

Since rad21$^{-/-}$ mice were embryonic lethal [286], we developed a genetically re-constructed Rad21 conditional KI (Wnt1-Cre x Rad21fl/fl) (Fig 12) carrying the Ala626Thr missense variant (position Ala626 in mouse is homologue of human amino acid Ala622). The Rad21 gene is located on mouse chromosome 15; fourteen exons have been identified, with the start ATG start codon in exon 2 and the TGA stop codon in exon 14.

The mutation Ala626Thr was introduced in rad21 to be expressed in response to Cre-driven recombination of the floxed alleles, driving its expression into subsets of ENS neurons in response to Wnt1 expression at any developmental points.

For the Conditional KI model, a reverse exon 14 cassette containing the mutation GCA to ACA (determining the missense substitution) Ala626Thr was inserted into intron 13. The WT and reverse exon 14 have been flanked with the LoxP and Lo2272 sites, in order to insert in exon 14 the desired mutation after the Cre LoxP recombination event (Figure 13).

To engineer the targeting vector, homology arms were generated by PCR using BAC clone RP23-128I22 and RP23-284G10 from the C57BL/6J library as template.

In the targeting vector, the Neo cassette was flanked by Rox sites. DTA was used for negative selection and C57BL/6 ESC (embryonic stem cells) were used for gene targeting.

After confirming the correct targeting (via Southern Blot and sequencing, Fig 14), ESC positive clones were selected for blastocyst microinjection, followed by founder (F0) production. Founders were confirmed as germline-transmitted via crossbreeding with WT and double heterozygous for Rad21 and Wnt-Cre have been generated by specific mating.

Thus, we generated a C57BL/6 mouse model (Wnt1-Cre x Rad21fl/fl) in which all neural crest-derived cell lineages, including the ENS, express the Rad21 Ala626Thr mutation.

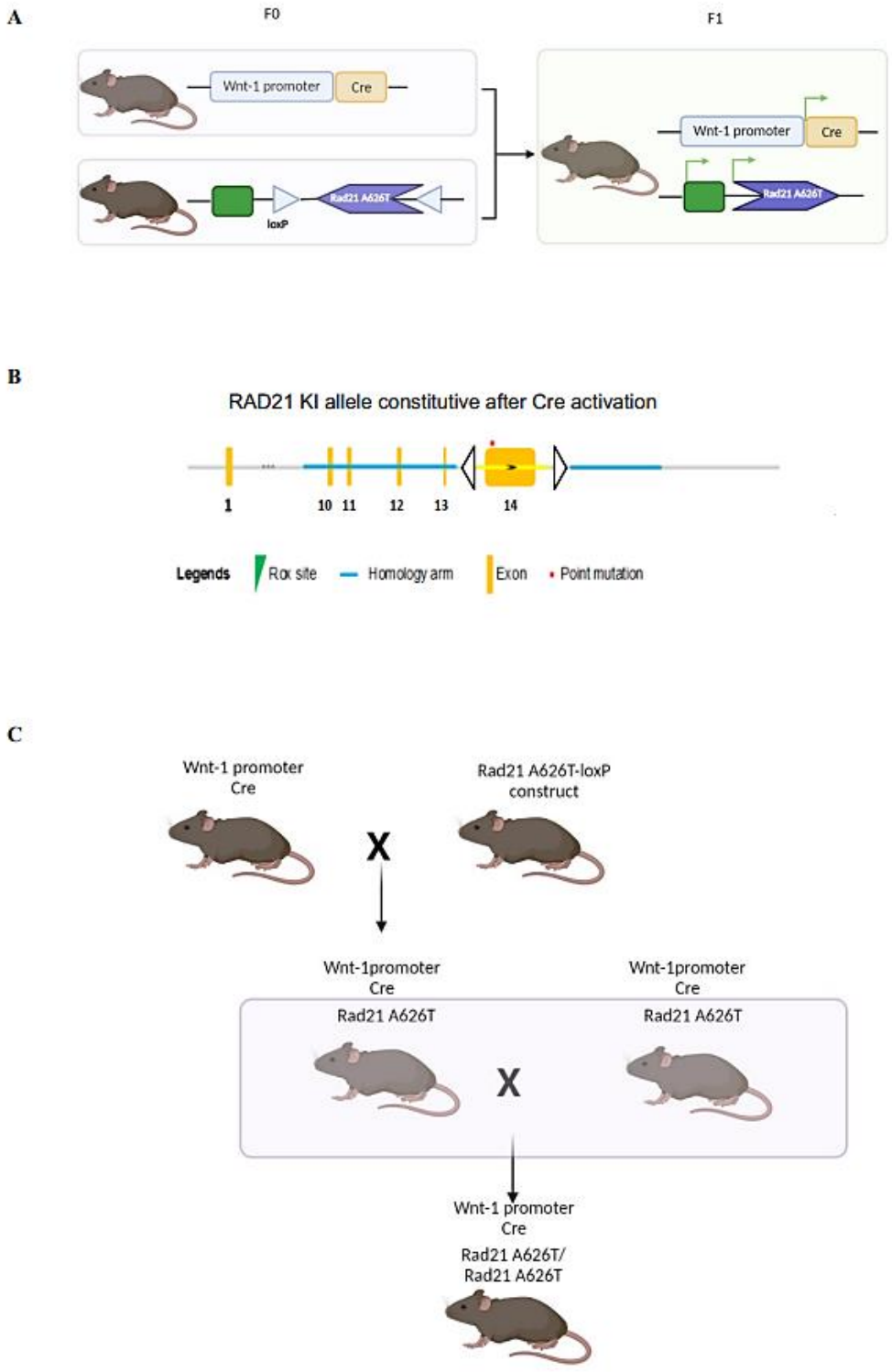


Figure 12. Generation of the Rad21^{A626T} conditional KI mouse. (A) Scheme of the constructs for the tissue-specific expression of the Cre protein and the construct carrying the Rad21 missense change flanked by the loxP sites for genomic integration in the cells expressing Cre, after the crossing of F0 mouse lines. The Rad21 construct was created and inserted in embryonic stem cells and heterozygous mice generated at Cyagen (USA). (B) The genomic locus of mouse Rad21 after Cre-driven recombination. (C) Scheme of the crosses to generate the homozygous conditional Rad21 ki mice carrying the A626T missense change. All mice were genotyped to confirm the correct genomic profiles.

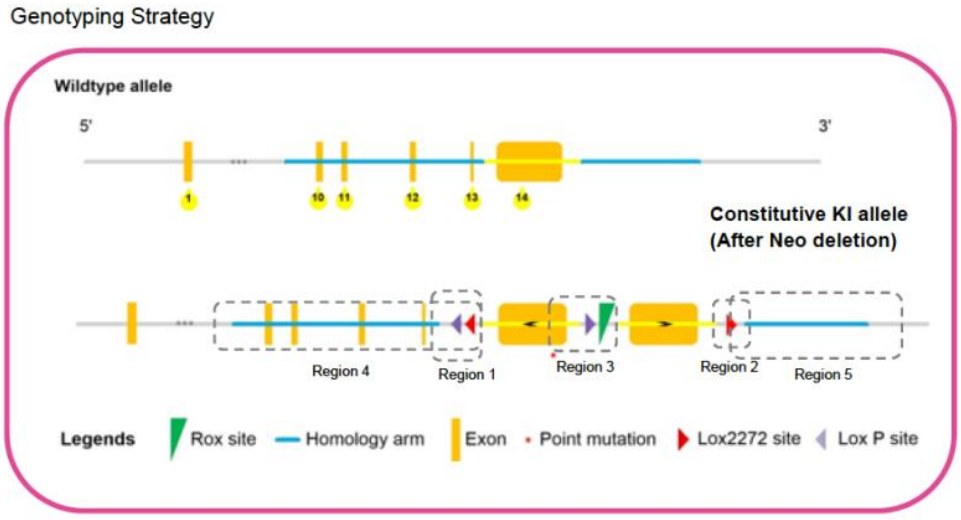


Figure 13: Genotypyng strategy of rad21A626T conditional KI mice. Outline of the modification introduced to insert the mutant allele in the genomic region of the rad21 gene, with the LoxP sites for inducible expression (Cre crossing).

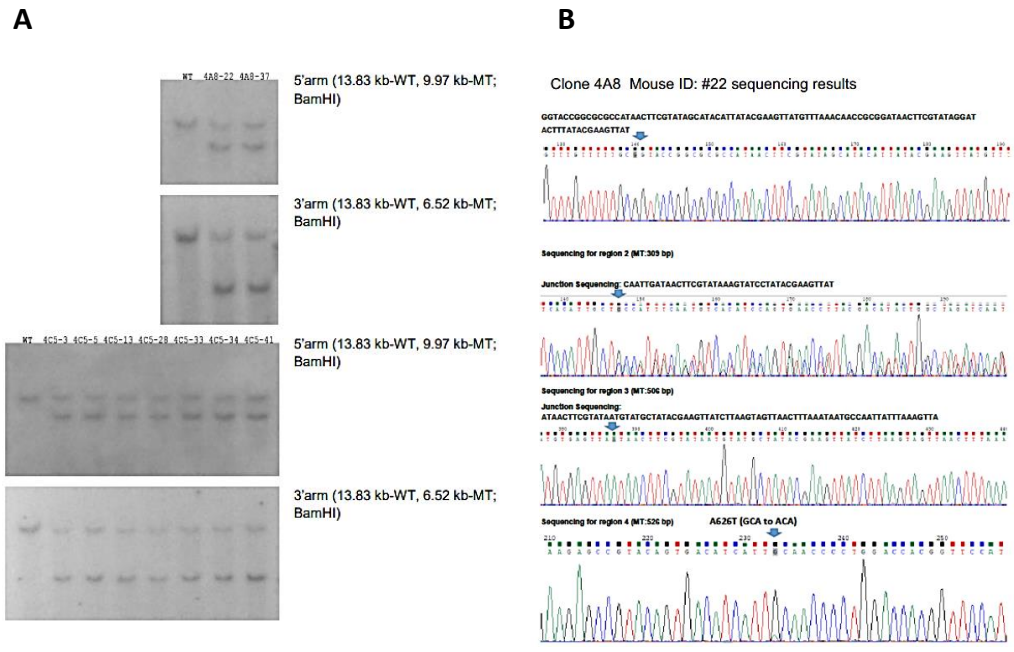


Figure 14: Example of the sequence electropherogram profiles of different regions (A) illustration of sequencing analysis. (B) DNA samples was confirmed correct by southern blotting.

Mouse genotyping

Mice were genotyped by PCR using genomic DNA from mouse tail. A small, minced tail biopsy (±2 mm) was treated with hot shot lysis buffer (25 NaOH, 0.2 mM Na2EDTAx2H2O) 1 hour at 99° C then 5 minutes on ice and lysates were collected by centrifugation at 16000xg 1 minute. 1 volume of neutralizing buffer (40 mM Tris Acid) was added, and DNA samples stored at -20°C until genotyped. 1µL of DNA was run on a 1% agarose gel for quality check. Rad21 Ala626Thr is a complex construct. Recent generations indicated that this was transmitted intact. However, we genotyped for 5 regions of the construct to remove mice with unwanted rearrangements.

Genotyping PCR was performed for the different regions using PCR conditions and PCR primers as indicated in the Supplementary material. Regions 1, 2, 3, Wnt1 and Wnt-internal control region were amplified using the KAPA HotStart Extra Mastermix 2X (Roche, Basel, Switzerland), according to Bonora et al, 2021 [287]. Regions 4 and 5 were genotyped using the Platinum Superfi Mastermix 2X (Thermo Fisher, Waltham, Massachusetts, United States).

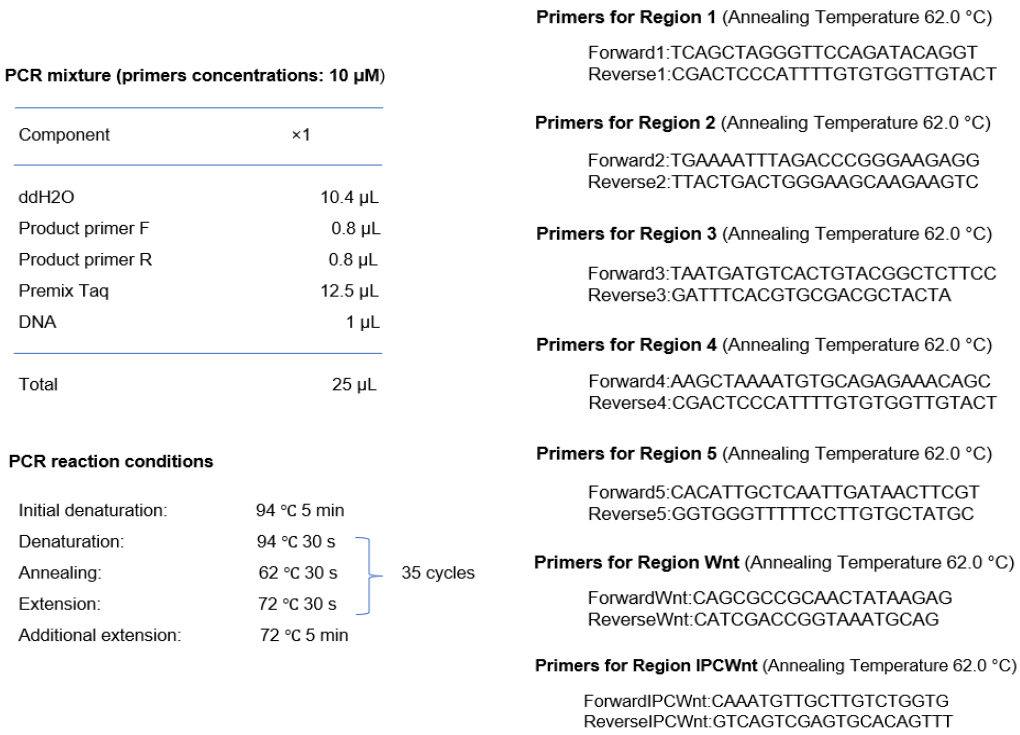


Figure 15: Supplementary material: PCR mixture/ conditions and primers for all regions used to specifically genotyping new construct all regions.

Carmine red dye assay

Total GI transit time was measured as previously described [288]. Rad21KI^{A626T} and WT animals were placed individually in cages devoid of bedding, and after fasting for 1 hour, 0.3 ml of 6% (w/v) carmine red dye solution (Sigma-Aldrich) in 0.5% (w/v) methylcellulose (Sigma-Aldrich) was administered to each mouse by oral gavage. The time period from gavage until the emergence of the first red-colored pellet was recorded as total intestinal transit time. Average total weight per stool was measured by placing individual animals in a clean cage and collecting all stools produced over a 1-hour period (9-10 am). Stools were desiccated at 75°C 16 hours to determine their dry weight. Water content per stool was assessed as the difference between wet and dry weight and expressed as a percentage. Intestinal transit time (n=11 Rad21KI; n=9 WT) was measured as the arrival time of coloured faeces after oral administration of a carmine red solution. The time period from gavage until the emergence of the first red-colored pellet was recorded as total intestinal transit time. Average stool weight was measured by placing animals individually in clean cages and collecting all stools produced over a 1-hour period (9- 10 a.m.). Stools was desiccated at 75°C 16 hours to determine their dry weight. Water content per stool was assessed as the difference between wet and dry weight and expressed as a percentage.

Intestinal contractile activity

For the measurement of isometric muscle contraction in isolated organ bath chamber, segments with the length of approximately 5-6 cm of the proximal colon of Rad21KI^{A626T} and WT mice were removed and immersed in modified Krebs-Henseleit Buffer (KHB, in mmol/L: 117.0 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 11.0 Glucose, 12.5 $NaHCO_3$, 15.0 Mannitol, pH 7.4, with constant 95% O_2 and 5% CO_2 oxigenation). Luminal contents was emptied from the preparations by gentle flushing with KHB and eight smooth muscle strips of equal size and weight (0.5-1.0 cm; 0.02 ± 0.001 g wet tissue) were prepared (these strips contained both muscle layers and MP in between).

After mucosa removal, full thickness smooth muscle strips were cut tangentially to the circular smooth muscle (CSM) (hence, CSM strips). The CSM strips were placed into an organ bath containing 12 mL KHB and connected to isometric force transducers (HBM) in order to record from the circular muscle. The initial tension of the smooth muscle strips was adjusted to 2 g. Contractile performance of CSM strips was registered using a Spider8 chart recorder (4.8 kHz/DC; HBM) combined with Catman Easy software (version 1.01; HBM). Isometric contractile performance of CSM strips, as assessed by frequency (F; Peaks/min), amplitude (A; mN) and mean active force (MAF; mN) of contractions, was defined as CSM strip contractility. Frequency, amplitude and mean active force of contractions were calculated for 2 min evaluation periods at regular intervals of 15 min. Active force of contractions, the arithmetic mean of all values describing the curve during an evaluation period, was assessed and data corrected by baseline values. Electrical force stimulation (EFS) was applied via two platinum electrodes placed into each chambers side by side with the tissue at 30 V, 10 Hz, 0,5 ms, 10 s, 250Ω at regular 15 min intervals.

Immunohistochemistry

Guts were exteriorized for tissue collecting and segments of the small (duodenum, jejunum and ileum) and large bowel were gently removed, opened along the mesenteric border, and vigorously flushed out with PBS and pinned on Sylgard®-coated Petri dish with the mucosal surface facing down and microdissected under a stereomicroscope (Leica S6E, Leica Microsystems, Italy) in order to obtain myenteric whole-mounts, specimens were subsequently fixed in 2% Zamboni fixative containing 0,2 M phosphate-buffered saline, 37% formaldehyde and 1,2% pitric acid, and stored overnight at 4 °C. Whole-mounts preparations were incubated in 10% normal donkey serum (Colorado Serum Co., Denver, CO, USA) in 1X PBS containing 0.3% Triton X-100 for 1 h at room temperature to reduce nonspecific binding of secondary antibodies and

permeabilize the tissue to the antisera. Preparations were then incubated at 4 °C in a humid chamber for 48 h in primary antibodies (Table 3). Labeled neurons were quantified in the small as well as large bowel of Rad21^{A626T}KI *vs* WT mice. After washing in PBS, the preparations were incubated for 1 h at room temperature in a humid chamber in a mixture of opportunely combined secondary antibodies indicated in Table 3 (fluorescein isothiocyanate (FITC)-conjugated and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated, Alexa Fluor 594-coniugated and Alexa Fluor 488-coniugated). Finally, after three more washes in PBS, preparations were mounted with SlowFade w/DAPI (Thermo Fisher Scientific). Microphotographs of 6 randomly fields small and large bowel whole-mount preparations were acquired with Nikon DS-Qi1Nc digital camera at 20× magnification, using NIS Elements software BR 4.20.01 (Nikon Instruments Europe BV, Amsterdam, The Netherlands). Fluorescent cell count was performed with NIS Elements software BR 4.20.01.

To quantify synaptophysin immunofluorescence five randomly selected fields per sample were acquired with a Nikon Ti-E fluorescence microscope coupled to an A1R confocal system and the NIS-Elements AR v.3.2 software. Images were acquired with oil immersion (x60) with an optical resolution of 0.18 micron, 3x3 scanner zoom, and 1024 x 1024 pixel resolution. 3D images were analyzed by the Imaris software (Bitplane, Belfast, United Kingdom). The software analyzes the volumes of all detected surfaces and calculates the mean value corresponding to the mean volume of interconnected synaptic buds per cell. This analysis allows measurement of the mean volume of single interconnected cell detected by DAPI directly linked to synaptic buds [289].

Table 3. List of primary and secondary antibodies used in this study.
Note: ChAT, choline acetylcholinesterase; nNOS, neuronal nitric oxide synthase

Primary Antibodies	Supplier	Host species	Dilution
HuC/D	A-21271 ThermoFisher Scientific	Mouse	1:200
Synaptophysin	Dako A0010	Rabbit	1:250
ChAT	Millipore Ab144P	Goat	1:100
nNOS	Novus NB100-8580	Goat	1:400
Secondary Antibodies			
AlexaFluor 594 anti-goat IgG	Jackson #705-605-147	Donkey	1:800
AlexaFluor 488 anti-rabbit IgG	Jackson #AB150073	Donkey	1:1000
AlexaFluor 488 anti-mouse IgG	Jackson #715-165-150	Donkey	1:1000

Statistics

The data are expressed as means ± SEM for n of animals or preparations. Statistical significance was determined by GraphPad Prism software application (version 6.0c, GraphPad Software, La Jolla, CA) using Student's t-test with Mann-Whitney test. P values of less than 0.05 are considered statistically significant. The sample size (N) of animals to be used was determined assuming a statistical power of 80% in detecting statistically significant differences with a significance level of 0.05.

Results

Phenotypic analysis of Rad21KI^{A626T} model

Intestinal transit time, determined as the time interval between marker infusion and the discharge of the first red coloured faecal pellet, significantly increased in Rad21KI^{A626T} compared with WT (260.5 ± 20.85 *vs.* 190 ±12.25; P≤0.005). Moreover, Rad21KI^{A626T} displayed no differences regarding wet and dry weight expressed as a percentage and the number of fecal pellets (Figure 15).

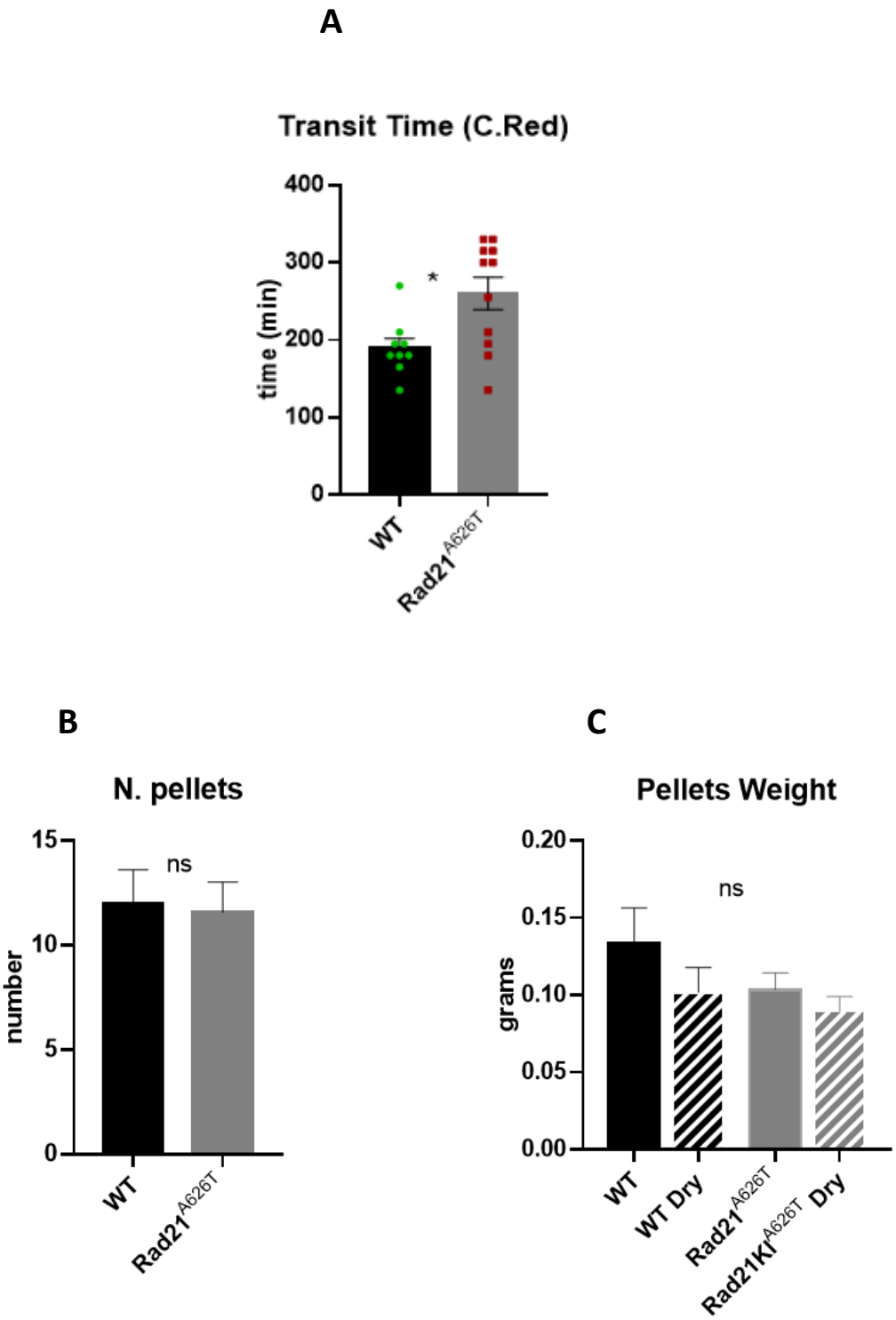
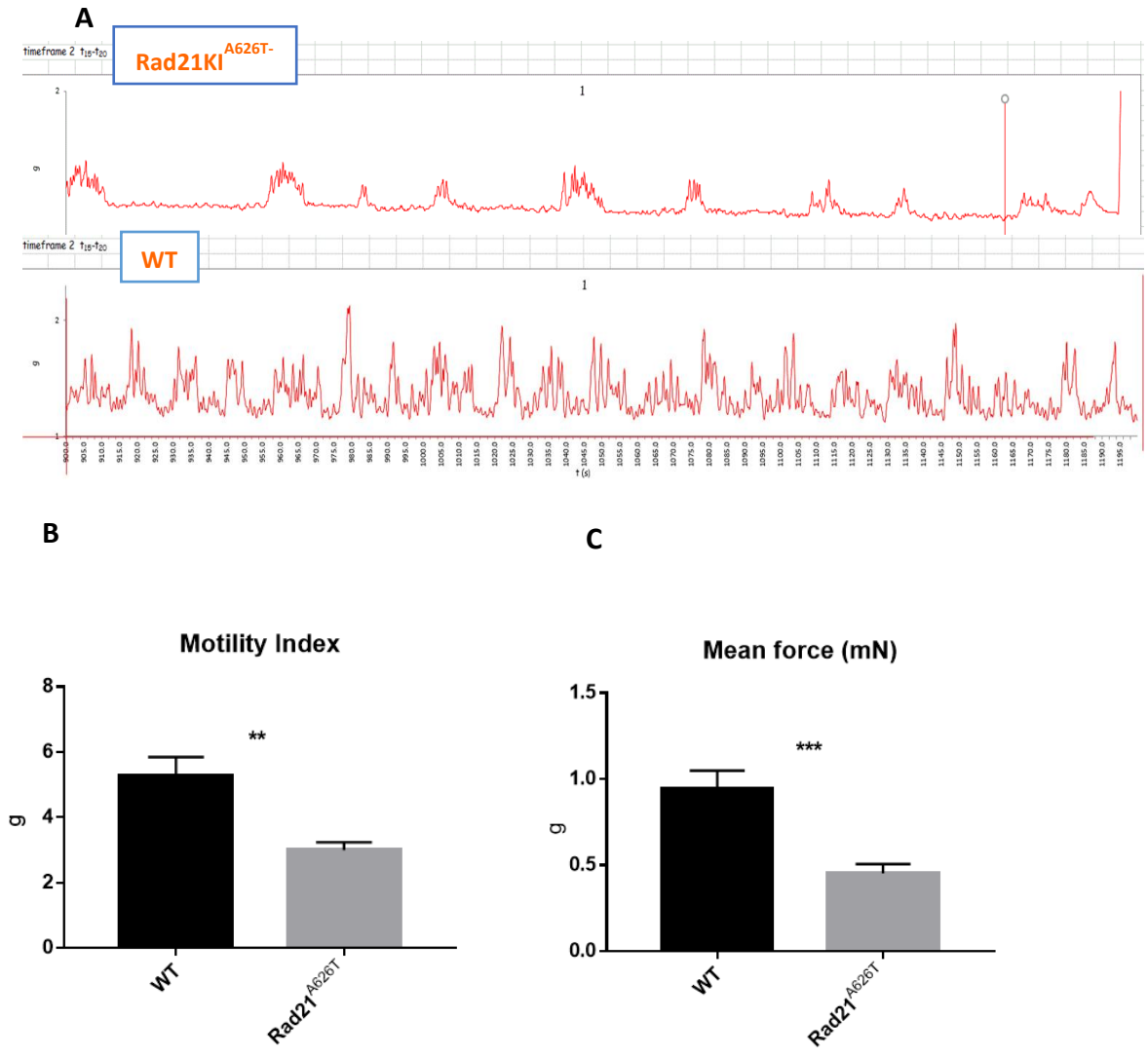


Figure 15: Intestinal transit time. In vivo study using Carmine red dye showed an increase of intestinal transit time in Rad21KI^{A626T} compared to WT adult mice (n=6; P ≤ 0.05) (A). Graph showing no statistical differences (ns) in total number of faecal pellets (B) and wet and dry weight pellets (C).

Evaluation of the Intestinal muscle contractility

Periodical rhythmic myogenic contractions were recorded in the circular smooth muscle of proximal colon preparations. The mean motility index and mean active force of these contractions was significantly decreased in Rad21KI^{A626T} *vs* WT in within 30 minutes interval of isometric force measurement (3±0.23 *vs* 5.26±0.58.; P≤0.005) (0.45±0.05 *vs.* 0.94±0.1; P≤0.005) (Figure 16). Considering the neuro mediated muscle activity, no statistical difference was evident in terms of impulse and amplitude between the wild-type and mutant mice (P= 0.3095) (P= 0.3939).



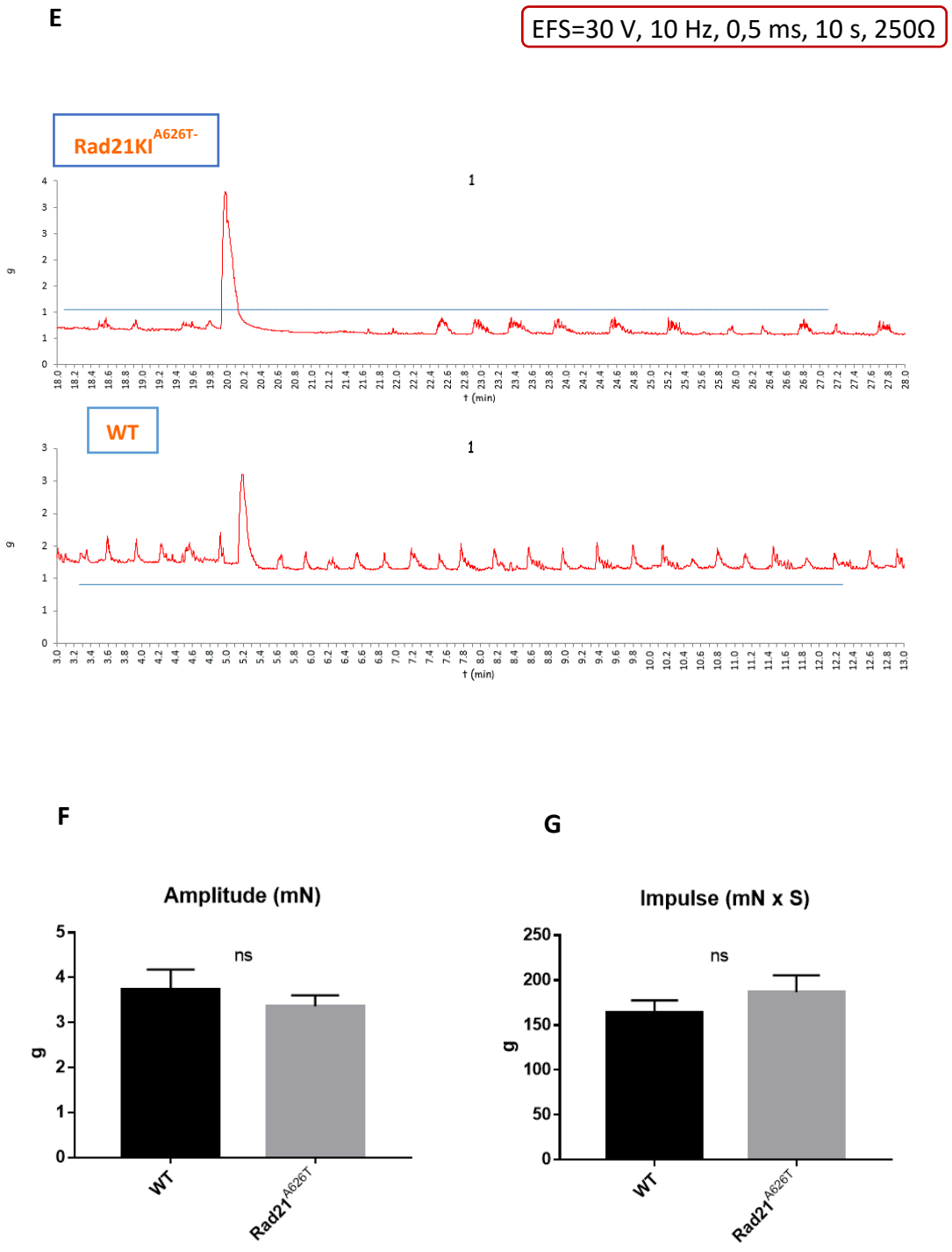


Figure 16. Large intestinal myogenic motility assay in WT *vs.* Rad21KI^{A626T} (A). Motility index (B), and mean force of contractions (C), of proximal colon adult mice tissues (n=6). *Value differs significantly (*P ≤ 0.05; Student's t-test followed by a Mann-Whitney test). Large intestinal nerve-mediated motility assay in WT *vs.* Rad21KI^{A626T} (E). Graph showing no statistical differences (ns) in amplitude (F), and impulse (G) after electrical stimulation of proximal colon adult mice tissues (n=6).

Qualitative and quantitative analysis of neurons in Rad21KIA626T

Whole-mount analyses were conducted in order to determine the total number of HuC/D and HuC/DnNOS-IR myenteric neurons. We performed HuC/D and nNOS double labeling immunohistochemical analysis of adult C57BL/6 mice (n = 6; age range: 4-5 months). The total number of HuC/D and HuC/D/nNOS-IR myenteric neurons did not significantly change in the small and large intestine of Rad21KI^{A626T} *vs* WT (figure 17). However, in the small intestine of Rad21KI^{A626T} we showed that HuC/D/ChAT IR myenteric neurons/field were significantly less than in WT mice (18.9 ± 1.4 *vs.* 32.4 ± 2, P≤0.005, Figure 16); likewise, in the large intestine HuC/D/ChAT immunoreactive myenteric neurons/field were 19.8 ± 2.1 in Rad21KI^{A626T} *vs.* 32.1 ± 3.8 in WT mice (P≤0.005, Figure 18).

Qualitative and quantitative analysis of synaptophysin in Rad21KI^{A626T}

Confocal images from different layers were processed with IMARIS software (see "Materials and Methods") to detect the "puncta" and establish the synapse density (number of synapses per unit area). The synaptic vesicle analysis showed a significant reduction of Syn immunoreactive in small and large intestine of Rad21KI^{A626T} *vs.* WT mice (22.12 ± 1.65 *vs.* 34.11 ± 2.07, P≤0.005) (22.53 ± 3.31 *vs.* 36.54 ± 3.54; P≤0.005, Figure 19).

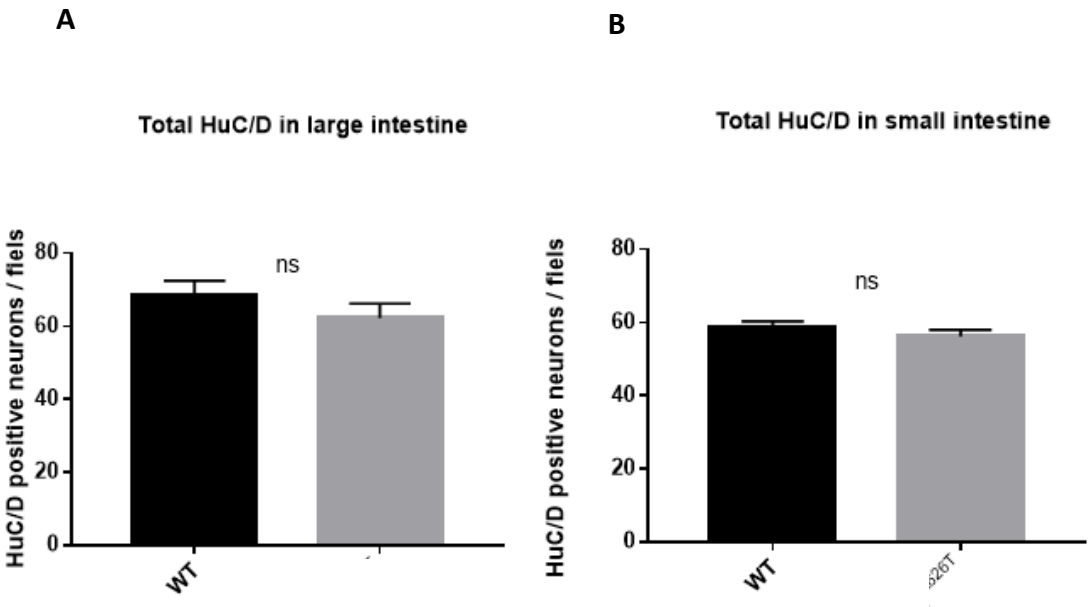
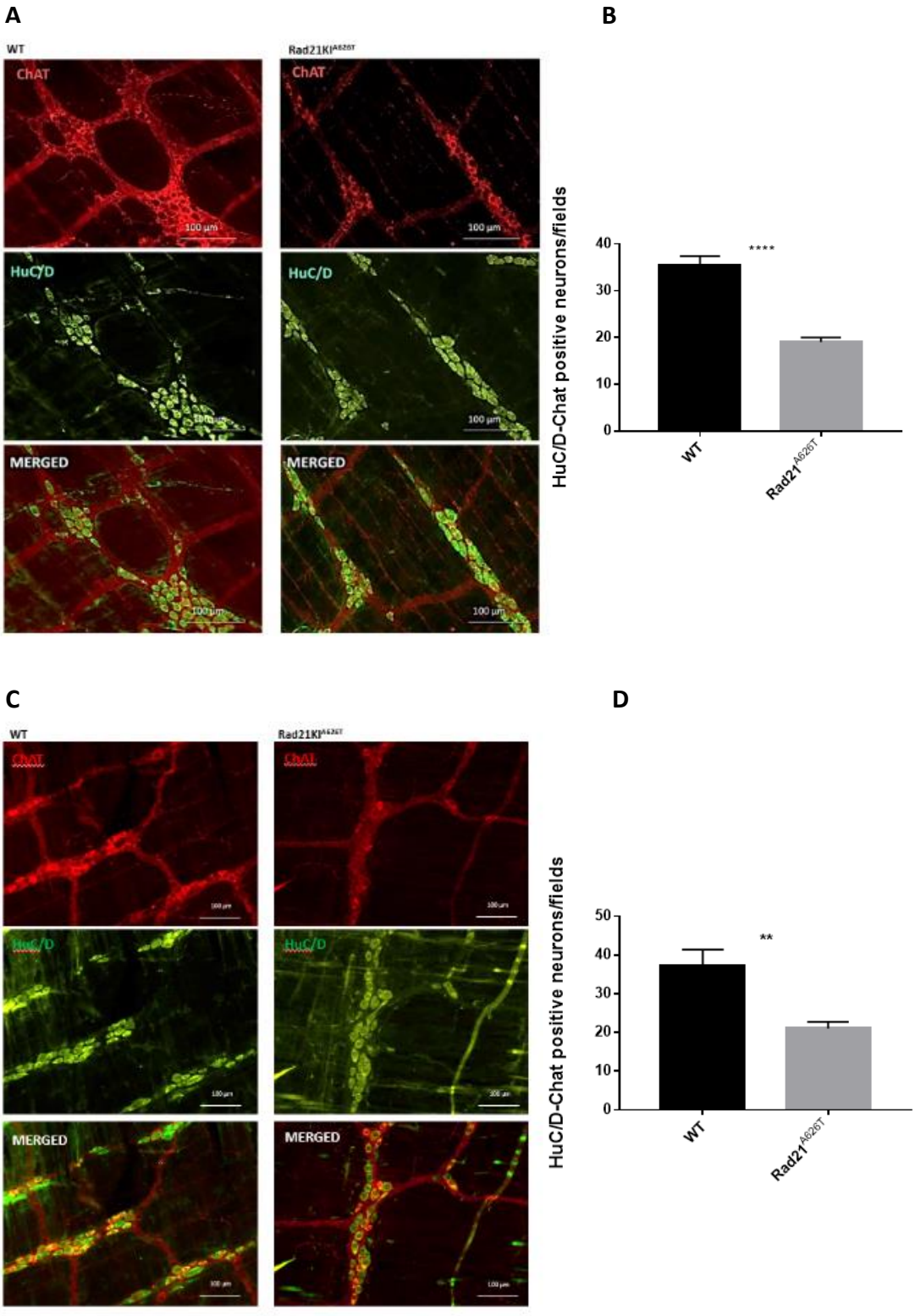


Figure 17: Quantitative analysis of total HuC/D in the large and small intestine of Rad21^{A626T} conditional KI and WT mice.

Graph showing no statistical differences (ns) in total number of HuC/D neurons of Rad21^{A626T} *vs.* WT in large intest ine (A) and small intestine (B).


A
B
WT
Rad21KI^A626T
ChAT
HuC/D
MERGED
100 µm
HuC/D-Chat positive neurons/fields
****
WT
Rad21^A626T
C
D
**

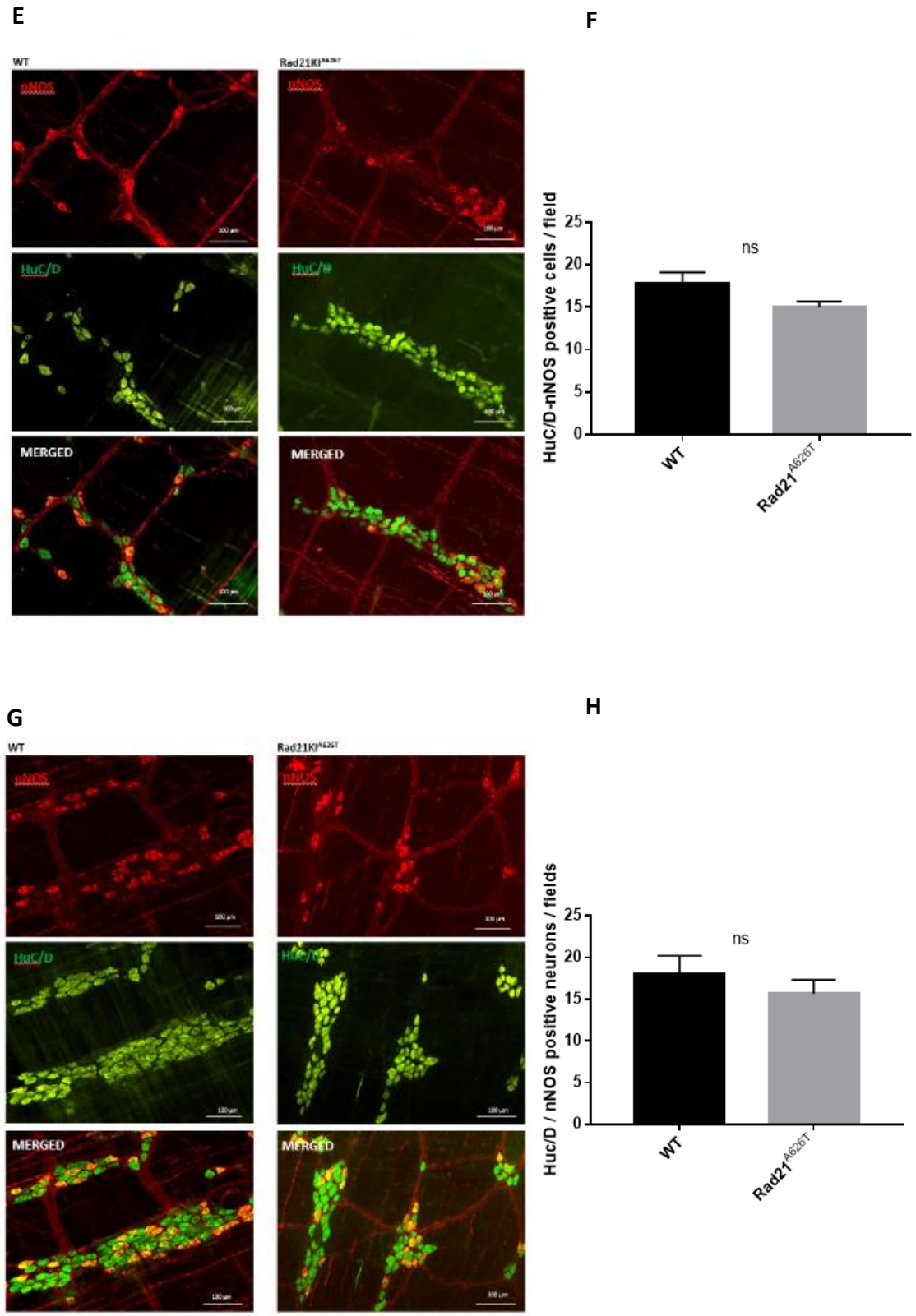

E
F
WT
Rad21KI
nNOS
HuC/D
MERGED
HuC/D-nNOS positive cells / field
ns
WT
Rad21A626T
G
H
Huc/D / nNOS positive neurons / fields

Figure 18: Analysis of neuronal markers in Rad21^{A626T} conditional KI and WT mouse small and large intestine (whole-mounts). (A) Immunolabeling of HuC/D (green) and ChAT (red) in whole-mounts from adult WT and Rad21^{A626T} mouse small intestine. The HuC/D/ChAT-IR neurons/field in the MP of Rad21^{A626T} were significantly lower compared to WT (18.9 ± 1.4 vs. 32.4 ± 2) (n=6 mice; P<0.0001, t-test). Scale bars:100 µm. **(B)** Graph showing the number HuC/D/ChAT-IR neurons/field in the small intestine of adult Rad21^{A626T} *vs.* WT mice (n=6; P < 0.05). **(C)** Immunolabeling of HuC/D and ChAT in whole-mounts of adult WT and Rad21^{A626T} mouse large intestine. The HuC/D/ChAT-IR neurons/field in the myenteric plexus of Rad21^{A626T} were significantly lower compared to WT (19.8 ± 2.1 vs. 32.1 ± 3.8) (n=6, P=0.048, t-test). Scale bars: 100µm. **(D)** Graph showing the number of HuC/D/ChAT neurons/field in large intestine of adult Rad21^{A626T} *vs.* WT mice (n=6; P< 0.05). **(E)** Double labeling of HuC/D (green) and nNOS (red) in Rad21^{A626T} conditional KI and WT mouse small and large intestine (whole-mounts). WT and Rad21^{A626T} mouse small intestine displayed HuC/D/nNOS neurons. Scale bars: 100µm. **(F)** Graph showing no statistical differences (ns) in HuC/D/nNOS neurons of Rad21^{A626T} *vs.* WT small intestine. **(G)** Also, HuC/D/nNOS positive neurons were readily visualized both in Rad21^{A626T} and WT mouse large intestine. Scale bars: 100 µm. **(H)** Graph showing no statistical differences (ns) in HuC/D/nNOS neurons of Rad21^{A626T} *vs.* WT large intestine. **(I, J)**

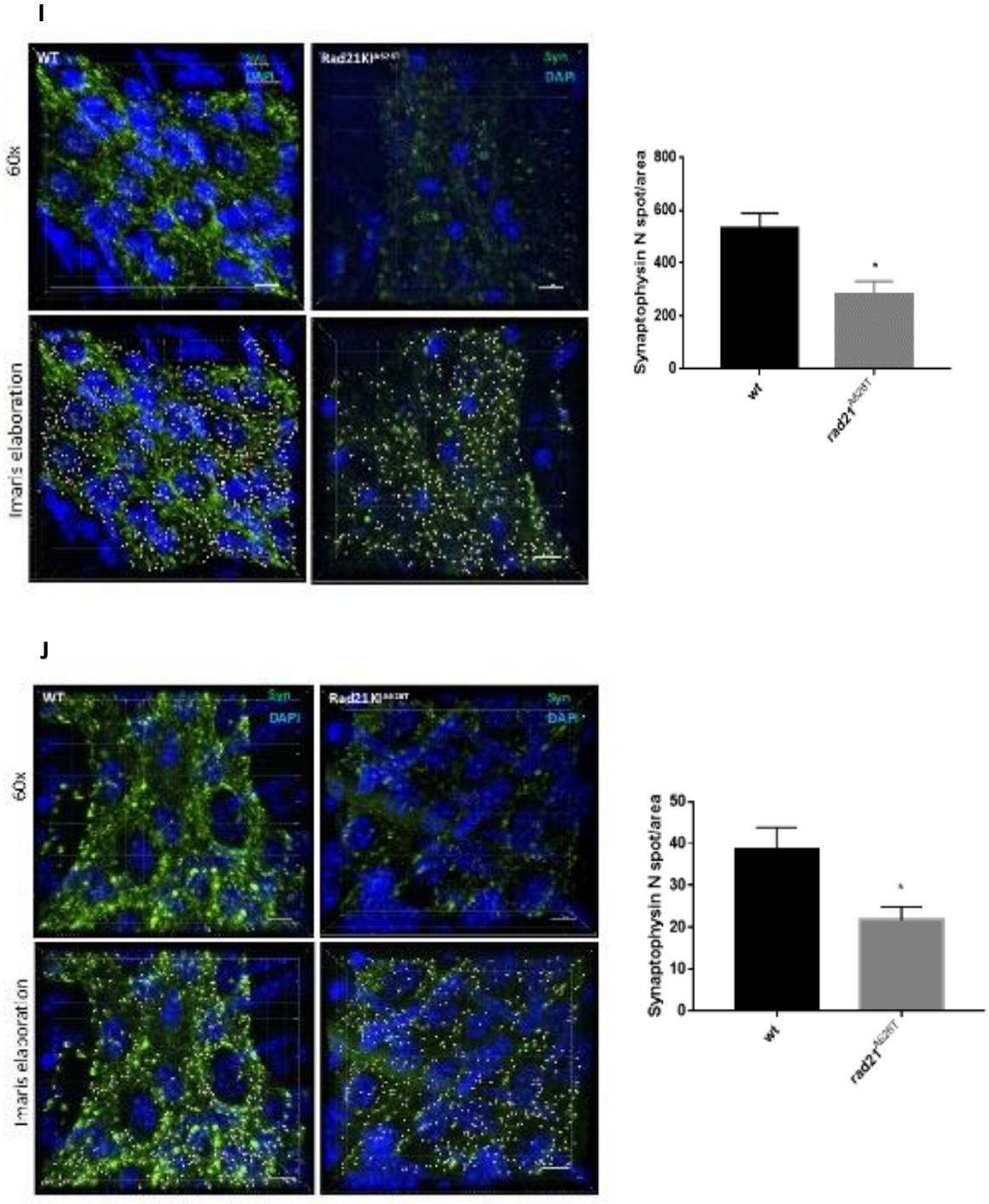


Figure 19: Qualitative and quantitative analysis of synaptophysin (Syn) in the small and large intestine of Rad21^{A626T} conditional KI mouse. Imaris software elaboration showed a reduction of Syn-IR in Rad21^{A626T} *vs*. WT in the small (P=0.012) and large intestine (P=0.011). Scale bars: 7 µm

Discussion

The main objective of this thesis was a deep characterization of the conditional Rad21KI^{A626T} mouse model in order to recapitulate the functional impairment caused by the RAD21 missense mutation in humans, to elucidate the CIPO-related pathophysiology and to fully understand the relative contribution of RAD21 in terms of pathophysiological impact and altered molecular pathways. Our group previous identified a novel homozygous mutation in a recessive form of CIPO, the damaging missense variant p.Ala622Thr in RAD21. RAD21 regulates different cell mechanisms as part of the cohesin, a multiprotein complex crucial for chromatin folding and transcriptional regulation. In order to study how the RAD21 mutation affects ENS functionality we developed a conditional KI mouse model (Rad21A626T) via Wnt-driven expression of Cre allowing the Rad21 missense mutation to be expressed in enteric neuronal lineages. This new KI mouse was engineered to reproduce the abnormalities identified in RAD21-mutated CIPO patients.

To our knowledge, the Rad21KI^{A626T} mouse is currently the only available model for investigating one peculiar form of CIPO, while representing a valuable tool to design *ad hoc* therapies of a still orphan disorder. Heterozygous and homozygous mice were identified by genotyping with PCR the Rad21 and the Wnt-Cre regions. Homozygous mice were viable and fertile, and we first investigated any GI-related defects in the adult animals.

Rad21 functional ablation in zebrafish generated a severe impairment of intestinal motility, with a significant depletion of enteric neurons in the gut of embryos at 5 days post fertilization (dpf), reminiscent of the CIPO phenotype observed in the patients with the RAD21 mutation. This latter finding is reminiscent of an enteric neuronal hypoganglionic phenotype observed in heterozygous retC620R/+ mice and shares similarities with the histopathology of some CIPO patients [267,290]. Full-thickness specimens of patients with CIPO showed that clinical manifestations and severity increase as the enteric neuron

number decreases. Compared to control tissues, a 50% loss of neuronal cells in the myenteric and submucosal ganglia may be a “critical threshold” to the occurrence of clinical manifestations [274].

Based on this data, we first assessed GI function in these animals, in terms of total GI transit time (by gavage of a carmine-red solution according to Nagakura et al., 1996), stool production (i.e. pellet output). Thus, we measured the total GI transit time by feeding a carmine-red solution to fasted animals, then assessing the time of retrieval of the coloured pellets and finally weighing and counting the number of pellets produced by each animal. Carmine red dye assay showed an increase of intestinal transit time in Rad21KI^{A626T} compared to WT, consistent with the CIPO phenotype. The number of pellets produced and the weight of the pellets with and without water content (indicated as dry pellet) were also evaluated during the study. Although the number of the fecal pellet did not show any statistical difference, the weight of dry pellet in Rad21KI^{A626T} *vs* WT displayed a small decreasing trend, but without statical evidence.

In parallel, since both excitatory and inhibitory enteric neurons are essential to generate and maintain propagating colonic motility, we performed an *ex vivo* analysis of the large intestinal myogenic and nerve-mediated motility (Nakamori et al 2021). To investigate neuro-muscular interaction in the intestine, we carried out measurement of isometric muscle contraction in organ bath chambers of the proximal colon of Rad21KI^{A626T} compared to the same preparations obtained from WT mice.

Smooth muscles have spontaneous contractions that can be measured based on of their motility index and mean active force. Analysis of myogenic contractility displayed a significant decrease of motility index and mean active force in proximal colon of Rad21KI^{A626T} comparing with WT mice.

This reduced myogenic motility together with the slowed intestinal transit in Rad21KI^{A626T} indicates that the mutation in Rad21 is impairing the GI functions.

To evaluate the nerve mediated motility, each tissue was electrical stimulated at regular intervals, leading to excitation of myenteric neurons with release of

neurotransmitters and consequently generation of muscle contractions. The electrical activity was evaluated in terms of amplitude and impulse and the peaks evocated after electrical field stimulation. Here, the mutant mice displayed no alterations compared to the wild-type animals, indicating no association between the mouse carrying A626T mutation and a possible alteration of nerve-mediated motility.

Another important point of this thesis was aimed at providing a characterization of the enteric neurological profile in Rad21KI^{A626T} vs. WT mice. Our results showed that HuC/D/ChAT (cholinergic) and HuC/D/nNOS (nitrergic) subclasses of neurons are expressed in Rad21KI^{A626T} mouse model. However, cholinergic, but not nitrergic myenteric neurons, were significantly reduced in the small and large intestine of Rad21KI^{A626T} compared to WT. The reduction of specific excitatory neuronal subsets suggests that deficit in this type of enteric neurons can contribute to the impairment of motility in CIPO patients with the RAD21 mutation. These data suggest significant changes in the neuronal type composition of the ENS in adult Rad21A626T mice. This is also in line with the experimental data obtained from *in-vivo* study, where we observed a slow transit in Rad21KI^{A626T} *vs* WT, followed by functional analysis, with an impairment of myogenic motility.

Considering that alterations in the number of neurons have been associated with the "synaptic dysfunction" observed in some mice models [291], we investigated synaptic connectivity in the intestinal whole-mounts preparation by immunohistochemistry and 3D morphometry. A significant reduction of synaptophysin immunostaining was observed in the small and large intestine of Rad21KI^{A626T} *vs.* WT mice, indicating a possible alteration of synaptic development / differentiation. Our previous data showed a decreased RUNX1 expression in homozygous RAD21 mutated CIPO patients and *in vivo* in zebrafish model [278]. The transcriptional downregulation of RUNX1, which controls neuronal subtype specification and axonal connectivity [292], argues in favour of a differentiation defect.

Taken together, these data support the basis that RAD21 mutation affects the ENS leading to the CIPO phenotype.

CONCLUSIONS

6. CONCLUSIONS

The ENS serves a vast array of regulatory functions through which the physiology of the GI tract occurs to preserve body homeostasis and ultimately life. Mounting evidence clearly indicates that the ENS is reminiscent of the CNS organization, not only for the neuro-bioelectrical properties and function, but also for its neurochemical profile and receptor expression, which provide a number of possible targets of therapeutic intervention. Thus, it should not be surprising that changes altering such a finely tuned and highly integrated system, such as the ENS, can lead to neuro-glia abnormalities and loss, thereby ensuing gut dysfunction. In this thesis, I tackled some aspects of enteric neuropathies particularly focusing on genetically driven molecular abnormalities. A clinical phenotype of enteric neuropathy is given by CIPO a severe dysmotility characterized by a markedly compromised quality of life and poor prognosis for affected patients. Notably, there is no effective treatment for this condition and from a pathophysiological standpoint there are no established animal models for studying the mechanisms contributing to neuropathy often underlying CIPO. The discovery of new genes in patients with enteric neuropathies and related CIPO represents a translational strategy aimed at better understanding the mechanisms leading to GI dysfunction and symptom generation. In CIPO, a rare and poorly investigated condition, thorough genetic analyses are expected to help developing novel therapeutic targets for affected patients. The lack of models for studying the physio-pathological processes of enteric neuropathies may account for the relatively slow progress in the management of CIPO and the minimally effective treatment options for these difficult cases. My first research work during this PhD program helped generating initial evidence that functional analysis is a crucial approach to highlight the molecular pathways involved in ENS morpho-functional changes, hence enteric neuropathy, and severe gut dysmotility. As it happens for CNS disorders, the technological advances in genomics, molecular phenotyping and regenerative medicine (using pluripotent

stem cells as possible treatment options) will represent the beginning of a new era in ENS disorders and related functional GI impairment.

In this line, the second study had as its main goal to perform a thorough research to understand the molecular cascades leading to the dysregulated ENS as a reliable model to gut dysfunction in patients with RAD21 mutation.

Furthermore, we developed and characterized a mouse model ($Rad21^{A626T}$) carrying the CIPO-specific variant p.Ala626Thr (homologous of the p.Ala622Thr in humans), in order to identify whether the genetically determined model reproduces the gut dysmotility in terms of GI transit and functional (i.e., electrophysiological) alterations.

Quantitative immunohistochemical results showed that HuC/D/ChAT (cholinergic), but not those expressing nitric oxide synthase (NOS, marker for inhibitory neurons), myenteric neurons were significantly reduced in mutant *vs.* wild type (WT) mice in the small intestine and colon. We also observed a significant reduction in synaptophysin-IR throughout the gut, indicating a decreased synaptic connectivity in $Rad21KI^{A626T}$ *vs.* WT mice suggesting a decreased connectivity. The reduction of excitatory neurons in $Rad21KI^{A622T}$ mice provides a solid basis to understand the involvement of this gene alteration in gut motility dysfunction including CIPO.

From functional analyzes it emerged an increase of intestinal transit time, therefore gut transit delay, accompanied by a decrease of myogenic motility, expressed as motility index and mean active force. These data suggest a correlation between the reduced number of excitatory / cholinergic neurons and the reduced intestinal transit.

In addition, taken together, these findings provide an accurate neurochemical basis to understand the neuropathic features of the RAD21-related CIPO patients. The mechanisms through which individual subsets of cholinergic myenteric neurons are affected in distinct gut segments of the $Rad21KI^{A626T}$ model require further analysis. It remains to be clarified and defined how the

cholinergic system appears to be primarily involved, and to establish when the defect arises during ENS development.

One of the future prospective will be to characterize the cell lineages and molecular cascades of Rad21KI^{A626T} to provide mechanistic insight into the pathogenesis of RAD21-mutated CIPO and identify potential targets for future therapeutic intervention.

In conclusion, changes to the neuro-functional aspects driven by genetic factors in the context of a severe dysmotility, i.e. CIPO, in an animal model emerge as new important and fascinating topics fuelling the interest of researchers in translational neurogastroenterology.

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ABSTRACT

Aquaculture is increasing the global supply of foods. The species selected and feeds used affects the nutrients available from aquaculture, with a need to improve feed efficiency, both for economic and environmental reasons, but this will require novel innovative approaches. Progress in the development of novel and sustainable aquaculture feeds to reduce reliance on wild fisheries, feed fortification to increase nutrient content, and expansion of the diversity of aquatic species produced are key areas for continued research and development. Ultimately, the degree to which aquaculture will contribute to nutrition depends largely on who can access the fish produced, which will be shaped by production technology as well as trade and price dynamics.

Nutritional strategies focused on the use of botanicals as modulators of several physiological responses and health promoters of the gastrointestinal tract have attracted interest in animal production. Previous research indicates the positive results of using essential oils (EOs) as natural feed additives for several farmed animals. In the last decades, these nutritional alternatives have been evaluated and reported in fish production in order to increase fish growth and feed utilization and to promote animal welfare. The contribution of aquaculture expansion to improving nutrition will be bounded by aquaculture's environmental sustainability. Therefore, the present study was designed to compare the effects of feed EO supplementation in two different forms (natural and composed of active ingredients obtained by synthesis) on the gastric mucosa in European sea bass. EOs decrease oxyntopeptic cells and increase somatostatin and ghrelin enteroendocrine cells. In addition, we showed that Na^+K^+-ATPase was expressed in oxyntopeptic cells (OPs) in the same way as H^+K^+-ATPase (typical marker for mammalian parietal cells) and, for this reason, consider Na^+K^+-ATPase a valid marker for OPs.

LIST OF ABBREVIATIONS

CTR Control diet

EOs Essential oils

EECs Enteroendocrine cells

GHR Ghrelin

GPCRs G protein-coupled receptors

OPs Oxyntopeptic cells

PYY Pancreatic polypeptide:

PPSS Pre-pro somatostatin

SOM Somatostatin

FI Feed intake

FCR Feed conversion rate

GI Gastrointestinal

IR Immunoreactive

N-EOs Natural EOs

PPSS Pre-pro somatostatin

ROI Region of interest

RAS Recirculating aquaculture system

RT Room temperature

S-EOs Synthesis

SGR Specific growth rate

SD Standard deviation

TRP Transient receptor potential

INTRODUCTION

Chapter I

1. The European Sea Bass background and biology

The European Sea Bass, a member of the Moronidae family (*Dicentrarchus labrax* L.), is a food fish, considered one of the first models for the intensive breeding in salt water. It is a coastal marine fish that lives in shallow waters (<100 m) from the north-eastern Atlantic Ocean to the Mediterranean and the Black Sea. The sea bass is euryhaline (0–40 ppt salinity) and eurythermal (2–32 °C) and is often found foraging in estuaries and lagoons from spring to fall, especially at the juvenile stage. During winter, juvenile and adult sea bass migrate from the coastline to deeper waters, where the temperature is more stable, as they prefer temperatures above 9–10 °C [1]. The sea bass normally matures at two to three years of age for males, and three to four years of age for females [2]. Reproduction happens in groups, between December and March in the Mediterranean and between March and June in the Atlantic. Females spawn an average of 200 000 eggs/kg, which are fertilized externally. Eggs hatch after three to five days, and larvae (4 mm at hatching) reach the post-larval stage (> 22 mm) in two to three months while migrating to inshore nursery areas and lagoons. The European sea bass is an opportunistic predator, feeding on plankton at the larval stage and on fish and crustaceans at the juvenile and adult stages.

From an evolutionary standpoint the Teleosts are relatively modern fish, appearing in the fossil record at the beginning of the Triassic period, around 250 million years ago [3]. Of the currently extant species, approximately 58% are exclusively marine spending their entire life in seawater, with 41% considered stenohaline freshwater species. A small minority (~1%) are capable of adapting to a range of salinities, and described as euryhaline[3]. The latter includes estuarine Teleosts that will experience frequent changes in salinity, such as the killifish (*Fundulus heteroclitus*), as well as those that undergo a more intermittent metamorphosis for spawning migrations into freshwater (anadromous species,

e.g. Atlantic salmon, Salmo salar) or seawater (catadromous species, e.g. European eel, *Anguilla anguilla*).

Chapter II

2. Anatomy and physiology of gastrointestinal tract

Gastroenterology in Osteichthyes, as defined bony fishes, which comprise most species of veterinary concern, such as salmon, koi, and catfish, and in Chondrichthyes continues to be a growing area for research and new clinical treatments.

Fish comprise the three largest extant classes of vertebrates, and, given the great diversity across these classes, comparative similarities and differences in the piscine gastrointestinal tract are highlighted. For veterinarians to better understand aquatic animal health, the most basic veterinary foundation begins with understanding fish anatomy and physiology.

The gastrointestinal (GI) tract of fishes can be subdivided into four topographical main regions: The headgut, foregut, midgut and hindgut. Further morphofunctional subdivisions can be superimposed on this basic plan. The GI begins with apprehension of food through the teeth, mouth, and pharynx, and then progresses down the esophagus, stomach, intestines (and relative pyloric ceca), with waste elimination out the cloacae, vent, or anus. Thus, the headgut is composed of the mouth and pharynx and its function is to acquire food and mechanically process it. The foregut follows and is comprised of the esophagus and stomach, where chemical digestion of food begins. In some fishes, the mechanical breakdown of food may also occur partially or fully in the stomach. The midgut accounts for the greatest proportion of the gut length and is where chemical digestion is continued and absorption mainly occurs. The hindgut is the final section of the gut, which includes the rectum; although in some cases there is no clear morphological distinction between midgut and hindgut. The foregut epithelium is of ectodermic and the hindgut of endodermic origin [4]. The liver, pancreas, and gallbladder are vital to digestion, as in other vertebrates. In this thesis I highlighted the following parts of the digestive tract: buccal cavity, pharynx, esophagus, stomach, intestine (or more specifically intestinal

enlargement or bulb, and intestine proper). There is no true stomach in the stone-roller, but there is an anterior enlargement of the intestine, which corresponds in position to the stomach of other fishes.

2.1 Buccal cavity

The buccal cavity is characterized by the presence of a rudimentary tongue, the anterior part of which resembles histologically the callous pad of the posterior pharynx. An area of the epithelium of the roof of the mouth just above the tongue shows a greater resemblance to the pad than does the tongue. On each side, and posterior to this specialized area of epithelium, is found the typical buccal mucosa with its numerous mucous cells and taste buds. A basement membrane separates the epithelium from the very definite stratum compactum. A submucosa is present.

The buccal cavity extends from the lips to the first gill slit, or to the pharynx. It begins as a small, narrow cavity, roughly about 3 or 4 mm. wide, and quite rapidly widens, reaching its maximum width just at the anterior boundary of the pharynx. Roughly, it can be divided into the roof and the floor. There is little difference between the two regions other than that the floor contains the rudimentary tongue, and the roof a large triangular fold. The epithelium of both regions is lifted up in a series of low longitudinal folds, the folds being more pronounced at the sides of the cavity. The following are the histological divisions of the mouth: a) mucosa (epithelium, basement membrane, and stratum compactum); b) submucosa.

Below the stratum compactum lies the submucosa, which is made up of rather fine strands of areolar connective tissue, capillaries, larger blood vessels, fat, and connective-tissue cells. As one goes away from the mouth, the fat cells increase in number, crowding the connective-tissue elements. The premaxillary bones, underlying the submucosa, have many slightly wavy collagenous fibers about them, as well as numerous capillaries. In the tip of the rudimentary tongue and in the lower lip is a membrane composed of large, uniform, very clear, stratified

cells and traversed by many vertically directed collagenous fibers. In the tongue, this membrane is found to occupy most of the space in the submucosa. The cone-shaped papillae on which the taste buds are borne are fairly numerous. In them are found capillaries, connective tissue, and nerve fibers. The technique employed did not differentiate the nervous elements, but the work on taste buds by others substantiates the statement [5].

2.2 Pharynx

For convenience, the pharynx is divided into two regions: the anterior, being the region of gill slits; and the posterior, being the region of the dorsal callous pad and the ventral pharyngeal teeth. The callous pad is topped by a very thick surface layer of dead cells. The base of the pad is raised up by a number of submucosal folds simulating papillae when seen in cross-section. Taste buds and goblet cells are abundant in the anterior pharynx and less conspicuous in the posterior. The submucosa is present in both regions but is greatly reduced by the invading pharyngeal muscles of the posterior part. Thyroid tissue was found in the submucosa of the anterior pharynx. A stratum compactum is present only in the anterior pharynx.

The division of the pharynx into an anterior and a posterior region is very marked, both anatomically and histologically. The anterior region extends from the first through the last gill slit; the posterior includes the region of the ventral pharyngeal teeth and a dorsal callous pad. These layers are present in the two divisions of the pharynx: u) mucosa (epithelium, basement membrane, and stratum compactum, the last in the anterior region only); b) submucosa, greatly reduced in the posterior region; c) muscularis. The surface of the roof has a wrinkled appearance, and just in front of the callous pad of the posterior pharynx are a few short, flattened folds. The floor also has a few folds, but of a different type from the hardened ones of the roof. The amount of uninterrupted surface is greater in the roof than in the floor because the gill slits are lateroventral in position[6].

2.3 Esophagus

The great amount of adipose tissue between the fasciculi leads to the peculiar appearance of the pharyngeal muscularis. A dozen major folds, their tips covered with stratified epithelial cells, and their sides with distended goblet cells, characterize the esophagus. Goblet cells attain their greatest development here. Taste buds are present, but not in great numbers. A true stratum compactum is absent, although in places the arrangement of the adjacent connective tissue simulates this structure. The submucosa is limited by the invasion of striated longitudinal muscle, which is regarded as the first layer of muscle. The outer muscular layer is circular in direction. These layers are somewhat disarranged in the region where the pneumatic duct leaves the esophagus. The outermost coat of the esophagus is the comparatively thick serosa.

The esophagus is a small, uniformly thick tube about 3 mm long. It is characterized by a large, anterior, longitudinal, dorsal crypt or pocket which is bordered by folds on each side. In addition, there are about ten tall longitudinal folds between which are present two or three small folds. The four layers present in the esophagus are: a) mucosa, b) submucosa, c) muscularis, and d) serosa. A true stratum compactum is absent.[7]

2.4 Stomach

Some fish species are stomachless (e.g. *Cyprinids*). Stomach (Figure 1) of fish is commonly fixed into a proximal (descending) and distal (ascending) limb, which should be termed the “corpus” and the “pyloric region” respectively, in order to avoid the false analogy with the mammals involved in the term “cardiac region” which is often given to the former; from the angle a caecum may extend back. The shape and proportions of these parts are so variable that much caution must be exercised in generalizing as to functional differences between them from observations based on only a few types. The lining epithelium commonly resembles that of mammals in being composed of slender mucous cells flich presumably produce, as in the latter group, the mucus which protects the

surface of the stomach from injury [8]. Certain fish possess an additional type of secretory cell, resembling pancreatic zymogen cells or the Paneth cells of the mammalian intestine; these, together with the presence of cilia [9], may perhaps represent the retention of primitive characters. The gastric glands are tubular, with oxyntopeptic cells, support in the lamina propria can be observed not only in the fundic region but also in the cardial region. The gastric glands of fish show a more primitive level of organization than those of mammals, for pepsinogen and acid are generally considered to be produced by the same cell instead of by separate chief and parietal cells, although the evidence for this is very incomplete; moreover, while the glands which contain these cells in the mammal (the 'proper gastric glands') have a neck region occupied by a special type of 'mucoid neck cell' differing from the surface cells, no such cell is found in fish, with certain possible exceptions amongst the more highly differentiated Teleostei [10]. Since the experimental part of this work focuses on the quantification of oxyntopeptic cells, the description of these gastric cells will be deepened in the following chapters. The glands ("chief glands") tend to be confined to the corpus, so-called "pyloric glands", as pointed out by Yung (1899), being often merely crypts of the surface epithelium, and not comparable with the true pyloric (or cardiac) glands, of mammals, which are composed of cells resembling the mucoid neck cells of the proper gastric glands. The chief glands may, however, extend into a part of the pyloric region, the extent to which they do so probably reflecting the course of embryonic differentiation, which appears to begin in the middle of the stomach and to proceed forwards and backwards[2]. In form it is typically spindle-shaped, but corpus and pyloric regions can be distinguished histologically, the former, occupying from four fifths to three-quarters of the total length, possessing the chief glands, which show an advance on the condition in fish in the presence of distinctive mucoid neck cells. In certain forms,[11], the pyloric region has characteristic glands which are formed of these same mucoid cells without the zymogen cells, and which thus recall the pyloric glands of mammals [12].

The cardiac stomach still shows a muscular tunica, composed of striated fibres that in the first part maintain the same disposition as in the oesophagus. Smooth fibres gradually substitute the striated ones and the longitudinal muscularis appears on the external wall and persists for all the rest of the digestive tract. The circular muscularis is heavily thickened in the pyloric stomach. The submucosa is much more developed in the adult. The gastric mucosa substitutes directly the oesophageal mucosa, without any transition form. In the stomach, the mucosa forms fold and cavities, and is constituted by columnar epithelium, with nuclei located at the base. The free surface of epithelial cells is smooth, without microridges, sometimes with small spheroidal or cylindrical blebs. Simple tubular gastric glands are present in the cardiac region, they increase in number in the fundus but are completely missing in the pyloric region. Glandular cells are of one type only, as in all Anamnia.

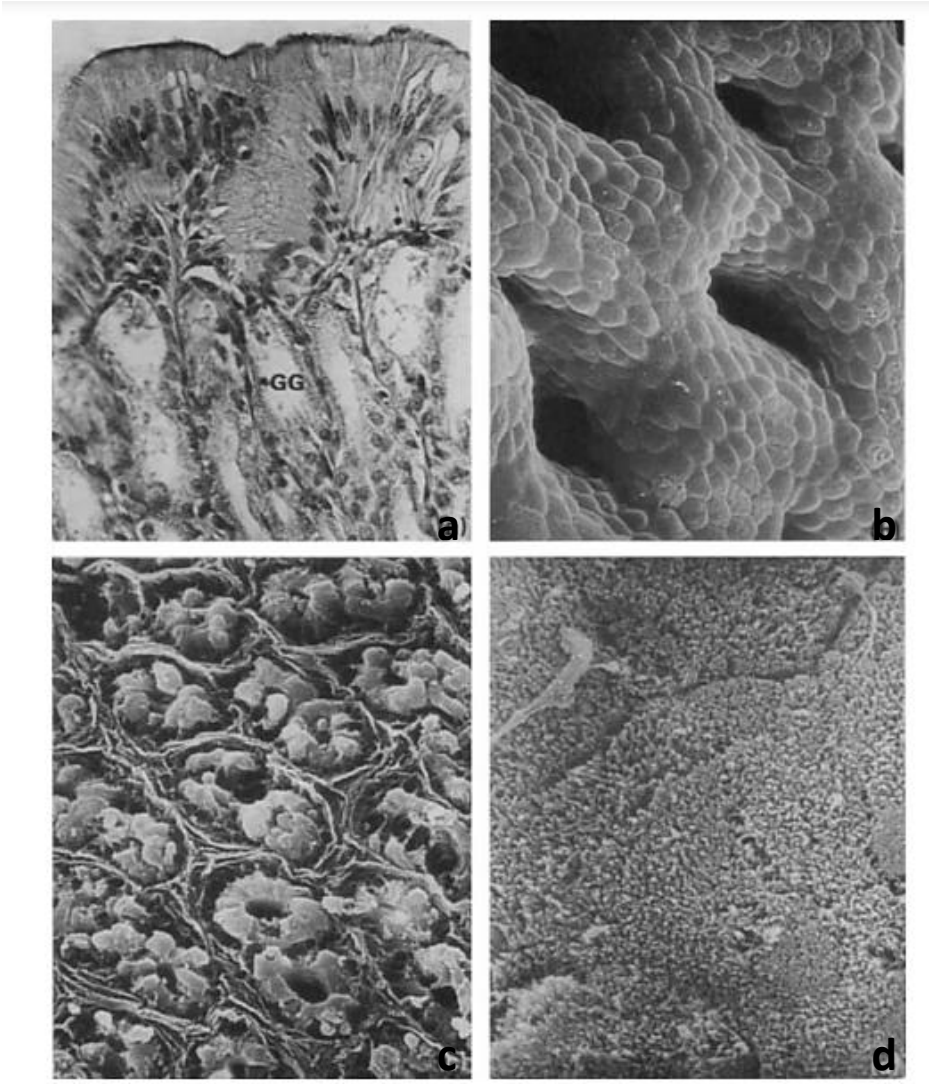

Figure 1. Stomach. Transverse section, gastric gland (GG); x 450 (a); SEM micrograph of gastric epithelium, note the emergence of gastric gland; x 1000 (b); SEM micrograph of a fracture at the submucosa level, note the gastric gland; x 700 (c); SEM micrograph of pyloric appendages epithelium; x 2800 (d)

2.5 Intestine

The intestine follows the pylorus or esophagus in gastric and agastric fishes, respectively. There is great variation in structure of the intestine of fishes. The majority of the authors subdivide the intestine into a cranial, middle (or intermediate) and caudal part that ends in the anus. Generally, the intestine of fishes is relatively long and undifferentiated throughout its length. The intestine of the longnose sucker is coiled; while the squawfish intestine is S-shaped and lacks coiling. Mohsin (1961) observed the intestine of the Indian gudgeon as

small and having two loops. The rectum is distinctly larger in diameter and is separated from the intestine by a sphincter. The intestine of the sea bass [13] forms "a loop and a half" and is about the same diameter as the rectum which is then distinguished from the intestine by somewhat thicker walls which taper slightly toward the anus. The walls of the intestine are much thinner and flabbier than those of the stomach [14]. In some stomachless fishes, the anterior intestine may bulge to form an intestinal bulb (*Cyprinidae*) and functions in temporary food storage (Figure 2). The wall of the intestine consists, from inside outward, of mucosa, submucosa, muscular and serosa tunica. Generally, the intestinal mucosa is lined by simple columnar epithelium. The epithelial cells have a striated border on the apical ends and the nuclei are round to ovoid, with each possessing a distinct nucleolus. Mucous secreting goblet cells, as indicated by a PAS-reaction, are few in number in the cranial intestine but increase in number caudally. In the cranial intestine, the submucosa is composed of loose connective tissue which, proceeding in the caudal direction, tends to thicken until it reaches the maximum thickness in the caudal intestine [15]. The muscle layer is divided into two well-defined layers of smooth muscle, an inner circular layer and an outer longitudinal layer. Connective tissue components and blood vessels penetrate into and through the two layers [16]. Nerve fibers form myenteric plexuses between the layers. The serosal coat of the intestine is composed of a thin layer of collagen fibers some of which project between the fibers of the longitudinal muscle layer. The serosal layer has composed of mesothelium: this serosal layer completely encircles the intestine.

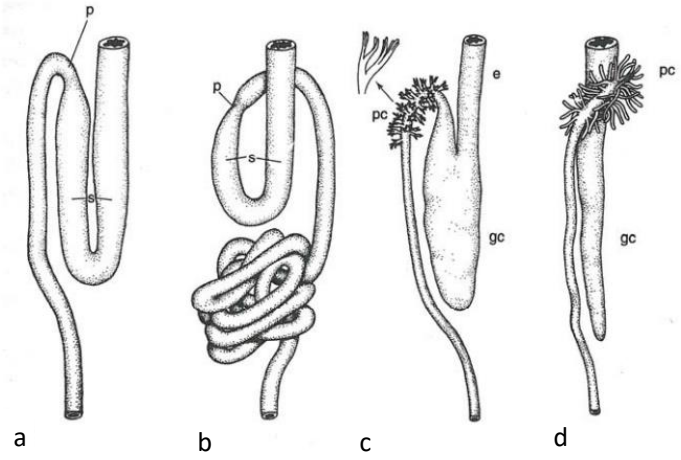

Figure 2: Alimentary canals of four different teleost fishes. Fundulus (a), Cyprinodon (b), Elops (c) and Trichiurus (d). e: esophagus, gc: caecumlike stomach, p: pylorus, pc: pyloric caeca, s: intestinal bulb (or stomach) (Kent and miller, 1997).

2.6 Liver and pancreas

Liver and the pancreas, two vitally important glands, are associated with the digestive tract. Regarding the liver, this is the largest gland in the body whose secretion, bile, is poured into the intestine. The liver is formed by an epithelial cell (hepatocytes), though which a network of vessels and sinusoids runs. The ductus hepaticus enters the first part of the intestine, after running parallel to a short ductus pancreaticus. The liver in fish is an always clearly visible gland that occupies the mid-cranial part of the coelomic cavity, whose shape and lobature are variable. For example, the liver in tuna appears to be three-lobed while in eels it is referred to as a single liver. Associated with the liver may be the gallbladder, an ovoid-shaped structure that represents a sort of bile storage point: in the gallbladder the bile is dehydrated and concentrated [17].

The second largest gland attached to the digestive system is the pancreas. Contrary as observed in mammals, the pancreas is rarely a well-defined organ in fish. Generally, it is disseminated in other organs: in the bream inside the liver tissue there are aggregates of pancreatic tissue [18]. Glandular tissue masses surround the intestine and vessels, and in the adult also infiltrate the liver.

The endocrine part of the pancreas is represented by the pancreatic or Langerhans islets which, in some Teleostei, organize themselves to form the so-called Brockmann bodies. In the endocrine pancreas there are different cells responsible for the production of different hormones. The α cells produce glucagon, the β cells are responsible for the production of insulin, the δ cells capable of producing somatostatin and finally the PP cells which instead produce the pancreatic polypeptide (PYY) [19].

2.7 Oxyntopeptic cells

In the fish, acid and pepsinogen secretions by the gastric mucosa are performed by one cell type, the oxyntopeptic cell (OP). This cell is present in the stomach of the fish, amphibians, reptiles, and birds [20]. Separation of the two functions in two cellular types seems to have occurred in mammals, where acid and pepsinogen secretions are performed by parietal and chief cells respectively. Acid secretion is a general feature in vertebrates with a stomach. Acid-secreting parietal cells and pepsinogen-secreting chief cells of mammalian stomachs are predominantly found in the mammalian corpus. In non-mammalian vertebrates, on the other hand, one cell type, the OP, secretes both acid and pepsinogen. The OP in the Atlantic cod, Gadus morhua, a saltwater teleost, and the rainbow trout, Oncorhynchus mykiss, a freshwater teleost, are mostly found in the cardiac part of the stomach [21]. Recently, through immunofluorescence techniques, immunoreactive OPs have been observed distributed over the entire surface of the mucosa of the sea bass [22] and sea bream (unpublished data): these cells were distributed along the adenomeres of the gastric tubular glands simple.

2.8 Enteroendocrine cells

GI endocrine cells are distributed in the mucosa of the GI tract and they synthesize various kinds of gastrointestinal hormones. In fish, as in mammals, feeding behavior is ultimately regulated by central effectors within feeding centres of the brain, which receive and process information from endocrine

signals from both brain and peripheral tissues. Although basic endocrine mechanisms regulating feeding appear to be conserved among vertebrates, major physiological differences between fish and mammals and the diversity of fish, in particular in regard to feeding habits, digestive tract anatomy and physiology, suggest the existence of fish- and species-specific regulating mechanisms [23]. The existence of endocrine cells has been immunohistochemically demonstrated in the gastrointestinal tract mucosa of different fish species (Diler et al, 2011). These cells have found throughout the epithelium of the digestive system [24].

In fish, enteroendocrine cells (EECs) have been observed mainly above the glandular adenomers, while a smaller number of these cells are found mixed with OPs. Generally, EECs intermingled between the mucous cells tend to reach the endoluminal side. These EECs located in correspondence of the endoluminal surface show an “open type” morphological aspect (“open type” EECs). These endocrine elements have an elongated or pyriform shape with two cytoplasmic extensions: one extension tends to reach the endoluminal side, insinuating itself between the epithelial cells, while the other, moving in the opposite direction, reaches the basement membrane. Other EECs exhibit a “closed type” morphological feature (“closed type” EECs). These cells, unlike the previous ones, do not show any cytoplasmic extension and have a rounded shape. Generally, SOM- and NPY-IR cells show a morphological appearance of "open type" EECs, while most GHR-IR cells show, on the contrary, appearance of "closed type" EECs [25]. Most EECs dispersed in the mucosa of the GI tract can directly sense nutrients at their apical pole facing the gut lumen, and can be induced to release hormones from their basal pole into circulation. Receptors belonging to the class of G protein-coupled receptors (GPCRs) have been implicated in protein/amino acid sensing in EECs [26 27].

2.8.1 Ghrelin and somatostatin

Somatostatin belongs to a family of peptide hormones that cause various effects on growth and metabolism. As for fish, several genes have been highlighted that

encode SOM which, in turn, give rise to different forms of somatostatin. Each of the somatostatin genes encodes a precursor or the pre-pro somatostatin (PPSS) and, depending on the gene involved, there can be various types of PPSS such as PPSS-I or PPSS-II. Somatostatin is produced in the cells of the endocrine portion of the pancreas and by the EECs (L cells) of the GI tract. This hormone exerts numerous direct and indirect effects, in particular on metabolism and eating behavior. Generally, SOM inhibits food intake and promotes catabolic processes such as the mobilization of stored lipids and carbohydrates. In addition, it has been shown that SOM carries out a coordination action between metabolism and reproductive processes [3].

As for GHR, its gene and peptide structures have been identified in several fish species. As in mammals, GHR is mainly produced in the stomach and, in the case of stomachless fish, in the intestine. GHR regulates the energy balance through its modulating action towards the release of pituitary hormones, regulating food intake and lipid metabolism. In mammals, GHR is made up of 28 amino acids: it derives from its precursor, pre-pro-ghrelin, made up of 117 amino acids. In tilapia and goldfish, the intracerebroventricular administration of GHR increases food intake and stimulates lipogenesis and the deposition of fat within the tissues. Furthermore, also in goldfish, it has been observed that ghrelin also acts on the metabolism of carbohydrates and glucose [28].

Chapter III

3. The European Sea Bass as a key marine fish model in the wild and in aquaculture

The European Sea Bass is highly regarded for capture and recreational fisheries. The fishing grounds are the north-eastern Atlantic Ocean, especially the English Channel, the Irish Sea and the Gulf of Biscay [29], as well as the Mediterranean. Captures in the two areas have been historically comparable, although in recent years the catches have been higher in the Atlantic Ocean. Recreational fisheries have a major share in capture fisheries, as they can account for 30% to 50% of the total catch in the Atlantic. However, in economic terms, European sea bass is now clearly an aquaculture species. Aquaculture has been producing more than fisheries since 1992 and has accounted for 96% of the total production in 2016 (aquaculture 165 915 tons vs. fisheries 6919 tons in 2016; [1,30]). Contrary to fisheries, sea bass culture is essentially located in the Mediterranean area, which accounts for 94% of the production, mostly in Turkey, Greece, Egypt and Spain. Aquaculture production is in two phases: first a hatchery-pregrowing phase, which produces fish of 1 to 20 g in three to eight months, and then an ongrowing phase to 250–450 g in 12 to 20 months. Hatcheries are typically inland, in temperature-controlled systems, whereas the bulk of the ongrowing is in sea cages in natural waters. The main market product is the 250–400 g pan-sized fish, but there is growing interest in the production of larger fish (800 g to 1 kg) to sell whole or as processed fillets (EUMOFA 2018).

The domestication of sea bass is very recent, like for most marine aquaculture species. It was the first non-salmonid marine species commercially cultured in Europe [31]. The first intensive rearing trials started in the early 1970s, based on wild-captured juveniles (Fig. 1). In the early 1980s, controlled reproduction in spawning tanks and larval rearing were developed, mainly in France and Italy, and made domestication possible [31]; [32]. Mostly wild-caught broodstock was used in the hatcheries, and the first selective breeding programs using exclusively

hatchery-born broodstock (and thus domestication in its genetic meaning) started only in the 1990s [32]. In 2016, it was estimated that approximately 50% of farmed sea bass came from selective breeding programs [33]. However, domestication remains recent, and in 2016, the sea bass strains with the longest history of domestication were eight generations away from the wild populations of origin [34].

Chapter IV

4. Current challenge

Global fish production is estimated to have reached about 179 million tonnes in 2018, of which 82 million tonnes, came from aquaculture production.

Of the overall total, 156 million tonnes were used for human consumption, equivalent to an estimated annual supply of 20.5 kg per capita. The remaining 22 million tonnes were destined for non-food uses, mainly to produce fishmeal and fish oil. Aquaculture accounted for 46 percent of the total production and 52 percent of fish for human consumption. Total fish production has seen important increases in all the continents in the last few decades. Global food fish consumption increased at an average annual rate of 3.1 percent from 1961 to 2017, a rate almost twice that of annual world population growth (1.6 percent) for the same period, and higher than that of all other animal protein foods (meat, dairy, milk, etc.), which increased by 2.1 percent per year. Per capita food fish consumption grew from 9.0 kg (live weight equivalent) in 1961 to 20.5 kg in 2018, by about 1.5 percent per year. Globally, fish provided more than 3.3 billion people with 20 percent of their average per capita intake of animal proteins, reaching 50 percent or more in developing countries.

Regarding the food fish consumption, live, fresh, or chilled fish still represented the largest share (44 percent) of fish utilized for direct human consumption as being often the most preferred and highly priced form of fish. It was followed by frozen (35 percent), prepared and preserved fish (11 percent) and cured at 10 percent.

The consumption of fish is extremely important, providing around 20% of their average per capita animal protein intake to more than 3.3 billion people, a percentage that is up to 50% higher in countries.

In 2018, aquaculture fish production was dominated by finfish (54.3 million tonnes – 47 million tonnes), Molluscs, mainly bivalves (17.7 million tonnes), and crustaceans (9.4 million tonnes).

Starting from these data and considering time as an important variable, it's not difficult to understand how aquaculture world production of fish had progressively and significantly exceeded the catches.

The broad trends that have driven growth in global fish consumption in recent decades have been paralleled by many fundamental changes in the ways consumers choose, purchase, prepare and consume fish products. The globalization of fish and fish products, propelled by increased trade liberalization and facilitated by advances in food processing and transportation technologies, has expanded supply chains to the point where a given fish may be harvested in one country, processed in another and consumed in yet another.

This development has allowed consumers to access species of fish that are caught or farmed in regions far from their point of purchase, and it has introduced new species and products to what were previously only local or regional markets. Although the choices available to an individual consumer have multiplied, at the global level they are increasingly similar among countries and regions Seasonal shortages of individual species in certain markets are also mitigated to some extent by the international diversification of supply sources and advances in preservation technologies. As a result, major supply shocks affecting key species are likely to affect consumption for a greater number of people in more geographically dispersed markets. Increasing consumer awareness of sustainability, legality, safety and quality issues is driving demand for traceability systems and certification schemes of a growing range of fish and fish products. While capture fisheries will remain relevant, aquaculture has already demonstrated its crucial role in global food security, with its production growing at 7.5 percent per year since 1970. Recognizing the capacity of aquaculture for further growth, but also the enormity of the environmental challenges the sector must face as it intensifies production, demands new sustainable aquaculture development strategies. Such strategies need to harness technical developments in, for example, feeds, genetic selection,

biosecurity and disease control, and digital innovation, with business developments in investment and trade.

A responsible use of available resources related to fishing and aquaculture activities is therefore necessary today.

However, although this issue is widely recognized, the protection of resources is not often placed at the heart of the sector's development strategies. In the past the resources were considered inexhaustible and, with the advent of scientific and technological progress after World War II, an intense development of fishing and aquaculture followed.

Only later the problem of the availability of some resources arised, although some are renewable. With technological and scientific progress, aquaculture production has reached unprecedented peaks. Only later did the problem arise of the availability of some resources, even if renewable. With technological and scientific progress, aquaculture production has reached unprecedented peaks. However, all these advances then had a negative impact on the environment.

Aquaculture also present problems in limited availability of sites, destruction of habitats and the release of harmful chemicals and veterinary medicines into the environment.

In this context of constant population growth, the fisheries and aquaculture sector has much to contribute to securing all the SDGs, but is at the core of SDG 14 – Conserve and sustainably use the oceans, seas and marine resources for sustainable development [30].

EXPERIMENTAL STUDIES

Chapter V

5. Effect of essential oils on the oxyntopeptic cells and somatostatin and ghrelin immunoreactive cells in the European sea bass (*Dicentrarchus labrax*) gastric mucosa

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Abstract: The current work was designed to assess the effect of feed supplemented with essential oils (EOs) on the histological features in sea bass's gastric mucosa. Fish were fed three diets: control diet (CTR), HERBAL MIX® made with natural EOs (N-EOs), or HERBAL MIX® made with artificial EOs obtained by synthesis (S-EOs) during a 117-day feeding trial. Thereafter, the oxyntopeptic cells (OPs) and the ghrelin (GHR) and somatostatin (SOM) enteroendocrine cells (EECs) in the gastric mucosa were evaluated. The Na^+K^+-ATPase antibody was used to label OPs, while, for the EECs, anti-SOM and anti-GHR antibody were used. The highest density of OP immunoreactive (IR) area was in the CTR group (0.66 mm^2 ± 0.1). The OP-IR area was reduced in the N-EO diet group (0.22 mm^2 ± 1; CTR vs. N-EOs, $P < 0.005$), while in the S-EO diet group (0.39 mm^2 ± 1) a trend was observed. We observed an increase of the number of SOM-IR cells in the N-EO diet (15.6 ± 4.2) compared to that in the CTR (11.8 ± 3.7) (N-EOs *vs.* CTR; $P < 0.05$), but not in the S-EOs diet. These observations will provide a basis to advance current knowledge on the anatomy and digestive physiology of this species in relation to pro-heath feeds.

Keywords: essential oil; oxyntopeptic cells; Na^+K^+-ATPase; somatostatin; ghrelin; enteroendocrine cells

1.INTRODUCTION

In the last decade, the application of natural feed additives has been able to support optimal gut health and function, thus enhancing growth, feed utilization, and disease prevention in the whole aquaculture sector [1, 2]. Essential oils (EOs) are extracted from plants raw materials and contain compounds produced during plant secondary metabolism. They are natural multicomponent systems of volatile, lipophilic, odoriferous, and liquid substances, obtained from complex mixtures of low-molecular-weight substances [3,4]. EOs contain compounds that are responsible for antimicrobial, antibacterial, anti-oxidant, antiviral, and antimitotic properties [5,6]. As a result, EOs have been the focus of aquaculture studies due to their diverse properties, and they are good candidates as they enhance the health, growth, and welfare of the fish[3]. In no-mammalian vertebrates, one cell type, the oxyntopeptic cells (OPs), secretes both hydrochloric acid and pepsinogen into the lumen to initiate protein digestion[7,8,9,10].

The hydrochloric acid promotes the conversion of pepsinogen into pepsin, an efficient proteolytic enzyme[9,11,12]. The gastric proton pump, Na^+K^+-ATPase, is responsible for stomach luminal acidification in vertebrates and is a characteristic feature of the gastric gland[13]. In addition to the H+K+ -ATPase, Na^+K^+-ATPase in the gastric mucosa was also detected in vertebrates (including humans)[14,15]. The Na^+K^+-ATPase was found in correspondence of the parietal cells[14,16,17,18,19].

Ghrelin (GHR) is a 28-amino-acid peptide[20,21,22] that is involved in the control of energy homeostasis and increases food intake in mammals[24,25,26,27]. In fish, GHR mRNA is highly expressed in the stomach/gut, and moderate levels are detected

in the brain[28,29,30]. GHR enteroendocrine cells (EECs) were detected in various tissues of no-mammalian vertebrates such as in the hypothalamus and stomach[30,31,32], as well as in the gastrointestinal tract of chicken[33], in the stomach of turtle[34,35], in the stomach of rainbow trout36 and in the gut of zebrafish[37].

Unlike GHR, somatostatin (SOM) inhibits food intake, promotes catabolic processes (e.g., mobilization of stored lipid and carbohydrate)[38,39], and promotes the reduction of basal and/or stimulated gastric acid secretion[40]. SOM EECs have been documented in the alimentary canal of northern pike (*Esox lucius*)[41], milkfish (Chanos chanos)[42], and the predatory longnose gar (Lepisosteus osseus)[43]. In order to assess the gut health status of fish in relation to different feeding and nutritional strategies, the intestine has been largely considered the main target tissue for histological evaluation including determination of the morphological/morphometric characteristics of salmonids and other species of commercial interest[44,45,46,47,48,49]. Indeed, sparse attention was devoted to exploring the morphological features of the gastric mucosa in response to different feeding conditions. In addition, there is no detailed information on the histological features of the OPs in the European sea bass.

In this context, the aim of the present work was to investigate the presence and distribution of the OPs and GHR/SOM EECs in the gastric mucosa of the European sea bass fed diets supplemented with natural EOs and artificial EOs composed of active ingredients obtained by synthesis. Finally, we saw that Na^+K^+-ATPase was expressed in OPs in the same way as H^+K^+-ATPase (typical marker for mammalian parietal cells) and that, for this reason, it can be considered a valid marker for OPs.

2. MATERIALS AND METHODS

2.1. Rearing Conditions and Tissue Sampling

A commercial diet (43% protein, 21% lipid, pellet diameter 4.0 mm VRM, Verona, Italy) was coated with HERBAL MIX®, a blend of essential oils, natural essential oils (NEOs), or EOs obtained by synthesis (S-EOs). N-EOs contained a blend of

natural essential oils of thyme, garlic, rosemary, and cinnamon, while S-EOs was a blend of thymol and carvacrol, diallyl sulfide, cineol, and cinnamaldehyde (main components of N-EOs). The concentration ratio between molecules was the same in N-EOs and in S-Eos, and the inclusion rate was 1000 g ton^{-1} for both blends. A diet without supplementation was kept as the control (CTR) group. Table 1 reports the fatty acid composition of the diets. The inclusion of the blends did not affect the overall fatty acid composition of the different diets. European sea bass juveniles obtained from an Italian commercial hatchery were reared in a recirculating aquaculture system (RAS) at the Laboratory of Aquaculture, Department of Veterinary Medical Sciences of the University of Bologna, Cesenatico, Italy. At the beginning of the trial, 50 fish (75.0 ± 0.2 g) per tank were randomly distributed into 9, 900 L conical-bottom tanks provided with natural sea water (oxygen level 8.0 ± 1.0 mg L^{-1}, temperature 23 ± 1.0 ◦C, salinity 25 g L^{-1}). Each diet was fed twice a day to triplicate groups to satiation for 117 days using the overfeeding approach as described in Busti et al. Fish were individually weighed at the beginning and at the end of the trial. At the end of the trial, four fish per tank (total of 36 specimens mean weight 270.0 ± 4.6 g) were sampled for gastrointestinal tract histology. After euthanasia with a lethal dose (300 mg L^{-1}) of MS222, the stomach was gently isolated and fixed in 10% buffered formalin. After fixation, the stomach was cut symmetrically along the major axis to obtain two equal halves and embedded in paraffin.

2.2. Immunohistochemistry

Stomach sections were processed for single- and double-labeling immunofluorescence. Sections were deparaffinized, rehydrated, and incubated for 1 h in a humid chamber at room temperature (RT) with appropriate normal serum (5% normal goat or donkey serum) and 1% BSA diluted in PBS (phosphate buffered saline, 0.01 M pH 7.4) to reduce the nonspecific binding of the secondary antibodies. The sections were then incubated at 4 ◦C in a humid chamber for 24 h with the primary antibody rabbit anti-Na^+K^+-ATPase (Abcam,

Cambridge, UK) diluted to 1:200. After washing, the sections were incubated at RT for 1 h with the goat anti-rabbit Alexa Fluor® 488. For the EECs, rat anti-somatostatin (Enzo Life Sciences, New York, NY, USA) diluted to 1:300, was used in association with mouse anti-ghrelin (Acris/OriGene, Herford, Germany) diluted to 1:300 in PBS. The sections were incubated at 4 ◦C overnight. After washing, the sections were incubated at RT for 1 h with donkey anti-rat Alexa Fluor® 488 and donkey anti-mouse Alexa Fluor® 594 secondary antibody (Invitrogen, CA, USA) diluted in PBS and then coverslipped with buffered glycerol, pH 8.6.

2.3. Threshold Binarization Method

In order to characterize the area occupied by the immunoreactive (-IR) OPs in the gastric mucosa, the following method was applied. The slides were scanned with the Nikon DS-Qi1Nc digital camera at 20× magnification, using NIS Elements software BR 4.20.01 (Nikon Instruments Europe BV, Amsterdam, The Netherlands). Automated Image Binarization was applied to an area of each selected gastric image by means of the NIS Elements software BR 4.20.01. Image binarization is a widely used method that allows to distinguish objects of interest from the background. Indeed, it determines a gray threshold and assigns each pixel of a digital image to one class (image objects). If it is a gray value, it is greater than the determined threshold compared to the other class (image background). Specifying correct threshold limits is a crucial procedure of the automated image analysis used to determine which pixels will or will not be included in the binary layer. In our case, using binarization, we were able to separate the pixels that represented the OP-IR cells of the gastric surface (brighter pixels) from those that represent the rest of the section (Figure 3 A, B). The area of measurement can be restricted by a user-defined region of interest (ROI). Within the ROI, it is possible to binarize only the selected part and not include other parts of the section (Figure 3C). This allowed the quantification of the gastric surface covered

by OP-IR cell area. The gastric morphometric assessments were performed in a blind fashion by two investigators.

2.4. Antibody Specificity

Specificity for GHR and SOM antibodies was demonstrated by the lack of immunostaining when the antibodies were pre-adsorbed with an excess of the homologous peptide. The Na^+K^+-ATPase antibody is specific for zebrafish. Furthermore, the recognized antigenic sequence of the Na^+K^+-ATPase antibody has a structural homology of 95% with that of sea bass. In addition, omission of the primary antibody excluded inappropriate binding of the secondary antibody.

2.5. Validation of the Na^+K^+-ATPase/H^+K^+-ATPase Antibodies as a Marker of Oxyntopeptic Cells (OPs)

Serial sections (4 µm thick) of sea bass stomach were used to validate the use of anti-Na^+K^+-ATPase antibody as a marker of OPs cells. In one section, the primary antibody H^+K^+-ATPase (Aviva System, San Diego, CA, USA) was used, while the other section was incubated with the Na^+K^+-ATPase primary antibody. Subsequently, the sections were incubated with donkey anti-rabbit Alexa Fluor® 488. A total overlap of the OPs was observed (Figure 4).

In addition, in the esophagus–stomach and stomach–intestine junctions, it was observed that the OPs tended to decrease until disappearance: this feature has been highlighted with both ATPase antibodies (Figure 4A–D).

2.6. Morphometric Evaluations

Preparations were examined on a Nikon Eclipse Ni microscope, and the images were recorded with a Nikon DS-Fi2 (for ordinary histology) and Nikon DS-Qi1Nc (for immunofluorescence) digital camera and NIS Elements software BR 4.20.01 (Nikon Instruments Europe BV, Amsterdam, The Netherlands).

Slight adjustments to contrast and brightness were made using Corel Photo Paint, whereas the figure panels were prepared using Corel Draw (Corel Photo Paint and Corel Draw, Ottawa, ON, Canada).

The 20× objective was used for the morphometric evaluation. In the gastric mucosa, the area occupied by the OPs-IR in 4.1 mm2 (0.410 × 10 fields) was measured by binarization (described above). Furthermore, in the gastric mucosa EECs, the number of GHR- and SOM-IRs in 4.1 mm2 were counted.

2.7. Statistical Analysis

All fish growth data are presented as mean ± standard deviation (SD). The tank was used as the experimental unit for analysing growth performances. Data were analyzed by a one-way ANOVA and Tukey's post hoc test. The differences among treatments were considered significant at $p < 0.05$. The values obtained from the OP-IR area and the EEC number were grouped for each experimental group (CTR, N-EOs and S-EOs), and the means were calculated.

Results were expressed as mean ± SD. Data were analyzed by one-way ANOVA (GraphPad Prism 4, GraphPad Software, Inc., La Jolla, CA, USA): we considered the experimental group as the main effect. In addition, means were subsequently separated by using Tukey—HSD test. P value < 0.05 was considered statistically significant.

The gastric morphometric assessments were performed in a blind fashion by two investigators.

3. Results

Data on growth performances (final body weight and specific growth rate, SGR), feed intake (FI), and feed conversion rate (FCR) at the end of the trial are summarized in Table 2. No significant differences were observed in final body weight, SGR, FCR, and FI during the overall trial ($P < 0.05$).

The gastric mucosa is lined by a simple columnar epithelium composed of poorly stained epithelial cells with a central elongated nucleus: these elongated cells

(mucins cells) were positioned above the gastric glands. Below the gastric glands, the presence of the lamina muscularis mucosae limits the mucosa from the submucosa. The submucosa is composed of loose connective tissue without the presence of glands. The muscular layer presents circular internal and longitudinal external bundle orientations. By immunofluorescence, we observed OPs in all parts of the stomach: these cells showed intense immunoreactivity. The OPs were distributed along the adenomere of the simple tubular gastric glands (Figure 5). The immunoreactivity of the OPs was interrupted in the transition from esophageal epithelium to the gastric mucosa (Figure 6A–D). Similarly, in correspondence with the passage from gastric mucosa to the first part of the intestine, the positivity of the gastric glands was interrupted. The highest density of the oxyntopeptic IR area was in the CTR group (0.66 mm2 ± 0.1).

Oxyntopeptic IR area was significantly reduced in the N-EO diet (0.22 mm2 ± 1, CTR vs. N-EOs, $P < 0.005$), while in the S-EO diet (0.39 mm2 ± 1) a decreasing trend was observed (Figure 7). EECs were mainly distributed over the glandular adenomeres: few cells were located along the glandular adenomere. Generally, the EEC cells are intermingled between mucous cells and, in some cases, tend to reach the endoluminal side (Figure 8A–D). Some SOM- or GHR-IR cells had the morphological appearance of “open-type” EECs with an elongated (“pear-like”), homogenous cytoplasm and two cytoplasmic prolongations, one reaching the lumen and the other the basal lamina. Other SOM or GHR-IR cells had the “closed-type” EEC appearance with a round shape without cytoplasmic prolongations. In particular, the EEC-IR cells located in the endoluminal side showed an “open-type” appearance, while those located in inner part of the mucosa displayed a “closed-type” shape (Figure 8 C, D). We observed that SOM-IR cells co-expressed GHR and vice versa.

In the gastric mucosa, the N-EOs group (15.6 ± 4.2) exhibited a significant change in the mean number of SOM-IR cells compared to the CTR group (CTR 11.8 ± 3.7) (CTR vs. N-EOs; $P < 0.05$), while there were no significant differences in the mean number of the SOM-IR cells with respect to the S-EO diet (13.8 ± 3.4). We did not

observe significant differences regarding the mean number of GHR-IR cells in the three experimental groups (CTR 11.9 ± 6, N-EO diet 13.8 ± 6.4 and S-EO diet 14.2 ± 7.8, respectively) (Figure 9). The percentage of colocalized IR cells/total of GHR-IR cells was 34% in the CTR (438/1297), while the percentage decreased in the N-EOs (28%, 397/1430) and S-EOs (28.3%, 394/1391) groups. Similarly, the percentage of colocalized IR cells/total of SOM-IR cells was 34% in the CTR (438/1288), while the percentage decreased in the N-EOs (28.6%, 397/1387) and S-EOs (25.9%, 394/1519).

4. DISCUSSION

Previous studies have demonstrated the efficacy of herbal extracts on fish to attenuate stress response [1], improved immune system, enhanced gut tissue integrity [51,52,53], and increase feed digestibility and fish growth performance [1,54]. In this context, EOs have been studied for their several beneficial characteristics, such as antibacterial and antioxidant properties [55], and their ability to improve the feed conversion ratio by improved feed palatability [56,57,58] feed digestibility, and nutrient transport[57].

In addition, excess use of various antibiotics, hormones, and other synthetic drugs to control diseases and improve fish growth in aquaculture is the reason behind the emergence of drug-resistant bacteria, suppressed immunity in the host, and production of toxic substances harmful to the environment and human health[59]. For this reason, of late, the World Health Organization (WHO) encourages supplemented diets incorporated with medicinal herbs or plants that minimize the use of chemicals in the diet of fish[52].

In the present study, supplementing a basic diet with EOs containing thymus, rosemary, cinnamon, and garlic reduced the expression of OPs and increased the number of SOM and GHR-IR EECs in the gastric mucosa. In the literature, no author reports were found on the morphometric quantification (IR area and/or number) of OPs and EECs in the fish gastric mucosa. For this reason, we have made several assumptions. To explain the reduction in OPs, natural small

molecules produced from medicinal plants have been used for a long time to treat and prevent various pathologies such as peptic ulcer. Several phytocomponents such as flavonoids, tannins, terpenoids, and saponin have been reported in distinct anti-ulcer findings as possible gastro-protective agents[59]. However, some of these phytocomponents (i.e., tannin and saponin) are also known as potential antinutritional factor in fish species[60].

Thymus was used in the prevention/treatment of some gastrointestinal disorders. Several researchers report that thyme contains numerous phenolic compounds, especially thymol and carvacrol, which are found in its essential oil. Additionally, in wild thyme, many other abundant phenolic compounds have been found such as caffeic and rosmarinic acid derivatives. The anti-ulcerogenic effect of thymus extracts were demonstrated in rats stimulated with ulcer-inducing substances (e.g., HCL/ethanol, indomethacin) [59, 61, 62].

Rosemary, which is used in folk medicine, has many therapeutic properties: antifungal, antiviral, antibacterial, anti-inflammatory, antitumor, antithrombotic, antinociceptive, antidepressant, anti-ulcerogenic, and antioxidant activities [63 ,64, 65, 66, 67, 68]. Two groups of compounds are primarily responsible for the biological activity of this plant, the volatile fraction and phenolic constituents as rosmarinic acid [66] and fractions of flavonoids and diterpenes, which are structural derivatives of carnosic acid [67]. Amaral et al.[69] report that rosemary extracts play a protective action in ethanol-induced gastric ulcers in rats. Similar results on the anti-ulcerogenic activity of crude hydroalcoholic extract of Rosmarinus officinalis were obtained by Dias et al. in rats[70].

Cinnamon is a traditional herb used for many diseases, and it has effects as an antioxidant, anti-inflammatory, antispasmodic, and anti-ulcerative agent. In the rat, the oral administration of cinnamon aqueous extracts for two weeks significantly improved gastric juice volume and decreased the gastric juice acidity and the gastric ulcer index[71]. In this regard, in the rat, intragastric administration of Oleum cinnamomi reduced gastric pH levels: the authors indicated that Oleum

cinnamomi prevents ulcerative lesions and has beneficial effects on the gastric mucosa[71].

The beneficial effects of garlic on decreasing blood pressure, triglyceride levels, and oxidative activities and its anticarcinogenic, antibacterial, antifungal, and antiviral properties have been proved[72,73]. Garlic has abundant chemical compounds such as allicin, alliin, S-allyl cysteines, thiacremonone, diallyl-disulfide, diallyl sulfide, and others. Lee et al.[74] indicate that diallyl disulfide, a secondary organosulfur compound derived from garlic, prevents gastric mucosal damage induced by acute ethanol administration in rats. In this regard, the gastroprotective effects of garlic extract were shown by El-Ashmawy and El-Bahrawy[75] in rats treated with indomethacin-induced gastric ulcers.

Some authors claim that mammalian parietal cells are functionally more capable of secreting acid than OPs present in fish and lower vertebrates[10, 75, 76, 77]. For this reason, it is conceivable that essential oil compounds performed a similar action to that of acidifiers by inhibiting acid secretion. Dietary acidifiers (organic and inorganic acids) have been broadly applied worldwide in the diets of animals (in order to replace antibiotic growth promoters), because of their potential to reduce both gastrointestinal-pH[78] and parietal cells[79, 80]. Some researchers also claimed that dietary acidifiers in the feed of fish reduce the pH in the stomach, which helps improve pepsin activity, enhancing the protein metabolism and mineral intake of the intestines[10, 80, 81, 82].

Previous studies have shown that European sea bass maintain a slightly acidic gastric pH (4.5–5) during fasting followed by a strong acidification (pH below 3) stimulated by the ingestion of food[82], possibly indicating the specific need for this species to reach a low gastric pH for optimal pepsin activity. However, a recent study has also hypothesized that the reduction of feed buffering capacity induced by the low fish meal content of current aquafeed formulations might lead to relatively low pH in the intestinal tract with consequences on feed utilization[83].

SOM acts as a potent inhibitor of gastric acid secretion. In gastrin gene knockout mice, a reduction in the number of parietal cells was observed, whereas the EEC number was not affected by gene deletion[84, 85, 86, 87].

In our study, we showed an increase in SOM-IR cells in fish fed a diet supplemented with EOs. It is plausible that EOs reduce acid secretion by the stimulation of EECs (probably D cells). In mammals, the stimulatory effects of gastrin, histamine, and acetylcholine tightly regulate gastric acid secretion and the inhibitory actions of SOM on their respective receptors located in the parietal cells [87.] When gastric pH becomes too low, SOM secretion increases to inhibit not only acid production by parietal cells but also gastrin secretion by G cells[88].

In the piglet, Mazzoni et al.[80] observed an increase of the SOM-IR cells after feed supplementation of sodium butyrate in the post-weaning period. In the rat, intraperitoneal administration with thymoquinone significantly increased the number of SOM-positive cells[89], while, in piglets, intragastric administration of thymol upregulated SOM and SOM receptor (SSTR1 and SSTR2) genes in the gastric mucosa[90]. Another hypothesis could be that the phytocompounds, contained in the EOs, could have interacted with the OPs and EECs by means of transient receptor potential (TRP) channels. TRPV1 and TRPV4 receptor families displayed a ubiquitous distribution in mammals[91], zebrafish, and sea bass[92,93]. In the rainbow trout, TRPV1 (and TRPV4) is distributed in several organs, but its expression in the intestine was twofold higher than in the other districts such as retina, brain, pineal organ, spleen, heart, and blood cells[94]. TRPV1 (so-called "capsaicin receptors") plays a key role in many other sensory functions and in detecting a large array of noxious stimuli and was also found in vagal, splanchnic, and pelvic visceral afferents, implicated in gastrointestinal mechanosensory functions and visceral hypersensitivity[95]. In addition, TRPV1 is expressed in parietal cells[96], endocrine G cells, gastric epithelial cells[97, 98, 99, 100], as well as the esophageal, small intestinal, and colonic epithelial cells. Besides the endogenous agents, TRPV1 is activated by several spices, such as capsaicin, cinnamaldehyde, allyl-isothiocyanate, and allicin [99, 100, 101].

Another hypothesis may be that the different components of EOs may have modulated gastrointestinal bacteria communities. The normal microbiota of the gastrointestinal tract surfaces contains saprophytic and potential pathogenic bacteria species, and both types are capable of multiplying and infecting the fish when conditions become favorable. Under normal conditions, fish maintain a dynamic microbial equilibrium in defense against these potential invaders using a repertoire of innate and specific defense mechanisms [102, 103, 104]. This bacterial community can modulate expression of genes in the digestive tract involved in the stimulation of epithelial proliferation, promotion of nutrient metabolism, and innate immune responses, while preventing the potential development of intestinal disorders and imbalances in intestinal homoeostasis [104]. Due to the chemical diversity and possible interactions among the molecules, EOs not only may modulate gut bacterial composition by their effects directly on the bacterial cell, but they also can affect the host in a number of other ways, mainly modulating the immune and other physiological responses.

Recently, in European sea bass, a dietary blend of organic acid (citric acid, sorbic acid) and essential oils (thymol and vanillin) was able to induce a potential functional reconfiguration of the gut microbiome, promoting a significant decrease in several inflammation-promoting and homeostatic functions [50]. Regarding GHR, several authors report that phytochemical compounds of essential oils (e.g., cinnamaldehyde) decrease ghrelin secretion in mouse ghrelinoma 3-1 cell lines but, at the same time, upregulated the ghrelin gene expression [105]. Conversely, we observed an increase (not significant) of the GHR-IR cells in the gastric mucosa: the increase of the GHR is a positive aspect considering the ample evidence that GHR is an orexigenic peptide in several fish species. We do not have an explanation for the results obtained; probably the mechanisms that regulate the expression, presence, and distribution of GHR in the gastrointestinal tracts of fish are different from those in mammals.

In mammals, acid secretion in the stomach is mediated by parietal cells. The gastric H^+K^+ -ATPase, a member of the P2-type ATPase family, is the integral

membrane protein responsible for gastric acid secretion. P-type ATPases comprise five groups: Type I ATPases, Type II ATPases (Ca^{2+}-ATPases, Na^+K^+ -ATPases and H^+K^+ -ATPases), Type III ATPases, Type IV ATPases, and Type V ATPases [106, 107, 108]. In addition to the H^+ -K^+ -ATPase, Na^+K^+ - ATPase was also detected in the gastric mucosa in vertebrates including humans. Some authors have found Na^+K^+ -ATPase in the gastric mucosa, in correspondence to parietal cells [14,16–18] and oxyntopeptic cells [19, 109].

Present in all animal cells, Na^+K^+ -ATPases appear at a higher concentration and more actively in seawater Teleosts110. In the present study, we have shown colocalization between Na^+K^+ -ATPase and H^+K^+ -ATPase in the sea bass OPs. On the other hand, Gonçalves et al.[109] showed, by means double immunofluorescence, Na^+K^+ -ATPase/H^+K^+ -ATPase co-expression in the gastrointestinal tract of some Cypriniformes.

5. CONCLUSIONS

For the first time, it has been shown in sea bass that the administration of a diet supplemented with the EO blend HERBAL MIX® affects the distribution of OP, SOM, and GHR-IR cells in the gastric mucosa. It is possible that the EOs carry out directly or indirectly (by means the SOM EECs) an acidifying-like action.

In intensive and semi-intensive farming, it has long been known that various biotic and abiotic factors, as well as aquaculture procedures (handling, transport, or stocking density), activated a stress system that induces negative effects on different physiological processes in fish (growth, reproduction, and immunity). Before reaching the intestine, the food undergoes numerous transformations within the stomach. In this context, EOs could represent a promising strategic alternative method to antibiotics/chemicals for maintaining and promoting health, as well as preventing and potentially treating some diseases and/or improving growth also in the gastric context.

Further studies are needed to gain more insights to understand the mechanisms of action of various EOs on the morphology of the fish gastric mucosa. The

observations obtained in this study will provide a basis for a better understanding of the digestive physiology and help pathologists and nutritionists in future studies on diet and diseases affecting this species.

Table 1: Fatty acid composition of the three experimental diets.

Fatty Acid Composition (g/100 g)	CTR	N-EOs	S-EOs
Caprinic acid (10:0)		0.006 ± 0.001	0.006 ± 0.001
Lauric acid (12:0)	0.027 ± 0.006	0.031 ± 0.007	0.028 ± 0.006
Myristic acid (14:0)	0.163 ± 0.035	0.146 ± 0.031	0.150 ± 0.032
Pentadecanoic acid (15:0)	0.020 ± 0.004	0.019 ± 0.004	0.019 ± 0.004
Palmitic acid (16:0)	1.570 ± 0.190	1.550 ± 0.180	1.540 ± 0.180
Isoheptadecanoic acid (17:0 iso)	0.013 ± 0.003		0.011 ± 0.002
Hexadecenoic acid (16:1)	0.220 ± 0.630	0.193 ± 0.041	0.210 ± 0.630
14-Methylhexadecanoic acid (17:0 anteiso)	0.016 ± 0.003	0.015 ± 0.003	0.014 ± 0.003
Margaric acid (17:0)	0.026 ± 0.005	0.025 ± 0.005	0.024 ± 0.005
Heptadecenoic acid (17:1)	0.028 ± 0.004	0.029 ± 0.005	0.029 ± 0.004
Stearic acid (18:0)	0.425 ± 0.060	0.422 ± 0.061	0.418 ± 0.060
Octadecenoic acid (18:1)	8.250 ± 0.680	9.090 ± 0.760	8.930 ± 0.750
Octadecadienoic acid (18:2)	3.900 ± 0.370	4.070 ± 0.380	3.960 ± 0.380
Arachidic acid (20:0)	0.090 ± 0.019	0.091 ± 0.020	0.090 ± 0.019
Octadecatrienoic acid (18:3)	1.550 ± 0.180	1.440 ± 0.170	1.480 ± 0.170
Eicosenoic acid (20:1)	0.446 ± 0.063	0.374 ± 0.056	0.395 ± 0.058
Stearidonic acid (18:4 n-3)	0.050 ± 0.011	0.043 ± 0.009	0.044 ± 0.009
Behenic acid (22:0)	0.047 ± 0.010	0.050 ± 0.011	0.048 ± 0.010
Docosanoic acid (22:1)	0.267 ± 0.042	0.229 ± 0.036	0.234 ± 0.038
Lignoceric acid (24:0)	0.062 ± 0.013	0.061 ± 0.013	0.037 ± 0.008
Polyunsaturated fatty acids (>C20)	0.386 ± 0.049	0.342 ± 0.046	0.337 ± 0.046
Polyunsaturated fatty acids	6.210 ± 0.420	6.160 ± 0.420	6.100 ± 0.420
Monounsaturated fatty acids	9.260 ± 0.800	9.960 ± 0.760	9.840 ± 0.850
Saturated fatty acids	2.470 ± 0.210	2.420 ± 0.200	2.400 ± 0.200
Fatty acids ratios			
Polyunsaturated fatty acids/monounsaturated fatty acids	0.671 ± 0.074	0.618 ± 0.064	0.620 ± 0.069
Polyunsaturated fats/saturated fatty acids	2.510 ± 0.280	2.550 ± 0.280	2.540 ± 0.280
Volatile organic acids (mg/kg)			
Acetic acid	627 ± 94	700 ± 110	700 ± 110
Butyric acid	67 ± 22	62 ± 22	66 ± 22

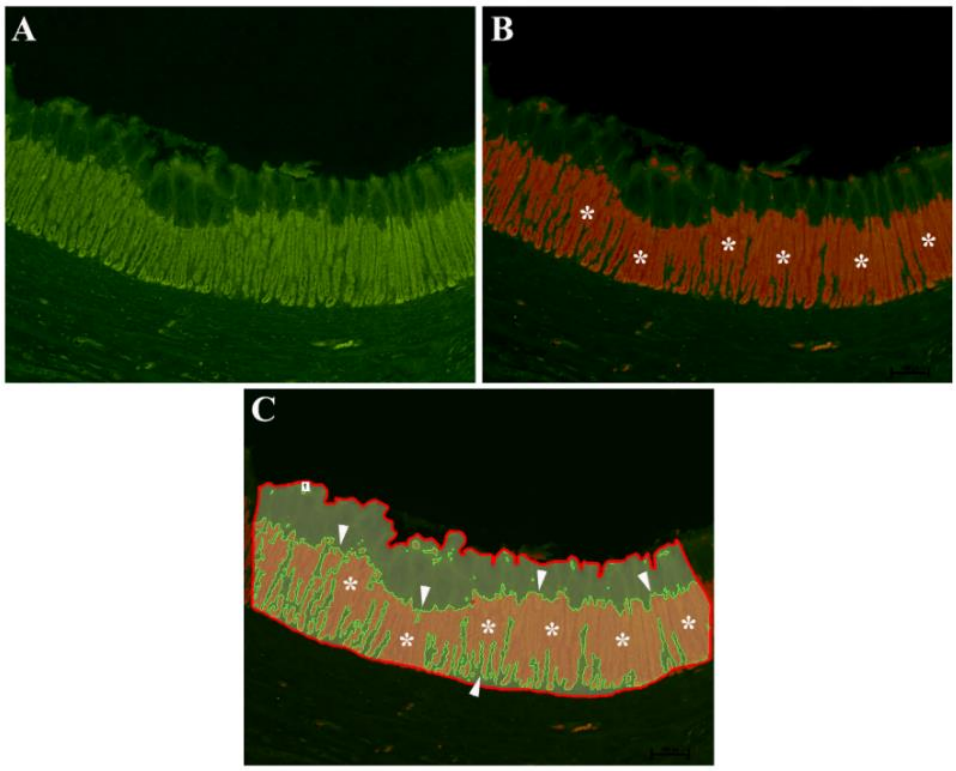

Figure 3. Threshold binarization method of European sea bass gastric mucosa. (A) Original acquired image. (B) Specific threshold to highlight the OP-IR cell area (asterisks). (C) Represents the region of interest (ROI) defined by the red line within which the binarized area is colored in red (asterisks) and delimited by a green line (arrowheads)

Table 2. Growth performance of European sea bass fed experimental diets over 117 days

Data are given as the mean (n = 3) ± SD. No significant differences among treatments (One-way ANOVA, p > 0.05). IBW = initial body weight. FBW = final body weight. SGR = specific growth rate (% day−1) = 100 × (ln FBW − ln IBW)/days. FI = feed intake (% average body weight−1, AWB day−1) = ((100 × total ingestion)/(ABW))/days. FCR = feed conversion rate = feed intake/weight gain.

Experimental Diet	CTR	N-EOs	S-EOs	*p* Value
IBW (g)	75.3 ± 2.88	74.9 ± 1.54	74.9 ± 2.42	0.835
FBW (g)	274.2 ± 7.81	267.9 ± 3.49	263.6 ± 4.19	0.137
SGR	1.10 ± 0.05	1.09 ± 0.03	1.07 ± 0.02	0.529
FI	1.47 ± 0.05	1.41 ± 0.02	1.42 ± 0.03	0.158
FCR	1.52 ± 0.08	1.54 ± 0.06	1.52 ± 0.02	0.894

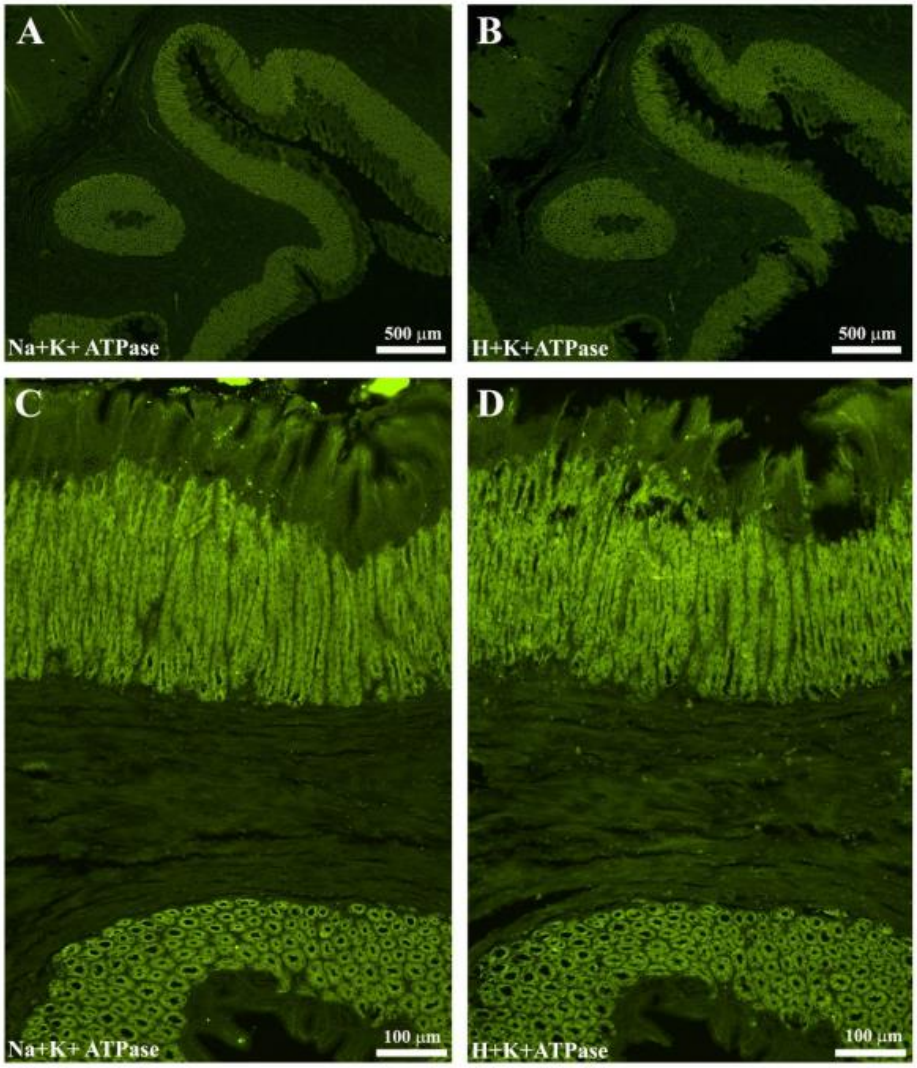

Figure 4. The images (A–D) show, in serial sections, a total overlapping of the oxyntopeptic-IR cells in the gastric mucosa both using Na^+K^+-ATPase (A,C) and H^+K^+-ATPase (B,D) primary antibodyes

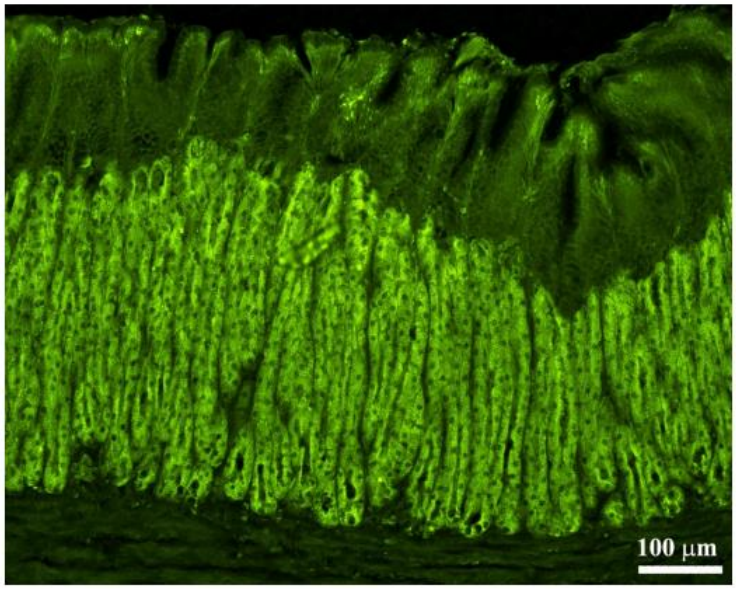

Figure 5. Localization of oxyntopeptic (OP) immunoreactive cells marked with Na^+K^+-ATPase antibody in the European sea bass gastric mucosa. The positive OP cells were observed along the tubular-like structure.

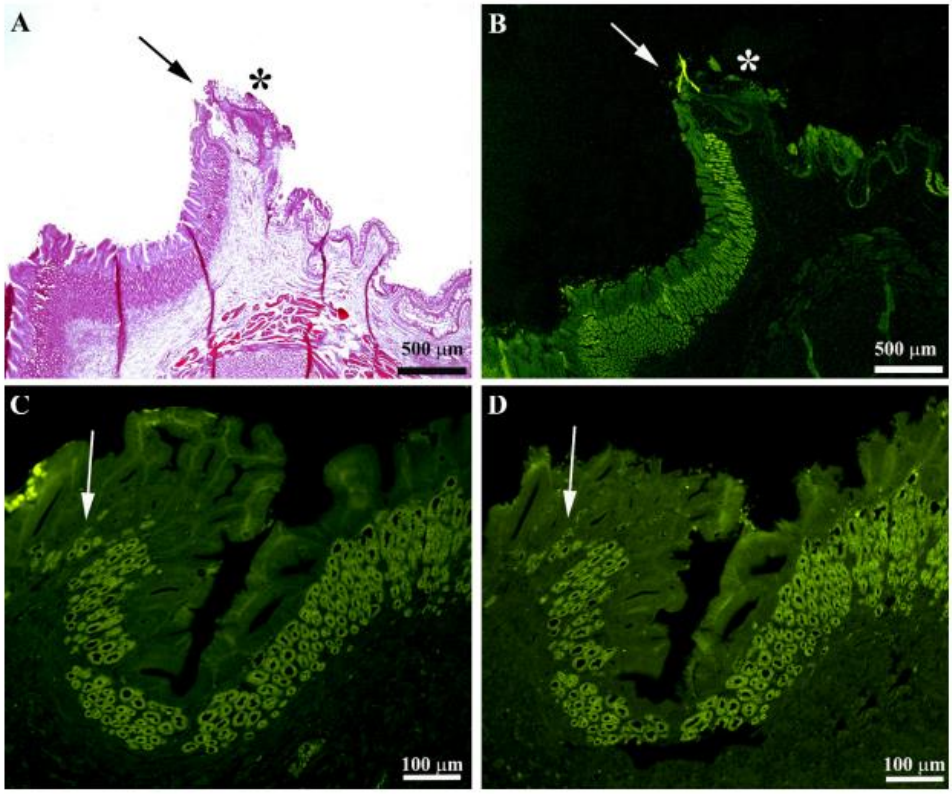

Figure 6. Gastric mucosa of the European sea bass. The images show the transition from esophagus to stomach in serial sections stained with hematoxylin–eosin (A) and Na^+K^+-ATPase (B). The simple esophageal columnar epithelium (asterisks) abruptly passes to the gastric mucosa with typical gastric glands (arrows). Images (C,D) show that the presence and distribution of the oxyntopeptic cells at the esophagus–stomach junction tend to decrease until they disappear (arrows): this feature was highlighted in both serial sections stained with Na^+K^+-ATPase and H^+K^+-ATPase antibody

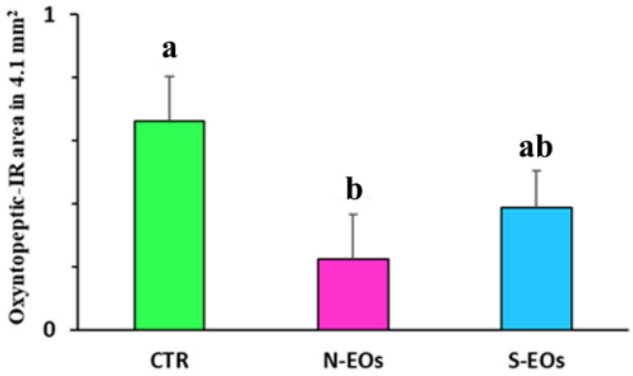

Figure 7. Graph showing the oxyntopeptic cells' immunoreactive area in the sea bass gastric mucosa. CTR (control group), N-EOs (natural EOs), and S-EOs (EOs obtained by synthesis). Different letters (a and b) indicate significantly different mean values at $P < 0.01$. Values are expressed as mean + SD.

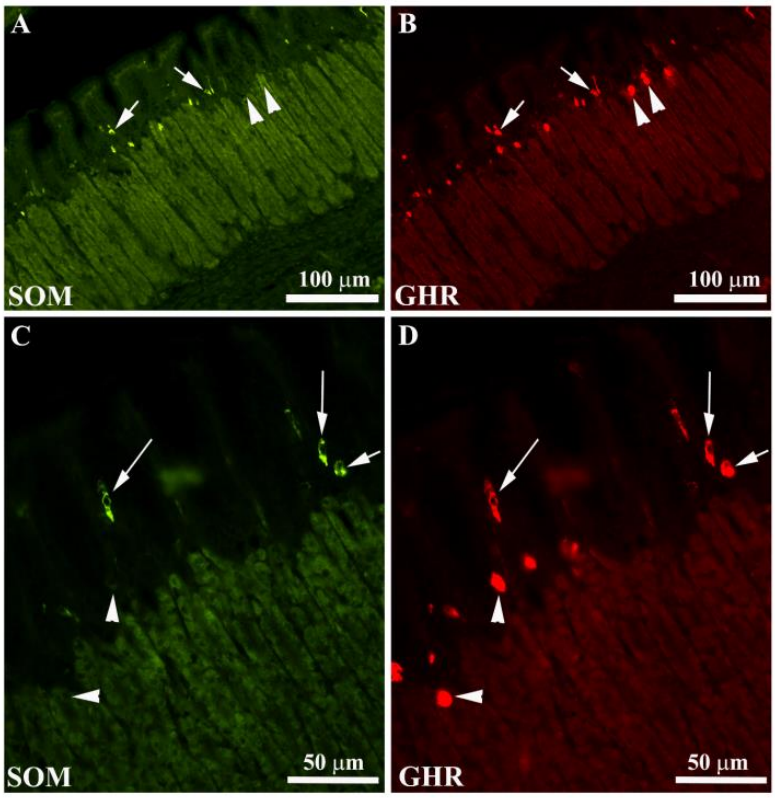

Figure 8. Localization of somatostatin (SOM) (A,C) and ghrelin (GHR) (B,D) enteroendocrine cells (EECs) in the European sea bass gastric mucosa. Some EECs co-expressing SOM/GHR-IRs (A,B, arrows). The arrowheads in (A,B) indicate GHR-IR cells (B) not containing SOM-IR (A). In some cases, both SOM and GHR-IR cells show a typical "open-type" EEC morphology (C,D, long arrows), while other SOM and GHR-IR cells were found lying close to the basal lamina of the glands and exhibiting typical "closed-type" EEC morphology (C,D, short arrows). Even in these higher-magnification images, some GHR-IR EECs were negative for SOM (C,D, arrowheads).

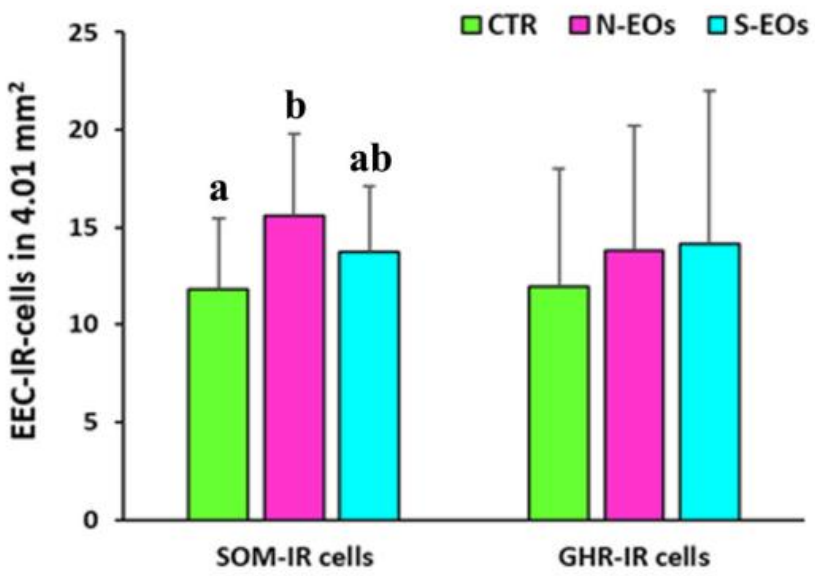

Figure 9. Graph showing the number of the somatostatin (SOM) and ghrelin (GHR) enteroendocrine (EEC) immunoreactive (IR) cells in the sea bass gastric mucosa. CTR (control group), N-EOs (natural EOs), and S-EOs (EOs obtained by synthesis). Different letters (a and b) indicate significantly different mean values at $P < 0.05$. Value are expressed as mean + SD.

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CONCLUSIONS

6. CONCLUSIONS

In the last decade, the application of natural feed additives has been able to support optimal gut health and function, thus enhancing growth, feed utilization, and disease prevention in the whole aquaculture sector. Global fish production that comes from aquaculture production is increasing dramatically.

The consumption of fish is extremely important, providing around 20% of their average per capita animal protein intake to billion people.

It's not difficult to understand how aquaculture world production of fish had progressively and significantly exceeded the catches.

The broad trends that have driven growth in global fish consumption in recent decades have been paralleled by many fundamental changes.

A responsible use of available resources related to fishing and aquaculture activities is therefore necessary today.

With technological and scientific progress, aquaculture production has reached unprecedented peaks. However, all these advances then had a negative impact on the environment. Moreover, in intensive and semi-intensive farming, it has long been known that various biotic and abiotic factors, as well as aquaculture procedures (handling, transport, or stocking density), activated a stress system that induces negative effects on different physiological processes in fish (growth, reproduction, and immunity).

In this context, EOs could represent a promising strategic alternative method to antibiotics/chemicals for maintaining and promoting health, as well as preventing and potentially treating some diseases and/or improving growth also in the gastric context.

These plant derivatives are most likely able not only to modify gastric morpho-functional characteristics but also modulating the intestinal microbiota, demonstrating great potential in acting as growth promoters and, at the same time, on the health of fish.

To understand how these new technologies can have a positive effect on the gastrointestinal tract and animal welfare, it was extremely important study and analyze the anatomy and physiology of the GI tract of fish.

For the first time, we showed how diet administration supplemented with the EO blend HERBAL MIX® could directly affects the distribution of OP, SOM, and GHR-IR cells in the sea bass gastric mucosa. This is possible through an EOs acidifying-like action on SOM EECs.

It remains to clarify and continue to perform studies on oxyntopeptic and enteroendocrine cells, to understand the role of EOs on the morphology of the fish gastric mucosa. The observations obtained in this study will provide a basis to better understand digestive physiology and help pathologists and nutritionists in future studies based on diet and diseases affecting this species.

Furthermore, other studies are needed to obtain more information in order to better understand the mechanisms of action of the various EOs and on the morphology of the gastric mucosa of fish. These results can be considered as a valid support for the feed formulation as well as for the health status of this widely farmed fish species.

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