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Lesions and targeting of neuro-glio-epithelial unit during irritable bowel syndrome

JURY

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NL. Lilli, P. Aubert, E. Coron, M. Neunlist, R. De Giorgio, S. Bruley des Varannes. ***In vitro* effect of *Lactobacillus reuteri* on impaired paracellular permeability of intestinal epithelial cells.**

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ABBREVIATIONS

5-HT	Serotonin
Ach	Acetylcholine
ACTH	Adrenocorticotropic hormone
AH	After hyperpolarisation
AJs	Adherent junctions
AP	Action potential
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BMP	Bone morphogenetic protein
CCK	Cholecystokinin
CD	Crohn's disease
CGRP	Calcitonin gene related peptide
CK	Casein kinase
CNS	Central nervous system
Cx43	Connexin-43
DCs	Dendritic cells
DSS	Dextran sodium sulphate
ECM	Extracellular matrix
EDNR	Endothelial receptor
EGC	Enteric glial cells
EGF	Epidermal growth factor
ENCDCs	Enteric neural crest derived cells
ENS	Enteric nervous system
EPSP	Excitatory post synaptic potential
ET	Endothelin
FAK	Focal adhesion kinase
FGID	Functional gastrointestinal disorders
FODMAPs	Fermentable oligo-, di- and monosaccharides and polyols
GABA	Gamma aminobutyric acid
GDNF	Glial cell derived neutrophic factor
GFAP	Glial fibrillary acidic protein
GI	Gastrointestinal
GS	Glutamine synthetase
GSK	Glycogen synthase kinase
GSNO	S-Nitrosoglutathione
HES	Hairy/Enhancer of Split
H1R	Histamine 1 receptor
HPA	Hypothalamic-pituitary-adrenal
HSCR	Hirschsprung disease
IBD	Inflammatory bowel disease

Abbreviations

IBS	Irritable bowel syndrome
IBS-C	Constipation-predominant IBS
IBS-D	Diarrhoea-predominant IBS
ICC	Intestinal cells of Cajal
IEB	Intestinal epithelial barrier
IECs	Intestinal epithelial cells
IELs	Intraepithelial lymphocytes
Ig	Immunoglobulin
IHH	Indian hedgehog
IL	Interleukin
ILCs	Innate Lymphoid cells
INF	Interferon
IP3	Inositol triphosphate (IP3)
IPANs	Intrinsic primary afferent neurons
IPSP	Inhibitory post synaptic potential
JAM	Junctional adhesion molecule
Lactobacillus reuteri	*L. reuteri*
LPS	Lipopolysaccharides
MAPK	Mitogen activated protein kinase
MLC	Myosin light chain
MLCK	Myosin light chain kinase
NC	Neural crest
NGF	Nerve growth factor
NK	Natural killer
NLR	NOD-like receptor
NO	Nitric oxid
NT	Neurotrophic
PAR	Proteinase-activated receptor
PD	Parkinson’s disease
PI	Post infectious
PKC	Protein kinase C
ROCK	Rho kinase
S	Synaptic
SCFA	Short chain fatty acid
SEM	Semaphorin
SHH	Sonic hedgehog
SM	Smooth muscle
SP	Substance P
SSRI	Selective serotonin reuptake inhibitors
SUP	Supernatant
TCA	tricyclic antidepressant
TGF	Transforming growth factor
TJs	Tight junctions
TLR	Toll-like receptor
TNF	Tumore necrosis factor

Abbreviations

T_{REG}	Regulatory T
TSLP	Thymic stromal lymphopoietin
UC	Ulcerative colitis
VGF	Vascular endothelial factor
VIP	Vasoactive intestinal polypeptide
ZO	Zonula occludens

GENERAL INTRODUCTION

Digestion is a very complex and finely regulated mechanism that requires a variety of highly integrated regulatory mechanisms, finely tuned by different cell types, including:

i. endocrine cells, distributed throughout the gastrointestinal (GI) mucosa;

ii. smooth muscle (SM) cells that represent final effectors of contractility and/or relaxation;

iii. interstitial cells of Cajal (ICC) that are pace-makers of gut motility and regulators of neuro-muscular transmission;

iv. neurons of both intrinsic (enteric) and extrinsic (sympathetic and parasympathetic) origin (Furness 2012).

The neural network supplying the digestive system exerts a prominent regulatory role. Different types of reflexes are involved in the control of digestive functions including pathways that rely on sympathetic ganglia, those travelling from and to the gut via the central nervous system (CNS) and circuits of the enteric nervous system (ENS) located into the wall of the GI tract (Furness 2012).

The ENS, that is also called “brain in the gut” contains ~ 100-500 million of neuronal cell bodies organized in two major ganglionated plexuses (i.e., the myenteric and submucosal ones), neural processes connecting ganglia among them, and nerve fibers targeting effector cells, e.g. SM cells, epithelia, splanchnic blood vessels, immune cells and pancreato-biliary cells. In addition to neurons enteric glial cells (EGC) are fundamentals key players displaying a number of functions, ranging from

neurotransmission to enteric neuronal maintenance and survival. This complexity is the reason why ENS can function also independently of the CNS.

Over the last decades, the close relationship between brain and gut has inspired several studies aimed to understand how these two entities can affect one each other in physiological and pathological conditions.

The relationship between the two *brains* is bidirectional but, although the idea that the brain can alter intestinal functions has long been recognized and accepted, it is less common the concept that signals from the gut can affect mood, behavior and cognitive functions. In this line stress and anxiety are demonstrated to alter the gut function, while diet and intestinal disorders may be related to mood changes. In support of this concept, serotonin is one of the main neurotrasmitter involved in the brain/gut (or gut/brain) axis being produced by enteroendocrine cells of the intestinal mucosa. In physiological conditions serotonin controls digestive and nervous functions (such as peristaltic reflex and satiety, respectively). During inflammation, the excess of serotonin produced results in the saturation of its receptors and up-regulation of the specific brake-down enzymes.

Among intestinal diseases, the irritable bowel syndrome (IBS) is an example of alteration in which psychosocial and genetic factors may have together a role in the onset of the disorder. For many years IBS has been a *clinical enigma*, being considered linked to less severe dysfunctions strongly amplified by psychological and sociocultural factors. Over recent years, the conception of mind and body as a "*unique system*" has led to consider IBS as a bio-psychosocial disorder associated to a dysregulation of *brain-gut axis*, resulting in the alteration of visceral pain and autonomic functions perception and visceral stimuli processing at central level. In this

manner, several intestinal factors (motility, visceral sensitivity and alteration of intestinal permeability and intestinal flora composition), in association with central factors (stressful or traumatic experiences, anxiety and depression) would act concomitantly to generate IBS (Vaiopoulou et al., 2014).

However, recent evidences have assigned a central role of the intestinal epithelial lesions in the pathophysiology of IBS. The intestinal epithelial barrier (IEB) represents the window through which the body “tastes” the external environment, so its integrity is necessary to protect against pathogens. Lesions of the intestinal barrier alter intestinal permeability and favour the passage of luminal contents, including pathogenic bacteria. The activation of molecular pathways in response to intestinal barrier injuries springs from the physical proximity between intestinal barrier, bacterial flora and enteric nervous and immune system (Zhang et al., 2015).

A large population of microorganism composing the intestinal flora (*microbiota*) inhabits the gut. The intestinal microbiota are acquired at birth during the contact between the newborn and maternal flora or after the exposure to environmental microbes. Gut microbiota composition changes depend on dietary habits (breastfeeding and nutrition in early childhood) and the balance between the different species existing regulates several intestinal functions (such as digestion and absorption) and immune activity, such as defence against pathogenic invasion (de Vrese and Schrezenmeir, 2008). Intestinal infections, antibiotic use or stress can alter this balance causing the so-called *dysbiosis*; in these cases probiotics play a central role in the regulation of the immune system. Probiotics are specific strains of microorganisms, especially lactobacilli and bifidobacteria, able to colonize the intestinal flora with positive effects on the health of the host. Interestingly, studies

have recently shown positive effects of some strains of probiotics in the treatment of IBS symptoms (Lee et al., 2011).

The maintenance of intestinal barrier homeostasis and integrity is highly regulated by glial and neuronal components of the ENS along with intestinal microbiota and immune cells. This mutual interaction in the gut defines a ***neuro-glio-epithelial unit*** similar to the neuron-glial barrier in the brain, highlighting even more the analogy existing between these two great systems. The regulation of the ENS components in order to strength the intestinal barrier is a promise in the treatment and prevention of a number of digestive diseases, including IBS (Neunlist et al., 2013).

In this context, the objectives of my PhD thesis were twofold:

1. To investigate the effects of a probiotic strain in preventing IEB injuries.

2. To assess the putative alterations of enteric glial cells (EGC) and mediators produced by the colonic mucosal microenvironment involved in pathophysiology of IBS.

The main results that I have obtained during my PhD thesis are summarized as follows:

1. The strain *Lactobacillus reuteri* was able to prevent the protease activated receptor 2 agonist-induced increase of paracellular permeability in Caco-2 monolayers, probably in part via a mechanism involving modulation of tight junction proteins expression.
2. The EGC network was reduced in biopsies from IBS patients compared with HC. The supernatant from colonic biopsies of IBS patients reduced the proliferation and the amplitude of Ca^{2+} response induced by adenosine

triphosphate (ATP) in EGC cultures compared to control. The modification of the amplitude of Ca^{2+} response induced by ATP in EGC treated with histamine allowed to find a possible soluble mediator responsible for the changes of EGC electrophysiological proprieties. Finally, a pharmacological approach has demonstrated that the effects of histamine and IBS supernatants upon ATP induced Ca^{2+} responses were mediated via activation of histamine receptor 1.

This work resulted in two papers recently submitted and presented in this thesis. The following introduction aim to present:

- The intestinal epithelial barrier and the regulation of its homeostasis
- The enteric nervous system and its neural and glial components
- The irritable bowel syndrome
- The gut microbiota

INTRODUCTION

1. THE DIGESTIVE SYSTEM

1.1 Overview

The digestive system is composed of a set of organs comprising the oral cavity, the gastrointestinal (GI) tract as well as the related glands. The digestive tract is approximately eight meters long and includes pharynx, esophagus, stomach, small intestine (duodenum, jejunum, and ileum) and colon (cecum, ascending colon, transverse colon, descending colon, sigmoid colon and rectum) terminating in the anus. Small intestine and colon are separated by the Bauhin's ileocecal valve. The glands include the salivary glands, liver, gallbladder and pancreas connected to the digestive tract by specific channels. All of digestive tract organs have the same histological organization with a wall consisting of four distinct layers:

1) *mucosa* that lines the intestinal lumen is composed by intestinal epithelial barrier (IEB) and lamina propria. The IEB is a monolayer of polarized cells based on basal lamina. The lamina propria or stroma is a connective tissue rich in blood and lymphatic capillaries, containing numerous lymphoid follicles. It is separated from the submucosa by the *muscularis mucosae* composed of two thin layers of smooth muscle (SM) fibers, the inner circular and outer longitudinal;

2) *submucosa*, a loose connective tissue layer containing the submucosal plexus (Meissner's plexus) and blood and lymph vessels;

3) *muscularis* composed by an internal layer of SM cells and an outer longitudinal layer with between the two the myenteric plexus (Auerbach's plexus);

4) *external adventitia*, the ends of the digestive tract, is a loose connective tissue called adventitious and for the rest, it is a connective tissue lined by a simple epithelium called serous (Timmermans et al., 1992) (Figure 1).

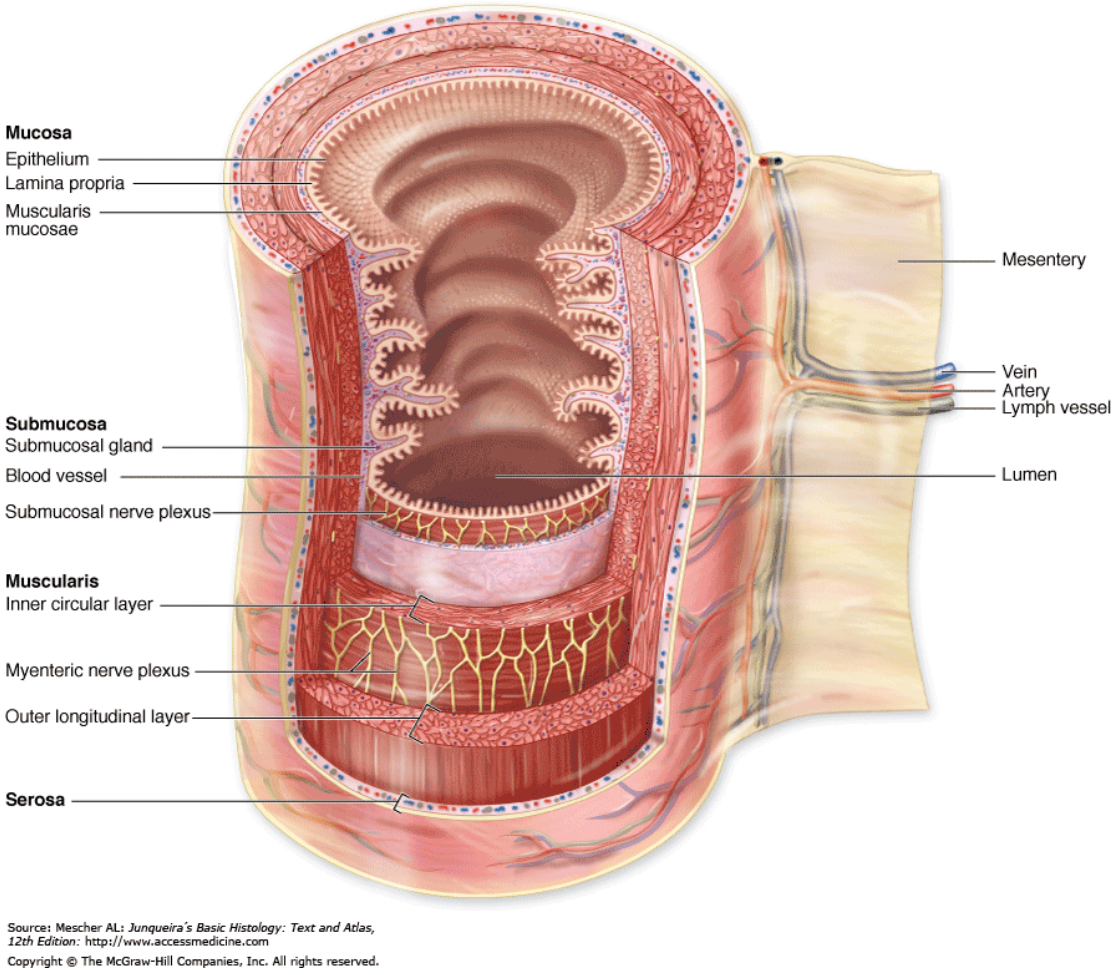

Figure 1. ***Major layers and organization of the digestive tract.*** *Schematic diagram showing the four layers and their major components listed on the left (13th edition of Junqueira's Basic Histology,Mescher,A. 2013)*

1.2 The intestinal epithelium

The digestive epithelium is a layer of cells, which composes a protective barrier against the outside environment from the esophagus to the anus, and, the organization of this barrier depends on the function performed by each component of the digestive tract. The esophagus is composed by a stratified squamous epithelium, which protects the wall friction of the bolus. Stomach is composed by a single-layered epithelium composed of mucus-secreting cells in order to form a protective film against gastric acidity (Figure 2A).

In this study, only the colon and intestinal epithelium, called IEB will be detailed. This dynamic barrier will provide two roles: to allow the absorption of nutrients, electrolytes and water and preventing the passage of pathogens (viruses, toxins, bacteria) (Zhang et al., 2015).

The small intestine is the site of absorption of nutrients, water, electrolytes and vitamins, while non-digestible food residues of alimentary bolus are transformed into feces through the reabsorption of water and soluble salts in the colon. These different functions are made possible by a specialized epithelium in terms of morphology and cells. The mucosa of the small intestine has a very important exchange surface between the intestinal lumen and internal environment that increases the efficiency of absorption of nutrients. Epithelia are folded into repeat units comprising invaginations (crypts of Lieberkuhn) associated with numerous protrusions (villi). Three levels can be distinguished: transversal circular folds or valves conniventes of 1 cm high, the villosities of 0.5 to 1 mm high and microvilli of the brush border of 1 to 2 μm high. In the colon the mucosa is flat and punctuated with cryptic invaginations (Figure 2).

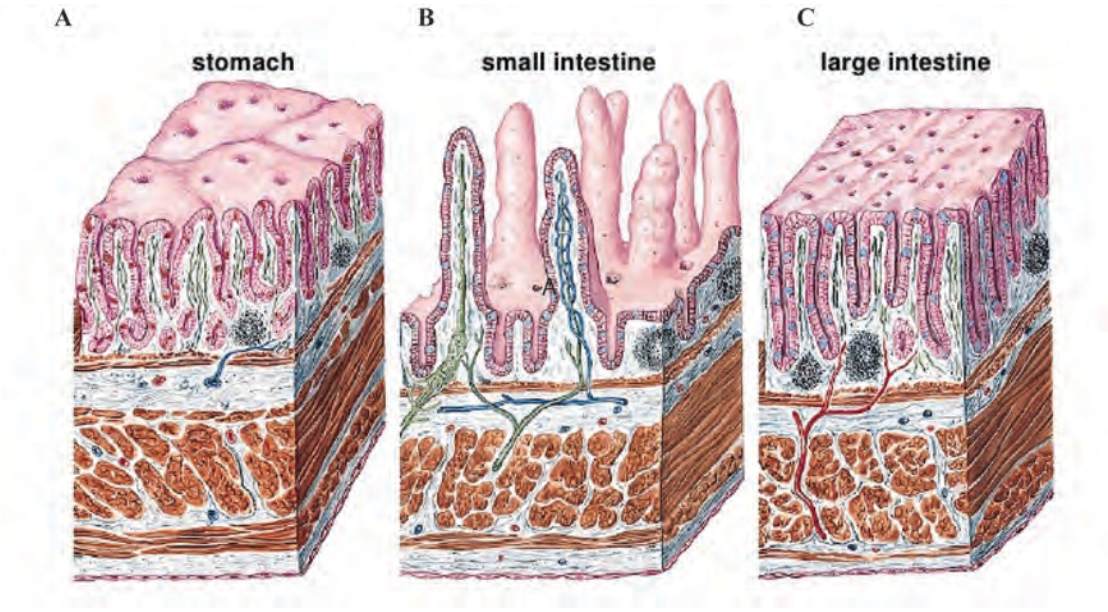

Figure 2. Comparison of histology of stomach, small intestine and large intestine. *The mucosa of the small intestine has a very important exchange surface. Conversely, the mucosa of the stomach and large intestine (colon) is flat and rich in cryptic invaginations.*

1.2.1 Intestinal epithelial barrier cells

The intestinal epithelium cover approximately 400 m^2 of surface area with a single layer of cells organized into crypts and villi (Figure 3). Pluripotent intestinal epithelial stem cells residing in the base of crypts allow the continuous renewal of the surface every 3–5 days. These stem cells give rise to transit amplifying daughter cells that undergo 4–5 rounds of rapid cell division as they migrate apically towards the villus (van der Flier and Clevers, 2009), (Casali and Batlle, 2009). During this journey upper the crypt these cells start to differentiate and exit onto the villus epithelium as mature absorptive enterocytes, mucus-secreting goblet cells, or hormone-producing enteroendocrine cells. These cells subsequently continue migrating and generally reach the villus tip after 3–5 days, when they die and are exfoliated into the lumen

(Vries et al., 2010). A fourth differentiated cell type, the Paneth cell, evades this upward migration program, instead forcing its way to the base of the crypt (van der Flier and Clevers, 2009; Vries et al., 2010). Secretory intestinal epithelial cells (IEC), including enteroendocrine cells, goblet cells and Paneth cells, are specialized for maintaining the barrier function of the epithelium. Enteroendocrine cells secrete numerous hormone regulators of digestive functions, while goblet cells and Paneth cells are respectively involved in the luminal secretion of mucins and antimicrobial proteins. In addition, crypts also supply less common cell types such as the M-cells, brush/tuft/caveolated cells and cup cells, although their lineages are poorly understood (Lin and Barker, 2011).

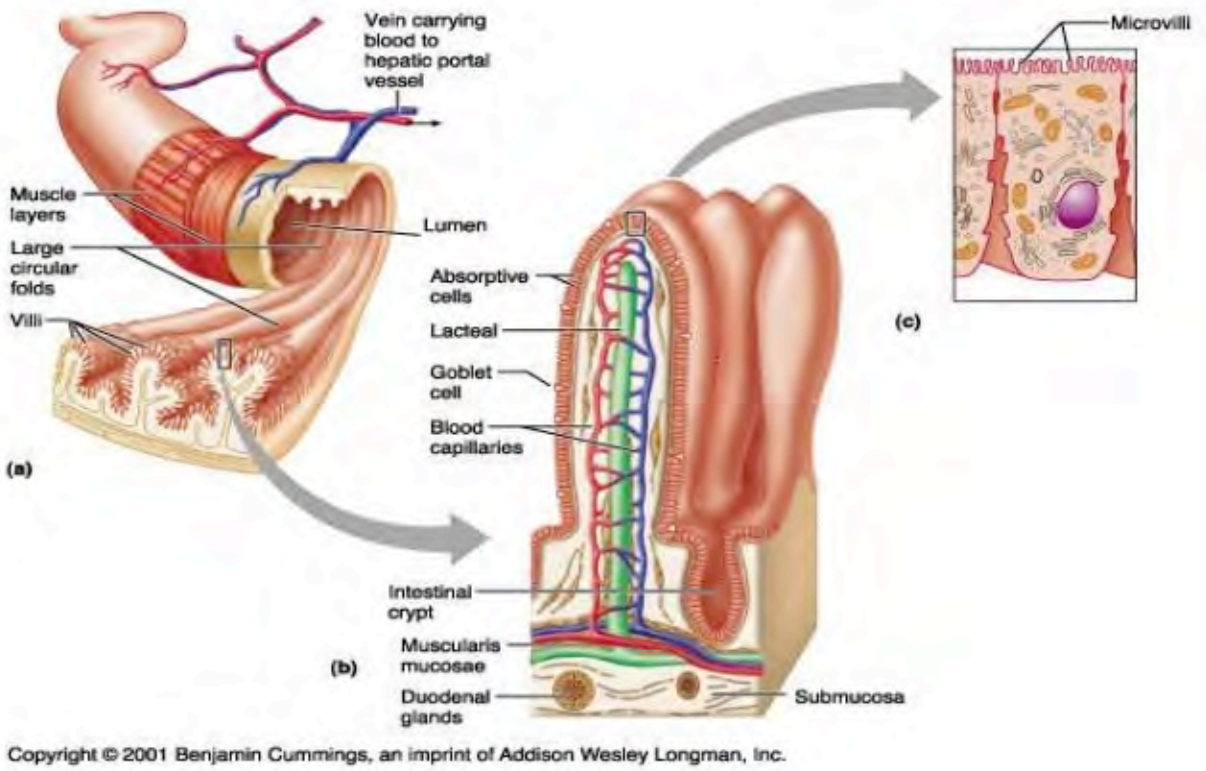

Figure 3. Diagram of the organization of the intestinal epithelium*. Section of digestive tube (left) showing the circular folds compounds of the intestinal villi. Structure of an intestinal villus (middle) and IECs with microvilli on their apical (right). ©2011 Benjamin Cummings, an imprint of Addison Wesley Longman, Inc.*

Introduction

Intestinal epithelial stem cells

Two schools of thought exist as to define the exact intestinal stem cell identity: the "*+4 position*" model and the "*stem cell zone*" model. In the past, crypt has been viewed as a tube of proliferating cells bounded from below by Paneth cells. Since the late1950s, the "*+4 position*" model, largely championed by *Chris Potten* and colleagues (Marshman et al., 2002), has therefore placed the stem cells at position *+4* relative to the crypt bottom, with the first three positions being occupied by the terminally differentiated Paneth cells. The second school of thought proposed by *Leblond* and colleagues in the early 1970s, has been based on the identification of crypt base columnar cells, small, undifferentiated, cycling cells hidden between the Paneth cells and identified by the presence of *Lgr5* gene (Cheng and Leblond, 1974; Barker et al., 2008) (Figure 4). A second pool of intestinal stem cells is composed by slower cycling Bmi1-expressing cells situated at position +4 above the crypt base (Sangiorgi and Capecchi, 2009). Between stem cells and differentiated cells there is an intermediate step where stem cells differentiate into progenitor that will become specialized cells. The first theory is that the stem cells give three kinds of progenitors that differentiate into enterocytes or secretory cells. The second theory is that stem cells provide a first line of common progenitors differentiate either into enterocytes either in a second generation of IECs progenitors with a secretory activity (Bjerknes and Cheng, 2006).

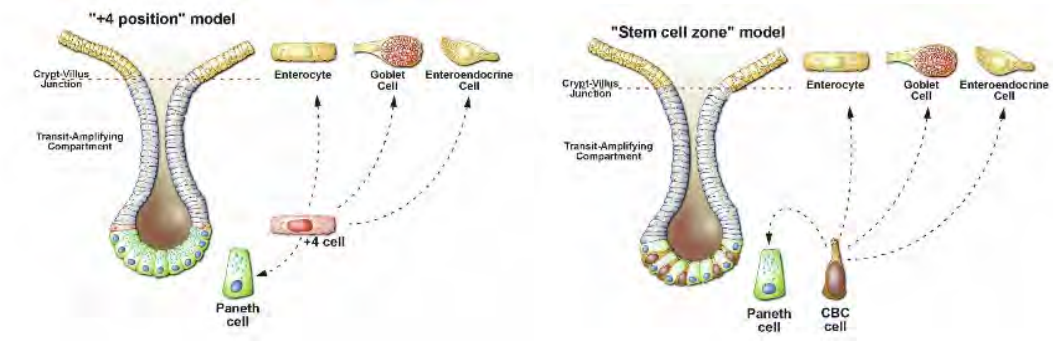

Figure 4. Two-opposing models of the identity of intestinal epithelial stem cells. *In the"+4 position" model the crypt base is exclusively populated by terminally differentiated Paneth Cells and the stem cells must therefore be located just above the Paneth cells at the +4 position. This model predicts that the enterocytes, goblet cells, and enteroendocrine cells are derived from +4 cell progeny that differentiate as they migrate out of the crypts onto the villi. In contrast, the Paneth cells differentiate as they migrate down from the +4 position toward the crypt base (left panel). The "stem cell zone" model states that small, undifferentiated, cycling cells (termed crypt base columnar cells) intermingled with the Paneth cells are likely to be the true intestinal stem cells (right panel) (Barker, N. 2008).*

Enterocytes

The enterocytes are the most represented cells in the IEB (> 80% of all small IECs). They are located all along of villi and at the top of crypts and they are longer and more numerous in the small intestine rather than colon. At their apical pole, these cells exhibit a dense array of microvilli whose diameter of about 100 nm remains constant among the enterocytes. This brush border is ordered very regularly on the apical side of the enterocytes (Crawley et al., 2014) (Figure 5). Estimations based on morphometric data suggest that this array of microvilli allows to very significantly increasing from 9 to 16 times the area of the small intestine, enhancing the surface of exchange (Helander and Fändriks, 2014). Moreover, microvilli are covered with glycocalyx rich in glycoproteins and glycolipids, playing a protective role and

containing enzymes necessary for digestion and nutrient transport (Johansson et al., 2011).

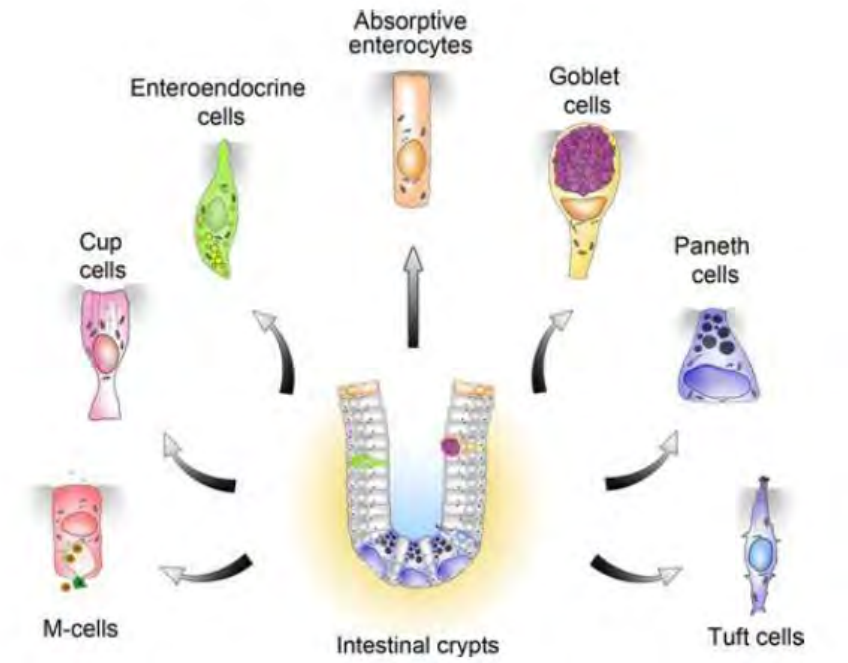

Figure 5. Representation of intestinal crypt and classes of intestinal differentiated cells*. Intestinal epithelial stem cells Lgr5^{+} located at the bases of the crypts are at the origin of many epithelial cells types, the enterocytes, goblet cells, enteroendocrines cells, Paneth cells, M-cells, "Tuft"cells and "Cup"cells (Gerbe, F. 2011).*

Goblet cells

Goblet cells appear early in development (in the human fetal small intestine, at 9–10 weeks' gestation). Their morphology is shaped by the distended theca containing mucin granules located below the apical membrane responsible for the production and preservation of a protective mucus blanket (Figure 5). Goblet cells synthesize and secrete, by exocytosis into the intestinal lumen, high molecular weight glycoproteins known as mucins (Kim and Ho, 2010). The proportion of goblet cells

among epithelial cell types increases caudally from duodenum (4%) to distal colon (16%), similar to the increasing number of microbial organisms present in the proximal intestine to colon (Karam, 1999). Mice lacking the MUC2 mucin or with MUC2 mutations develop severe inflammation with bloody diarrhoea, weight loss, rectal prolapse, and for the mice lacking Muc2 also colon cancer after 3–6 months, directly reminding of ulcerative colitis (UC). These results suggest that the inner mucus layer formed by the goblet cells is necessary to make an anchored layer that excludes bacteria (Velcich et al., 2002; Johansson et al., 2008; Van der Sluis et al., 2006).

Enteroendocrine cells

Enteroendocrine cells are found scattered throughout the epithelium of the GI tract from the stomach to the rectum. They release gut hormones in response to meal-related stimuli and thereby exert actions ranging from the local control of gut motility and secretion to the regulation of insulin release and food intake. Although they are located in the epithelial layer and many are open type, having microvilli-covered apical surfaces that make direct contact with the luminal constituents (Gribble and Reimann, 2015). However, a large proportion of enteroendocrine cells are closed-type cells that do not make contact with gut lumen (Figure 5). Enteroendocrine cells are classified according to the principal hormone they produce. In the stomach, for example, enteroendocrine cell populations include cells producing 5-hydroxytryptamine (serotonin [5-HT]) (enterochromaffin cells), histamine (enterocromaffin-like cells), somatostatin (D-cells), gastrin (G-cells) and grelin (P- or D1-cells). In the duodenum, jejenum and ileum enteroendocrine cells produce

glucose-dependent insulinotropic polypeptide (K-cells), cholecystokinin (CKK) (I-cells), motilin (M-cells) and secretin (S-cells) in addition to enterochromaffin cells, G- and D-cells. More distally, these cell types drop in numbers, and there is an increase in the number of cells producing neurotensin (N-cells) and glucagon-like peptides 1 and 2 but also peptide YY, 5-HT and insulin-like peptide 5 (L-cells) toward the distal intestine (Stengel and Taché, 2009; Ku et al., 2003). Interestingly, in recent years it has been shown that there is considerable co-expression of hormones within individual enteroendocrine cells (Svendsen et al., 2015; Cho et al., 2015).

The interaction between enteroendocrine cells is important for the regulation of hormonal secretion. For example D-cells can interdigitate at their baso-lateral pole with neighboring G-cells by elaborate processes, enabling the close paracrine regulation of gastrin secretion by somatostatin. Recent 3-D images of enteroendocrine cells producing peptide YY and CKK have shown a basolaterally located process termed a neuropod which extends toward the enteric nervous system (ENS) and glia as well as toward myofibroblasts probably enabling a unidirectional or bidirectional communication between enteroendocrine cells and the ENS (Bohórquez et al., 2015).

Enteroendocrine cell secretory products accumulate in intracellular granules and are secreted by exocytosis at the basolateral membrane into the interstitial space where they can act locally or remotely via the bloodstream. For example, CKK is secreted in response to the intake of a meal and its release is controlled by the presence of lipid and protein degradation products. CKK acts on the pancreas to induce the secretion of digestive enzymes in the intestinal lumen and the gallbladder to induce the release of bile salts (Mawe, 1991).

Paneth cells

Described for the first time by *Josef Paneth* and *Gustav Schwalbe*, Paneth cells are secretory cells based principally at the base of crypts in the small intestine and, in lower concentrations, in the stomach and colon. The life span of Paneth cells in the crypt is much longer (approximately 30 days) than that of epithelial cells along the small intestine villi (approximately 3 to 5 days) (Clevers and Bevins, 2013). Paneth cells are specialized in the production of antimicrobial peptides (defensins) and proteins in response to various stimuli including components of the bacterial surface. They have the ultra-structural hallmarks of professional secretory cells, including an extensive endoplasmic reticulum and Golgi network and apical clustering of the secretory abundant and large granules secreted into the crypt lumen. Evidences have shown that proteins and antimicrobial peptides released by Paneth cells not only protect from infections by enteric pathogens but also help shape the composition of the indigenous microbiote (Petnicki-Ocwieja et al., 2009). The contents of granules depend on the condition of the intestinal lumen and, consequently, the expression of certain antimicrobial peptides as Reg III is inducible while others such as alpha-defensins or lysozyme are expressed constitutively (Bevins and Salzman, 2011).

M-cells

M-cells play a crucial role in the intestinal immune system by sampling and uptake of antigens, and then transporting them from the gut lumen to the underlying immune system. They are predominantly present on the follicle-associated epithelium in the small intestine, colon and rectum. In the colon, M-cells are embedded in the

IEB and, thanks to microvilli that compose them, prevent access of microorganisms to their apical surface and they are responsible for the recognition and presentation of certain luminal pathogenic to the immune cells (Wang et al., 2014).

Cup cells

Cup-cells are characterized by a shorter brush border with linear arrays of particles in their microvillous membrane. Like M-cells, cup cells express vimentin, but they differ from M-cells in the glycosylation pattern of their plasma membrane and do not transport antigens and pathogens to mucosal immune cells (Gerbe et al., 2012).

Tuft cells

Tuft cells represent a minority of the murine intestinal epithelium (0.4%), located at the apex of the crypts. These cells have a «sensor» function of nutrient contents and can secrete opioids. Tuft cells are characterized by a unique tubule-vescicular system and a microfilament bundle connected to microvillosities penetrating the intestinal lumen (Gerbe et al., 2012).

1.3 The homeostasis of the IEB

Intestinal epithelial homeostasis is maintained by a strict equilibrium between cell proliferation in the crypt and cell shedding from the villus tip. Differentiate enterocytes are constantly removed and replaced by new cells originated by

undifferentiated adult intestinal stem cells located at the base of crypts. These new cells migrate toward the apical zone of the intestine during maturation. The epithelial layer is the result of a strict balance between cell proliferation and death to maintain the intestinal barrier (Negroni et al., 2015). This dynamism allows to a cell turnover every 4-5 days in human intestine, requiring 10 billion cells per day.

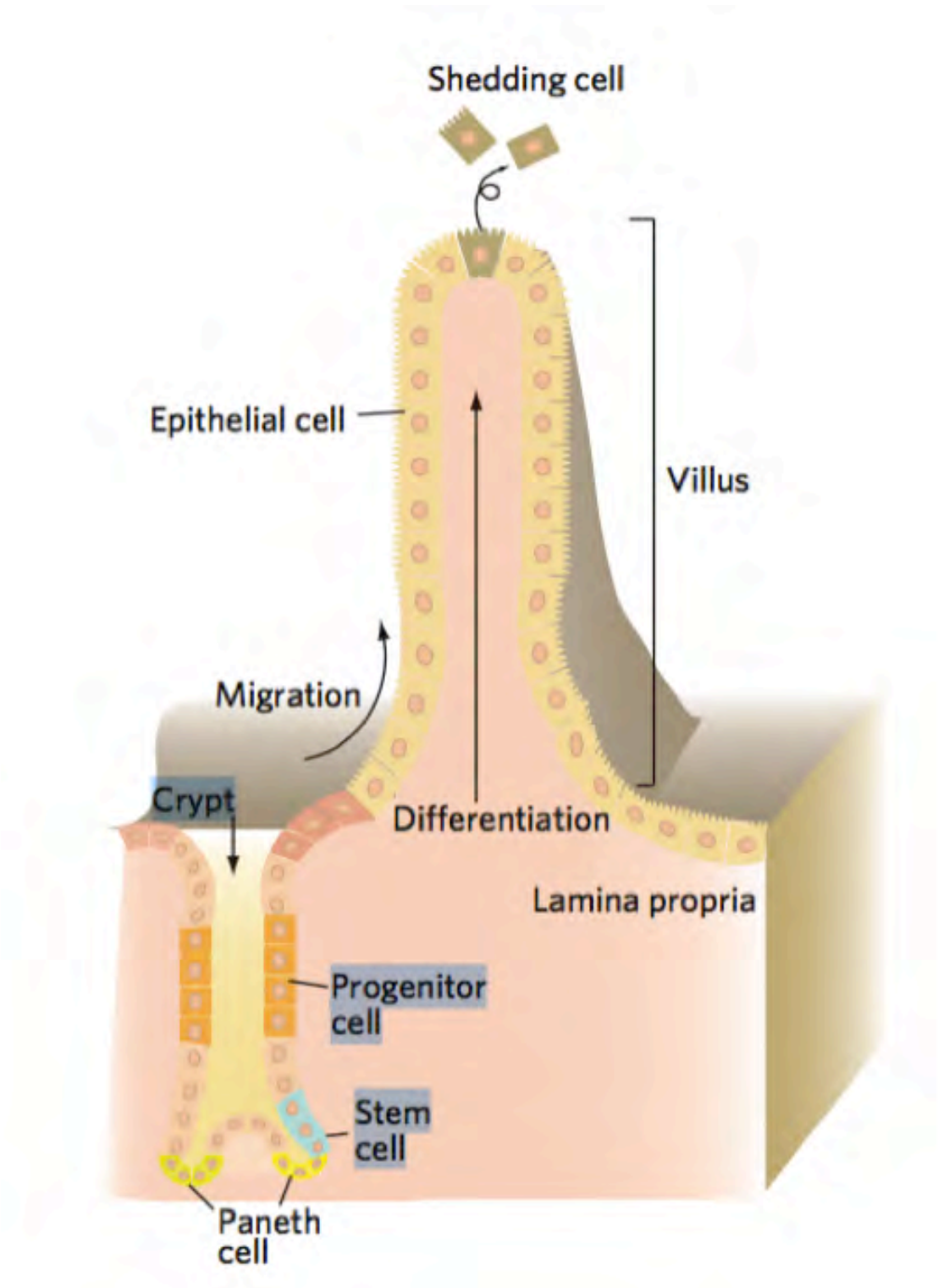

Figure 6. Intestinal epithelial barrier homeostasis. *The dynamics of the renewal of the IEB is controlled by a balance between the compartments proliferation, differentiation, and programmed cell death leading to epithelial cells exfoliation (Ashida, H. 2011).*

The intestinal stem cell fate, including self-renewal, proliferation and differentiation, is modulated by signals that regulate the architecture of the crypts and villi such as Wnt/β-catenin (proliferation and maintenance of crypts), transforming growth factor (TGF) β/bone morphogenetic protein (BMP) (inhibition of proliferation and induction of differentiation) and Hedghog (proliferation, repair, anti-inflammatory action) pathways. Notch (inhibition of proliferation and differentiation) and Hippo (inhibition of proliferation and apoptosis) pathways control stem cells fate. Recently, the contribution of Paneth cells secreting various important niche ligands (such as EGF, Wnt3a and Notch) in the stem cell niche, has received intense attention (Qi and Chen, 2015; Sato et al., 2011).

1.3.1 The IECs proliferation

The term “proliferation” refers to the process by which precursor cells replicate to expand the cellular pool. The canonical Wnt/β catenin pathway is a key regulator of proliferation of the stem cell in intestinal crypts. In the absence of Wnt, cytoplasmic β -catenin protein is constantly degraded by the action of the Axin complex, which is composed of the scaffolding protein Axin, the tumor suppressor *adenomatous polyposis coli* (APC) gene product, casein kinase (CK) 1, and glycogen synthase kinase (GSK) 3. CK1 and GSK3 sequentially phosphorylate the amino terminal region of β-catenin, resulting in β-catenin recognition by an E3 ubiquitin ligase subunit, and subsequent β-catenin ubiquitination and proteasomal degradation (He et al., 2004) (Figure 7a). Wnt proteins induce intracellular inactivation of GSK3β, a component of the destruction complex. The resultant dephosphorylation and

stabilization of β-catenin, a substrate of GSK3β, lead to the nuclear translocation of β-catenin and activation of target genes by the complex consisting of β-catenin and the TCF family of transcription factors (Logan and Nusse, 2004) (Figure 7b).

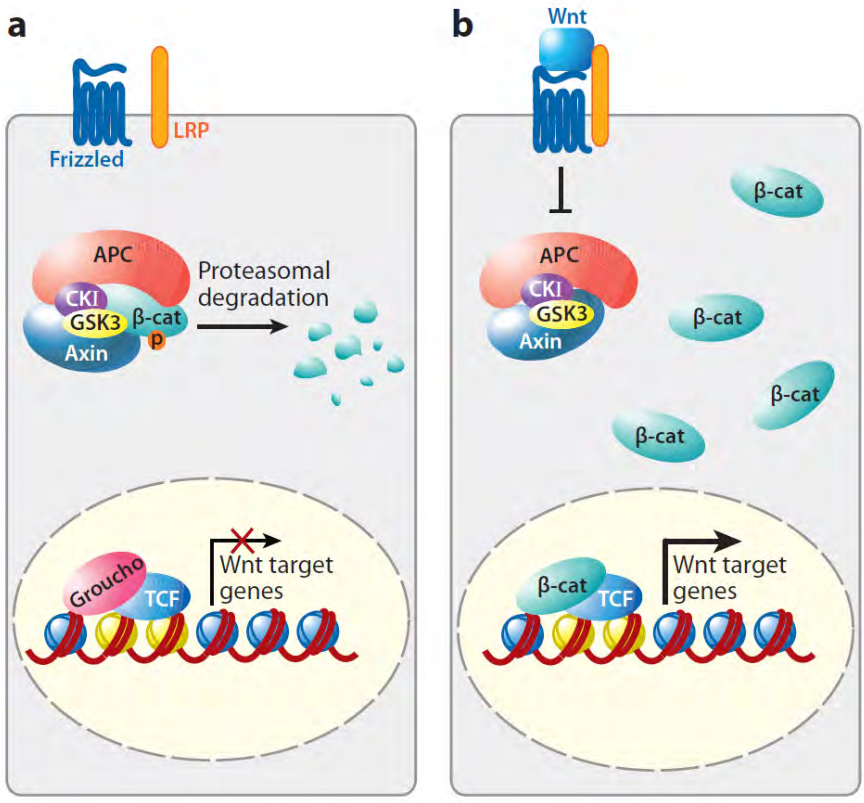

Figure 7. Wnt/β-catenin signalling pathway. *In the absence of active WNT (a), β-catenin is degraded, and prospective target genes are in a repressed state. If WNT signalling (b) is active, β -catenin degradation is reduced. As β -catenin accumulates it enters the nucleus and activates transcription. APC, adenomatous polyposis coli; beta-cat, β -catenin; CBP, CREB-binding protein; CK, casein kinase; DKK, Dickkopf; DSH, Dishevelled; GBP, GSK3-binding protein; GSK, glycogen synthase kinase; LRP, LDL-receptor-related protein; P, phosphorylation; sFRP, secreted Frizzled-related protein; TCF, T-cell factor (van der Flier, LG. 2009).*

However, constitutively activated β-catenin signalling, due to APC deficiency or β -catenin mutations that prevent its degradation, leads to excessive stem cell

renewal/proliferation that predisposes cells to tumorigenesis (Polakis, 2007). Moreover, Wnt/β catenin signalling is also indispensable for intestinal morphogenesis playing a pivotal role in the development of non-neoplastic GI disorders, such as chronic inflammatory bowel disease (Kuhnert et al., 2004).

1.3.2 The IECs differentiation

In addition to Wnt/β-catenin signalling, the Notch pathway plays a central role in intestinal cells differentiation. The interaction between one of four Notch receptors and any one of five Notch ligands results in proteolytic cleavage of the receptor. The free Notch intracellular domain translocates in the nucleus and binds to the transcription factor RBP-J_K (CSL or CBF1) to activate target genes (Artavanis-Tsakonas et al., 1999) (Figure 9a). In the intestinal epithelium Notch signals are mediated by Notch1 and Notch2 receptors, which work redundantly. The conditional inactivation of both these receptors results in the conversion of proliferative crypts into post mitotic goblet cells (Riccio et al., 2008). Contrarily, gain of function of Notch1 receptor causes the depletion of Goblet cells and the reduction of enteroendocrine and Paneth cells differentiation (Fre et al., 2005). So the Notch pathway is essential to maintain the crypt compartment in its undifferentiated state and to control absorptive versus secretory differentiation in intestinal epithelium.

Typically, Notch pathway results in the activation of Hairy/Enhancer of Split (Hes) genes that encode transcriptional repressors. In intestine, the direct Notch target gene *Hes1* represses transcription of bHLH transcription factor *Math1*, whose expression is required for the differentiation of secretory cells (Jensen et al., 2000)

(Figure 9b). *Yang et al*. have shown that epithelium of Math1 mutant mice is populated only by enterocytes (Yang et al., 2001). The zinc finger transcriptional repressor Gfi1 is important to the differentiation of Paneth cells and Goblet cells, as demonstrated by mice $Gfi1^{-/-}$ which have no Paneth cells and a significantly reduction of Goblet cells (Shroyer et al., 2005).

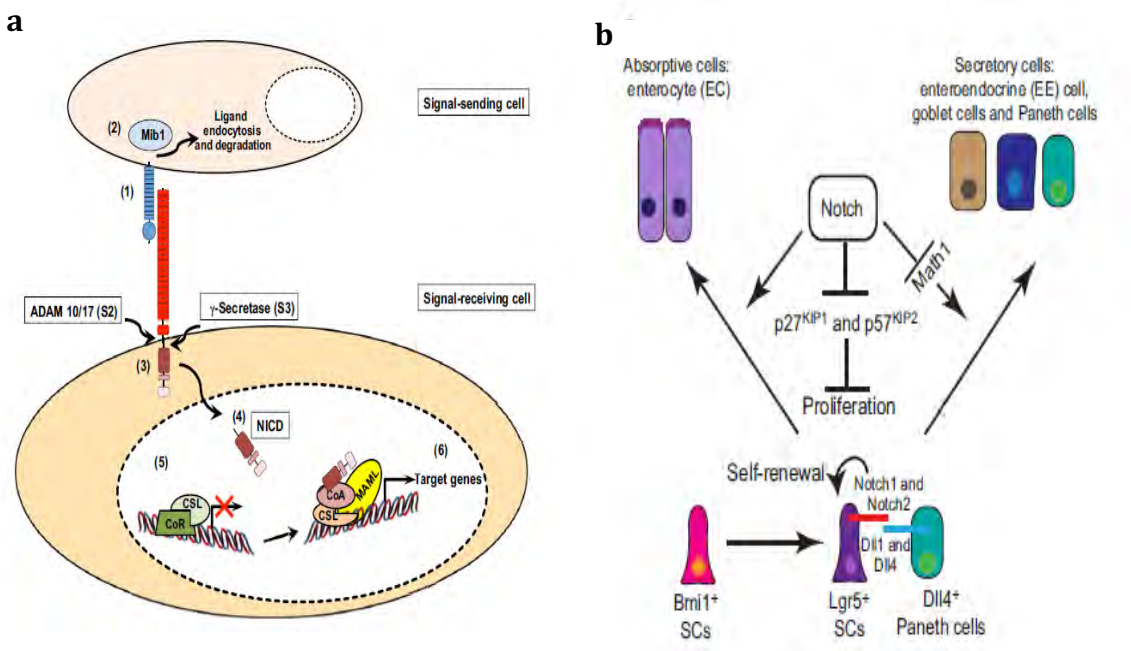

Figure 9. Notch signalling and function in the IECs differentiation*. a) A Notch ligand expressed on the surface of a signal-sending cell interacts with a Notch receptor on the signal-receiving cell, triggering Notch receptor activation cleavages lead to the generation of a free intracellular domain (NICD), which translocates to the nucleus of the signal-receiving cell. In the absence of NICD a transcriptional repressor complex composed of CSL and additional co-repressors, keeps Notch target genes silent. The interaction of NICD with CSL dissociates the repressor complex and leads to the recruitment of MAML and additional co-activators to the complex. The assembly of this transcriptional activation complex on the promoter regions of Notch target genes leads to an up regulation of gene expression. b) Activation of Notch signalling in Lgr5+cells maintains stem cells self-renewal and proliferation by negative regulation of the cyclin-dependent kinase inhibitors p27KIP1and p57KIP2, and the transcription factor Math1, which is necessary for differentiation of the secretory cell lineages. Notch thus biases cell fate choice towards the absorptive lineage (Koch, U. 2013).*

1.3.3 The IECs death

The intestinal epithelium undergoes continuous and rapid self-renewal that need to be tightly regulated because inappropriate cell death responses inexorably

lead to the development of diseases, like inflammatory disorders and cancer (van der Flier and Clevers, 2009). Along the length of villus, the epithelial cell death can occur at the level of the stem and early transit cells in the crypt (referred as *spontaneous apoptosis*) and at the villus tip or to the surface epithelial cuff in the colon, where epithelial cells, after travelling from the crypt base, differentiate and then die from anoikis (Bertrand, 2011).

Patterns of spontaneous apoptosis have been described within the crypt region with a difference between the large and small intestine, concerning stem cell regions in the small intestine but rarely the colonic crypts. Accordingly, the anti-apoptotic gene Bcl-2 is barely expressed in the small intestine and strongly expressed at the base of the colonic crypts (Merritt et al., 1995).

Necrosis is considered as an accidental and uncontrolled cell death, frequently associated to disorders. Cytotoxic bacteria were shown to induce necrosis in IECs, which indicates that this cellular death process has an important role in infectious GI disease (Watson, 1995).

Autophagy constitutes a self-degradation process through which cell recycles its own nonessential organelles. This is induced under conditions of nutrient starvation, bacterial infection, liberating energy stores and promoting cellular survival. Defects in autophagy have been associated with intestinal bowel disease (IBD) and several types of GI cancers (Yang and Klionsky, 2010). Genome-wide association studies have identified a relation between IBD and autophagy-related genes such as ATG16L1 and IRGM which play a role as restriction factors for pathogens through autophagy (Brest et al., 2011; Lassen et al., 2014). NOD2, the first gene associated with IBD, encodes for a cytoplasmic pattern recognition receptor that

recognizes bacterial peptidoglycan in the cell cytoplasm. Although not an autophagy gene, NOD2 directs autophagic proteins through recruitment of ATG16L1 to the plasma membrane at bacterial entry sites. Several Crohn's disease (CD)-associated risk polymorphisms have been identified in NOD2, including a truncated version of the protein that cannot recruit ATG16L1 to the plasma membrane (Ogura et al., 2001; Hugot et al., 2001).

1.3.4 The IEB reparation

Dysfunction of the IEB may result in systemic penetration of toxins and other factors leading to a generalized inflammatory response and remote organ pathology. For that reason, sophisticated and multiple mechanisms have evolved to allow a rapid repair (Mammen and Matthews, 2003). During the process of restitution, normally polarized cells bordering the zone of injuring undergo an important change in cell shape and phenotype. Cells flatten and take on a squamoid appearance then begin to spread by extending pseudopod-like structures known as lammelipodia. Then, cells loose their apical-basolateral polarity and become polarized along a leading-trailing edge axis after brush border and junctional disassembly. Then, a contractile "purse-string" of actomyosin cables connected via intercellular adherents junctions forms within minutes of the injury to draw the flattened migratory epithelial cells forward over their underlying basal lamina. Finally, epithelial repolarization and re-differentiation ensue and epithelial proliferation is stimulated to restore the functional capacity of the mucosa (Basson, 2001; Jacinto et al., 2001) (Figure 10a).

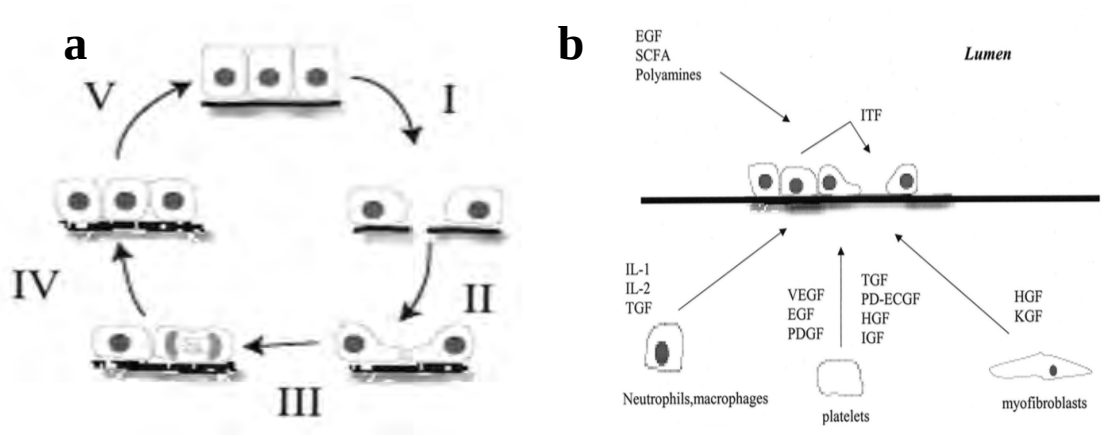

Figure 10. Model of intestinal epithelial restitution. *a) After injury cells bordering undamaged zone flatten and take on a squamoid appearance then begin to spread by extending lammelipodia to cover the defect. Epithelial cells differentiate and repolarize. Mitosis occurs if additional repair is needed. Once junctions between cells are re-established, monolayer is intact. b) Numerous cytokines and growth factors present in the lumen and in the submucosal locations are involved in the epithelial barrier repair. Cell types participating in the repairing process include neutrophils, macrophages, myoepithelial cells, platelets and epithelial cells. EGF, epidermal growth factor, SCFA, short chain fatty acid, ITF intestinal trefoil factor, IL, interleukin, TGF transforming growth factor, VEGF vascular epidermal growth factor, PDGF, platelet-derived growth factor, PD-ECGF, platelet-derived endothelial factor; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; KGF, keratinocyte growth factor (Mammen, JM. 2003)*

Numerous peptide and non-peptide factors regulate the process of epithelial repair. Myofibroblast immediately secretes hepatocytes and keratinocyte growth factors, which interact with basolateral membrane receptors of epithelial cells stimulating cell migration and proliferation. Macrophages and mononuclear cells in the submucosa secrete pro-inflammatory cytokines interleukin (IL)-12, IL-2 and tumor necrosis factor (TNF) as well as members of TGF family. Platelets cells release factors including vascular endothelial growth factor (VGF), epidermal growth factor (EGF), TGF-β1, platelet-derived growth factor, platelet-derived endothelial factor, hepatocyte growth factor and insulin-like growth factor from cytoplasm and granules. These growth modulating factors interact with their epithelial cell surface receptors

and start intracellular signalling pathways that promote restitution (Assoian and Sporn, 1986; Karey and Sirbasku, 1989; Koehne et al., 2000) (Figure 10b). For superficial lesions, the repair is fast, from several hours to a few days (Podolsky, 1999). In cases of severe and deep lesions, it may take from several days to several weeks and involves the remodelling of the extracellular matrix of the tunica submucosa and sometimes even lower layers. These steps repair and healing can be altered, for example during fibrosis, where an excess production of collagen and matrix elements is observed. Repair defects can lead to the formation of ulceration of the mucosa (Sturm and Dignass, 2008).

1.4 The molecular components of the IEB

The IEB integrity is ensured by cell-cell and cell-matrix interactions allowing close monitoring of the intestinal permeability. The integrity of this barrier is important to prevent permeation of pro-inflammatory molecules, such as pathogens, toxins and antigens from the lumen into the tissues and circulatory system (Turner, 2009). Barrier defects have been associated to several human intestinal diseases, including IBD, celiac disease and irritable bowel syndrome (IBS) (Dunlop et al., 2006; van Elburg et al., 1993) but also to not-intestinal pathologies such as acquired immunodeficiency syndrome (AIDS), type I diabetes, multiple sclerosis and rheumatoid arthritis (Bosi et al., 2006; Martínez-González et al., 1994; Sharpstone et al., 1999; Yacyshyn et al., 1996). Epithelial tight junctions (TJs), adherent junctions and desmosomes mediate the cell-cell interactions.

1.4.1 Cell-cell interactions

The TJ is a multi-protein complex that forms a permeable trimming between adjacent cells and separates the apical and the basolateral domains of epithelial cells (Turner, 2006). Four integral trans-membrane proteins compose them: occludin (Furuse et al., 1993), claudins (Furuse et al., 1998), junctional adhesion molecule (JAM) (Martìn-Padura et al., 1998) and tricellulin (Ikenouchi et al., 2005) (Figure 11). The intracellular domains of these proteins interact with cytosolic scaffold protein, such as zonula occludens (ZO) proteins, which in turn joint trans-membrane proteins to the actin cytoskeleton. The interaction between TJs and actin cytoskeleton allows the integrity of the barrier and regulate the intestinal permeability (Figure 11). The contraction of actin is regulated by the phosphorylation of the myosin light chain (MLC) that is mediated by kinases such as MLC kinase (MLCK) and Rho-associated kinase. This activation induces the contraction of actin and the opening of the paracellular pathways (Turner et al., 1997).

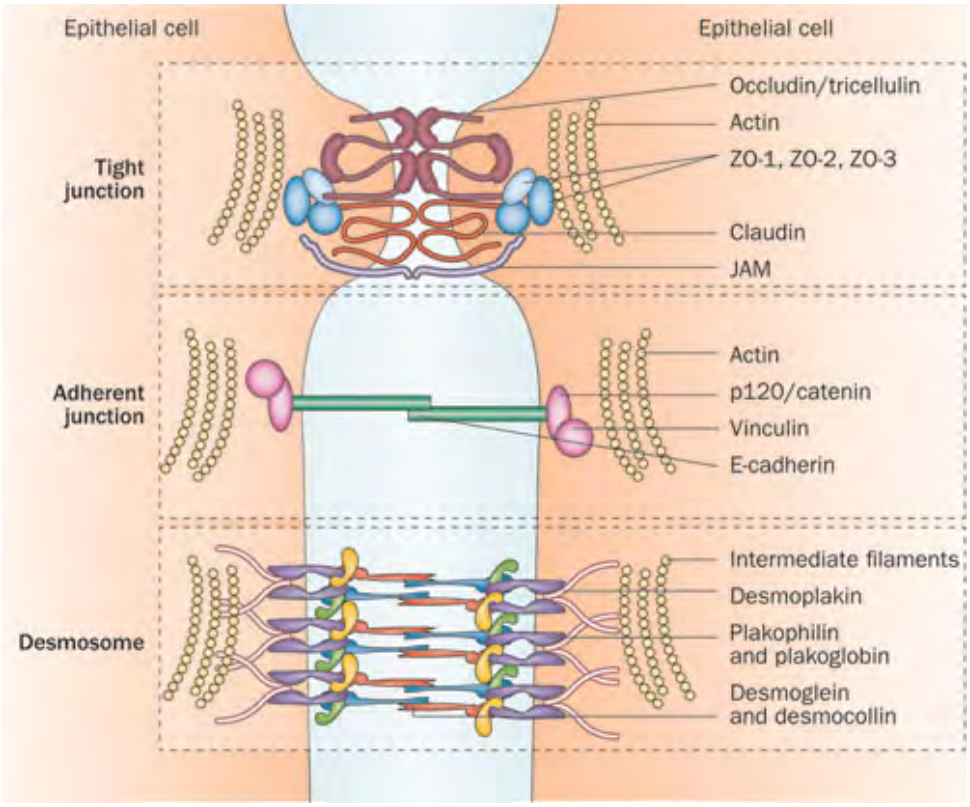

Figure 11 Junctional complexes regulating the intestinal epithelial cells interactions. *Tight junctions regulate IEB paracellular permeability; adherent junctions and desmosomes anchor epithelial cells and confer mechanical strength to the IEB. JAM, junctional adhesion molecule; ZO, zona occludens (Neunlist, M. 2013).*

Occludin occupies the first position in the TJ. Its function is not yet full understood but recent studies show that the knockdown of this protein increases paracellular permeability to macromolecules, suggesting its role in the maintenance and assembly of TJs (Al-Sadi et al., 2011). The phosphorylation of tyrosine residues is involved in the regulation of epithelial barrier disassembly. Several studies show that the tyrosine phosphorylation of occludin attenuates the interaction with ZO-1, leading to dissociation from the junctional complex. It would seem that several substances such as hydrogen peroxide and acetaldehyde induce TJ disruption through the phosphorylation of occludin tyrosine (Atkinson and Rao, 2001; Elias et al., 2009).

Claudins are a multigene family with at least 24 members in human. As occludin, some claudin isoforms are phosphorylated in association with protein localization and paracellular permeability (Fujibe et al., 2004). It has been shown that claudin-1 knockout mice die within 24 hours of birth because of a dramatic loss of fluid and electrolytes through the impaired epidermal barrier, highlighting the role of claudins in the barrier formation and paracellular permeability (Tamura et al., 2011)

The JAM family belongs to immunoglobulin (Ig) superfamily, expressed in different cellular types including epithelial, endothelial and immune cells. JAM-A and JAM-4 are involved in the TJ regulation and maintenance of intestinal barrier. A study has shown that JAM-A knockout mice exhibit higher permeability to dextran and most severe colonic injury and inflammation induced by dextran sodium sulphate in JAM-A knockout mice compared to wild type (Laukoetter et al., 2007). A role of JAM-A was also found in IBS by *Wilcz-Villega et al.* which have recently shown that, in IBS, JAM-A expression was significantly reduced in the cecal epithelium and associated with abdominal pain severity (Wilcz-Villega et al., 2013).

The first TJ-specific proteins identified were ZO proteins and ZO-1, -2 and -3 have been identified to date (Haskins et al., 1998). They are multi-domain proteins that interact with TJ proteins through their N-terminal domain and with actin cytoskeleton and cytoskeleton-associated protein through their C-terminal domain. ZO-1 protein is the most characterized among ZO proteins, playing a pivotal role in the regulation of intestinal permeability. It has been demonstrated that the significantly higher intestinal permeability of IBS patients was associated with significantly lower expression of ZO-1 mRNA in biopsies of IBS patients compared to healthy controls (Piche et al., 2009).

Tricellulin is the first integral membrane protein that is concentrated at the vertically oriented TJ strands of tricellular contacts. When tricellulin expression is suppressed with RNA interference, the epithelial barrier is compromised and TJs are disorganized suggesting the critical function of tricellulin for formation of the epithelial barrier (Ikenouchi et al., 2005).

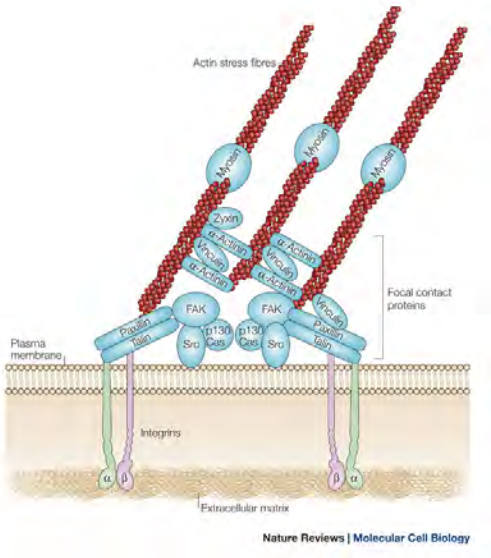

Figure 12. Architecture of focal adhesions. *The extracellular matrix, integrins α and β and the cell cytoskeleton interact at sites called focal contacts. Focal contacts are dynamic groups of structural and regulatory proteins that transduce external signals to the cell interior. The integrin-binding proteins paxillin and talin recruit focal adhesion kinase (FAK) and vinculin to focal contacts. α-actinin is a cytoskeletal protein that is phosphorylated by FAK, binds to vinculin and crosslinks actomyosin stress fibres and tethers them to focal contacts. Zyxin is a α-actinin- and stress-fibre-binding protein that is present in mature contacts. The composition of a focal contact is therefore constantly varying depending on external signals and cellular responses (Mitra, SK. 2005).*

The major cell adhesion molecules in adherent junctions (AJs) are cadherins. These proteins connect adjacent cells through homophilic interactions and are linked

to the cytoskeleton through catenins proteins. To initiate the adhesion process, extracellular domains of cadherins engage in the Ca^{2+}-dependent homophilic trans-interaction with identical cadherin molecules on an adjacent cell, while theirs cytoplasmic tails bind to p120- and β- (or its homolog γ-) catenin proteins (Figure 11). In turn, β-catenin interacts with α-catenin, which contains an actin-binding domain and physically links AJ complexes to the actin cytoskeleton (Capaldo et al., 2014). Mechanical forces and Rho-family of small GTPases regulate the interactions between the actomyosin cytoskeleton and the AJs, facilitating not only the coupling but also the detachment of cadherin-catenin complexes from actomyosin cytoskeleton, allowing cell-cell separation, cell sorting, and cell migration (Klezovitch and Vasioukhin, 2015).

Desmosomes are junctions that provide strong adhesion or hyper-adhesion mediated by the desmosomal cadherin, desmocollin, desmoglein and plaque proteins such as periplakin, envoplakin, plakoglobin, p0071, plakophilins and the NH2-terminus of desmoplakin (Figure 11). They are connected to the keratin intermediate filament cytoskeleton forming a network that confers mechanical resistance to tissues (Cirillo, 2014).

Finally, gap junctions forming intercellular channels in specialized regions of the plasma membrane mediate another type of communication between the cytoplasm of two cells. These channels allow exchange of ions, second messengers, and small metabolites promoting electrical and biochemical coupling between cells (Meşe et al., 2007). In chordate animals, a family of genes called connexins, each of which has four trans-membrane domains that constitute the wall/pore of the channel, encodes gap junction channels. These domains are connected by two extracellular loops that

play roles in the cell–cell recognition and docking processes (Krutovskikh and Yamasaki, 2000). N- and C-termini cytoplasmic domains are susceptible to various post-translational modifications, such as phosphorylation, which are believed to have regulatory roles (Cruciani and Mikalsen, 2002).

1.4.2 Cell-matrix interactions

The extracellular matrix (ECM) provides much of the structural support to parenchymal cells in tissue. Components of ECM are produced intracellularly and then secreted by exocytosis in the existing matrix, where they then aggregate and compose an ordered network of polysaccharides and fibrous proteins, such as collagens, elastin, fibronectin and laminins (Kim et al., 2011). Polysaccharides are linked to protein forming proteoglycans, the most important structural and functional bio-macromolecules of ECM. They interact with growth factors, cytokines and chemokines, cell surface receptors and ECM molecules either via their core proteins or mainly, through their glycosaminoglycan side chains participating in cell signalling, proliferation, migration, differentiation, apoptosis and adhesion (Iozzo and Schaefer, 2015).

Collagens are the main structural fibrillar proteins present in basement membrane and ECM. This protein superfamily is composed by 28 different types, constituting up to 30% of the totals proteins in humans. The main function of collagen is to act as structural support and binding partners for others ECM proteins, growth factors, signal receptors and adhesion molecules.

Elastic fibers are present within tissue as large network. These structures are composed by two main components, elastin and microfibrils, providing recoil and elasticity to tissue, which undergo repeating stretching forces (Theocharis et al., 2015).

Fibronectin binds cell surface integrins and connects cells with collagen fibers allowing a reorganization of cytoskeleton to facilitate cell movement during dynamic tissue remodelling, formation or repair and embryonic development. Laminins are heterotrimeric glycoproteins present in basement membrane that interact with each other as well as with other ECM components and resident cells participating in the organization of ECM and cell adhesion (Theocharis et al., 2015) .

The extracellular matrix of the the IEB consists of two layers: the *basement membrane* located at the basal pole of the epithelial cells and primarily formed by a network of collagen IV, laminins and proteoglycans, but also fibronectin or tenascin; the *reticular lamina* underlying basal lamina, is composed mainly of fibrillar collagens produced by fibroblasts, connective tissue, but also to fibronectin and proteoglycans (Basson, 2003). The cell-matrix interaction (focal adhesion) is mediated by integrins composed of two sub-units α and β associated non-covalent manner. These proteins are assembled in clusters and linked to the matrix by their extracellular domain interacting with the actin cytoskeleton via several types of intermediate proteins such as paxilline, taline, vinculine, FAK (Focal Adhesion Kinase) and α-actinine (Figure 12). The binding of the integrins to their extracellular ligands is dependent on divalent cations such as calcium or magnesium. When integrins interact with laminins at the extracellular level and with intermediate filaments at the intracellular level, specific structures named hemidesmosomes are

formed (Theocharis et al., 2015). The components of the IEB and ECM are involved in the regulation of the intestinal permeability.

1.5 The intestinal permeability

The intestinal permeability is defined as ‘‘*the facility with which intestinal epithelium allows molecules to pass through by non-mediated passive diffusion*’’(Travis and Menzies, 1992), allowing solute and fluids exchange between the lumen and tissues through the intestinal epithelial layer. This barrier prevents loss of water and electrolytes, entry of antigens and microorganisms into the body while allowing exchange of molecules between host and environment and absorption of nutrients (Brandtzaeg, 2011). The *transcellular* compartment of the IEB is composed of the apical plasma membrane of enterocytes while the *paracellular* space is sealed by TJs (Hossain and Hirata, 2008). In this manner, the intestinal transport of molecules from the intestinal lumen to the *lamina propria* can occur through two distinct mechanisms: paracellular diffusion through TJs between IECs and transcellular transport involving endocytosis / exocytosis (transcytosis) mediated or not by membrane receptors (Ménard et al., 2010) (Figure 13).

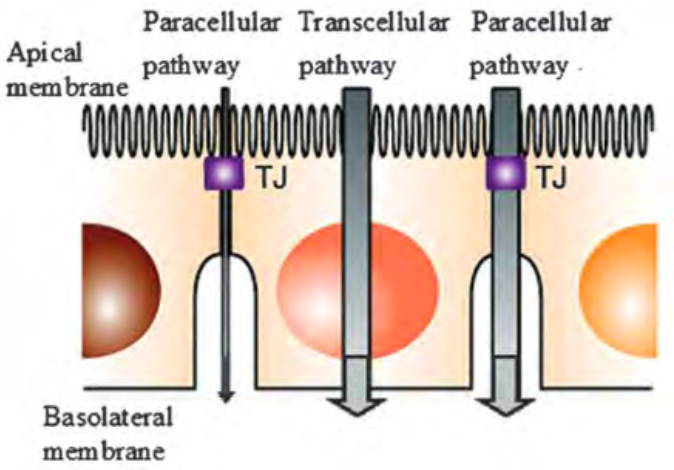

Figure 13 Paracellular and transcellular permeability across the IEB. *The intestinal transport of molecules from the intestinal lumen to the lamina propria can occur through TJs between IECs in a paracellular pathway or through mechanisms of endocytosis / exocytosis (transcytosis) in a transcellular pathway (Hossain, Z. 2008).*

1.5.1 Paracellular permeability

The paracellular pathway allows the passage of molecules with molecular mass < 600 Da through the pores in the epithelial TJs driven by water movements due to transepithelial electrochemical or osmotic gradients. Paracellular permeability is determined by pore size in TJs, with most pores being in the range 8-9 Å in diameter (Watson et al., 2001). TJs are highly dynamic structures and their permeability is regulated by several physiological and pathophysiological conditions causing the disruption of TJs acting on the actomyosin cytoskeleton. The dysfunctions of TJs mediate by cytokines result in immune activation and tissue inflammation. In contrast, several growth factors protect and maintain the integrity of TJs (Turner, 2009). Several signalling proteins, including protein kinase C (PKC), mitogen-activated protein kinases (MAPK), MLCK and Rho family of small GTPase, control the interactions between transmembrane proteins and the actomyosin ring at TJs sites

(Ulluwishewa et al., 2011). Contractions in the actomyosin ring are largely regulated by phosphorylation of MLC by MLCK. This phosphorylation stimulates perijunctional actomyosin contraction, leading to the distension of transmembrane TJ and increases paracellular permeability (Ma et al., 2005) (Figure 14). The Rho family of small GTPases, RhoA, Rac, and Cdc42 is implicated in the regulation of TJ structures and functions. Downstream effectors of Rho, known as Rho kinases (ROCK), mediate the phosphorylation of MLC inducing contraction of the actomyosin ring (Nusrat et al., 1995; Kimura et al., 1996).

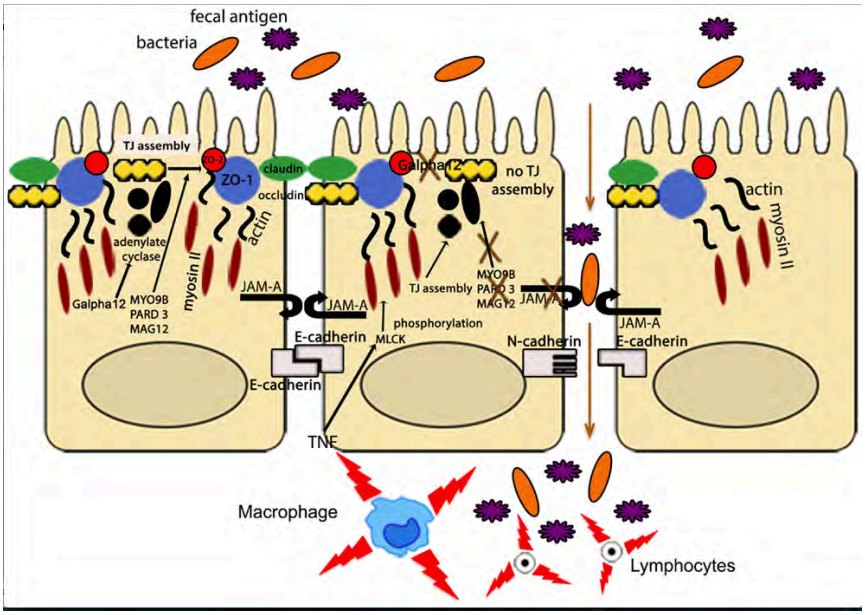

Figure 14 Defective TJs assembly induced by pro-inflammatory cytokines. *Phosphorylation of myosin II through MLCK activation by TNF leads to TJ disassembly. Lack of junctional proteins, such as JAM-A, or altered expression and/or pairing (e.g., dominant negative N-cadherin, claudin-2 overexpression) leads to increased epithelial permeability, facilitating translocation of luminal bacteria, and antigens and exposure to the mucosal adaptive immune system (Pastorelli, L. 2013).*

Several studies have demonstrated the effect on the intestinal permeability of pro-inflammatory cytokines such as interferon (INF)-γ, TNF-α and different ILs. *Bruewer* and collaborators showed that INF-γ increased intestinal permeability by inducing a endocytosis process that targets into an endosomal compartment TJ transmembrane proteins, such as occludin, JAM-A and claudin-1 (Bruewer et al., 2005). Recent studies have demonstrated that TNF-α impairs the intestinal TJs in intestinal cell cultures by mechanisms that involve the activation of MLCK. Contrariwise, the anti-inflammatory cytokines IL-10 and TGF-β have protective/promotive effect on the intestinal barrier functions.

Clinical and animals studies have reported the deleterious effect of total parenteral nutrition upon the intestinal barrier integrity. Increase in intestinal permeability and decreased levels of TJ proteins showed by mice administered with total parenteral nutrition is reversed by IL-10 treatment (Sun et al., 2008). Farther, pre-treatment of T84 cells with TGF-β reduces the disruption of the TJ barrier induced by enterohemorrhagic *E. coli* (Howe et al., 2005).

1.5.1.1 Protease-activated receptor 2 and intestinal permeability

The GI epithelium is repeatedly exposed to an array of proteinases involved in digestion and host defense acting as signalling molecules via proteinase-activated receptors (PARs also known as thrombin receptors). PARs are expressed on epithelia of every region of the GI and accessory organs including the salivary glands (Kawabata et al., 2000), stomach (Kawao et al., 2002), intestine (Kong et al., 1997), pancreas and liver (Bohm et al., 1996). PARs are seven-transmembrane G-protein-

coupled proteins, activated by an enzymatic cleavage of the N terminus of the receptor by a serine protease. This cleavage is mediated by a well-characterized family of enzymes requiring a serine within the active site and generates a new tethered ligand that interacts with the receptor within extracellular loop-2 (Figure 15). Four subtypes of receptors (PAR1-4) have been identified: PAR-1, -3 and -4 are alternative thrombin receptors with different tissue distributions involved in the activation of platelets (Vu et al., 1991) while PAR-2 is widely distributed throughout the body, especially in the GI tract and is activated by trypsin, mast cell tryptase and coagulation factors VIIa and Xa, but not thrombin (Compton et al., 2001; Nystedt et al., 1994).

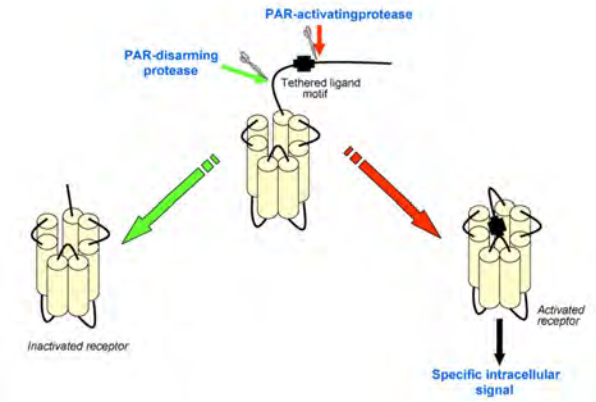

Figure 15 Mechanism of PARs activation. *Activating protease cleaves the N-terminal extracellular domain of PAR exposing the tethered ligand, which binds and activates the cleaved receptor. Disarming protease cleaves the N-terminal extracellular domain upstream from the activation site silencing the receptor, which cannot be activated by activating proteases (Vergnolle, N. 2008).*

PAR-2 is a protein of 397 amino acid residues with a trypsin cleavage site (SKGR/SLIG) resistant to thrombin at N-terminus (Bohm et al., 1996). Synthetic

peptides such as SLIGRL-amide and SLIGKV-amide can activate PAR- 2 (as well PAR1 and -4) also by non-enzymatic mechanism. These peptides (of five or six amino acids) are able to mimic the actions of the endogenous activators binding directly the body of PAR-2 (Kawabata et al., 2000).

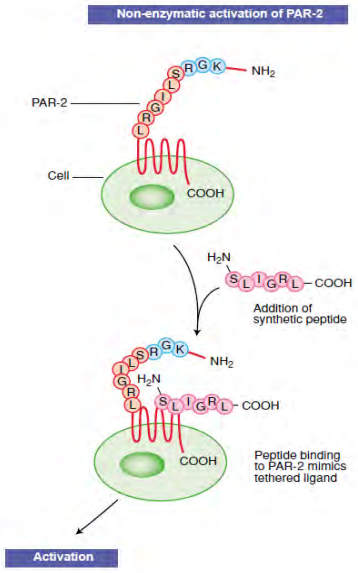

Figure 16 Non-enzymatic activation mechanism of PAR-2. *A synthetic SLIGRL peptide, based on the amino acid sequence of the tethered ligand directly binds to the body of PAR-2 without cleaving the N-terminal peptide, mimicking the effect of the PAR-2 activating protease (Kawabata, A. 2002).*

In IECs, PARs are expressed at apical and basolateral side suggesting that they can be activated by luminal, circulating, secreted, of even membrane-associated proteinases (Vergnolle, 2008). PAR-2 activation in intestinal tissues or epithelial cells provokes chloride secretion and, consequently, diarrhoea associated with fluid transports. *In vivo* response to PAR-2 agonist affects contractility of intestinal smooth increasing GI transit (Corvera et al., 1997; Kawabata et al., 2001). Several studies have also demonstrated the activation of epithelial cells lines proliferation (Darmoul

et al., 2001) and evocation of inflammatory response in the colon of mice (Cenac et al., 2002), induced by PAR-2 activation.

The pathophysiological role of PAR-2 in the development of colonic inflammation is strengthened by the finding of PAR-2 over-expression in biopsies from patients with IBD (Kim et al., 2003). However, numerous studies demonstrate the effect of PAR-2 activation on the intestinal permeability. Several studies have demonstrated that PAR-2 activation from the luminal side either *in vivo* or *in vitro* in epithelial cells increases paracellular permeability through a mechanism dependent on MLCK activation and cytoskeleton rearrangement. The disruption of intestinal barrier mediated by PAR-2 activation could allow the passage of food antigens and luminal bacteria, leading to activation of innate immunity and maybe to the development of food allergy and/or bacterial infection (Cenac et al., 2004; Vergnolle, 2008). In 2002 *Coelho et al*. showed that intra-colonic infusion of the PAR2-activating peptide SLIGRL triggered a dose-related increase in colonic paracellular permeability (Coelho et al., 2002).

1.5.2 Transcellular permeability

Nutrients from the diet are absorbed into blood vessels across mucosa of the small intestine. In addition, intestinal mucosa plays a critical role in maintenance of body water and balance absorbing also ions, water and electrolytes. The transcellular permeability pathway mediates these processes of absorption via intestinal enterocytes. Three mayor mechanisms of transport through the intestinal barrier are possible: passive or active transport and exocytosis or endocytosis. Passive transport

is driven by the concentration gradient for the nutrient across the membrane and can be divided in simple or facilitated diffusion. Small soluble molecules as lipids are capable of crossing the lipid bilayer of membrane and to be transported by simple diffusion. However, glucose and amino acids transport is facilitated by transport protein driven by the concentration gradient. Another mechanism that uses a transport protein is the active transport requiring energy in the form of adenosine triphosphate (ATP) to move nutrients against concentration gradients. Na^+/K^+ pump is the best example of active transport but also glucose and amino acids are actively transported. Big molecules can get across the membrane or exit a cell through endocytosis or exocytosis mechanisms that provide an invagination of cell membrane surrounding and engulfing molecules and fusing with cell membrane to release the contents to the opposite side, respectively.

Monosaccharides

Na^+-glucose symporter is a protein transporter Na^+-dependent responsible for the glucose and galactose absorption. Being an active transport, the energy is supplied by Na^+/K^+ pump embedded in the basolateral membrane maintaining low intracellular sodium. Exporting 3 sodium ions from the cell in exchange for 2 potassium ions, this pump establishes a gradient of both charge and sodium concentration across the basolateral membrane. Glucose is then got out from the cell by a facilitate diffusion through a protein transporter GLUT2 (Cheeseman, 1993). Contrarily, GLUT5 at apical side and GLUT2 at the basolateral side regulate the absorption/secretion of fructose through a facilitated diffusion mechanism (Jones et al., 2011).

Amino acids

Amino acid absorption uses a mechanism similar to glucose but several transport proteins are needed to accommodate the wide range of amino acids that must be absorbed (Silk et al., 1985).

Fats

Fats are soluble in the lipid bilayer and they are absorbed by simple diffusion. Once in enterocytes, lipids must be made water miscible for transport in blood. Bile acids are used to emulsy them in order for digestive enzymes such as lipase to act. Then, a series of steps including re-esterifying and packing with others lipids and proteins in a water miscible complex called lipoprotein, follow. Finally, to get it out of the cell, exocytosis is used (Hussain, 2014).

Water and electrolytes

Being a small molecule, water can pass through pores within the membrane or between the cells. The passage of water takes place passively driven by osmosis, a spontaneous movement of solvent molecules through a semi-permeable membrane into a higher solute concentration compartment. The absorption of nutrients makes gut contents hypotonic and water is absorbed. Indeed, water absorption is highly dependent from nutriments absorption. Na^+ absorption takes place at the apical side through co-transport Na^+/glucose, Na^+/amino acids and with the anti-porters Na^+/H^+ and Cl^-/HCO_3^-, according to a concentration gradient maintained by the Na^+/K^+ pumps. The descent movements of Na^+ into the cell provide the energy required for

the passage of H^+ from the cell to the lumen. Similarly, the descent movements of HCO_3^- out of the cell provide the energy for the entry of Cl^- into enterocytes. Absorption of K^+ takes place by passive diffusion driven by the difference between lumen and blood K^+ concentration. The absorption of water results in an increased concentration of K^+ in the lumen and consequent absorption of K^+ in the intestine. In the crypts, potassium ions are secreted via K^+ channels at basolateral side while Cl^- ions are secreted by the apical CFTR channel causing the secretion of Na^+ through the paracellular way (Figure 17).

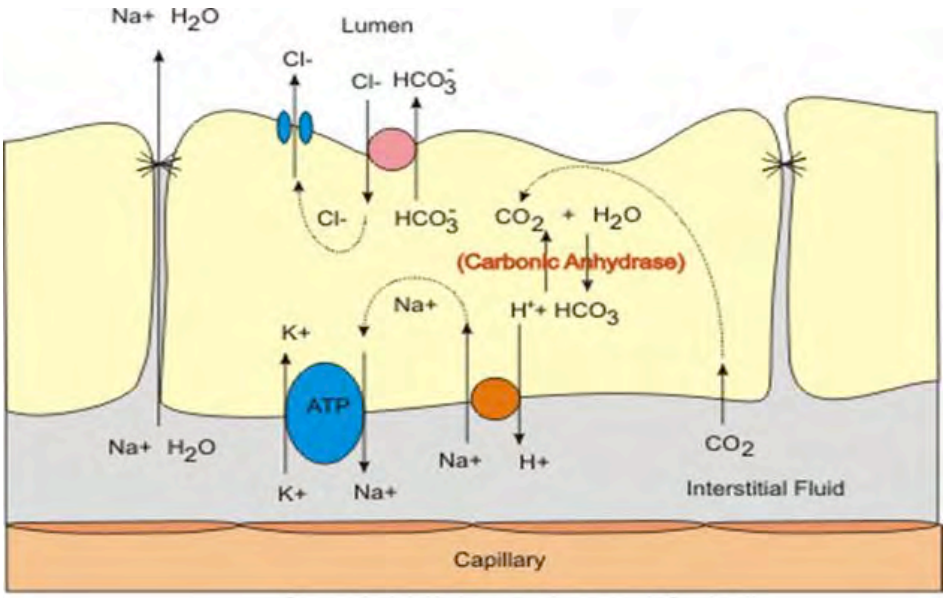

Figure 17 Absorption and secretion of electrolytes and water. *In enterocytes, low intracellular Na^+ is maintained by a large number of Na^+/K^+ ATPases pumps embedded in the basolateral membrane. These pumps export 3 Na^+ ions from the cell in exchange for 2 K^+ ions establishing a gradient of both charge and Na^+ concentration across the basolateral membrane. In the upper part of the small intestine Cl^- absorption is mainly by passive diffusion. The absorption of Na^+ ions through the epithelium creates apical electronegativity and basolateral electropositivity. Then Cl^- ions move along this electrical gradient to follow the Na^+ ions. When Na^+ sodium ions are absorbed, moderate amounts of H^+ ions are secreted into the lumen of the gut in exchange for some of the Na^+. These H^+ ions in turn combine with the HCO_3 ions to form H_2CO_3 that dissociated to form H_2O and CO_3. The water remains part of the chyme in the intestines while carbon dioxide is readily absorbed into the blood and subsequently expired through the lungs.*

1.6 Regulation of immune cells by IECs

The commensal flora and luminal antigens can occasionally breach the epithelial barrier and invade the mucosa. However, innate and adaptive immune components predominantly in the subepithelial lamina propria are normally capable of preventing excessive, inflammatory reactions.

1.6.1 Innate immune regulation

The capacity of the IECs to maintain barrier and immunoregulatory functions depends on their ability to act as sensor for pathogens and integrate commensal bacteria-derived signals into anti-microbial and immunoregulatory responses, via pattern-recognition receptors, including Toll-like receptor (TLR) (Abreu, 2010), NOD-like receptor (NLR) (Chen and Núñez, 2011; Elinav et al., 2013), and RIG-I-like receptor (Li et al., 2011; Broquet et al., 2011) families. The recognition of conserved microbial-associated molecular motifs and pathogen-specific virulence proprieties by TLRs and NLRs implies the activation of NFkB signalling pathway involved in the epithelial homeostasis and repair. Furthermore, reactive oxygen species (ROS) produced in response to commensal or pathogenic bacteria also promote the formation by IECs of focal adhesion necessary for cell migration and wound healing (Swanson et al., 2011; Leoni et al., 2013).

Numerous immunoregulatory signals produced by IECs are necessary for tolerating immune cells, limiting steady-state inflammation and activation of innate and adaptive immune cell response. Most of them depend on the translation of commensal bacteria-derived signals by IECs to immune cells. Thymic stromal lymphopoietin (TSLP), TGF-β and retinoic acid produced in response to commensal bacteria-derived signals further the development of dendritic cells (DCs) and macrophages. DCs promote the differentiation of naïve regulatory T (T_{REG}) cells and the maturation of B cells into IgA-secreting plasma cells (Schulz et al., 2009; Macpherson and Uhr, 2004). In contrast, resident macrophages lack migratory properties in the steady state and form trans-epithelial dendrites that penetrate into the intestinal lumen to taste exogenous antigens (Niess et al., 2005; Rescigno et al.,

2001), promoting survival and expansion of T_{REG} cells and tolerance in intestinal lamina propria through IL-10 production. Recently, innate lymphoid cells (ILCs) have been identified at barrier surface functioning as regulators of tissue homeostasis, early innate response to infection and inflammation. Groupe 1 ILCs include natural killer cells (NK cells) and ILC subset 1 (ILC1) which produce INF-γ and TNF-α ⓘ●✕🎁?🚋☁●?🎁 👟 IL-12 and or IL-15 (Bernink et al., 2013; Fuchs et al., 2013). Contrarily to NK cells that can directly kill target cells, ILC1 produce cytokines in response to stimulation. Groupe 2 ILCs produce cytokines IL-5 and IL-13 invoking a protective epithelial response, including goblet cell hyperplasia and enhanced mucus secretion (Moro et al., 2010; Neill et al., 2010). Groupe 3 ILCs produce IL-17A and IL-22 in response to stimulation by IL-1β and IL-23. IL-22 has an important role in protecting the intestinal epithelium following injury or infection by bacterial pathogens (Zenewicz et al., 2008). Intestinal epithelial cell-derived-cytokine-IL-25 and TSLP induce the expansion and differentiation of basophil progenitors and multipotent progenitors type 2 cells, respectively. IL-25 and IL-33 stimulate ILC2 whereas IL-25 suppresses ILC1 and ILC3 limiting macrophage production of IL-1β, IL-23 and IL-12 (which activate ILC1) (Figure 19).

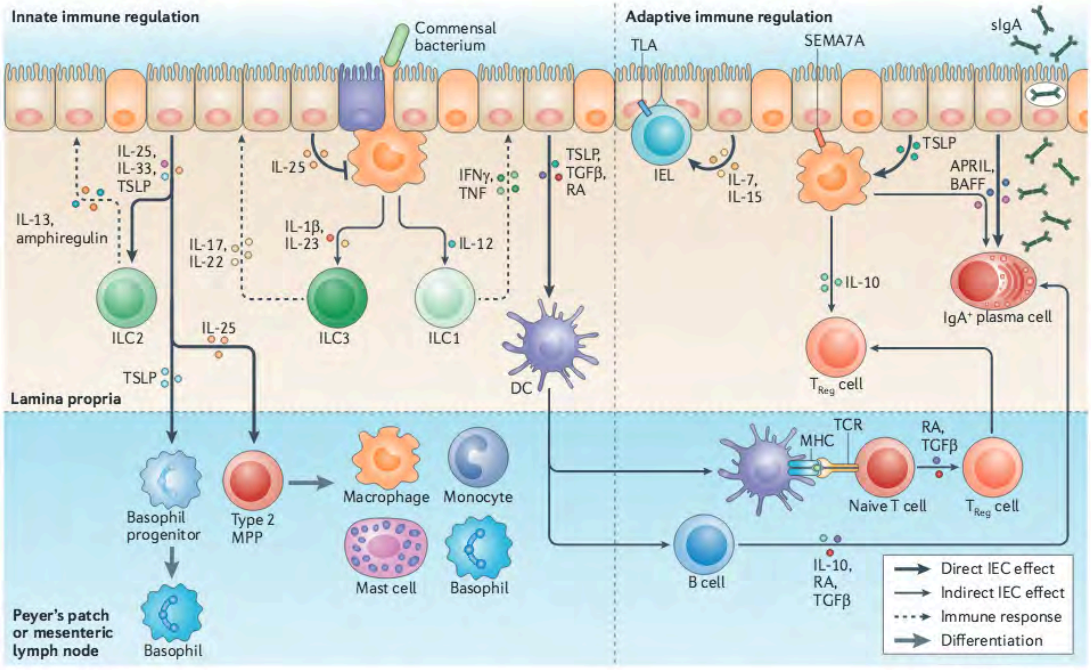

***Figure 19 Innate and adaptive immunity regulation by IECs**. IEC-derived cytokines IL-25 and thymic stromal lymphopoietin (TSLP) induce the expansion and differentiation of basophil prognitors and multipotent progenitor type 2 (type 2 MPP) cells. IL-25 and IL-33 stimulate group 2 innate lymphoid cells (ILC2s); IL-25 suppresses ILC1 and ILC3s. IECs condition dendritic cells (DCs) and macrophages towards through the production of TSLP, transforming growth factor-β (TGFβ) and retinoic acid (RA). DCs promote the differentiation of naive CD+T cells into regulatory T (T_{REG}) cells and the maturation of B cells into IgA-secreting plasma cells. Mucosal cell-derived DCs also imprint a gut-homing phenotype on primed B cells and T cells through the production of RA. After trafficking to the intestine, T_{REG} cells are expanded in number by macrophages that are conditioned to produce IL-10 by TSLP-mediated stimulation and through contact-dependent interactions with IEC-expressed semaphorin 7A (SEMA7A). The production of a proliferation-inducing ligand (APRIL) and B cell-activating factor (BAFF) by IECs and by TSLP-stimulated macrophages and DCs promotes class-switch recombination and the production of IgA by B cells in the intestinal lamina propria. IEL, intra-epithelial lymphocyte; IFNγ, interferon-γ; sIgA, secretory IgA; TCR, T cell receptor; TLA, thymus leukaemia antigen; TNF, tumour necrosis factor (Peterson, LW. and Artis, D. 2014).*

1.6.2 Adaptive immune regulation

Mucosal B-cell system collaborates with the secretory IECs to provide a first line of defence by the selective transport of polymeric IgA across epithelial cells

lining the mucosal surface. Mucosal B cells are subject to a dominant class switch to IgA, and thereafter undergo terminal differentiation to plasma blasts and plasma cells.

Mucosal plasma cells constitute humoral defence in the gut, secreting immunoglobulin as IgA secreted and transported across the epithelial layer. IgA bind to microorganisms and reduce their motility and adhesive properties limiting the bacterial penetration through the epithelial cell layer (Kaetzel et al., 1991, Crottet and Corthésy, 1998, Macpherson and Uhr, 2004). The majority of commensal bacteria that can pass through the intestinal lamina propria are rapidly phagocyted by local tissue macrophages, without induction of an immune response. Some bacteria can be seized by DCs which thereafter migrate to lymph nodes where they induce a adaptive immune response secreting IgA-secreting plasma cells that migrate to mucosal sites (Macpherson and Uhr, 2004). A large number of differentiated T cells are present within the epithelial surfaces of the body, contributing to the mucosal immune system. These intraepithelial lymphocytes (IELs) are probably involved in local immunosurveillance through bidirectional interactions between IECs (Cheroutre et al., 2011; Edelblum et al., 2012). Following trafficking in the intestine, T_{REG} cells are expanded in number by macrophages that produce IL-10 via stimulation mediated by TSLP and through contact-dependent interactions with IECs expressing semaphorin (SEMA) 7A (Figure 19).

1.7 The regulation of the IEB

Secretory IECs, including, Goblet cells, Paneth cells and enteroendocrine cells are specialized for maintaining the digestive or barrier function of epithelium. As already discussed in previous paragraphs, the secretion of mucins and antimicrobial

preoteins by goblet and Paneth cells, respectively, establishes a physical and biochemical barrier to microbial contact and underlying immune cells (Kim and Ho, 2010). Enteroendocrine cells are the link between central and enteric neuroendocrine system through the secretion of hormone regulators of digestive function (Peterson and Artis, 2014), while, Goblet cells-derived products further contribute to the regulation of the intestinal barrier acting as a signal that promotes epithelial repair, migration of IECs and resistance to apoptosis (Taupin et al., 2000; Dignass et al., 1994).

Function

Two major regulatory factors of intestinal barrier could be identified: *diet* (nutrients and prebiotics) and the *intestinal microbiota* (probiotics), both related to life style, indicating that the environmental factors influence the function of the intestinal barrier. Vitamin A and its derivatives regulate growth and differentiation of intestinal cells. Vitamin A-deficient diet changes mucin dynamics and expression of defence molecules such as MUC2 and defensin 6 (Amit-Romach et al., 2009). Vitamin A deficiency is associated with a decreased small bowel villus height and, in experimental model of enteritis, is associated to more several injury (Warden et al., 1997). Also vitamin D play a role for the intestinal barrier and its deficiency is a characteristic of IBD (Ulitsky et al., 2011).

Short chain fatty acid (SCFA), including acetate and butyrate, are produced by intestinal microbial fermentation of undigested dietary carbohydrates. In IBD a butyrate deficit causes TJ lesions and impaired intestinal permeability (Plöger et al., 2012). Several animal studies have showed that western style diet and plant-derived

flavonoids, e.g. quercetin present in grapes and onions, enhanced intestinal permeability (Moreira et al., 2012; Serino et al., 2012), epithelial resistance and claudin-4 expression in epithelial cells (Amasheh et al., 2009; Amasheh et al., 2012).

Commensal bacteria and probiotics are known to enhance and protect intestinal barrier integrity *in vitro*. The probiotic *E.coli Nissle 1917* is able to prevent barrier disruption in T84 and Caco-2 cells induced by an enteropathogenic *E. coli* strain (Zyrek et al., 2007). *In vivo* studies have also demonstrated the beneficial effects of probiotics on IEB. For example, administration of *Lactobacillus plantarum* into the duodenum of healthy human volunteers significantly increased ZO-1 and occludin in TJ structures (Karczewski et al., 2010).

A key regulator of IEB functions is the ENS. It has been demonstrated that the activation of enteric neurons reinforces IEB functions. In particularly, vagus nerve stimulation, which ultimately activates enteric neurons, enhances IEB functions (Neunlist et al., 2003). However, enteric neuromediators can exert different effects on IEB. For example, acetylcholine (Ach), neurokinin A and substance P (SP) increase transcellular and paracellular permeability (Boudry et al., 2011; Cameron and Perdue, 2007; Hällgren et al., 1998). In a clinical trial, treatment of diarrhoea-predominant (IBS-D) patients with a neurokinin antagonist reduced symptoms associated with increased permeability, such as pain and/or discomfort (Zakko et al., 2011). Conversely, vasoactive intestinal peptide (VIP) decreases intestinal permeability in different epithelial cell lines (Neunlist et al., 2003; Boudry et al., 2011; Blais et al., 1997) or increased paracellular permeability induced by SP or pathogens (Hällgren et al., 1998; Conlin et al., 2009).

Enteric glial cells (EGC) also play a pivotal role in the control of intestinal permeability. Fulminant jejuno-ileitis caused by *in vivo* ablation of enteric glia evidence the importance of glial component in the maintenance of IEB integrity (Bush et al., 1998). Interestingly, in a noncontact model of EGC and IEC monolayer, EGC reduced paracellular permeability in a S-nitrosoglutathione-dependent pathway (Savidge et al., 2007). In addition, glial cell derived neutrophic factor (GDNF) maintains mucosal homeostasis during colitis and can restore IEB function *in vivo,* in dextran sodium sulphate (DSS)-induced colitis (Steinkamp et al., 2003; Zhang et al., 2010).

Proliferation and differentiation

Enteric neurons and EGC also modulate IEB proliferation and differentiation. Enteric neurons have a direct anti-proliferative effect on IECs via the liberation of VIP (Toumi et al., 2003), while Ach and SP stimulate intestinal proliferation (Cheng et al., 2008; Goode et al., 2003). Glial cells also are involved in the epithelial restitution, at least in part by proEGF release and by an increase of FAK expression and activity in IECs (Van Landeghem et al., 2011).

Another important role of EGC in the IEB homeostasis is the regulation of IECs proliferation, exerting an important anti-proliferative effect mediated by glial derived mediators, such as TGFβ1 or lipid mediators, such as 15dPGJ2. The mechanisms involved in the glial anti-proliferative effect are cell–cell contact inhibition and blocking at G0–G1 of the cell cycle *in vitro* (Neunlist et al., 2007). In addition, the production of soluble ligands of PPARγ, such as omega 6 derivatives and 15dPGJ2, by EGC leads to the activation of PPARγ pathways in IECs that is

associated to anti-proliferative and pro-differentiative effects (Bach-Ngohou et al., 2010).

2. THE ENTERIC NERVOUS SYSTEM

2.1 Organisation of the ENS

The ENS is a quasi-autonomous part of the nervous system including neural circuits that control motor local blood flow functions, mucosal transport and secretions, and modulates immune and endocrine functions. The neural circuits are composed of enteric neurones and EGC arranged in networks of enteric ganglia connected by interganglionic strands (Costa et al., 2000). In humans, the ENS contains an estimated ≈ $5x10^8$ neurons in two major ganglionated plexuses that extend the entire length of the bowel in two major layers: the myenteric plexus between the longitudinal and circular muscle and the submucosal plexuses associated with the mucosal epithelium (Figure 20).

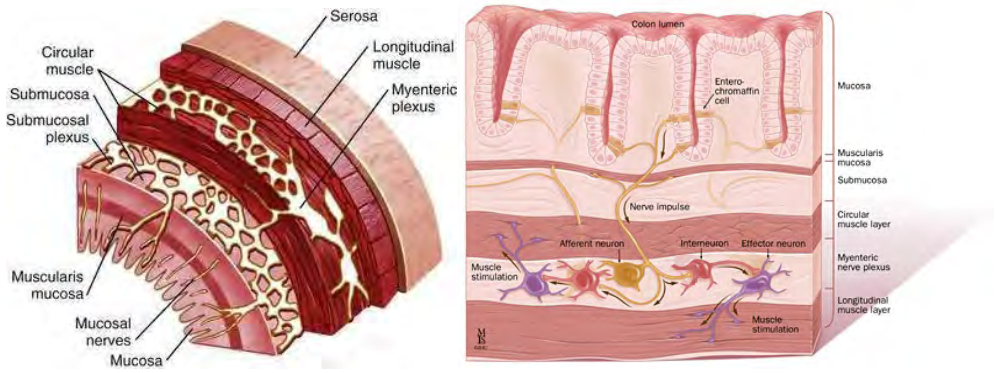

Figure 20 Organization of the ENS. *The ENS is composed of two major plexuses, one submucosal and one located between the circular and longitudinal SM layers. These neurons receive and coordinate neural transmission from the GI tract and central nervous system.*

Introduction

The first investigation of the ENS dating back to 1860 when *Meissner* and *Auerbach* described for the first time two different nerve plexuses present in the intestinal wall: Meissner's plexus or submucosal plexus and Auerbach's plexus or myenteric plexus (Meissner, 1857; Auerbach, 1864). Subsequently, *S.R. y Cajal* established the existence of sub-types of neurons, classified in 1899 according to their morphology by *Dogiel*. About fifty years after, *Bayliss and Starling* made the functional discovery of the ENS. Isolating a loop of dog intestine, they observed a muscular movement (the peristaltic reflex), which caused forward movement of the contents. Interestingly, cutting all nerve-mediated communication between the gut and the CNS, the reflex activity was unaffected and they introduced the term "the local nervous mechanism" (Hansen, 2003).

The myenteric plexus is positioned between the outer longitudinal and circular muscle layers, the submucous plexus is positioned in the submucosa consisting, in large mammals, of more than one layer: an inner network (Meissner's plexus) and an outer (Schabadasch's) plexus. In the human intestine, a third intermediate plexus lies between Meissner's and Schabadasch's plexuses. Non-ganglionated plexuses also supply all the layers of the gut (Timmermans et al., 1992; Furness, 2000; Balemba et al., 2002; Costa et al., 2000).

The human ENS is composed by 100 millions of neurons and comprises many different types of neurons with a higher density in the myenteric plexus than submucous plexus (Wood et al., 1999).

2.2 Development of ENS

The ENS is derived from the neural crest (NC), a highly proliferative and migratory cell population originating at the junction of the neural plate and the ectoderm. The precursors of ENS originate mainly in the vagal segments of the neural tube, while sacral and anterior trunk NC make a small contribution to the distal bowel and the foregut ENS, respectively (Yntema and Hammond, 1954; Le Douarin and Teillet, 1973; Burns and Douarin, 1998; Durbec et al., 1996).

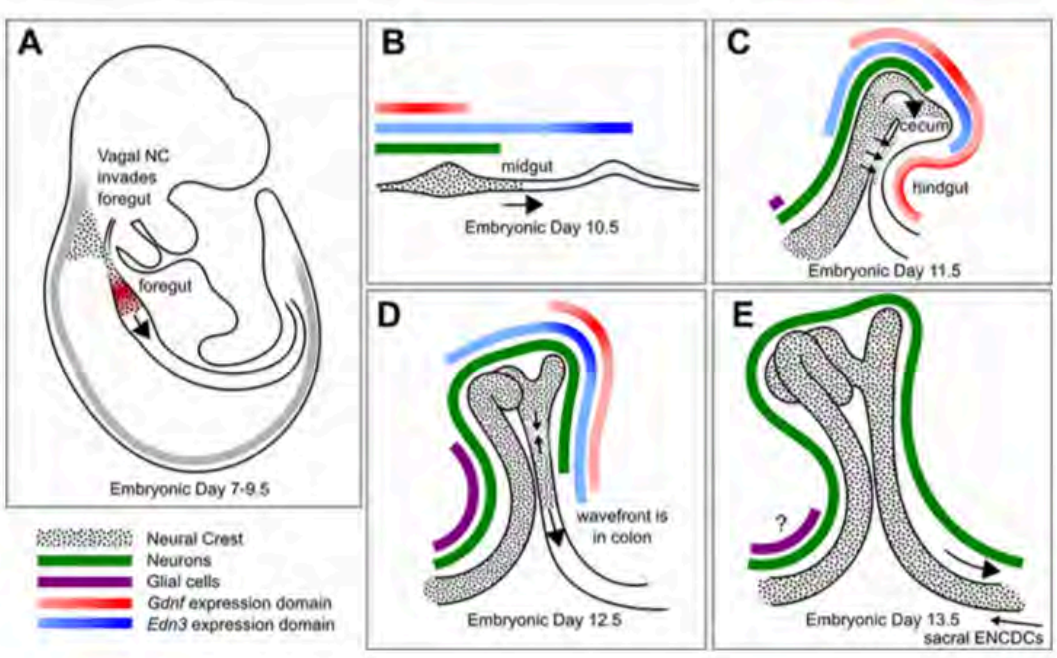

Figure 21 Colonization of the mouse GI tract by enteric neural crest-derived cells (ENCDCs). *A) NC cells (black) exfoliate from the vagal region of the dorsal neural tube and migrate in the ventral stream to the region adjacent to the foregut, which express GDNF. B-E) ENCDCs migrate rostrocaudally, proliferate and differentiate first into neurons (green) and later in the glia (purple). The cecal appendage grows and at the embryonic day 11 and 12, ENCDCs invade the colon by crossing the mesentery and transiting the cecum (C). Cecal and transmesenteric populations fuse to form the ENS in the rostral colon (D) and the transmesenteric population populates the terminal colon as the smaller sacral ENCDC population enters the bowel and migrates caudorostrally (E). Human ENS development proceeds through a similar process (Lake, JI 2013).*

At embryonic day 9.5 in the mouse and prior to week 4 in human embryos, preenteric neural crest-derived cells (pre-ENCDCs) invade the foregut and start the rostrocaudal migration completed at day 14 in the mouse and week 7 in humans (Kapur et al., 1992; Fu et al., 2003, 2004a) (Figure 21). In addition, ENCDCs undergo inward radial migration forming the myenteric and submucosal plexuses. During migration, ENCDCs extensively migrate, proliferate and differentiate into neurons and glia condensing in ganglia to form a network in the gut. Failure of ENCDCs to

colonize the distal bowel causes Hirschsprung disease (HSCR), a common and life-threatening developmental disorder.

The control of ENS development is complex and must be guided by specific molecular signals. Several cell surface receptors and ligands, transcriptional factors, morphogens and proteins transmitting signals from the cell surface to the cytoskeleton and nucleus are involved.

2.2.1 Molecular mediators of ENS development

RET/GFRα1/GDNF pathway

RET is a transmembrane tyrosine kinase receptor expressed in ENCDCs. RET is activated by GDNF, neurturin, artemin and persephin by binding to the glycosylphosphatidylinositol-linked GDNF family of receptors (GFRα1, GFRα2, GFRα3 and GFRα4, respectively) and supports ENS precursor survival, proliferation, migration and neurite growth (Gianino et al., 2003; Heuckeroth et al., 1998; Taraviras et al., 1999; Young et al., 2001). Genetic mutations affecting the genes of this pathway are involved in the onset of HSCR and several type of hereditary cancer (Lake and Heuckeroth, 2013; Emison et al., 2005).

Endothelin receptor type B, endothelin-3, and endothelin- converting enzyme 1 pathway

Endothelial receptor (EDNR) type B, a G protein-coupled receptor expressed in NC derivatives, and its ligand endothelin (ET)-3 are required for ENS development

in the colon. Mutations in EDNRB, ET-3 or the ligand-processing protease ET-converting enzyme can cause HSCR (Lake and Heuckeroth, 2013).

Transcription factors

Several transcription factors play an important role in early the ENS development. Sox-10 is a transcription factor expressed in the neural tube prior NC delamination, in migratory ENCDCs and in mature glia. Apoptosis of NC cells prior their migration in the foregut it has been showed in homozygous Sox10-null mice (Kapur, 1999). PHOX2B is expressed in the developing ENS and adult enteric neurons and is required for RET expression in mouse pre-ENCDCs.

Several others transcription factors are involved in the ENS development such as PAx3, ZFHX1B, ASCL1 and HAND2 but for some of them, mutant phenotypes are unexpected and the relevance to human disease is unknown (Lake and Heuckeroth, 2013).

2.2.2 Morphogenesis in ENS development

The organisation of the ENS network occurs in precise positions within the bowel wall and requires several molecules controlling morphogenesis. The *hedgehog pathway* is directly and indirectly involved in the developing ENS. Localized expression of hedgehog proteins in the epithelium is essential for concentric patterning of the bowel wall (Ramalho-Santos et al., 2000). The hedgehog ligands, Sonic hedgehog (Shh) and Indian hedgehog (Ihh) are expressed by the gut epithelium

during ENS development. Shh promotes proliferation, inhibits neuronal differentiation and prevents premature invasion of ENCDCs into future submucosa (Fu et al., 2004b; Sukegawa et al., 2000). Probably because of the importance of spatial and temporal expression required for these proteins, loss of Shh or Ihh has very different effects. Mutation of Shh excessively increases the enteric neurons number, while loss of Ihh results in dilatation of gut segments and aganglionosis in part of GI tract. Indirectly, instead, hedgehog signalling induces bowel mesenchyme to secrete the BMP-4, a modulator of ENS development. BMP-4 enhances neuronal aggregation and regulates the clustering of ENCDCs into definitive ganglia.

Other molecules involved in ENS development are netrins, which regulate the migration of ENCDCs and SEMs which are involved in the axon growth cone repulsion (Jiang et al., 2003; Anderson et al., 2007). Retinoic acid is a diffusible morphogen produced locally in tissues required for the efficient migration of ENCDCs and to reduce levels of a negative regulator of ENCDCs proliferation and migration, phosphatase and tensin homolog protein (Sato and Heuckeroth, 2008).

2.3 Extrinsic innervation of the GI tract

Digestive functions require communication links between the intrinsic system and the CNS. These links take the form of sympathetic and parasympathetic fibers that connect either the central and ENS or connect the CNS directly with the digestive tract.

Efferent pathways transfer information from CNS to GI, while *afferent pathways* follow the opposite direction. The extrinsic innervation of the gut is

supplied by the autonomic sympathetic and parasympathetic nervous system, typically antagonists and represented by splanchnic nerves, the vagus nerve and the sacral parasympathetic. This communication, also known as brain-gut axis, provides the control of digestive functions such as motility, immunity, sensitivity and satiety.

2.3.1 Sympathetic and parasympathetic *efferent* pathways

Efferent fibers carry the information from the CNS to digestive tube. Cell bodies of efferent sympathetic fibers are located within the pre-vertebral sympathetic ganglia, synapsing with post-ganglionic neurons of the sympathetic chains and splanchnic nerves. Stomach, small intestine and a part of the proximal large intestine are innerved by fibers from celiac-mesenteric ganglia. The inferior mesenteric ganglia give fibers to the large intestine, and rectum is innerved by fibers originating from the pelvic ganglia (Figure 22).

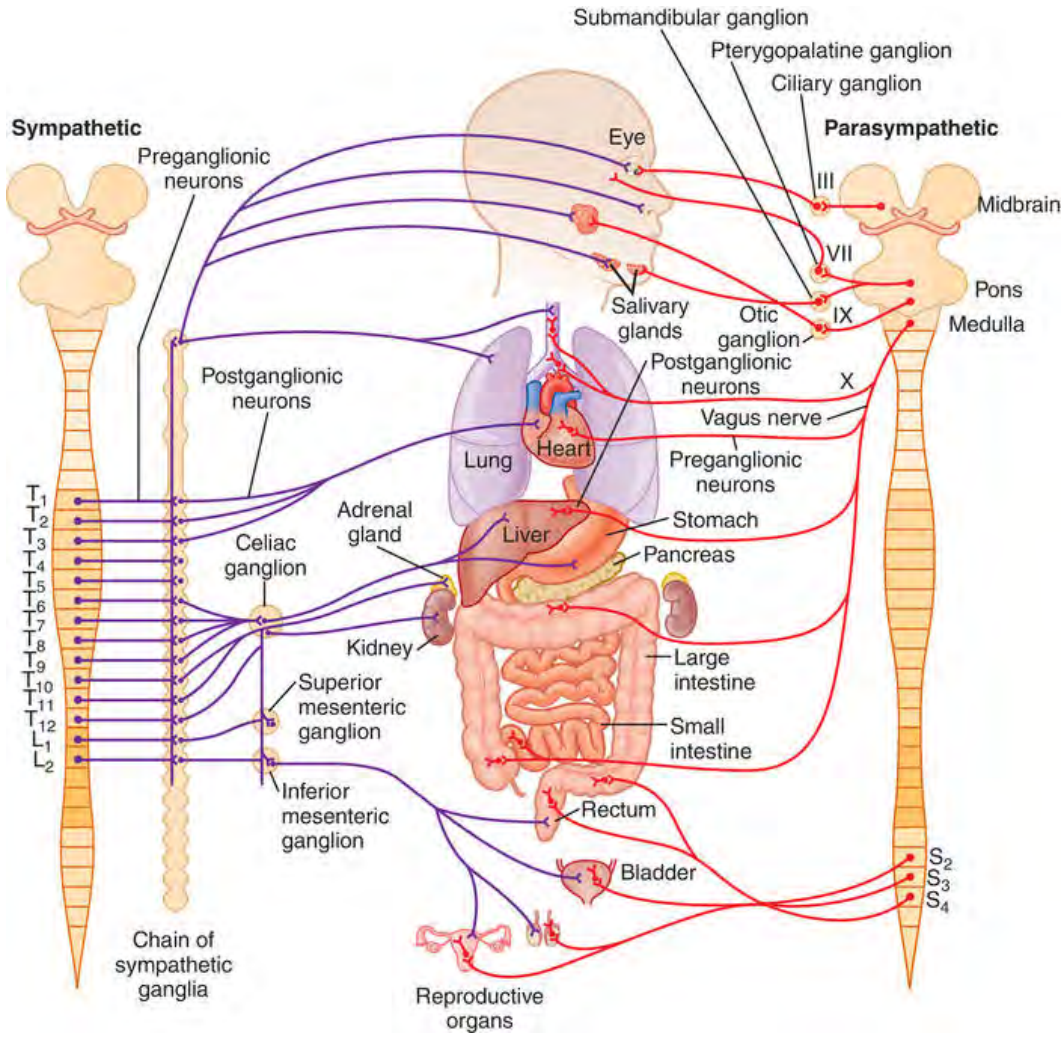

Figure 22 Autonomic nervous system. *Schematic representation of the autonomic nervous system, showing distribution of sympathetic and parasympathetic nerves to the head, trunk, and limbs.*

The sympathetic innervation from pre-vertebral ganglia provide the SM wall, ganglia of the mucosal plexus (MP) and submucosal plexus (SMP), and arteries of the GI tract (Furness and Costa, 1974). Sympathetic nerve fibers terminate in vessel walls or enteric plexuses in order to control vascular tone or secromotor neurons (Furness, 1970). The Ach and norepinephrine mediate pre-ganglionic and post-ganglionic transmissions, respectively. The adrenal gland is also considerate a sympathetic ganglion specialized in the release of adrenaline and noradrenaline.

The parasympathetic system includes the vagus and the pelvic nerves. The vagus nerve controls secretory and motor functions of the upper parts of the digestive tube while pelvic nerves control distal colon, rectum and bladder functions (Kirchgessner and Gershon, 1989). The upper vagal efferent innervation starts in the dorsal nucleus of vagus nerve and in the nucleus ambiguous (Hopkins et al., 1996). The nucleus ambiguous contains the cell bodies of nerves that innervate the muscles of the soft palate, pharynx, and larynx. Visceral and motor pre-ganglionic neurons present in the dorsal motor nucleus of the vagus nerve innervate myenteric and submucosal plexuses of the ENS and, therefore, they synapse with postganglionic neurons directly near the concerned organ (Hopkins et al., 1996) (Figure 22). Pre- and post-ganglionic neurotransmitter is the Ach.

2.3.2 Sympathetic and parasympathetic *afferent* pathways

The brain is constantly informed by the digestive tract via the afferent pathway that carries the information from the digestive tube to the CNS. The responses generated by the integration of information originating from the gut traverse the same nerves in the reverse directions. Vagal afferents innervate mucosa, enteric plexuses and *muscularis mucosae*, especially in the esophagus and stomach. Cell bodies of visceral sensory neurons, from which vagal afferents depend, are located in the plexiform ganglion of the neck and they synapse in the nucleus of the solitary tract in the medulla oblongata. Vago-vagal reflexes started by these neurons affect swallowing, intestinal motility and secretions. Vagal afferents are sensitive to nutrients in the intestinal lumen and respond to physiological, mechanical and

chemical stimuli through mechano- and chemo-receptors, as well as, temperature- and osmo-sensor (Berthoud and Neuhuber, 2000).

Sympathetic afferents essentially convey digestive visceral pain pathways to the spinal cord. Cell bodies of sympathetic afferents are located in the dorsal root ganglion and their short axons synapse in the lamina V of the dorsal horn, where a convergence with sympathetic afferents explains the phenomenon of referred pain of visceral origin (Mei, 1985). The neurotransmitters are neuropeptides, such as calcitonin gene related peptide, VIP, somatostatin and dynorphin including the family of tachykinins (SP, neurokinin A).

2.4 Enteric neurons

Enteric neurons can be classified according to their morphological, neurochemical, or functional properties.

2.4.1 Morphological classification

According to their morphology, neurons are classified into Dogiel type I to type VII and giant neurones but most of all are types I-III.

Dogiel type I neurons are characterized by an elongated body, several dendrites and a single axon. Dogiel type II neurons are composed by a spheroidal body and from 3 to 7 axons. Dogiel type III neurons have a elongated body, short dendrites and a single long axon (Furness, 2000) (Figure 23).

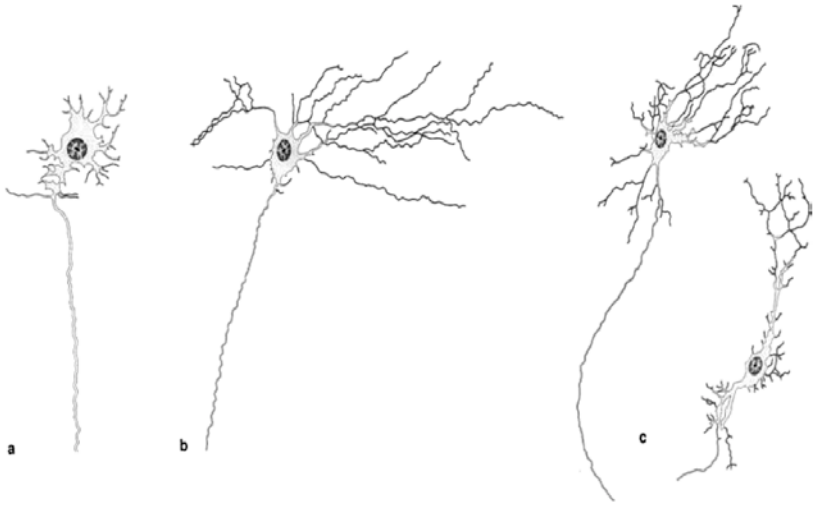

Figure 23 Different morphology of Dogiel types I-III enteric neurons. *a) Dogiel type I; b) Dogiel type II; c) Dogiel type III.*

2.4.2 Electrophysiological classification

Electrical classification includes type S (synaptic) and AH (afterhyperpolarisation) enteric neurons. Type S neurons have the morphology of Dogiel type I or III while type AH neurons have Dogiel type II morphology (Wood, 1994; Furness, 2000). Type S neurons show short action potential (AP) followed by hyperpolarization potential of short duration (range from 20 to 100 ms) with a rapid excitatory postsynaptic potential (EPSP). Type AH enteric neurons are characterized by AP followed by a large hyperpolarization potential (range from 75 to 110 mV) without EPSP (Wood et al., 1999).

More than 30 neurotransmitters have been identified in the ENS, such as small molecules (norepinephrine and 5-HT), larger molecules (peptides) and gases (nitric oxid [NO] and carbon monoxide). The major excitatory transmitter of the ENS is the

Ach that mediates fast excitatory postsynaptic potentials on nicotinic/cholinergic receptors.

Several signal substances are linked to the slow EPSP, which seem to be the mechanism for gating and prolonged neural excitation or inhibition of effectors. Several chemical messenger substances also produce inhibitory postsynaptic potentials (IPSP) by an autoinhibitory mechanism (Furness et al., 1999; Kunze and Furness, 1999). More than thirty neurotransmitters have been identified in the ENS and more than ten molecules can be found in the same neuron (Schemann and Neunlist, 2004).

The presynaptic inhibition is mediated by Ach, 5-HT, histamine, norepinephrine and neuropeptide Y; some of the same neurotransmitters such as Ach, 5-HT, histamine together with CCK, calcitonin gene-related peptide (CGRP), ILs, pituitary adenylate cyclase-activating polypeptide (PACAP), TNF-α and VIP mediate the EPSP. Finally, CCK, 5-HT, norepinephrine transmit also the IPSP together with adenosine, ATP, opioids and somatostatin (Hansen, 2003).

2.4.3 Functional classification

Functional classification of enteric neurons distinguishes sensory neurons, interneurons, motor neurons, muscle motor neurons and secretor and vasomotor neurons.

Sensory neurons

Different types of functional neurons exist in the gut. *Intrinsic primary afferent neurons* (IPANs) have their cell bodies within the GI tract and originate either in the myenteric or in the submucosal plexus, innervating both the mucosal and muscular layers of the gut (Brookes, 2001; Schemann et al., 2001). These two groups of intrinsic neurons are complemented by two groups of extrinsic afferents whose cell bodies lie either in the jugular and nodose ganglia (vagal afferents) or in the dorsal root ganglia (spinal afferents). IPANs are essential for the ENS control of the digestion, whereas extrinsic afferents inform the brain about processes that are relevant to energy and fluid homoeostasis and the sensation of discomfort and pain (Holzer, 2002) (Figure 24).

Interneurons

Interneurons are present in all regions of the digestive tract and receive synaptic inputs from IPANs, extrinsic neurons (sympathetic and parasympathetic neurons) and other interneurons, making synaptic outputs onto other class of enteric neurons. Interneurons are mainly Dogiel type II neurons, both AH and S types.

One type of orally directed (ascending) and three types of anally directed (descending) interneurons have been described in the small intestine of guinea pig (Kunze and Furness, 1999). The ascending interneurons are mainly cholinergic, whereas descending ones have more complex signalling mechanism: Ach/NO/VIP/somatostatin neurones are involved in local motility reflexes, while Ach/5-HT are involved in the local secretomotor reflexes. Two non-cholinergic, fast

excitatory postsynaptic potentials, one mediated by ATP and the other by 5-HT, mediate interneuronal transmission (Grundy and Schemann, 2006; Hansen, 2003; Furness, 2000) (Figure 24).

Motor neurons

Muscle motor, secretomotors and vasomotors neurons are S/Dogiel I type neurons. Muscle motor neurons innervate the longitudinal and circular muscles and the *muscularis mucosae* in the gut. Muscle motor neurons may mediate excitatory and inhibitory stimuli. Excitatory transmission is mainly muscarinic, cholinergic and tachykinergic, while inhibitory stimuli are mediated through NO, VIP, ATP, pituary adenylate cyclase-activating polypeptide, gamma aminobutyric acid (GABA), neuropeptide Y and carbon monoxide (Furness, 2000). Inhibitory neurons discharge continuously and the contraction function is regulated by their state of activity. Contractile activity propagates when they are switched in the aboral direction and when vomiting, the opposite takes place. Chronic idiopathic constipation and oesophageal sphincteral achalasia are related to the inhibitory motor neurons dysfunction (Wood et al., 1999; Hansen, 2002) (Figure 24).

Secretor and vasomotor neurons control secretion and blood flow, respectively and they are directly controlled by IPANs through the release of Ach and VIP (Hansen et al., 1998). Most of secretor neurons have their cell bodies in the mucosal plexus, while some other neurons, such as vasomotor neurons, project from the submucosa to the myenteric plexus or provide the muscularis mucosae (Furness, 2000). There are two types of secretomotor neurons: cholinergic neurone releasing Ach that acts on muscarinic receptors on the mucosal epithelium and non-cholinergic neurons releasing VIP to mediate local reflex responses (Furness, 2000) (Figure 24).

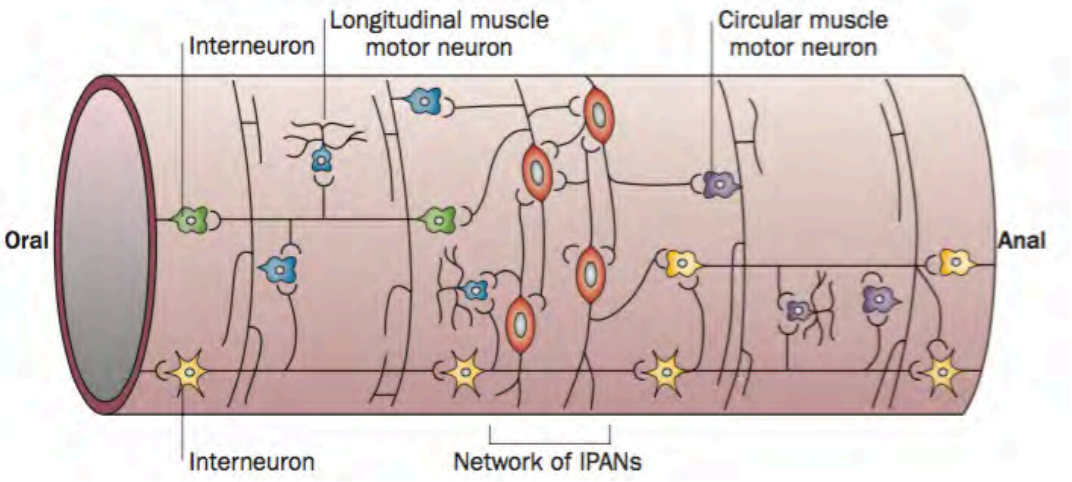

Figure 24 Nerve circuit in the small intestine. *Simplified circuit diagram showing the major circuit features. Networks of interconnected intrinsic sensor neurons (IPANs; red) detect luminal chemistry and mechanical distortion. IPANs synapse with ascending (green) and descending (yellow) interneurons, excitatory muscle motor neurons (blue) and inhibitory muscle motor neurons (purple) (Furness, JB. 2012).*

2.5 Enteric glia

EGC were discovery at the end of the 19th century (Dogiel, 1899) and they have been considered, for a long time, as supportive cells for neurons. Enteric glia form a large and widespread network at all levels of the GI tract. The majority of enteric glia is found in the ganglia of myenteric plexus and the submucosal plexus, but also in the interconnecting nerve strands of the ganglionated and in all non-ganglionated plexuses (where they represent the only cellular elements), in the mucosal plexus in the mesentery, accompanying the extrinsic nerves to the gut and in close contact with the epithelial cell layer (Endo and Kobayashi, 1987; Wedel et al., 1999; Mestres et al., 1992; Bernstein and Vidrich, 1994).

2.5.1 Morphological classification and glial markers

There are at least four morphologically distinct subclasses of EGC based on their localization. Type I EGC have a star-shaped morphology and are located within the ganglia, whereas type II includes more elongated inter-ganglionic EGC. Mucosal and intramuscular EGC have type III and IV morphology, respectively (Gulbransen and Sharkey, 2012; Boesmans et al., 2015) (Figure 25).

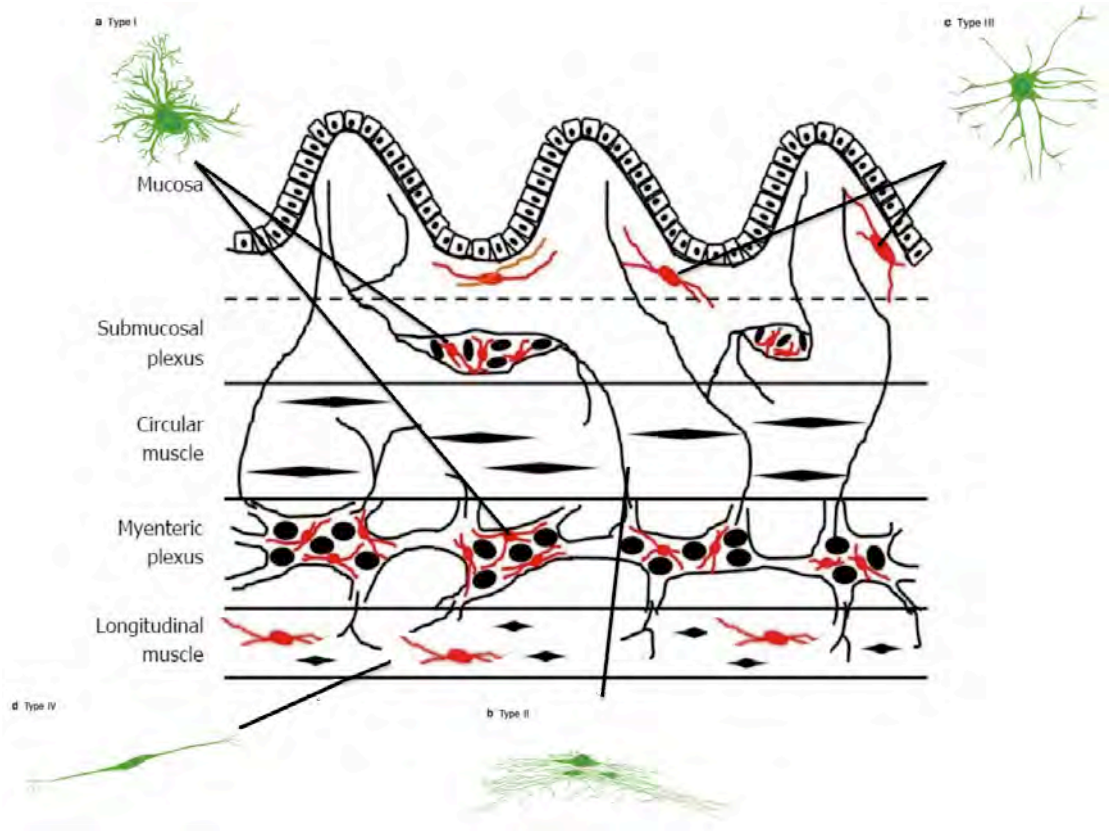

Figure 25 Subpopulations of enteric glia. *a) Type I or protoplasmic EGC are star-shaped with irregular extensions and located within the ganglia; b) type II or fibrous EGC have long extensions and are present at the level of interganglionic fibers; c) type III or mucosal EGC have many extensions; d) type IV or intramuscular EGC are elongated and run along the nerve fibers in the longitudinal muscle. Glial cells are represented in red. (Modified images from Yu, YB. 2014 and Gulbransen, BD. 2012).*

Similarly to astrocytes in the CNS, the main constituent of the EGC is the glial fibrillary acidic protein (GFAP) but a host of immunohistochemical markers is nowadays available to label EGC.

GFAP

Mature enteric glial cells express GFAP (Jessen and Mirsky, 1980) and vimentin (Jessen and Mirsky, 1983) but, while GFAP is a specific glial cell marker, vimentin is also expressed in myofibroblastes (Rühl et al., 2001). In the CNS, the expression levels of GFAP are modulated by astrocyte inflammation, differentiation and injury. In the peripheral nervous system nerve injuries induce the expression of GFAP in myelinating and non-myelinating Schwann cells (Jessen and Mirsky, 1992) and, in the ENS evidences report that GFAP levels increase during intestinal inflammation (Bradley et al., 1997) (Figure 25).

Sox-10

Sox-10 belong to the SOX family of transcription factors involved in many developmental processes (Bowles et al., 2000), such as differentiation, neurogenesis, NC development and skeletogenesis, controlling stamness and cell fate (Akiyama and Lefebvre, 2011; Wegner, 2010). Sox-10 is expressed in NC cells and, in the developing gut, is found in all the ENS progenitors migrating in a rostro-caudal direction to colonise the foregut, midgut and hindgut and form the ganglionic

plexuses (Bondurand et al., 2003; Kuhlbrodt et al., 1998) (Figure 25).

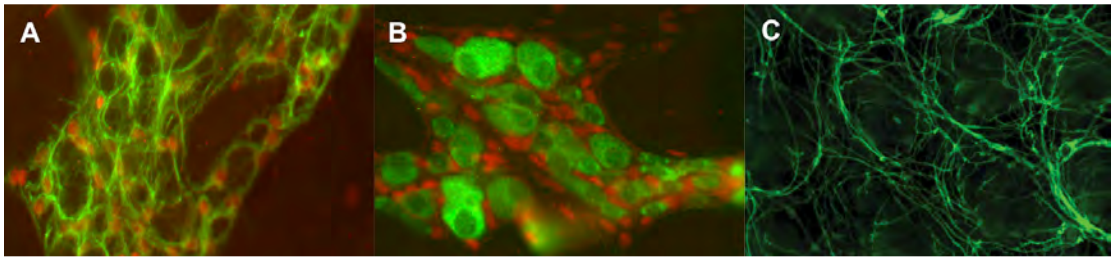

Figure 25 Immunohistochemical staining of EGC and neurons of the ENS. *A) EGC co-expressing Sox-10 (nuclei in red) and GFAP (cytoplasm in green) and B) EGC nuclei displaying Sox-10 (red) immunoreactivity and enteric neurons cell bodies (HuC/D, green) immunoreactivities in myenteric ganglia of the rat small intestine (De Giorgio, R. 2012). C) S100β immunoreactivity in the mucosa from human colonic biopsies (Lilli, NL. unpublished data).*

S100β

The S100β belongs to the family of small and acidic Ca^{2+}-binding S100 proteins, localized in the cytoplasm and/or nucleus of both nervous and non-nervous several tissues (Zimmer et al., 1995). In the GI tract, S100 protein is exclusively expressed in the cytoplasm of EGC. The predominant isoform is *ββ* –homodimer (S100β) that regulates cytoskeletal structure and function, as well as Ca^{2+} homeostasis (Heizmann, 2002). In the CNS, S100β has neurotrophic activity, modulates cell growth/differentiation and responds to neuroinflammatory/neurodegenerative processes (Rothermundt et al., 2003; Huttunen et al., 2000).

Other markers

Among many other glial markers, *glutamine synthetase* (GS) and surface antigen *rat neural antigen* (Ran)-2 are specific markers particularly for enteric intrinsic glia (Jessen and Mirsky, 1983; Bartlett et al., 1981). Brain specific fatty acid binding protein (B-FABP) and the low affinity neurotrophic (NT) receptor p75 are expressed in mature enteric glia (Young et al., 2003).

Enteric glia vs CNS microglia markers

Enteric glia and CNS microglia are entirely unrelated. All EGC have neuroectodermal origins, whereas microglia are mesodermal cells of monocyte-macrophage lineage. Consequently, CNS microglia express monocyte and macrophage markers, such as Mac-1, CD11b/CD18 and ED (Lehrmann et al., 1998). In the gut, resident macrophages form a dens network in close apposition to ENS and express typical monocytes/macrophages markers (Kalff et al., 1998).

2.5.2 Differential expression of glial markers

Boesmans et al., have recently demonstrated differential expression of glial markers GFAP, Sox10 and S100β in EGC subtypes within the myenteric plexus. They compared the expression of S100β or Sox10 to GFAP and they showed that majority of type-I EGC residing in the myenteric ganglia co-expressed GFAP/S100β or GFAP/Sox10, while the remainder cells of this subtype population

expressed either S100β *($S100\beta^{+}/GFAP^{-}$)*, either Sox10 *($Sox^{+}/GFAP^{-}$)* or GFAP only. In addition, the relative percentage of *$Sox10^{+}/GFAP^{-}$* in three types of EGC reflected the relative percentage of *$S100\beta^{+}/GFAP^{-}$* EGC, indicating that the majority of $S100\beta^{+}$ EGC co-expressed Sox10. However, a small population of type-I/II and a higher population of type-III enteric glia were *$S100\beta^{-}/Sox10^{+}$*. All these results suggest that glia-specific marker expression in gut is highly dynamic (Boesmans et al., 2015) (Figure 26).

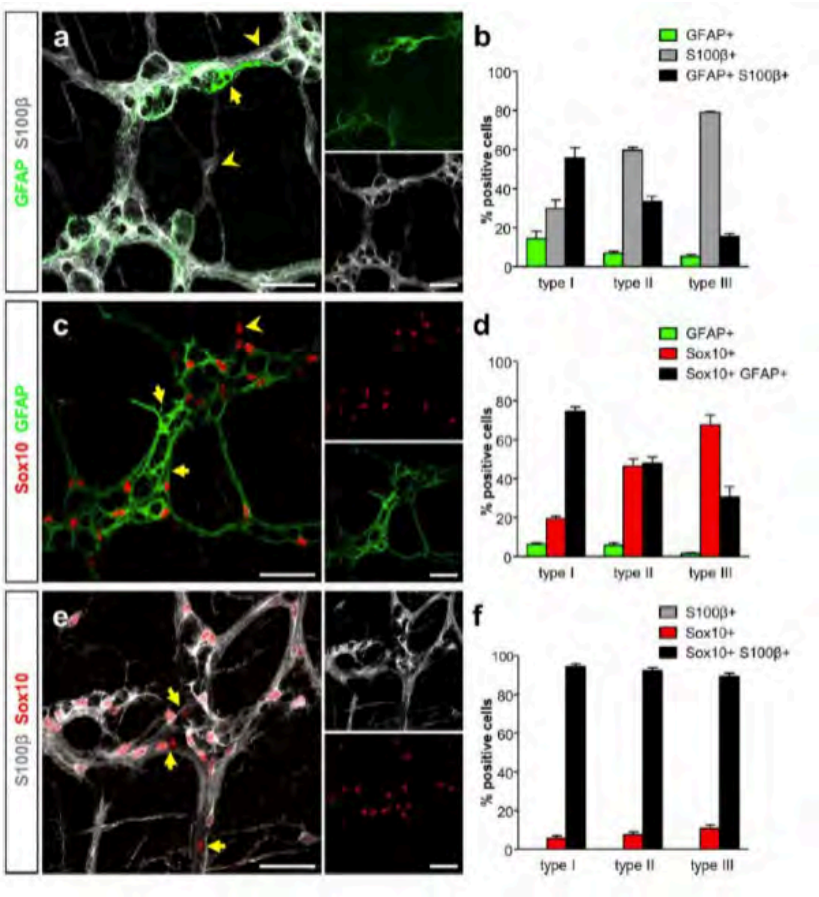

Figure 26 Differential expressions of enteric glial markers in the myenteric plexus of the rat ileum. *a) Double immunostaining for GFAP (green) and S100β (grey): EGC expressing only GFAP or S100β are indicated by arrow and arrow-heads, respectively; c) Double immunostaining for GFAP (green) and Sox10 (red): EGC expressing only GFAP (arrows) or Sox10 (arrowhead); e) Double immunostaining for S100β (grey) and Sox10 (red): arrows indicate EGC expressing only Sox-10; b, d, f) quantification of single and double positive cells in the EGC subpopulations of rat myenteric plexus (Boesmans, W. 2015).*

2.6 Neuron-Glia communication

Neurones in the CNS induce development of astrocytes, while, these latters, regulate neuronal plasticity, differentiation, synaptic transmission (Bezzi and Volterra, 2001; Gomes et al., 1999) and heterocellular signalling through the release of gliotransmitters via increased cytosolic fluid [Ca^{2+}] (Araque et al., 2002). Several

inhibitory and excitatory gliotransmitters exist, such as amino acids (D-serine, glutamate and GABA) (Kang et al., 2013; Lee et al., 2011), nucleotides (ATP) (Blum et al., 2008), neurotrophins [nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and NT3] (Bergami et al., 2008) and cytokines (ILs and TNFs).

Several evidences support the idea that, as astrocytes in the CNS, enteric glia modulate neurons. First of all, enteric glia closely interact with neurons in the ganglia; secondly, EGC have several similarities with astrocytes, such as neurotransmitters production/degradation and trophic factors supporting interactions with neurons (von Boyen et al., 2004; Cirillo et al., 2011). The activation of enteric glia by neurons causes increased intracellular [Ca^{2+}] levels, as demonstrated in a model of guinea pig myenteric plexus stimulation with ATP (Gulbransen and Sharkey, 2009). In addition, a recent study shows that Ach and 5-HT also increased intracellular [Ca^{2+}] levels in EGC (Boesmans et al., 2013), suggesting that enteric glia respond to different excitatory neurotransmitters by changes in intracellular Ca^{2+} levels.

2.6.1 Connexin-43 hemichannels mediate Ca^{2+} responses

Purines released from neurons in the myenteric plexus recruit Ca^{2+} responses in the surrounding EGC *in vitro* (Gomes et al., 2009) an *in situ* (Gulbransen and Sharkey, 2009). In a recent study, *McClain et al.*, found that selectively inhibition of EGC Ca^{2+} responses slowed gut transit in mice colon and that enteric glia Ca^{2+} responses were associated with changes in Connexin-43 (Cx43) expression/transcription (McClain et al., 2014).

Connexins are integral membrane proteins containing 4 transmembrane domains, two extracellular loops, a cytoplasmatic loop and cytoplasmic N- and C-termini and, more than 20 isoforms have been described. The docking of two hemichannels originates complete channels, or *connexons*, forming a gap junctional channel. Each connexon is formed by 6 connexin subunits and a homomeric channel consists of 12 connexin proteins either of the same (*homodimeric connexon*) or of different (*heterodimeric connexon*) isoforms (Figure 27).

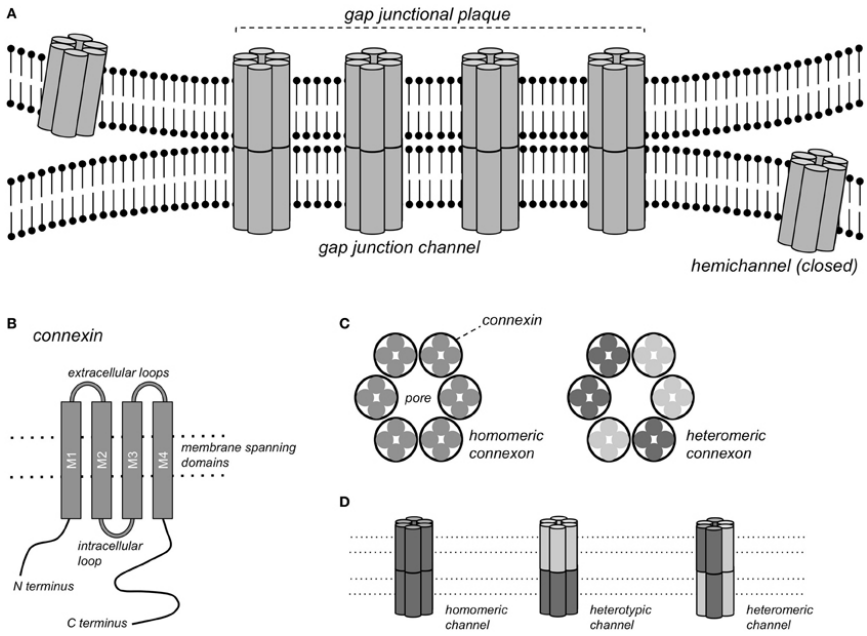

Figure 27 Gap junction channels. *A) Representation of a gap junctional plaque. B) Structure of single connexin subunit with 4 transmembrane domains. C) A hemichannels (connexon) is formed by a hexagonal arrangement of 6 connexin subunits of the same (homodimeric connexon) or different (heterodimeric connexon) isoforms. D) Homodimeric and heterodimeric connexons (Verheule, S. 2013)*

Many of connexins are phosphoproteins and phosphorylation generally regulates connexin biosynthesis, trafficking, assembly/membrane insertion, channel gating and internalization/degradation. Cx43 contains several serine residues in C-terminus phosphorylated by PKC, MAPK and cdc2 kinase (Saez et al., 2003).

In the ENS, Cx43 expression is confined to EGC and is required to propagate Ca^{2+} responses among enteric glia, acting to regulate GI motility (McClain et al., 2014). *In vitro* models of cultured enteric glia demonstrate the capacity of EGC to respond to neurotransmitters, such as ATP (Kimball and Mulholland, 1996; Zhang et al., 2003).

ATP plays a pivotal role in the regulation of synaptic transmission not only in CNS astrocytes but also in the ENS, where is involved in controlling GI motility, secretomotor function, blood flow and synaptic transmission (Bornstein, 2008; Christofi, 2008; Ren and Bertrand, 2008). Recently, *Gulbransens* and *Sharkey* have shown that enteric glia detect neuronally release of ATP via pannexin channels through ATP receptors, P2Y4, and respond with increases in $[Ca^{2+}]_i$, providing the evidence of neuron-to-glia signalling in the ENS (Gulbransen and Sharkey, 2009) (Gulbransen and Sharkey, 2012) (Figure 28).

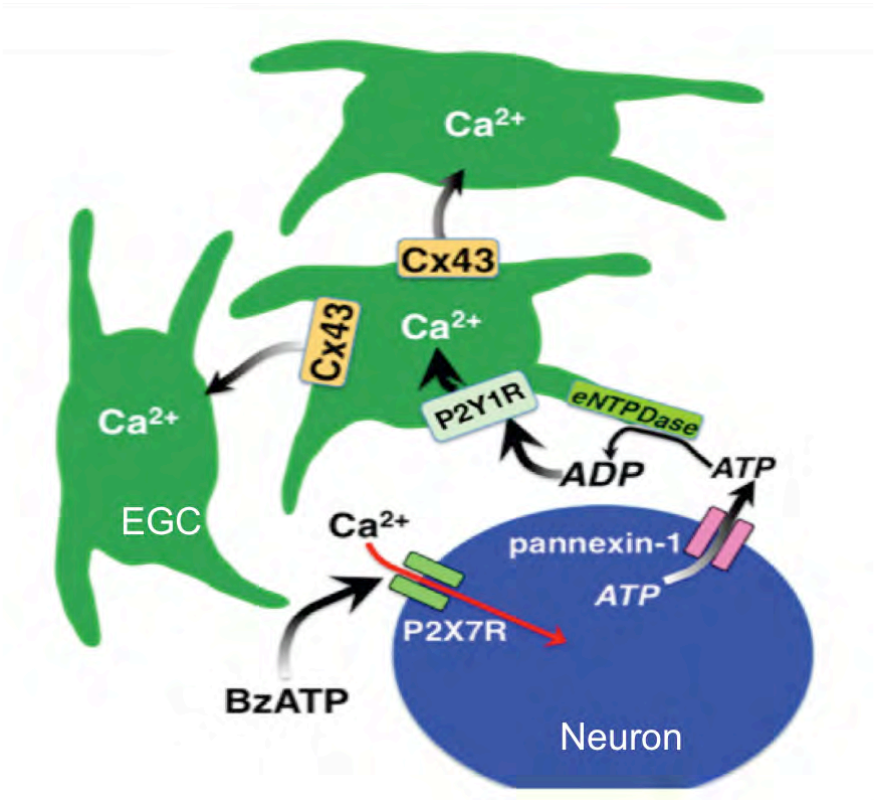

Figure 28 Enteric glia Ca^{2+} waves evocated by enteric neuron-to-glia communication depend on Cx43*. Model of pharmacological enteric neuron activation with the neuronal P2X7 receptor selective agonist BzATP. Glial Ca^{2+} responses generated by released purines diffuse between EGC through Cx43 (McClain, JL. 2014).*

2.6.2 Functional interactions between glia and neurons

As mentioned above, loss of EGC in genetically modified mice causes neuronal degeneration and fulminant jejuno-ileitis, suggesting that enteric glia are crucial for maintenance of neuronal and bowel integrity (Bush et al., 1998). Recently, it has been demonstrated that EGC produce lipid-derived mediators, such as 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) that exerts a role in the IEB maintenance and in the ENS neuroprotection leading to an increase in intracellular neuronal glutathione production (Abdo et al., 2012).

Enteric glia are anchored to the surface of enteric ganglia and nerve strands through intermediate filaments of GFAP stabilizing the ENS (Gabella, 1981, 1990). As astrocytes in the CNS, enteric glia sustain neurons creating a protective microenvironment in the ENS. For instance, astrocytes have been postulated as an additional K^+-uptake to help neurons in the re-uptake of large amounts of K^+ ions released in extracellular space during neuronal activity. The expression of voltage-activated inward and outward K^+-channels related to the astrocytic K^+-channels, indicates that EGC could have a role to prevent extracellular K^+-accumulation in the ENS (Zhang et al., 2003; Walz, 2000).

Enteric glia could have two functions in enteric neurotransmission: 1) participate in neurotransmitter synthesis and/or inactivation, 2) respond to signalling molecules and directly participate in synaptic transmission. Immunoistological studies prove the involvement of enteric glia in the neurotransmission:

GS is an enzyme that catalyzes the conversion of glutamate plus ammonia to the amino acid glutamin. In the ENS, GS is exclusively expressed by EGC, suggesting that they could have a pivotal role in the glutamatergic signalling by detoxification of waste ammonia and neuronally released glutamate (Rühl, 2005; Jessen and Mirsky, 1983).

Data showing predominantly expression of GABA transporter GAT2 in EGC suggest that enteric glia may be involved in the detoxification of GABA via rapid GABA-removal from extracellular space (Fletcher et al., 2002).

Immunoreactivity for L-arginine, a precursor of NO, has been exclusively found in EGC suggesting that enteric glia may partially provide L-arginine for neuronal NO-synthesis (Nagahama et al., 2001).

Enteric glia may also play a role in the nucleotide signalling controlling availability of purinergic agonists ATP and ADP (Braun et al., 2004). In addition, enteric glia also express purinoreceptors, such as P2Y7 or P2Y2, and respond to ATP and uridine triphosphate (UTP) increasing cytosolic Ca^{2+} (Vanderwinden et al., 2003; Sarosi et al., 1998). These evidences suggest that not only enteric glia participate in neurotransmitter synthesis and inactivation, but also have an active role in the neurotransmission. In fact, EGC respond to several neurotransmitters (5-HT, ET family members) and molecules implicated in neuro-immune cross-talk (histamine, PAR) (Kimball and Mulholland, 1996; Zhang et al., 1997, 1998).

2.7 Enteric glia in diseases

Glial dysfunctions are implicated in several neurodegenerative diseases such as Parkinson's disease (PD). Aggregates of α-synuclein, the pathological hallmark in PD, were found not only the CNS but also in the ENS that, according to Braak's theory (Braak et al., 2003), would be the initial site of α-synuclein aggregation. In addition, the increase in the expression levels of pro-inflammatory cytokines and glial markers (GFAP, Sox10 and S100β) found in PD patients suggests an association between glial dysregulation and enteric inflammation (Devos et al., 2013; Clairembault et al., 2014).

Prion diseases, a neurodegenerative condition caused by dissemination of pathological isoforms of the cellular prion protein, is another example of neurodegenerative pathology correlated with alteration in the performance of EGC (Albanese et al., 2008).

Fatal inflammation and enterocolitis result from genetic ablation of EGC and autoimmune mechanisms of enteric glia destruction, respectively (Bush et al., 1998; Cornet et al., 2001). In IBD, such as CD and UC, the proliferation of enteric glia and the expression of glial markers GFAP and S100β are altered. In CD patients, the expression of GFAP is increased in inflamed regions of the intestine and reduced in non-inflamed regions, suggesting a link between injury to EGC and continuous inflammation in these patients (von Boyen et al., 2011).

During inflammation enteric glia are activated by proinflammatory cytokines that modulate proliferation and, when operating for long periods (TNF-α and INF-γ), may also cause apoptosis of EGC. Treatment with anti- TNF-α antibodies in CD patients reverses cell-death condition allowing the glial structure to recover and heal mucosa (Colombel et al., 2010).

2.8 ENS control of IEB

Electron microscopy images have revealed close proximity (in the range of 1 μm) between EGC, axons and IECs setting the anatomical basis for paracrine communication between cells. This *neuronal–glial–epithelial* unit is reminiscent of the *neuronal–glial-endothelial unit* of the blood–brain barrier (Neunlist et al., 2013; Van Landeghem et al., 2011) (Figure 29).

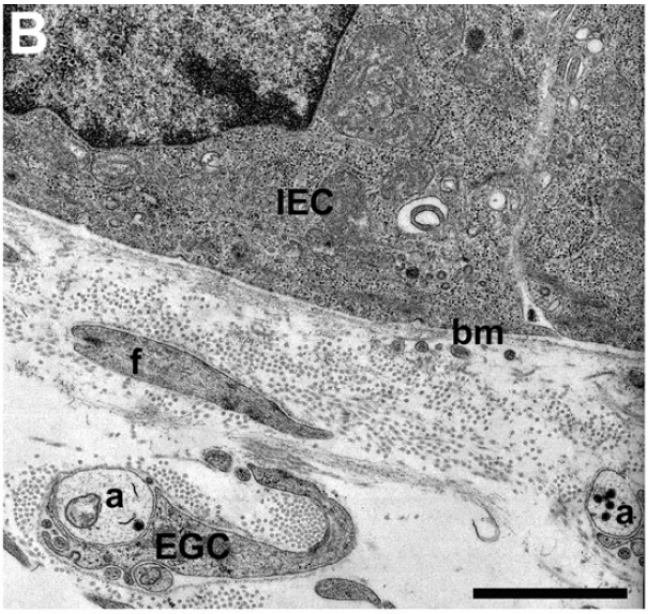

Figure 29 Neural-glial-epithelial unit. *Electron microscopy images of human colonic mucosa highlighting the close proximity (~ 1 µm) between enteric glial cell (EGC) ensheathing axon (a) and intestinal epithelial cell (IEC), the basal membrane (bm) and myofibroblasts (f) (x20.000; scale bar 1 µm) (Van Landeghem, L. 2011).*

2.8.1 Effects on IEB permeability

The activation of enteric neurons results in the reinforcement of IEB functions. Using an *in vitro* model of co-cultures composed of human colonic submucosa, containing the enteric neuronal network, and human colonic epithelial cell lines, *Neunlist et al*. showed that the VIPergic pathways were involved in the control of IEB, reducing intestinal permeability and regulating of TJ proteins, (Neunlist et al., 2003). VIP is increasingly being recognized an enteric neuromediator involved in the maintenance of IEB functions. Several studies show that VIP prevents or reduces the increase of intestinal permeability induced by other neuromediators, or inflammatory/pathogens mediators (such as *Citrobacter rodentium*) and that the reduction of intestinal permeability, induced by enteric neurons stimulation, can be

reversed by VIP antagonists (Neunlist et al., 2003; Hällgren et al., 1998; Conlin et al., 2009).

The indirect activation of enteric neurons through vagal and sacral stimulation, also have an effect on the intestinal permeability in cases of inflammation (Costantini et al., 2010; Luyer et al., 2005; Ghia et al., 2006). Several enteric neuromediators increase intestinal permeability, such as SP, neurokinin A (Hällgren et al., 1998) and Ach. The activation of cholinergic pathways is involved in the alteration of IEB functions in animal models of maternal separation, in UC and a bile induced increase in paracellular permeability (Cameron and Perdue, 2007; Gareau et al., 2007; Wallon et al., 2011).

Several evidences highlight the essential role of mucosal EGC for the integrity of the gut epithelium suggesting that EGC are a major constituent of the IEB protection (Bush et al., 1998). In a mouse model of intestinal injuries caused by severe burns, the increase in intestinal permeability was prevented by the activation of EGC through the vagus nerve stimulation (Costantini et al., 2012). S-nitrosoglutathione (GSNO) is a molecule having a central role in the mediation of EGC effect on the IEB functions. In a noncontact co-culture model of EGC and a confluent IEC monolayer, EGC reduced intestinal permeability and, GSNO was able to reproduce this effect suggesting the involvement of nitrosylation-dependent pathways in the control of paracellular permeability (Savidge et al., 2007). The involvement of GSNO was confirmed in a study showing that EGCs and GSNO prevented *Shigella flexneri*-induced increase in paracellular permeability *in vitro* and mucosal lesions *in vivo* (Flamant et al., 2011).

GDNF is another factor synthesized and secreted by EGC to have reinforcing proprieties of IEB. In a model of colitis induced by DSS, GDNF restores IEB functions *in vivo* and enhances the protective effect of enteric neurons on the IEB, exerting direct protective effects on neurons (Zhang et al., 2010; Anitha et al., 2006; Baudry et al., 2012). Enteric glia also produce polyunsaturated fatty acid metabolites implicated in the regulation of intestinal permeability. Recent evidences have shown that the inability of EGC to control IEB permeability in CD patients could be due to their low 5-hydroxyeicosatetraenoic acid (15-HETE) production (Pochard et al., 2016).

2.8.2 Effect on IEB reparation and IECs proliferation

Enteric neurons have the ability to modulate IEB proliferation and differentiation via the secretion of several neuromediators. The neuromediator VIP mediates the anti-proliferative effect of enteric neurons on IECs (Toumi et al., 2003), whereas SP or Ach stimulate IECs proliferation (Cheng et al., 2008; Goode et al., 2003). Glucagon like peptide 2 also have a trophic effect on IECs mediated by the activation of enteric neurons *in vivo* (Bjerknes and Cheng, 2001).

The healing and IECs proliferation are largely controlled also by EGC. The effect of EGC in the recovery of IEB is in part mediated by pro-EGF or by the increase in IEC FAK expression and activity (Van Landeghem et al., 2011). Enteric glia have also an important anti-proliferative effect on IEC mediated by various glial derived mediators (TGFβ1) or lipid mediators (15dPGJ2). In addition, anti-proliferative and pro-differentiative effects of EGC are mediated in part by the

activation of PPARγ pathways in IECs, through the synthesis of soluble ligands of PPARγ, such as omega 6 derivatives and 15dPGJ2 by EGC (Neunlist et al., 2007; Bach-Ngohou et al., 2010).

2.9 ENS control of digestive functions

2.9.1 Control of intestinal motility

The ENS is essential to coordinate muscle function in digestive tract. Movements of the esophagus are largely determined by neural pattern of CNS, whereas the rather extensive ENS of the esophagus has a subsidiary role. Gastric propulsive movements are primarily myogenic, whereas CNS monitors the state of the stomach controlling its volume, strength of contractions, and acid secretion (Schubert and Peura, 2008). With the exception of defecation, controlled through the defecation centers in the lumbosacral spinal cord, the control of the motility of the small and large intestines is dominated by the ENS (de Groat et al., 1981).

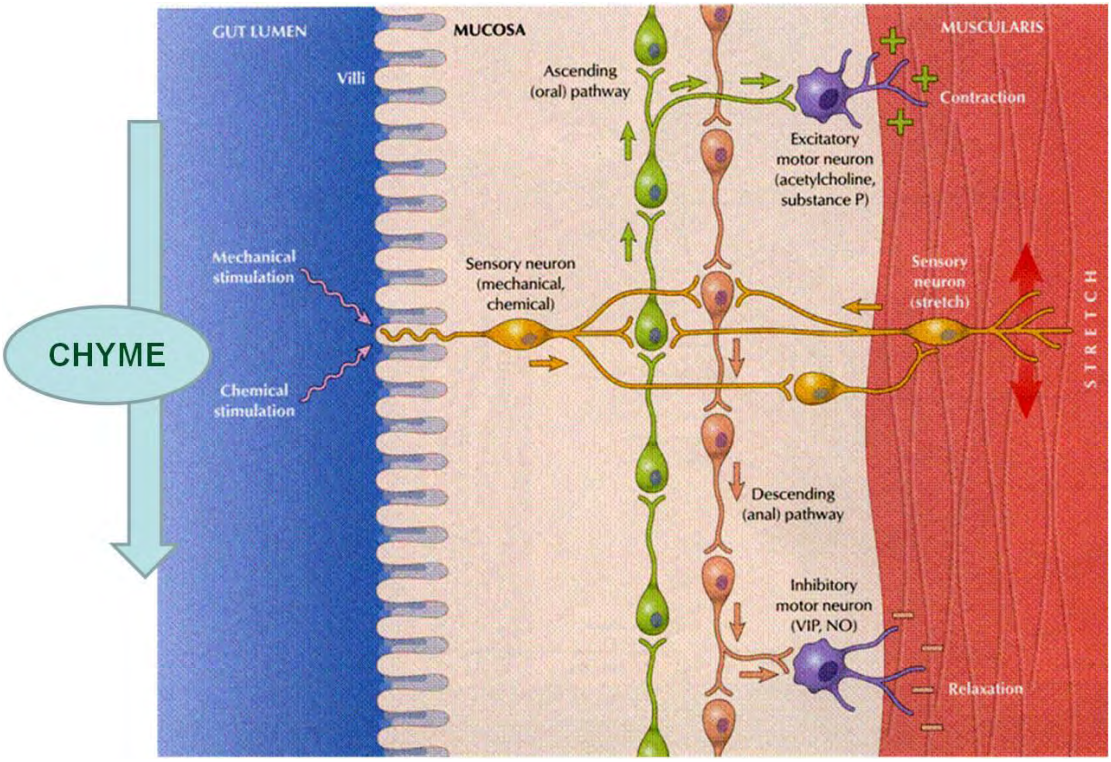

Figure 30 Mechanism of peristalsis. *Sensory neurons of the ENS are chemically and mechanically stimulates by the chyme in the intestinal lumen; these sensory neurons synapse with two sets of interneurons, activating excitatory motor neurons above the bolus and inhibitory motor neurons below the bolus. Ach/substance P and VIP/NO/ATP mediate excitation and inhibition reflexes, respectively.*

Two basic patterns of motility are conducted by the digestive tract: *propulsion*, through which chyme is pushed along the digestive tube during disassembly/absorption, and *segmentation contractions*, segmental rings of contractions to cut and mix bolus and digestive enzymes.

The principal type of propulsive motility is peristalsis, a ring of SM contractions that moves the bolus distally through the oesophagus and intestines. First described by *Bayliss* and *Starling* at the end of XIX century, this type of motility is characterized by a contraction above and relaxation below a segment being stimulated. Mechanical distension actives afferent enteric neurons that synapse with

two sets of cholinergic interneurons, leading to two distinct effects: 1) one group of interneurons activates excitatory motor neurons above the bolus. These neurons contain Ach and SP and stimulate contraction of SM above the bolus. 2) Another group of interneurons activates inhibitory motor neurons that stimulate relaxation of SM below the bolus using NO, VIP and ATP neurotransmitters (Figure 30).

Enteroendocrine cells compose the system of sensor to monitor and respond to changes in luminal pressure, pH or nutrients. The best characterized of these cells are enterocromaffin cells which store 5-HT. Over 95% of 5-HT is located in the gut and over 90% of that store is present in enterocromaffin cells and dispersed in the enteric epithelium, from the stomach through the colon. The remaining part of the store is located in the ENS as neurotransmitter of descending myenteric interneurons. Interestingly, serotonergic signalling is diminished in mucosa during inflammation and potentiation of 5-HT and ⁄ or desensitization of its receptor could explains symptoms seen in IBS-D and constipation-predominant IBS (IBS-C), respectively (Gershon, 2004).

In addition, cholinergic transmission depends on synaptic junctions between motor neurons and a specific class of interstitial cells of Cajal (ICC) interposed between enteric neurons and SM cells in GI muscles. During stimulation, Ach released from enteric motor neurons, binds primarily to receptors expressed by ICC and, the activation of neighboring SM cells occurs by conduction of excitatory stimulus via gap junctions between ICC and SM cells. Thus, terminals of enteric motor neurons, intramuscular ICC, and SM cells form functional units releasing signals and transducing neural inputs into mechanical responses (Ward et al., 2000).

The enteric glia also play a pivotal role in the regulation of intestinal motility. *Nasser* and collaborators have demonstrated that mice treated with gliotoxin fluorocitrate showed alteration of colon contractility *in vitro* and GI transit *in vivo* (Nasser et al., 2006). *Aubé et al*. also showed a significant impairment of digestive motility in mice whose enteric glia was depleted (Aubé et al., 2006).

The role of EGC in the control of GI motility has been confirmed also in human. For instance, in a study on idiopathic slow transit constipation, one of the most severe and intractable forms of constipation, 26 severely constipated patients displayed a significant decrease in EGC and ICC compared to healthy control (Bassotti et al., 2006).

2.9.2 Control of intestinal blood flow

The GI vascular system is regulated by an elaborated system of metabolic, endocrine and neural control mechanisms. The GI vessels are innervated by primary afferent neurons, symphatetic efferent neurons and receive inputs from parasymphatetic efferent end enteric neurons. Neurogenic constriction of GI blood vessels results from activation of extrinsic sympathetic nerve fibers of the celiac and mesenteric ganglia, whereas vasodilatation is regulated by enteric neurons that relay regulatory input from parasympathetic efferent neurons through CGRP and SP, and extrinsic primary sensory neurons. These afferent neurons originate from dorsal ganglia roots and can regulate vessel diameter indirectly via autonomic reflexes and directly via release of vasoactive neuropeptides. Enteric neurons innerving arteries and arterioles in the GI tract originate in the submucosal plexus. One population of

these neurons is cholinergic, whereas the other population is VIPergic and exhibits a similar distribution of sympathetic neurons. Mechanical stimuli induce vasodilatation after a cholinergic reflex and chemical stimuli involve neurons releasing CGRP and SP (Vanner and Surprenant, 1996; Vanner and Bolton, 1996; Vanner, 1994).

2.9.3 Control of intestinal secretion and absorption

Secretomotors reflexes in the GI tract are important for lubrication and movement of digested products, absorption of nutrients or during diarrhoea to flush out microbes. Mechanical stimulation of enterochromaffin cells allows to 5-HT release and activation of IPANs of the submucous plexus. These signals are sent to interneurons or secretomotor neurons to stimulate chloride and fluid secretion via Ach or VIP neurotransmitters and activate chloride-secreting crypt epithelial cells expressing muscarinic and VIP receptors (Christofi, 2008). However, stimulation of submucosal plexus neurons reduces the absorption of Na^+ ions suggesting that enteric neurons are also implicated in intestinal absorption (Hayashi et al., 2003)

2.10 The role of histamine and histamine receptors in the gut

Histamine (2-[4-imidazole]-ethylamine) is a short-acting endogenous amine isolated for the first time in the 1910 by Barger and Dale (Barger et al., 1910). Histamine is known to be a mayor mediator of acute anaphylactic reactions. Since its discovery, numerous other physiological functions have been attributed to this biogenic amine exerting its biological actions through four different G- protein

coupled receptors (H1R–H4R) (Tanaka et al., 2002). Briefly, histamine H1R mediates sensorineural signalling, vascular dilatation / permeability and airway SM contraction; H2R is involved in gastric acid secretion and also exerts immune modulatory properties (Jutel et al., 2009); H3R is abundantly present in the CNS and is involved in several nervous complaints, such as sleep–wake disorders, attention-deficient hyperactivity disorders, epilepsy, cognitive impairment and obesity (Singh et al., 2013). Finally, H4R is predominantly expressed on lymphocytes, mast cells and DCs is probably involved in immune-mediated disorders such as allergic rhinitis, asthma and pruritus (Liu 2014). Histamine is present in virtually all organs, with high concentrations in the stomach, lymph nodes and thymus (Kumar et al., 1968). Histamine is synthesized from the L-histidine by L-histidine decarboxylase and stored in the main sources of histamine, the granules of mast cells and basophils (Endo et al., 1982). L-histidine decarboxylase is also expressed by enterochromaffin-like cells, histaminergic neurons, lymphocytes, monocytes, platelets and neutrophils, without retaining high amounts of histamine by these cells (Jutel et al., 2009). In the GI tract histamine is involved in several physiological processes, including immunological responses, visceral nociception, modulation of intestinal motility, gastric acid secretion (Black et al., 1972; Dawicki et al., 2007; van Diest et al., 2007), and in several GI disorders such as IBD, IBS, neoplasia, food allergies, gastric ulcers and systemic mastocytosis (Black et al., 1972; He et al., 2004; Barbara et al., 2006). H1R is largely expressed in the GI on enterocytes, immune cells, blood vessels, monocytes and enteric nerves (Sander et al., 2006). H2R is expressed on gastric parietal cells, enterocytes, lymphocytes, myenteric ganglia and SM cells. Although the presence H3R is present in GI tissues of guinea pig (Poli et al., 2001), the human GI tract seems to be deprived of H3R (Sander et al., 2006). H4R is expressed in liver,

pancreas, bile ducts and, in the GI tract, is similarly distributed in duodenum, colon, sigmoid and rectum (Sander et al., 2006).

Several evidences reported the involvement of histamine in the pathophysiology of IBS. The study of *Sander et al*., showing an altered expression of H1R and H2R receptor in mucosal biopsies from the terminal ileum and large intestine of patients with symptoms of food allergy and/or IBS (Sander et al., 2006) is consistent with the current concepts for the involvement of histamine release from enteric mast cells and inflammatory granulocytes and its paracrine signalling function in the ENS. The plasticity in histamine receptor expression in patients with food allergy and IBS emphasizes the potential clinical benefit of drugs that act on histaminergic pathways (Sander et al., 2006). Microscopic colitis, intestinal infections, IBS, and others functional GI disorders including abdominal pain, watery diarrhoea and defecation urgency are associated to mastocytosis and increased levels of histamine (Wood 2002; Barbara et al., 2004). Mast cells are located in close proximity to nerves and the proximity of activated mast cells to nerves correlates with the frequency and severity of abdominal pain, suggesting a functional mast cell mediators–nerve interface relevant for immunoneural signalling (Barbara et al., 2004). The potent excitatory effect of histamine in the human ENS is well known (Breuing et al., 2007). Recently, *Bunher et al*. showed that mediators released from colonic mucosal biopsy samples of IBS patients (including histamine) excited neurons of the human submucosal plexus (Buhner et al., 2009). In addition, several animal studies have shown the involvement of histamine in visceral hypersensitivity and sensitization of extrinsic afferent neurons, suggesting that similar mechanism may be operative also in the human gut (Jiang et al., 2011). However, the effect of histamine on the enteric glia is still unknown, although several histamine functions have been

described in the astrocytes, such as the regulation of growth and proliferation (Rodriguez et al., 1989), glycogenolysis via H1R and H2R (Arbonés et al., 1990), Ca^{2+} signalling mediated by H1R (Jou et al., 1996; Jung et al., 2000; Barajas et al., 2008) which promotes the release of gliotransmitters (Shelton et al., 2000) and the production of neurotrophic factors (Lipnik-Štangelj et al., 2004; Jurič et al., 2011).

3. THE IRRITABLE BOWEL SYNDROME

3.1 Definition and epidemiology

In 1849 Cumming said of the IBS "*The bowels are at one time constipated another lax, in the same person. How the disease has two such different symptoms do not profess to explain*" (Horwitz and Fisher, 2001). The IBS is a GI disorder typically characterized by discomfort and abdominal pain associated with stool form and frequency changes (Camilleri et al., 2012; Altayar et al., 2015). IBS patients suffer from altered bowel habit, which varies in both degree and time pattern between subjects. The contrasting nature of these associated symptoms, however, may indicate significant heterogeneity of underlying pain mechanisms (Altayar et al., 2015). Although it is not a "life threatening illness", IBS afflicts 9 – 23% of adult population across the world (10–15% in Europe and North America) and may severally alter quality of life. The prevalence of IBS is increasing in countries in the Asia–Pacific region, particularly in countries with developing economies (*World Gatroenterology organization 2009)*. The highest prevalence of IBS occurred in South America (21.0%) and the lowest in Southeast Asia (7.0%). The prevalence according to each individual country is shown in Figure 31 (Lovell and Ford, 2012).

The high onset of IBS is accompanied by large societal economic charges due to the fact that patients visit doctor more frequently, use more diagnostic tests, consume more medications, miss more workdays, have lower work productivity and hospitalized more frequently than patients without IBS. All these factors cause negative effects on the quality of life in affected patients (Lee and Bak, 2011; Chaudhary and Truelove, 1962).

Introduction

IBS mainly occurs between the ages of 15 and 65, with a greater prevalence in women. The first presentation of patients to a physician is usually in the 30–50-year-old age group; in some cases, symptoms may date back to childhood. There were systematic approaches that attempted to classify the area of functional GI disorders: in the 1962 *Chaudhary* and *Truelove* published a retrospective review of 130 patients at Oxford in whom they had made the diagnosis of IBS (then called *irritable colon syndrome*, ICS) (Chaudhary and Truelove, 1962). Afterwards, in the 1978 *Manning et al* first published the criteria based on a questionnaire to establish the presence of 15 symptoms thought to be typical of the IBS. They found that only distension, relief of pain with a bowel movement and more frequent and looser stools at onset of pain were significantly more common in IBS than in organic disease (Manning et al., 1978). In 1984 *Kruis et al* defined IBS by a logistic regression model that described the probability of IBS. The symptoms, which needed to be present for more than 2 years, included abdominal pain, flatulence and alternating constipation and diarrhoea while signs that excluded IBS were abnormal physical findings and/or history specific for any diagnosis other than IBS, erythrocyte sedimentation, leucocytosis, anaemia and impression by the physician that the patient had rectal bleeding (Kruis et al., 1984).

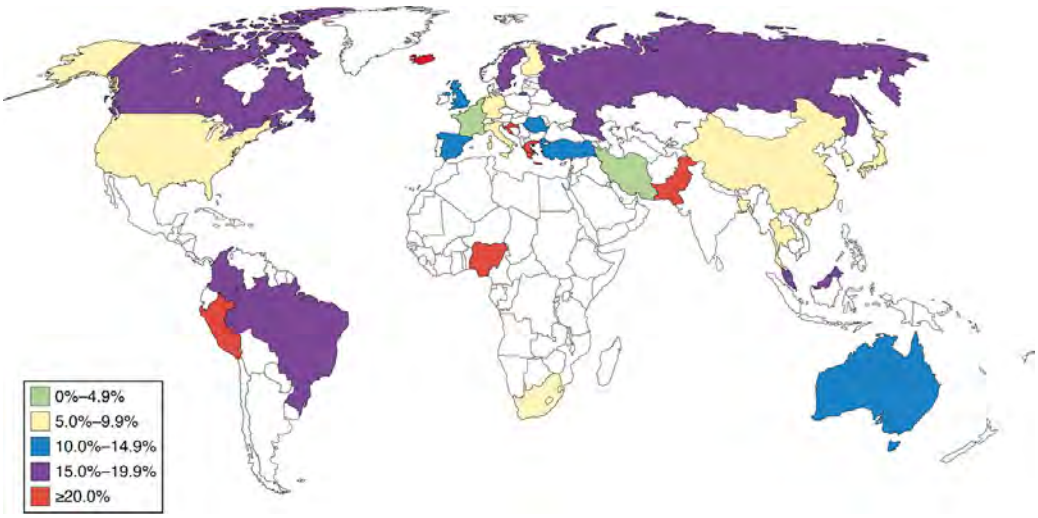

Figure 31: Prevalence of IBS according to country (Lovell, RM. 2012).

3.2 Diagnosis of IBS: *Roma criteria*

Currently, the diagnostic criteria followed in the field of the functional GI disorders (FGIDs) are the "*Roma Criteria*", a series of criteria established by an international commission, to establish the diagnosis and guide treatment of FGIDs, included IBS. These diagnostic criteria emerged thanks to the Rome Foundation, non-profit organization, under the professional management of Degnon Associates based in McLean, Virginia (USA). The Rome criteria were defined, for the first time, during the "XIII International Congress of Gastroenterology and the VI European Congress of Digestive Endoscopy" held in Rome on September 1988, and subsequently revised after four years. The first set of criteria (*The Rome Guidelines for IBS*) was published in 1989 followed by the *Rome Classification System for FGIDs* in the 1990. The *Rome I criteria for IBS* were published in 1992 with a first revision in 1999 (*Rome II criteria*) and a third revision made in 2006 (*Rome III criteria*) during the *Digestive Diseases Week* held in Los Angeles (USA).

Introduction

According to *Rome I criteria for IBS* abdominal pain or discomfort must be present continuously, for at least three months with at least one of the following findings associated: 1) attenuation with the evacuation, 2) variations in the frequency of evacuations and 3) changes in stool consistency; with at least 2 or more of the following findings (associated with at least 25% of evacuations): 1) altered stool frequency (from more than 3 daily discharges to less than 3 discharges per week), 2) altered stool consistency, 3) altered stool passage, 4) presence of mucus and 5) swelling or feeling of abdominal distension. Other symptoms that may be associated: nausea, vomiting, tiredness and feeling of fullness, even after small meal. In order to make a diagnosis of IBS according to *Rome II criteria*, pain or abdominal discomfort must have been present for at least 12 weeks (not necessarily consecutive), or more, over the last 12 months; also they have to be accompanied by at least two of the following symptoms: 1) the pain is eased by the evacuation, 2) variations in the frequency of bowel movements or 3) variations in stool consistency. The other suggestive symptoms (altered frequency/consistency of stools, altered passage of stool, mucus, bloating or abdominal distension) may be present, but they are not fundamental symptoms.

The most recent Rome III criteria (2006) state that a patient must have recurrent abdominal pain or discomfort at least 3 days per month in the last 3 months with onset of symptoms for more than six months (or more) before diagnosis, associated with at least 2 of the following findings: 1) improving with defecation; 2) occurring in association with changes of bowel movements; 3) appearing in association with changes in the form of feces. May occur additional symptoms: 1) less than three bowel movements per week, 2) more than 3 daily bowel movements, 3) hard or lumpy stools, often difficult to expel, 4) untrimmed, soft, mushy or watery

stools, 5) tension and stress during the evacuation, 6) urgency to have a bowel movement, 7) feeling of incomplete evacuation, 8) emission of white mucus (*mucorrea*) during the evacuation, 9) feeling of abdominal fullness, bloating or distension (American College of Gastroenterology Task Force on Irritable Bowel Syndrome et al., 2009). The committee of Rome II tried to subclass the IBS based on several criteria (frequency of the evacuation, form of stools, symptoms of defecation), but this classification was complex and it lacked of an evidence-based approach. For this reason, the sub-classification was revised, basing it solely on the stool consistency. With the Rome III criteria the *Bristol Stool Scale* has been validated, as a useful and easy tool both for the diagnosis of IBS, in its clinical variants, both for an assessment of the response to various therapeutic treatments. The new proposed classification provides four subcategories of IBS based on the prevailing character of stool.

3.3 Patients classification

IBS patients can be divided in two subsets: sporadic (nonspecific) and post infectious (PI)-IBS)/inflammatory bowel disease (IBD)-associated (IBD-IBS). Sporadic subset includes patients who have had symptoms for a long time without GI or other infections. PI-IBS is defined as a sudden onset of IBS symptoms following gastroenteritis in individuals who previously had no GI complaints, with a prevalence of 6-17% of IBS patients (El-Salhy, 2012) . IBD-IBS is defined in IBD patients in remission who display IBS symptoms IBD-IBS and occurs in 33-46% of UC patients and 42-60% of those with CD (Kumar and Wingate, 1985). Figure 32 describes the four possible bowel pattern subtypes of sporadic form of IBS at a particular point in

the time. Individuals with neither diarrhoea nor constipation have an unsubtyped IBS (IBS-U) instead patients not fitting the IBS with diarrhoea (IBS-D, untrimmed or watery stools in ≥ 25% and lumpy stools in <25% of bowel movements) or IBS with constipation (IBS-C, lumpy or hard stools in ≥ 25% and untrimmed or watery stools in <25% of bowel movements) subtypes as having either mixed IBS (IBS-M, lumpy or hard stools in ≥ 25% and untrimmed or watery stools in ≥ 25% of bowel movements) (Longstreth et al., 2006).

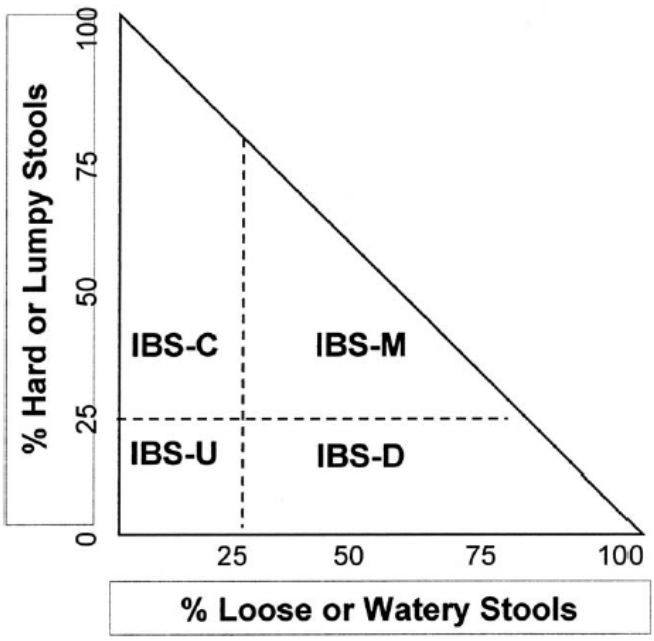

Figure 32: IBS subtypes. *Two-dimensional display of the 4 IBS subtypes according to bowel form at a particular point in time. IBS-C, IBS with constipation; IBS-D, IBS with diarrhoea; IBS-M, mixed; IBS-U, un-subtypes IBS (Longstreth, GF. 2006).*

3.4 Symptoms of IBS

IBS is characterized by symptoms that, periodically vary from mild to severe, may have important effects on the quality of life and the stress of patients (Longstreth

et al., 2006). Typical clinical manifestations in IBS are *discomfort* relieved by defecation, associated with a change in stool form, which can lead to a prevalence of constipation or diarrhoea. The most important symptoms of IBS are altered bowel motility and abdominal pain. Over the past 50 years alterations in the contractility of the colon and small bowel have been described in patients with IBS. Intestinal contractility may be affected by the ingestion of food or from psychological and physical stress; in 1985 *Kumar et al*. showed that fasting small bowel motor activity in IBS differed significantly from that in the healthy control and stress totally abolished the migrating motor complex or provoked prolonged abnormal irregular contractions (Kumar and Wingate, 1985). Moreover, the presence of discrete, clustered, prolonged and propagated contractions has been described in patients with IBS (Kellow and Phillips, 1987). Later, *Camilleri et al*. proved that in a group of IBS patients (predominantly female), colonic transit was the most prevalent physiologic abnormality (32%) (Camilleri et al., 2008).

Balloon distension studies of the rectosigmoid and ileum have showed that IBS patients report bloating and pain at balloon volume and pressure significantly lower than those that induce pain in healthy control, a phenomenon referred as visceral hypersensitivity (Kellow 1987, 1988), reported in up to 60% of IBS patients (Keszthelyi et al., 2015). Visceral hypersensitivity may account for symptoms of urgency, bloating, and abdominal pain experienced by patients with IBS and is an accepted biological marker in some IBS patients (Zhou et al., 2010a). Other commonly reported symptoms in IBS are urgency, straining, bloating, a sensation of incomplete evacuation and mucus in the stools (Khan and Chang, 2010).

Moreover, it is not uncommon to find non-GI symptoms including headache, fatigue, myalgia, dyspareunia, urinary frequency, dizziness and psychological factors (Whitehead et al., 2002) which may influence the persistence and severity of symptoms (Drossman et al., 1988) . About 60 % of IBS patients have psychiatric symptoms such as somatization, depression and anxiety; patients with a diagnosis of IBS are more likely to have these symptoms than other persons who have never received medical treatment for intestinal problems (Drossman et al., 1988; Whitehead et al., 1988). Additionally, there is a correlation between the presence of abuse during childhood (sexual or psychological) and severity of symptoms (Drossman, 1995).

3.5 The pathophysiology of IBS

The understanding of the pathophysiology and pathogenesis of IBS has been slowed due to descriptive studies based on heterogeneous symptoms and the lack of animal models. Although it is a disease that affects a large portion of the adult population, a lack of understanding of pathogenesis and evaluation strategies results in diagnostic uncertainty, and in turn frustration of both the physician and the patient (Sayuk and Gyawali, 2015). Recent developments in the understanding of the interactions between the gut, immune system, and nervous system have led to an extension of the horizons in the field of therapeutic options (Camilleri et al., 2012; Mayer et al., 2001).

Owing the presence of GI and non-GI symptoms, the pathogenesis of IBS appears to be multifactorial. Factors that have a central role in defining the pathophysiology of IBS are inheritance and genetics, environmental, dietary or

intestinal microbiota; in addition, recent evidences have shown the involvement of the alterations of ENS parallel to changes of the digestive tract functions (Figure 33).

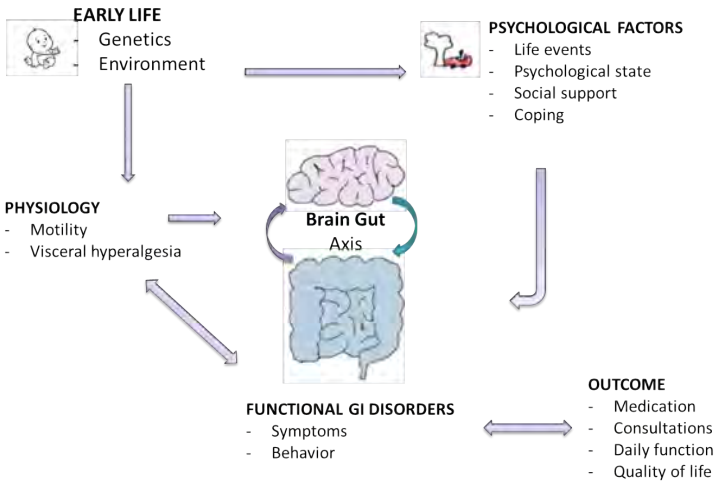

Figure 33 Schematic representation of the pathophysiology of IBS.

3.6 Genetic factors

Psychological factors and stress appear to be primary symptoms in IBS patients and it is supposed that IBS patients have a certain personality with predisposition to develop the disease and these behaviours may have a genetic and heritable basis (Vaiopoulou et al., 2014). Every day clinical practice shows a familial clustering of IBS, with 37% of patients reportedly having a family history of the disorder (Whorwell et al., 1986). The cross talk between brain and gut is a complex communication system that not only regulates the maintenance of GI functions but can also have an effect on the regulation of higher cognitive functions (Levy et al., 2001).

Polymorphisms

Evidences have shown that IBS is also associated with an alteration of the brain-gut bidirectional communication network and polymorphisms in the genetics factors can affect this relationship (Lembo et al., 2007). The majority of the twin studies also confirms a genetic component in IBS with a concordance significantly greater in monozygotic than dizygotic twins (Levy et al., 2001; Lembo et al., 2007).

Polymorphism, a genetic variation that alters gene expression and causes phenotypic diversity, plays an important role in disease susceptibility; in the IBS the genetic variation involves genes encoding proteins with immunomodulatory and/or neuromodulatory properties and the serotonergic, adrenergic and opioidergic systems. Serotonin is involved in the regulation of GI secretion, motility and visceral sensation through the action mediated by the activation of at least five types of receptors. A study of *Yeo et al.* showed that only one among 9 polymorphisms (insertion/deletion polymorphism) in promoter region of 5-HT reuptake transporter gene was associated with diarrhoea in women with IBS (Yeo et al., 2004). Moreover it has been showed that this polymorphism correlates with depressive episodes in IBS patients homozygous for the deletion (Jarrett et al., 2007) .

The GI motility is controlled by the autonomic system and several polymorphisms in adrenergic and opioidergic systems are associated with abdominal pain (Jarrett et al., 2007; Park et al., 2011), colonic transit and sensation rating of gas (Camilleri et al., 2013). Also cytokines IL-10, IL-8 (Romero-Valdovinos et al., 2012), TNF-α (Romero-Valdovinos et al., 2012), IL-1R (Barkhordari et al., 2010), IL-4 and IL-6 (Villani et al., 2010) gene polymorphisms are frequently reported in IBS patients and they are associated with increased risk of IBS (Vaiopoulou et al., 2014).

Micro RNA

Recently it has been shown that other important factors likely involved in the IBS are micro RNA (miRNA), small (21-23 nucleotides) single-stranded RNA molecules, not translated into proteins and which have regulatory function, such as translational repression of targeted mRNAs (Dinan, 2010). Particular sets of miRNA are regulators of gene expressions in GI tract (Dinan, 2010; Wu et al., 2008). In 2008 *Kapeller et al*. published the first association of miRNA and IBS (Kapeller et al., 2008); afterwards *Zhou et al.* showed a link between miRNA29a (Zhou et al., 2010b) and miRNA199 (Zhou et al., 2015) respectively with intestinal membrane permeability and visceral pain in IBS patients.

In conclusion, IBS is a multifactorial and heterogeneous disorder without a well-established molecular pathway or biomarkers. However, twin studies suggest that some genes have a role in the development of IBS but environmental factors are clearly important in determining the clinical manifestations of IBS.

3.7 Environmental factors

Various environmental factors play a key role in the development of IBS. It has been shown that early adverse life events, such as trauma or abuse, can impact neural network and neuroendocrine system development (Anda et al., 2006; Videlock et al., 2009). The exposure to severe wartime conditions in the first two years of life was associated with an increased risk of developing IBS (Surdea-Blaga et al., 2012). The animal model of maternal separation is an example of the effect of stress on the appearances of IBS symptoms (Coutinho et al., 2002). A large Norwegian population-

based twin study shows that the twins with a birth weight below 1500 g were significantly more likely to develop IBS, 7.7 years earlier than in higher weight groups (Surdea-Blaga et al., 2012). Even verbal, sexual, or physical abuse can contribute to the development of the IBS via brain-gut and mucosal immune alteration, and women with IBS are more likely to have experienced these episodes (Drossman, 1995). Additionally, a number of studies show a relationship between IBS and psychiatric disorders, particularly anxiety, depressive disorders and somatization (Fond et al., 2014). It has been showed an increase in adrenocorticotropin hormone, cortisol and catecholamine levels in IBS suggesting a link between stressor factors and the alteration of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (Coutinho et al., 2002; Fond et al., 2014; Elsenbruch and Orr, 2001, Esler and Goulston, 1973; Heitkemper et al., 1996).

Infectious gastroenteritis can also be included among environmental factors as it has been reported for the first time in 1962 by *Chaudhary* and *Truelove* which showed cases of post-infectious IBS (PI-IBS) patients with proven or supposed episode of bacterial or amoebic dysentery (Chaudhary and Truelove, 1962). IBS symptoms can appear after acute gastroenteritis and several factors as genetic, microbiome, immune function, severity of infection and treatment with antibiotics, as well as psychological status (such as stress) could lead to their chronicization (Spiller and Lam, 2012). Post-infectious IBS may explain only a minority of cases of IBS but it represents a link between exposure to an environmental agent, inflammation and IBS in individuals (Morcos et al., 2009).

Interestingly, IBS symptoms may develop following exposure to food-born pathogens (Morcos et al., 2009). Patients with IBS often associate the precipitation of

symptoms with food ingestion. Effectively, there is a correlation between food intake and visceral sensation in IBS patients that can lead to onset of symptoms. Several studies show the effect of a dietary fat on the occurrence of IBS symptoms, such as the increase in colonic sensitivity and alteration of the viscerosomatic pain referral in IBS (Simrén et al., 2001, 2000; Caldarella et al., 2005). Milk and wheat products, caffeine, fructose and certain meats are among the foods most often mentioned by IBS patients as responsible for occurrence of the symptoms after their ingestion (Simrén et al., 2001; Nanda et al., 1989). Bloating and diarrhoea are potentiated by poorly absorbed, highly fermentable short-chain carbohydrates and polyols (described by the acronym FODMAP: fermentable oligo-, di- and monosaccharides and polyols) including fructans, raffinose, lactose, polyols and galactans. By virtue of their osmotic effects in the distal small bowel and colon, these FODMAPs have the potential to enhance IBS symptoms (Morcos et al., 2009). Studies of the impact of FODMAP-free diets in IBS symptoms improvement suggest that this approach may be of value in some patients (Shepherd et al., 2008, 2006).

3.8 Treatments of IBS

In the past IBS patients were treated targeting predominant individual symptoms, such as bloating, diarrhoea, constipation and abdominal pain. Due to the heterogeneity of IBS population and associated symptoms, the physician first must carefully perform a detailed history before discussing treatment options to exclude other diagnoses with similar signs. Currently, the majority of treatments aimed at reducing symptoms and conventional pharmacological interventions (antispasmodics plus stool consistency modifiers) are the main treatments used to improve symptoms

and defecation by their anticholinergic properties (like dyciclomine and hyoscyamine) (Darvish-Damavandi et al., 2010; Saha, 2014). Tricyclic antidepressants (TCAs) and selective 5-HT reuptake inhibitors (SSRIs) appear to reduce abdominal pain and relieve global IBS symptoms (Saha, 2014). Intermittent abdominal pain with known exacerbating factors is better treated with antispasmodics, whereas chronic/constant abdominal pain may have a better response from TCA or SSRIs (Occhipinti and Smith, 2012).

Other conventional treatments include antidiarrheal, laxatives or bulking agents (Regnard et al., 2011; Longstreth et al., 2006), 5-HT_3 receptor antagonists, 5-HT_4 receptor agonists (Ford et al., 2009), TCAs (Rahimi et al., 2009), selective 5-HT reuptake inhibitors (Bundeff and Woodis, 2014) and antibiotics (Rezaie et al., 2010). Lubiprostone is a locally acting chloride channel activator that enhances chloride-rich intestinal fluid secretion and it has been approved for use in IBS-C women (Saha, 2014) whereas, in the case of IBS-D, loperamide increases GI transit time by interacting with the GI musculature, thus allowing for more water absorption (Talley, 2003).

It has been showed that small intestinal bacterial overgrowth (SIBO) is prevalent in IBS patients but is unclear whether SIBO causes IBS (Spiegel, 2011). Antibiotics treatments are the principal approach for treating IBS patients with SIBO. *Pimentel et al*. found that out of 202 of IBS patients, 75% had abnormal lactulose hydrogen breath test results signifying bacterial overgrowth and successfully eradication had significantly improved abdominal pain and diarrhoea (Pimentel et al., 2000). The same authors subsequently showed that the normalization of the lactulose breath test with antibiotics in IBS patients significantly reduced IBS symptoms (Pimentel et al.,

2003). However, the use of these medications has been limited by high recurrence rates, expensive costs and side effects.

As alterations of GI microbiota are involved in the pathogenesis of IBS, probiotics can be beneficial for IBS patients (Ortiz-Lucas et al., 2013). The probiotic strain *Bifidobacterium infantis 35624* has been shown to reduce pain, bloating, and defecatory difficulty and to normalize stool habit (Brenner et al., 2009); *bacteriumlactis DN-173 010* has been shown to accelerate GI transit and to increase stool frequency among IBS-C patients (Hussain and Quigley, 2006).

Among the several alternative options existing to improve IBS symptoms, the modification of lifestyle is the first treatment normally adopted. Physical activity, sharp reductions in drinking, dietary changes, abstinence from alcohol and coffee, and developing regular sleeping habits can improve symptoms of fatigue, bloating and abdominal discomfort but also defecation patterns and colonic transit times in IBS-C patient (De Schryver et al., 2005). Patients often report a correlation between the onset of signs and the ingestion of certain foods, so the elimination of certain foods from the diet may alleviate symptoms. For example, food high in soluble fibers can improve symptoms in some IBS-C patients (Li and Li, 2015). Furthermore, IBS patients have significantly more lactose intolerance complaints (bloating, diarrhoea and distension) than those without IBS, although IBS suffers failed to test positive for hydrogen breath test indicating lactose intolerance. Anyway, decreased intake of lactose can benefit some IBS patients (Gupta et al., 2007). The hypothesis is that the unabsorbed lactose could be subject to bacterial fermentation after ingestion and promote hydrogen gas production and gut distension.

A complementary and alternate medicine (such as traditional Chinese medicine) is often used for chronic medical conditions. Several techniques seem to be widely accepted by IBS patients to alleviate symptoms including acupuncture, hypnotherapy and relaxation. Acupuncture can regulate visceral reflex activity, gastric emptying, and acid secretion through affecting various endogenous neurotransmitter systems (Li and Li, 2015) and studies show that the application of acupuncture targeting in serotonergic, cholinergic, and glutamatergic pathways can increase the concentration of endogenous opioids, which reduces visceral and global pain perception in IBS patients (Ma et al., 2009; Zhou et al., 2009; Tian et al., 2008).

3.9 Gut-Brain axis interactions and IBS

The brain-gut bidirectional axis plays a prominent role in the modulation of gut functions. This communication is complex and involves CNS, brain and spinal cord, the autonomic nervous system, the ENS and the HPA. Numerous neuromodulatory peptides and neurotransmitters control neural transmission within the gut (ENS) and brain (CNS), including corticotropin-releasing factor (CRF), VIP, serotonin, CGRP, Ach, SP, NO, CKK and enkephalins (Drossman et al.; 2002; Kirkup et al., 2001; Konturek et al., 2003). HPA axis is considered as the core stress efferent axis coordinating the adaptive responses of the organism to stressors of any kind (Tsigos et al., 2002). HPA axis is activated by environmental stress, as well as elevated systemic pro-inflammatory cytokines, through secretion of the corticotropin-releasing factor (CRF) from the hypothalamus. CRF stimulates adrenocorticotropic hormone (ACTH) secretion from pituitary gland that, in turn, leads to cortisol release

from the adrenal glands. Cortisol is a major stress hormone that affects many organs, including the brain (Carabotti et al., 2015) (Figure 35).

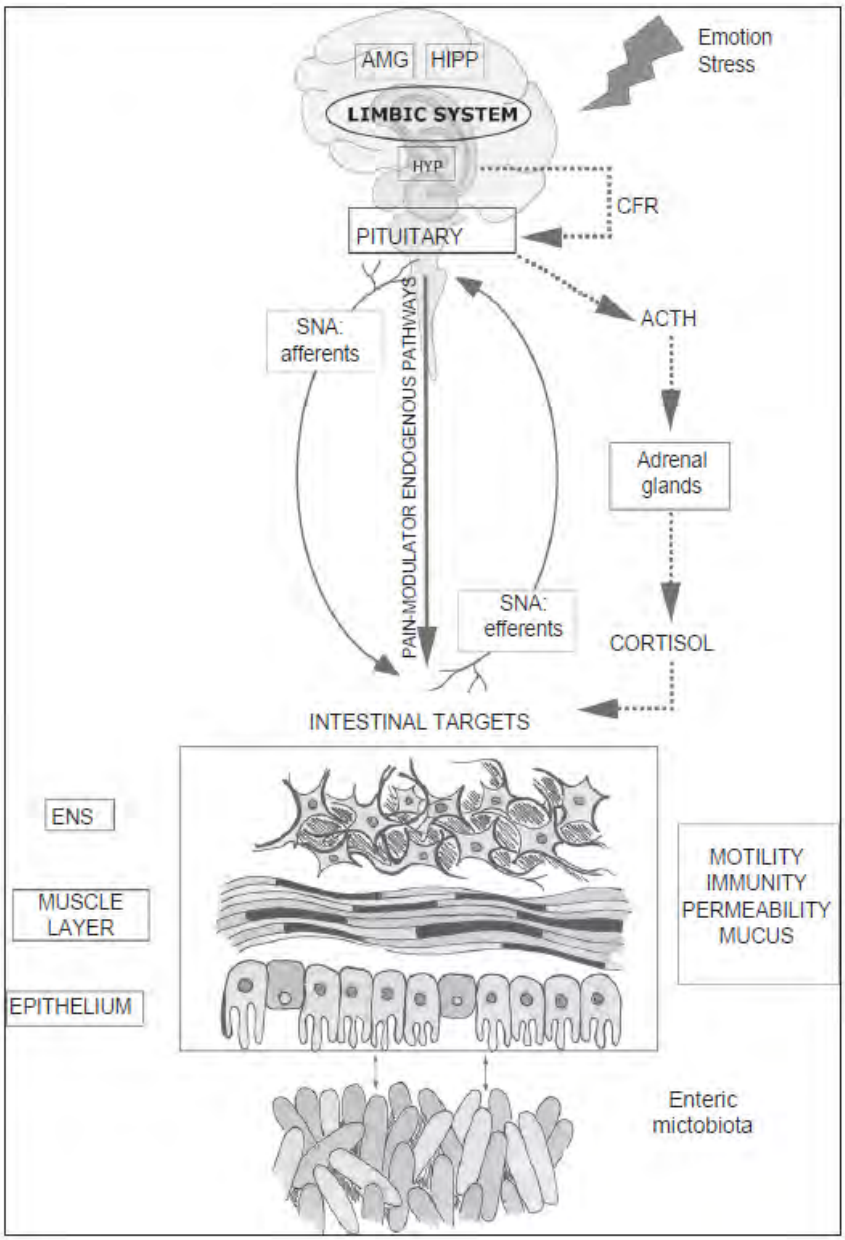

Figure 35 Gut-brain axis. *Environmental factors, such as emotion or stress, can activate the HPA axis. HPA, amygdala (AMG), hippocampus (HIPP) and hypothalamus (HYP) constitute the limbic system involved in the secretion of cortisol. Corticotropin-releasing factor (CRF) secreted by HYP stimulates adrenocorticotropic hormone (ACTH) secretion from pituitary gland that, in turn, leads to cortisol release from the adrenal glands. CNS communicates along both afferent and efferent autonomic pathways (SNA) with different intestinal targets such as ENS, muscle layers and gut mucosa, modulating motility, immunity, permeability and secretion of mucus. A bidirectional communication exists between gut microbiota and these intestinal targets, modulating GI functions (Carabotti, M. 2015).*

Studies in IBS patients have shown an impaired HPA axis that can lead to altered sensation, visceral hypersensitivity, cellular alterations of the entero-endocrine and immune system, changes in intestinal motility, or psychological distress (Koloski et al., 2012; Mayer et al., 2000). The altered intestinal motility reported in IBS could result from a dysregulation of motor nerves regulating GI-SM contraction, whereas enhanced awareness and hypersensitivity to abdominal distension, contraction, and discomfort could derive from a dysregulation of the sensory nerves linking intestinal receptors and nerve endings to the CNS (Drossman et al., 2002).

Often associated to IBS symptoms, anxiety and depression are also linked to alterations of CRF-HPA axis. Interestingly, as demonstrated in humans and rodents, CRF-antagonist attenuates the increase of anxiety, colonic motility, secretion and visceral hypersensitivity generated by CRF administration (Taché et al., 2009; Million et al., 2003; Trimble et al., 2007; Lembo et al., 1996; Sagami et al., 2004). In addition, using functional magnetic resonance imaging it has been shown that emotional arousal regions (hippocampus, hypothalamus, insula, and others) are inhibited by oral CRF-antagonist in a pain-expectation model, suggesting the CRF antagonism as an important target for IBS associated with stress responses including anxiety (Hubbard et al.; 2011).

A number of non-invasive techniques have become available to assess the brain-gut axis. Neuroimaging provides evidence of physiological differences in central processing between normal individuals and patients with functional digestive disorders. In general, although the results are often discordant, all the observations regarding abnormal functional responses to noxious rectal stimulation in IBS patients confirm the significant role of the CNS in the pathophysiology of this syndrome

(Silverman et al., 1997; Mertz et al., 2000; Baciu et al., 1999; Bonaz et al., 2002). The complexity of pathophysiological mechanisms probably depends on the complex role of the regions abnormally activated, such as cingulate cortex and amygdala. In fact, cingulate cortex is a critical pain center, integrates autonomic and endocrine functions, and is involved in recall of emotional experiences, while the amygdala is involved in the perception of emotions, particularly anxiety and fear (Mulak et al., 2004).

As highlighted by recent advances in research, bidirectional gut-brain interactions can be highly influenced by the gut microbiota, interacting with CNS through the regulation of brain chemistry and influencing neuro-endocrine systems associated with stress response, anxiety and memory function (Figure 35).

4. THE GUT MICROBIOTA

4.1 Definition, composition and functions of gut microbiota

The concept of the human microbiome was first introduced in 2001 by *Joshua Lederberg*, who defined it as 'the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease" (Lederberg and McCray, 2001).

The human body is inhabited by a vast number of bacteria, archaea, viruses, and unicellular eukaryotes but the GI tract is the most heavily colonized organ containing over 70% of all the microbes in the human body (Ley et al., 2006; Whitman et al., 1998). Gut microbiota are mainly composed by three of 50 bacterial phyla described: *Bacteroidetes* (Gram-negative), *Firmicutes* (Gram-positive) and *Actinobacteria* (Gram-positive) whereas *Proteobacteria*, *Verrucomicrobia*, *Fusobacteria*, and *Cyanobacteria* are present in minor proportions (Eckburg et al., 2005). In humans, the gut microbiota represent approximately 1 kg of our body weight and microbes in our gut are now recognized as an organ with many metabolic, immunologic and endocrine-like actions that influence human health (O'Hara and Shanahan, 2006). Sequencing of variable regions of ribosomal 16S RNA (the small subunit of prokaryotic ribosomes) gives information about the species presents in the studied samples, whereas metagenomics sequencing gives access to the entire metagenome sequences. Nowadays, all these bioinformatics capacities allow to estimate that the human GI tract contains approximately 10^{14} microorganisms (ten times more cells than the whole human body) and that each gut microbiota are

composed of 500–1000 distinct bacterial species (Eckburg et al., 2005; Gill et al., 2006).

The colonization of human gut starts immediately at birth although, contrary to the idea that the fetal intestine is sterile, some recent studies indicate that colonization may already start before delivery, by bacterial transmission through the placental barrier (Funkhouser and Bordenstein, 2013; Aagaard et al., 2014). During the passage through the birth canal, infants are exposed to a complex microbial population and evidences show a similarity between intestinal bacterial population of infants and the vaginal microbiota of their mothers (Mändar and Mikelsaar, 1996) contrarily to infants delivered through caesarean section which have a different microbial compositions compared to vaginally delivered infants (Huurre et al., 2008).

During the first year of life, the gut microbiota are relatively simple and varies between different individuals, starting to stabilize and resemble that of an adult after 1 years (Mändar and Mikelsaar, 1996; Funkhouser and Bordenstein, 2013). The composition of infant's gut microbiota change in correlation with a shift in feeding mode from breast- or formula-feeding to weaning and the introduction of solid food (Mackie et al., 1999). The use of antibiotics also influence the early microbiota composition and maturation of the immune system, which in turn might have long-term effects on adult microbiota and health later in life (Scholtens et al., 2012). Moreover, microbial succession is also influenced by numerous external (type of food eaten and dietary and temperature-related stresses) and internal (intestinal pH; microbial interactions; environmental temperature; peristalsis; bile acids; host secretions and immune responses; drug therapy; and bacterial mucosal receptors) host-related factors (Mackie et al., 1999).

The intestinal microbiota are not homogenous: in the stomach there are 10^1 bacteria per gram of contents with the majority of *Lactobacillus*, *Veillonella* and *Helicobacter*; in the small intestine the population varies from 10^3 (in the duodenum) to 10^7 per gram of contents (10^4 in the jejunum and 10^7 in the ileum) with a prevalence of *Bacilli* class of the *Firmicutes* and *Actinobacteria*. In the human colon 10^{12} bacteria per gram of contents are present with a prevalence of *Bacteroidetes* and the *Lachnospiraceae* family of the *Firmicutes* (Frank et al., 2007). Moreover, it also exist a significant difference in bacterial population between the intestinal epithelial surface and intestinal lumen separated by the mucus layer. *Swidsinski et al*. showed that many bacterial species present in the intestinal lumen did not access to the intestinal lumen and epithelial crypts. *Clostridium*, *Lactobacillus*, and *Enterococcus* were detected in the mucus layer and epithelial crypts of the small intestine whereas *Bacteroides*, *Bifidobacterium*, *Streptococcus*, members of *Enterobacteriacea*, *Enterococcus*, *Clostridium*, *Lactobacillus* and *Ruminococcus* were all found in feces (Swidsinski et al., 2005).

Gut microbiota are in a symbiotic relationship with human body performing several essential functions (Bäckhed et al., 2005; Hooper et al., 2002) (Figure 34). Gut bacteria: 1) produce a variety of vitamins, synthesize all essential and nonessential amino acids and carry out biotransformation of bile (Vyas et al., 2014), 2) take part in the metabolism of nondigestible carbohydrate (cellulose, hemicellulose, pectins, and gums), unabsorbed sugars, alcohols from the diet and host-derived mucins (Cummings et al., 1987; Koropatkin et al., 2012), 3) regulate host protection/immune-system development producing antimicrobial compounds (such as bacteriocins) and competing for nutrients and sites of attachment in the gut lining (Guarner et al., 2003). The intestinal microbiota are also involved in the

prevention of allergies: allergic children have a different composition of intestinal bacteria than those who do not develop allergies, hypothesizing that the intestinal microbiota stimulate the immune system and train it to respond proportionately to all antigens. Alteration in the gut microbiota composition in early life can lead to an inadequately trained immune system that can overreact to antigens (Björkstén et al., 2001).

Once established, intestinal microbiota remain relatively stable with a high variability between individuals (Ding and Schloss, 2014). The major impact on the microbiota composition is exercised by the diet: regime high in plant-derived carbohydrates, typical of non-western populations, are more diverse and enriched in *Prevotella spp*. at the expense of *Bacteroides spp*. as compared with Western populations consuming a diet high in animal protein, sugar, starch, and fat (Salonen and de Vos, 2014). Interestingly, it has been shown that even within days after the transition to a regime completely composed of animal or plant products changes in microbial structure, gene expression and metabolic activity can be observed (David et al., 2014).

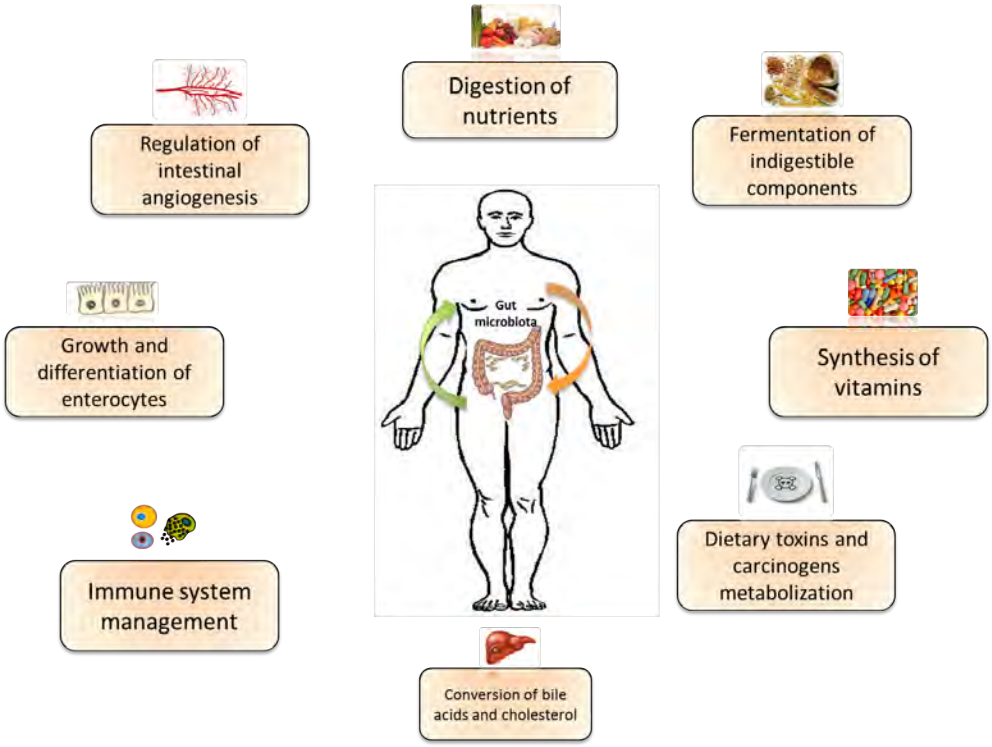

Figure 34 Schematic representation of relationship between gut microbiota and human body.

A perturbation in the composition of gut microbiota (also known as *dysbiosis*) has been associated with GI diseases such as IBS, immune disorders, and susceptibility to infections. Epidemiological observations show that the development of IBS symptoms is often preceded by a disruption of intestinal microbiota, suggesting that intestinal bacterial imbalance can be an important etiological factor in IBS (Ringel and Ringel-Kulka, 2015). *Jeffrey et al*. have described subsets of IBS patients, not corresponding to the traditional IBS subtypes, with an altered or normal-like microbiota composition and patients with normal-like microbiota blend had more adverse psychological factors. All these results suggest that the microbiota alteration could be relevant and central factors may predominate over microbiological factors for a part of IBS patients (Jeffery et al., 2012). The alteration of GI microbiota in the

IBS is confirmed by a faecal transplantation experience in which visceral hypersensitivity of IBS could be transferred via faecal sample to germ-free rats (Crouzet et al., 2013). In addition, it has been shown an alteration of intestinal permeability and an increase in GI transit of germ-free rats transplanted with microbiota of IBS-D patients compared to mice gavaged with the microbiota from healthy controls (De Palma et al., 2014). However, mechanisms by which the microbiota exert its effects are not yet well understood.

An increasing number of evidences suggest that perturbations of gut microbiota may increase predisposition to different disease phenotypes including non-intestinal pathologies, such as cardiovascular diseases, obesity, diabetes, liver or even brain diseases (Swidsinski et al., 2005; Mayer et al., 2015; Bäckhed et al., 2005).

Probiotics may restore the composition of the gut microbiome and introduce beneficial functions to gut microbial communities, resulting in amelioration or prevention of gut inflammation and other intestinal or systemic diseases (Hemarajata et al., 2013).

4.2 Definition of probiotics, prebiotics and synbiotics

According to the Food and Agricultural Organization of the United Nations and the World Health Organization, probiotics are defined as "*living microorganisms, which when administered in adequate amounts confer health benefits on the host*" (Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food, London 2002) whereas prebiotics are indigestible food ingredients that selectively promote the growth or activity of beneficial enteric bacteria, thereby benefiting the host (Hutkins et al., 2015). Synbiotics are

combinations of probiotics and prebiotics designed to improve the colonization of the intestinal tract by ingested microorganisms (de Vrese and Schrezenmeir, 2008). For their health-promoting qualities probiotics have been selected from the large spectrum of lactic acid bacteria and other microorganisms according to different criteria: free from toxic and pathogenic effects, safe for humans and best adapted to the ecosystem of the gut. They are resistant to gastric and bile acids, digestive enzymes, and able to adhering to IECs, survive reproduce in the human large intestine and produce of antimicrobial substances (de Vrese and Schrezenmeir, 2008). The majority of probiotics belong to the genera *Lactobacillus* and *Bifidobacterium*, gram-positive lactic acid-producing bacteria that constitute a major part of the normal intestinal microflora in animals and humans (de Vrese and Schrezenmeir, 2008).

Lactobacilli are non-spore forming rod-shaped bacteria strictly fermentative, aero-tolerant or anaerobic, aciduric or acidophilic. They are abundant in habitats where rich carbohydrate-containing substrates are available, such as human and animal mucosal membranes, on plants or material of plant origin, sewage and fermented milk products, fermenting or spoiling food (de Vrese and Schrezenmeir, 2008). In humans, *Bifidobacteria* compose the major part of intestinal microflora. They are non-motile, non-sporulating rods with varying appearance and most strains are strictly anaerobic. *Bifidobacteria* appear in the stools few days after birth and their number increases thereafter: in the adult colon there are 10^{10} - 10^{11} cfu/gram of *bifidobacteria* and this number decreases with the age (de Vrese and Schrezenmeir, 2008). In 1907 *Metchnikoff* and *Mitchell* showed a seminal report linking the longevity of Bulgarians with consumption of fermented milk products containing viable *Lactobacilli* (Metchnikoff and Mitchell, 1907) and, since then probiotics are often used as dietary supplement or functional foods to modulate gut functions.

4.3 Probiotic functions

Intestinal barrier reinforcement

One of probiotics function is the fortification of IEB via the production of protective heat shock proteins (hsp), which are synthesized in response to thermal, osmotic, oxidative or other stressors. These proteins help to maintain tight junctions between IECs and promote barrier function (Petrof et al., 2004).

Anti-inflammatory effect

The pro-inflammatory signalling pathway in IECs is mediated by NFkB pathway. Under non-stimulatory conditions, NFkB is inactive in the cytoplasm bound to the inhibitor molecule IkB. In response to pro-inflammatory stimuli IkB is phosphorylated by IKK and subject to ubiquitination and subsequent proteosomal degradation. Freed from IkB, NFkB is able to migrate into the nucleus to bind target promoters and activate the transcription of effector genes (Neish et al., 2000). Probiotics can prevent via different mechanisms the activation of NFkB and the subsequent secretion of pro-inflammatory cytokines. Avirulent *Salmonella typhimurium* PhoP and *Salmonella pullorum* in direct contact with polarized T84 epithelial cells decrease IL-8 and TNF-α secretion by inhibiting the polyubiquitination of IκB and subsequent proteasomal degradation (Neish et al., 2000). *Lactobacillus rhamnosus* generates increased quantities of reactive ROS able to inactivate the enzyme E3 ligase involved in the polyubiquitination of IκB preventing the activation of NFκB (Lin et al., 2009). *Lactobacillus plantarum* does not affect the ubiquitination of IκB but instead the chymotrypsin-like activity of proteasome, without causing cell death and toxicity like other proteasome-inhibitors

(Petrof et al., 2004). Several other studies demonstrate that probiotics have an inhibitory effect on NFκB pathway in IECs but fail to define how NFκB activation is prevented. Furthermore, not all probiotics strains inhibit NFkB activation. For example, *Bifidobacterium lactis* BB12 strain can transiently induce the activation of a transcriptionally active subunit of NFκB, to increase IL-6 secretion (Ruiz et al., 2005).

Probiotics can also prevent inflammatory response of IECs affecting MAPK signalling pathway independent of NFκB signalling and apoptosis modulating PI3K/Akt pathway (Resta-Lenert and Barrett, 2006). *Lactobacillus rhamnosus* GG ATCC 53103 can activate anti-apoptotic Akt/protein kinase B and inhibits pro-apoptotic p38 MAPK in TNF-α, IL-1α or IFNγ stimulated IECs (Yan and Polk, 2002). The capacity of probiotic to prevent apoptosis of IECs may be useful strategy to minimize deleterious effect of enteritis infections. For example, the probiotic yeast *Saccharomyces boulardii* is able to prevent apoptosis induced via caspase-3 activation during entero-pathogenic *Escherichia Coli* infection (Czerucka et al., 2000). PARγ, a nuclear hormone receptor that can regulate intestinal inflammation and homeostasis, is another target for probiotic modulation. It has been shown that PPARγ may reduce colitis inhibiting NFκB activity and this is confirmed also by data that demonstrate a reduction of PPARγ mRNA and protein expression in the colonic epithelial cells of patients with UC compared to healthy individuals and patients with CD (Voltan et al., 2008).

Immune regulation

DCs directly taste intestinal luminal contents through cellular processes extended between IECs into the lumen and orchestrate T-lymphocytes responses as a bridge

between microbes, innate and adaptive immunity (Macdonald and Monteleone, 2005). There are several examples of probiotic strains that promote the maturation or the survival of DCs. *Bifidobacterium breve* C50 produces a fermentation product, which induces DC maturation, increases DC survival and anti-inflammatory IL-10 production (Hoarau et al., 2008).

Intestinal immune function relies on several lymphocyte populations, including regulatory T cells specific to the intestine, a subset population of T cells that can suppress the function of Th effector cells and, probiotics may exert their anti-inflammatory effects by modulating this T-cell population (Thomas and Versalovic, 2010). In the lamina propria of the gut B cells differentiate into plasma cells and secrete dimeric IgA antibodies that complex with the polymeric Ig receptor on the basolateral surface of IECs to be transported to the apical cell surface and secreted into the intestinal lumen. Probiotics may also stimulate plasma to produce IgA in a strain-dependent manner (Delcenserie et al., 2008). In a study of *de Moreno de la Blanc et al.*, long-term oral administration of fermented milk containing *L. casei* DN-114001, *L. delbrueckii* subsp *bulgaricus* and *Streptococcus thermophiles* causes increase of the numbers of IgA cells in both the small and large intestine compared to controls (de Moreno de LeBlanc et al., 2008).

4.3.1 *Lactobacillus reuteri*

Lactobacillus reuteri (*L. reuteri*) is a heterofermentative bacterium considered to be one of the few true autochthonous (indigenous) *Lactobacillus* species in humans. Several *in vivo* and *in vitro* studies reported that *L. reuteri* is safe for human

consumption (Valeur et al., 2004; Urbańska and Szajewska, 2014) although plasmids encoding for antibiotic resistance genes can be found in some strains (Heavens et al., 2011; Rosander et al., 2008; Hou et al., 2014). According to the European Food Safety Authority, probiotics should not contain known antibiotic resistance traits. *L. reuteri* ATCC 55730 is a commercially available probiotic strain, which has been found to carry potentially transferable resistance traits for tetracycline and lincomycin. Therefore, it has been replaced by *L. reuteri* DSM 17938, a new probiotic strain where the two resistance plasmids have been removed without losing any probiotic characteristics (Rosander et al., 2008). Anyway, not all *L. reuteri* strains are the same or provide a beneficial response (Frese et al., 2011). *L. reuteri* DSM 17938, *L. reuteri* NCIMB 30242 and *L. reuteri* ATCC PTA 6475 are the most commonly of human origin used in dietary supplements.

L. reuteri is the most widely distributed *Lactobacillus* species among animals and considered to be one of a limited number of indigenous *Lactobacillus* species in the human intestine (Spinler et al., 2008). *L. reuteri* resides in the GI tract of mammalians such as humans, pigs, hamsters, mice, rats, dogs, sheep, cattle, and different bird (Mitsuoka, 1992). In pigs, rodents, and chickens *L. reuteri* is one of the most abundant species present in the gut (Valeur et al., 2004; Urbańska and Szajewska, 2014; Lee et al., 2009; Heavens et al., 2011); in contrast, its presence is much lower in humans, where the species is only occasionally found (Walter, 2008).

The ecological strategies of *L. reuteri* are different in human and animals (Walter, 2008). Tick cell layers of lactobacillus that line parts of the upper part of GI tract are presents in pigs, rats, mice and chickens and *L. reuteri* adheres to cells of the stratified squamous epithelium present at these sites (Frese et al., 2011; Spinler et al., 2008). In contrast, stratified squamous epithelia are absent in the human gut except

into the esophagus, and epithelial cell layers rich in lactobacilli have not been described (Walter, 2008).

Several human and animal trials have shown the beneficial effect of *L. reuteri* in different pathological conditions such as reduction of the duration of diarrhoea caused by rotavirus in children (Huang et al., 2014; Liu et al., 2013; Shornikova et al., 1997), reduction of IgE associated eczema in 2-y-olds children (Abrahamsson et al., 2007), improvement of gastric emptying, lowering of colic symptoms in 95% of infants and reduction of crying time in premature infants (Indrio et al., 2011; Xu et al., 2015; Mi et al., 2015). Colic is a poorly understood syndrome in which infants (from after birth to 6 months) have crying spells that last for at least 3 hours. In a prospective controlled trial *L. reuteri* was compared with simethicone treatment for efficacy in treating colic. After 28 days of treatment with *L. reuteri* 95% of infants had significantly reduced their daily crying times, compared with only 7% of infants receiving simethicone (Indrio et al., 2008). Many human studies report the beneficial effects of the *L. reuteri* treatment to prevent acute diarrhoea associated with virus infections and antibiotic use in children and adults (Francavilla et al., 2012; Shornikova et al., 1997; Cimperman et al., 2011). The majority of clinical trials show the beneficial effects of *L. reuteri* in the treatment of abdominal pain, functional constipation, GI motility, allergy, UC and regurgitation in new-borns and infants (Coccorullo et al., 2010; Oliva et al., 2012; Garofoli et al., 2014; Böttcher et al., 2008; Indrio et al., 2008; Romano et al., 2014). Rodents, turkeys, chickens, and pigs are the animal models used to study *L.reuteri* effects on the host. In a murine model of acquired immunodeficiency syndrome *L. reuteri* increased intestinal resistance to the eukaryotic pathogen *Cryptosporidium parvum* (Alak et al., 1997). Moreover, it has been shown that *L. reuteri* administration reduced mortality in chickens and turkeys

upon infection with Salmonella through a mechanism of competitive exclusion (Huang et al., 2014). Various *L. reuteri* strains produce a range of antimicrobial substances able to inhibit pathogens *in vitro*. *Reuterin* is the best characterized of those, a β-hydroxypropionaldehyde (3-HPA) derivative of glycerol. This substance is produced under anaerobic conditions and exhibits broad-spectrum effects against gram-positive and gram-negative bacteria, fungi, yeast and protozoa (Talarico et al., 1988). Although many bacteria strains are able to reduce glycerol, *L. reuteri* strains are unique because they produce high levels of *reuterin* and they are much more resistant to *reuterin* than most other bacteria, indicating that the antimicrobial activity of *reuterin* production has an ecological and evolutionary significance (Cleusix et al., 2007, 2008). In addition, *L reuteri* can produce *reutericyclin* (Gänzle et al., 2000), *reutericin 6* (Kabuki et al., 1997), vitamin B12 (Taranto et al., 2003; Morita et al., 2008) and encodes various antioxidant enzymes (Hou et al., 2014).

One of the main effects of *L. reuteri* is the modulation of the immune system. Several experimental models of colitis show an immunoregulatory effect of *L. reuteri* in reducing inflammation (Møller et al., 2005; Schreiber et al., 2009; Madsen et al., 1999; Peña et al., 2005; Fabia et al., 1993). It has also been shown that *L. reuteri* plays an important role in the induction of tolerance in human gut, inhibiting the induction of pro-inflammatory cytokines IL-12, IL-6, TNF-α in murine DCs (Christensen et al., 2002). The priming of DCs by *L. reuteri* was initiated by the binding of C-type lectin DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) and resulted in an induction of regulatory T cells *in vitro* (Smits et al., 2005) (Figure 36). The physiological relevance of the immune effects of *L. reuteri* was demonstrated *in vivo* using Lactobacillus-free mice where the administration of *L. reuteri* activated the production of pro-inflammatory cytokines

and chemokines by IECs in the jejunum and ileum. Elevated levels of IL-10, IL-2, TGF-β and Foxp3 (positive regulatory T-cells present in the supernatants from mice immune cells) could be explain that pro-inflammatory cytokine levels completely returned to normal after 21 days although high numbers of lactobacilli continued to be present in the gut. These results suggest that *L. reuteri* contributes to the immune regulation in the gut by modulating antigen-presenting cells toward favouring tolerance (Hoffmann et al., 2008) (Figure 36). A down-regulation of pro-inflammatory cytokines (e.g., TNF-α) by *L. reuteri* was also observed with macrophages, lipopolysaccharides (LPS)-activated monocytes, and primary monocyte-derived macrophages from children with CD (Lin et al., 2008). However, there are few studies about the effects of *L. reuteri* on IEB. In a recent study *Yang et al.* demonstrated that *L. reuteri* I5007 strain had beneficial effects on the expression of TJ proteins in newborn piglets and the *in vitro* results showed a positive effect on transepithelial electrical resistance of IPEC-J2 cells and the reduction of TJ proteins expression induced by LPS (Yang et al., 2015). *Liu et al.* also investigated the effect of lactobacilli from pigs with high mucosal expression of heat HSP27 and HSP72 in order to study how specific strains of lactobacilli (*L. rhamnosus* GG, *L. johnsonii* and *L. reuteri* P43-HUV) and their metabolites could influence gut barrier function and HSP expression in an IPEC-J2 cell model under an enterotoxigenic *Escherichia Coli* challenge. They found that *L. reuteri* treatment substantially thwarted detrimental effects of enterotoxigenic *Escherichia Coli* and preserved the barrier function (Liu et al., 2015).

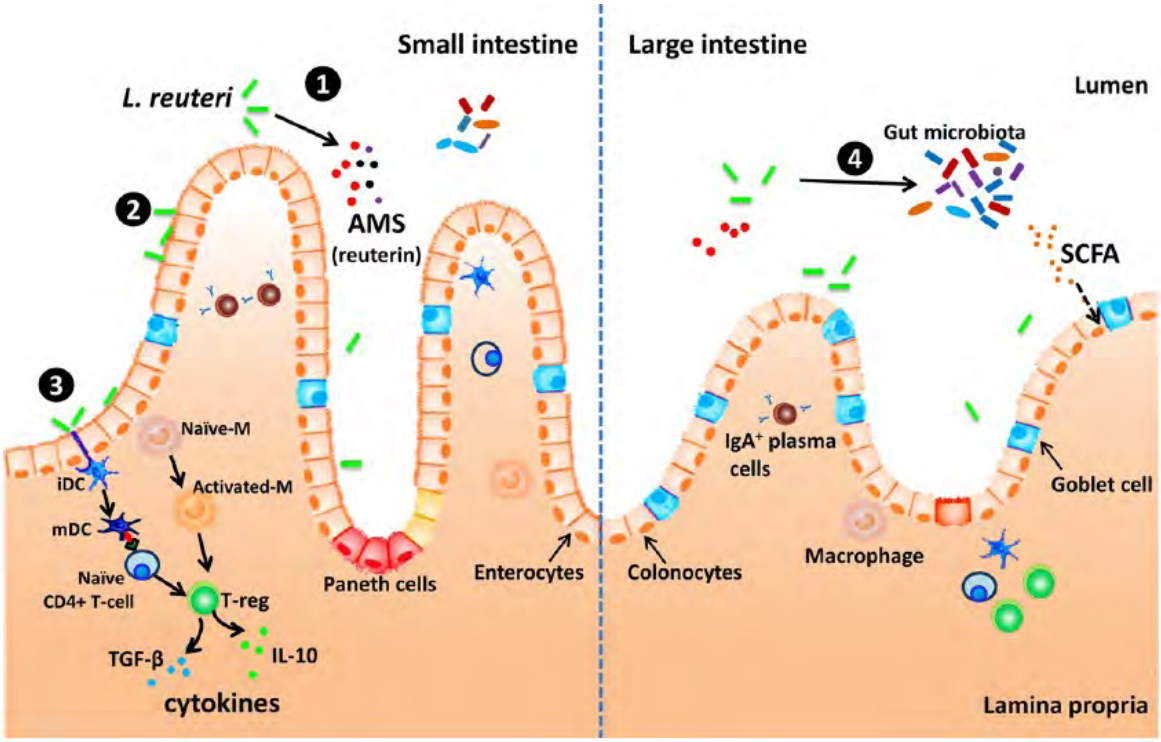

Figure 36. Modulation mechanisms of L. reuteri in the gut. *(1) L. reuteri produces a variety of antimicrobial substances (AMS) such as reuterin; (2) L. reuteri has the capacity to colonize and adhere to mucin and IECs; (3) L. reuteri stimulates or suppress innate immune responses affected by the production of cytokines in macrophages (M), monocytes, and dendritic cells (DCs). The modulation of DCs is mediated through DC SIGN and promotes development of regulatory T cells producing IL-10 and TGF-β; (4) L. reuteri has been reported affected the colonic microbial communities and short chain fatty acid (SCFA) concentration.(Hou, C. et al., 2015).*

4.4 Microbiome gut-brain axis

The most fascinating evidence of GI microbiome interactions comes from the observation that administration of oral antibiotics often dramatically worsened the clinical picture of patients with hepatic encephalopathy (Morgan, 1991). The involvement of dysbiosis was recently confirmed in several pathological conditions, such as anxiety and depressive-like behaviour (Foster et al., 2013; Naseribafrouei et al., 2014) and in autistic patients presenting specific microbiota alterations according

to the severity of the disease (Mayer et al., 2014; Song et al., 2004). Alterations of gut microbiota occurring during FGID, particularly in IBS, are highly associated with mood disorders and are linked to a disruption of gut-brain axis (Simrén et al., 2013; Mayer et al., 2011; Berrill et al., 2013). Germ-free animals show decreased anxiety and augmented levels of ACTH and cortisol associated to increased stress response (Clarke et al., 2013; Diaz et al., 2011). Interestingly, the normalization of gut-axis brain induced by microbial colonisation is age-dependent with reversibility only in very young mice, supporting the existence of a critical period during which the plasticity of neural regulation is sensitive to input from microbiota (Neufeld et al., 2001; Sudo et al., 2004).

Gut bacterial colonization is central to the development and maturation of both ENS and CNS, as proved by studies on germ-free animals. In fact, the absence of gut colonization is associated with alterations in neurotransmitters expression in both nervous systems (Barbara et al., 2005; Clarke et al., 2013; Diaz et al., 2011) and in modification of gut sensory-motor functions, such as delayed gastric empting and intestinal transit and reduced migrating motor complex cyclic recurrence and distal propagation (Iwai et al., 1973; Caenepee et al., 1989; Husebye et al., 2001).

Germ-free animals report also memory dysfunctions, probably associated with altered expression of BDNF, the most important factor involved in memory (Al-Qudah et al, 2014). Microbiota are essential for the modulation of serotoninergic pathway, as shown by increase in 5-HT turnover in germ-free animals (Diaz et al., 2011).

The communication between gut and microbiota is mediated by vagus nerve, since effects of dysbiosis are not present in vagotomised mice (Bravo et al., 2011).

This communication can be driven by different mechanisms. Data reported that *L. reuteri* modulates gut motility and perception of pain enhancing excitability of afferent sensory nerves inhibiting Ca^{2+}-dependent K^{+} channels opening (Kunze et al., 2009). Gut microbiota influence ENS function also by producing local neurotransmitters, including GABA, 5-HT, melatonin, histamine and Ach (Iyer et al., 2004). The ENS is also the target of bacterial products (SCFAs, butyric acid, acetic acid, propionic acid) able to stimulate 5-HT release (Grider et al., 2007), sympathetic nervous system (Kimura et al., 2011), memory and learning process (Vecsey et al., 2007; Stefank et al., 2009).

There are also several direct or indirect mechanisms by which gut-brain axis can regulate gut microbiota. The evidence that brain can modulate the secretion of signalling molecules is supported by the expression of neurotransmitter receptors on bacteria. These interactions can regulate the composition of microbiota, increasing the predisposition to infections and inflammation (Hughes et al., 2008). The capacity of brain to regulate gut functions inevitably involves gut microbiota, being many of these functions (such as motility, secretion of bicarbonate, acid and mucus) associated with the production of mucus layer and biofilm in the intestinal mucosa (Macfarlan et al., 2007).

Stress greatly influences gut microbiota, modulating, for example, size and quality of mucus (Rubio et al., 1992). In addition, the involvement of stress in GI transit alteration has important effects on the delivery of prebiotics and dietary fibers to enteric microbiota. Finally, stress increases intestinal permeability and gut alterations associated to the stress facilitate the expression of virulent bacteria (Alverdy et al., 2000; Cogan et al., 2007).

AIMS OF THE STUDY

The topics discussed in the introduction highlight the central role of the ***neuro-glio-epithelial unit*** in the pathophysiology of irritable bowel syndrome (IBS). The intestinal epithelial barrier (IEB) homeostasis is largely controlled by the enteric nervous system (ENS) as well as by the microenvironment at the IEB level. Abnormalities to the IEB, alteration of the ENS and dysbiosis are all elements that may contribute to the onset of IBS. Several evidences have shown the beneficial effects of probiotics in the treatment of IBS symptoms, even if the knowledge about mechanisms driving these effects is still poorly delineated. Moreover, many studies have shown the central role of the alteration of intestinal paracellular permeability and microenvironment during IBS. Although the effect of IBS supernatants has been largely tested upon the epithelial and neuronal component of the intestinal barrier and ENS, respectively, nothing is known about its effects on the enteric glia.

In this context, the aims of the first part of the work were:

I. to investigate the protective role of a probiotic strain, *Lactobacillus reuteri (L. reuteri)*, in an *in vitro* model of protease activated receptor 2 (PAR-2) agonist-induced increase of paracellular permeability of IECs (Caco-2) treated with three different probiotic forms: live bacteria, heat inactivated bacteria and supernatant from bacterial culture;

II. To analyze the *L. reuteri* effect on the expression of tight junction (TJ) protein components (ZO-1) and on the induction of cytokines secretion (IL-8) by Caco-2 cell monolayers;

III. To investigate the ability of *L. reuteri* to prevent IBS supernatant-induced increase of paracellular permeability of Caco-2 cells.

The second part of this work aimed to characterize:

I. putative alterations of EGC phenotype in a bio-collection of colonic biopsies from IBS patients and healthy controls, identifying mediators produced by the colonic mucosal microenvironment involved in these changes;

II. whether supernatants from colonic biopsies of IBS patients could induce EGC phenotypic and functional alterations;

III. soluble factor responsible for EGC changes.

Two papers presented hereafter and recently submitted were produced during this thesis work.

RESULTS

Paper 1: *In vitro* effect of *Lactobacillus reuteri* on impaired paracellular permeability of intestinal epithelial cells

INTRODUCTION PAPER 1

The alteration of intestinal permeability can play a central role in the pathophysiology of irritable bowel syndrome (IBS). The main actors in the regulation of permeability are tight junctions (TJs), multiprotein complexes that mediate cell-cell adhesion and regulate transportation through the extra-cellular matrix (Nusrat et al., 2000; Mitic et al., 1998). Colonic biopsies from IBS patients show an increased intestinal epithelial barrier (IEB) permeability. In addition, *in vitro* studies have proved that supernatant from the intestinal mucosa of IBS patients increases paracellular permeability of intestinal epithelial cell (IEC) cultures, suggesting the impact of the mucosal microenvironment in the IBS (Piche et al., 2009).

Evidences showing the onset of IBS frequently after intestinal infection or antibiotic treatment are consistent with the alteration of gut microbiota as potential factor contributing to the pathophysiology of IBS (Kerckhoffs et al., 2011; Lee et al., 2011). Several clinical trials have shown beneficial effects of probiotics composing the gut microbiota in alleviating IBS symptoms (Kianifar et al., 2015; Faghihi et al., 2015; Hu et al., 2015; Grandy et al., 2010). Probiotics are live friendly microorganisms that provide benefit to the host via different possible mechanisms, such as competition with pathogens and IEB reinforcement (Rijkers et al., 2011; Camilleri et al., 2012). *Lactobacillus reuteri* (*L. reuteri*) is one of the few true autochthonous Lactobacillus species in humans, for which have been reported numerous positive effects in treating of several disorders (Dinleyici et al., 2015; Holz et al., 2015; Savino et al., 2015). The knowledge about the effects of *L. reuteri* in IBS is currently limited to clinical trials (Niv et al., 2005; Amirimani et al., 2013).

Results

The aim of this study was to test *in vitro* the protective effect of a specific strain of *L. reuteri* on impaired colonic permeability.

To this purpose, Caco-2 cell monolayers previously treated with live, heat inactivated *L. reuteri* or bacterial supernatant were exposed to protease activated receptor 2 (PAR-2) agonist (SLIGRL) or supernatant of colonic biopsies from IBS patients.

We showed that *L. reuteri* was able to prevent SLIGRL-induced increase in paracellular permeability, reduction of ZO-1 expression and IL-8 release in Caco-2 cell monolayers, but not IBS supernatant-induced increase in paracellular permeability.

Our study demonstrates that *L. reuteri* is able to prevent IEB dysfunction induced by PAR-2 agonists, probably in part via a mechanism involving modulation of TJ protein expression, providing foundations for *ad hoc* designed clinical trials in IBS patients.

PAPER 1

In vitro effect of *Lactobacillus reuteri* on impaired paracellular permeability of intestinal epithelial cells

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Results

Abbreviations used in this paper:

DMEM: Dulbecco's modified Eagle's medium **GI:** Gastrointestinal; **HC:** Healthy control; **IBS:** Irritable bowel syndrome; **IBS-C:** Constipation-predominant irritable bowel syndrome; **IBS-D:** Diarrhoea-predominant irritable bowel syndrome; **IBS-M:** Mixed stool forms-irritable bowel syndrome; **IBS-U:** Undetermined-irritable bowel syndrome; **IEB:** Intestinal epithelial barrier; **IEC:** Intestinal epithelial cell; **IFN:** Interferon; **IL:** Interleukin; **PAR:** protease-activate receptor; **SUP:** supernatant; **TER:** trans-epithelial resistance; **TJ:** Tight junction; **TNF:** Tumor necrosis factor; **ZO:** Zonula occludens;

Key messages:

- Intestinal epithelial barrier (IEB) dysfunctions have a central role in the pathophysiology of irritable bowel syndrome.
- This study investigated the effects of the probiotic strain *Lactobacillus reuteri* on intestinal paracellular permeability using an *in vitro* model of IEB-induced dysfunctions.
- *L. reuteri* effectively prevented barrier dysfunction induced by protease-activate receptor 2 (PAR-2) *in vitro*

Abstract

BACKGROUND: Irritable bowel syndrome (IBS) is a complex disorder characterized by abdominal symptoms including chronic abdominal pain or discomfort and alteration of bowel habits. Increasing evidences demonstrate a central role of intestinal epithelial barrier (IEB) dysfunctions and in particular increased paracellular permeability, in the pathophysiology of IBS. Therefore, therapeutic approaches enhancing IEB might be of interest in IBS. **METHODS:** Caco-2 cell monolayers were pre-incubated for 8 hours with live, heat inactivated *Lactobacillus reuteri* (*L. reuteri*) or bacterial supernatant (SUP) and paracellular permeability, ZO-1 expression, and interleukin-8 (IL-8) levels were measured after 24 hours of incubation with PAR-2 agonist (SLIGRL) using sulfonic acid flux, western blotting and ELISA, respectively. Then, paracellular permeability of Caco-2 cell monolayers pre-incubated for 8 hours with live *L. reuteri* was measured after treatment with SUP from colonic biopsies of IBS patients and healthy controls (HC). **RESULTS:** Live or heat-inactivated *L. reuteri*, but not its SUP, prevented SLIGRL-induced increase of paracellular permeability. Pre-incubation of Caco-2 with live or heat inactivated *L. reuteri* but not bacteria SUP prevented SLIGRL-induced increase in basolateral IL-8 levels. In addition, live *L. reuteri* prevented SLIGRL-induced reduction of ZO-1 protein expression. However, live *L. reuteri* was not able to prevent IBS SUP-induced increase of paracellular permeability of Caco-2 monolayers. **CONCLUSIONS & INFERENCES:** *L. reuteri* was able to prevent barrier dysfunction induced by SLIGRL in part via a mechanism involving modulation of ZO-1 protein expression. Our data provide the basis to clinical studies with *L. reuteri*.

Keywords: IBS, probiotics, PAR-2, paracellular permeability

INTRODUCTION

Irritable bowel syndrome (IBS) is a gastrointestinal (GI) disorder characterized by abdominal discomfort or pain associated with changes in stool frequency and form (constipation-predominant [IBS-C], diarrhoea-predominant [IBS-D], alternating stool forms [IBS-M] and undetermined [IBS-U]), affecting up to 20% of the adult population (1). Emerging evidence suggests that altered intestinal permeability can be a prominent mechanism contributing to IBS and related symptoms. Intestinal paracellular permeability is regulated in part by the most apical epithelial intercellular junctions, the tight junctions (TJs) composed of transmembrane proteins, i.e. claudin or occludin, connected to multiprotein complex such as zonula occludens-1, and -2, linked to actomyosin ring (2), (3). Piche et al., showed an altered epithelial barrier / increased permeability of colonic biopsies from IBS patients compared to healthy controls (HC) and a significantly increased paracellular permeability of Caco-2 cell monolayers incubated with supernatants (SUP) containing soluble mediators released by the intestinal mucosa of IBS patients (4). Recently, alteration of gut microbiota has emerged as an additional potential factor contributing to the altered epithelial barrier / permeability, and thereby to low-grade inflammation / immune activation, in IBS. Changes in the composition of the normal microbiota and abnormal colonic fermentation may play an important role in the development of IBS symptoms (5). In this line, IBS occurs more frequently after intestinal infection or antibiotic treatment. Thus, manipulation of gut microbiota by probiotics appears to be a treatment modality for IBS (6).

Probiotics are live microorganisms that confer a health benefit to the host (7). They are able to resist to chemical insults and can compete with other enteric

microorganisms for survival (1). Probiotics exerts several effects including binding to the intestinal epithelial cells (IECs), inhibition of pathogenic bacteria, acidification of the colon by nutrient fermentation and suppression of the growth of pathogens by secreting antimicrobial. Lactobacilli and Bifidobacteria are natural components of the colonic microbiota and they have been shown to exhibit beneficial effects in IBS clinical trials (8), (9), (10), (11). Even if the great variety of species, strains and doses of probiotics make difficult any conclusion about the optimum probiotic strategy to be used in IBS (12) and further good-quality randomized clinical trials are required, data obtained so far indicate that probiotic therapy is a promising strategy to treat IBS. Lactobacillus reuteri (*L. reuteri*) is a heterofermentative bacterium considered to be one of the few true autochthonous Lactobacillus species in humans (13). Evidence has shown that *L. reuteri* is effective in the infantile colic, alleviation of eczema, and suppression of H. pylori infection (14), (15), (16), (17). However, only few double blind, placebo controlled randomized studies showed the effects of *L. reuteri* in patients with IBS (18), (19).

The aim of this study was to test the protective effect of a specific strain of *L. reuteri* on colonic permeability in Caco-2 cells incubated with a proteolytic buffer mimicking the mean effect of IBS SUP.

MATERIALS AND METHODS

Experimental design

In this study, we assessed the protective role of the *L. reuteri* on the intestinal paracellular permeability using an in vitro model relevant to IBS. In order to mimic intestinal epithelial barrier (IEB) dysfunction, a finding detectable in IBS patients, Caco-2 monolayers were exposed to a protease activated receptor (PAR)-2 agonist (SLIGRL) pre-treated with *L. reuteri* to verify whether the probiotic was able to prevent SLIGRL-induced IEB abnormalities. Preliminary experiments were conducted to determine the concentration and the incubation time of SLIGRL for the treatment of Caco-2 monolayers. Two concentrations (25 and 100 μM) of SLIGRL were used at 6 hours and 24 hours. Only SLIGRL 100 μM applied to Caco-2 monolayers for 24 hours significantly increased paracellular permeability and reduced monolayer transepithelial resistance (TER). Secondly, a pH dose-response assay (10^6, 10^7 and 10^8 cells 100 μl^{-1}) was performed to determine the optimal concentrations of bacteria to be used in the present study. Two different concentrations of *L. reuteri* (10^6 and 10^7 cells 100 μl^{-1}) were tested. The concentration of 10^7 cells 100 μl^{-1}, with a higher effect on TER and paracellular permeability, was chosen to test the protective role of probiotic. In addition, the effect of three different probiotic forms was investigated: live bacteria (Lr 10^7), heat inactivated bacteria (Lr 10^7 inact) or SUP from bacterial culture (Lr Sup 10^7). The aim of the work was to verify whether the pre-treatment of Caco-2 cells monolayers with *L. reuteri* was able to prevent IEB lesions induced by SLIGRL or SUP from biopsies of IBS patients, exploring the probiotic effect on the expression of TJ protein components, such as ZO-1, and on the induction of cytokines secretion (IL-8) by Caco-2 cell monolayers.

Cell culture

Human IEC line Caco-2 were plated onto porous filters (12-well Transwell Clear, 0.40 mm porosity, 1.1 cm of diameter; Corning, ATGC, Marne la Valle´e, France), at a density of 200 000 cells/filter and cultured in Dulbecco's modified Eagle's medium (DMEM) (4.5 g l^{-1} glucose; Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Abcys, Paris, France), 2 mmol l^{-1} glutamine (Invitrogen) and 50 IU ml^{-1} penicillin, and 50 mg ml^{-1} streptomycin (Invitrogen) or DMEM GlutaMaxTM-I (4.5 g l^{-1} glucose, pyruvate; Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Abcys, Paris, France) and 50 IU ml^{-1} penicillin. The culture medium was changed every 2 days. All cells were maintained in incubators (95% air, 5% CO2) at 37°C. Polarised and differentiated monolayer formed by Caco-2 cells was used after 14 days of culture.

Bacterial strain preparation

The probiotic strain, *Lactobacillus reuteri* DSM 17938 (Biogaia, Stockholm, Sweden) was cultured in Man, Rogosa and Sharpe medium (MRS) (Biokar Diagnostics, Beauvais, France). Stock cultures were maintained in cryobeads (Cryogene), stored at -80°C. For all experiments, the pre-culture consisted of 10 mL of growing medium inoculated with 2.14 X 10^8 CFU g^{-1}. Purity of pre-culture was assessed by microscopic observation of methylene blue (Réactifs Ral, Martillac, France) stained slides as well as plate spreads. The optical density measured at λ580 nm has been used to calculate the adequate volume of culture to centrifuge. Bacteria have been harvested by centrifuging an appropriate volume of 10^8 bacteria ml^{-1} (3000 g, at room temperature for 15 minutes). The cell pellet was washed in phosphate buffered saline

(PBS) and re-suspended in 3 ml of Caco-2 cells medium (DMEM-serum and antibiotics free). After 15 days, confluent wells of Caco-2 cells were incubated for 8 hours with *L. reuteri* suspension. Each experimental condition was performed at least in duplicate. Inactivated bacteria were prepared by heating *L. reuteri* to 100℃ for 20 minutes prior washing in PBS and re-suspension in DMEM serum and antibiotics-free; inactivation was confirmed by overnight culture in appropriate culture broths and on MRS agar plates. Bacterial SUP were prepared by washing a volume of 10^7 bacteria 100 μl^{-1}. The cell pellet was washed in phosphate buffered saline (PBS) and re-suspended in 5 ml of Caco-2 cells medium (DMEM-antibiotic free). The suspension was incubated for 13 hours and then centrifuged at 2000g for 15 minutes at room temperature; the SUP was filtered and used to incubate 15 day-confluent wells of Caco-2 cells for 8 hours.

Determination of TER and paracellular permeability

Caco-2 monolayer TER was measured with an epithelial volt–ohm meter (WPI, Stevenage, UK) before and after 8 hours of incubation with *L. reuteri* and after 24 hours of incubation with PAR-2 agonist (SLIGRL). Results (in ohms) were presented as differences compared to basal values. Paracellular permeability studies were performed using the sulfonic acid flux through Caco-2 monolayer. After 8 hours of incubation, 50 µl of sulfonic acid were added to the apical compartment. Basolateral aliquots were taken after 30 minutes and the fluorescence level was measured every 30 minutes in a 96-well fluorescent plate reader using a fluorimeter at λ496 nm (Varioskan, Thermo Scientific, Waltham, Massachusetts, United States).

ELISA

The concentration of IL-8 was quantified in SUP of Caco-2 cell cultures with the BD OptEIA human IL-8 ELISA kit, according to the manufacturer's instructions (BD OptEIA ELISA Set, BD Biosciences, Franklin Lakes, New Jersey, United States). Absorbance measurements were performed at λ450 nm on a spectrophotometric enzyme-linked immunosorbent sandwich assay (ELISA) plate reader (Varioskan, Thermo Scientific, Waltham, Massachusetts, United States) using the SkanIt software (Thermo Scientific).

Western blot analyses for ZO-1

Caco-2 cells were lysed in buffer containing 0.5 M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA, (Merck Millipore, Molsheim, France), 2 mM orthovanadate, phosphatase inhibitor cocktail II (Roche, Neuilly sur Seine, France) and a protease inhibitors cocktail (Roche). BCA Protein Assay Kit (Thermo Scientific, Villebon sur Yvette, France) was used to quantify total protein concentration then measured at λ562 nm using a standard spectrophotometer or plate reader (Varioskan, Thermo Scientific, Waltham, Massachusetts, United States). Proteins were separated using the Invitrogen NuPage Novex Bis Tris MiniGels before electrophoretic transfer with the iBlot Dry Blotting System also from Invitrogen. Membranes were blocked for 1 hour at 25 °C in Tris-buffered saline (TBS) (100 mM NaCl, 10 mM Tris, pH 7.5) with 5% non-fat dry milk. Membranes were incubated overnight at 4°C with the primary antibodies for mouse monoclonal anti-ZO-1 (1:500, Invitrogen). Bound antibodies were detected with horseradish peroxidase-conjugated anti-mouse antibodies (1:5000, Amersham, Les Ulis, France) and visualized by

enhanced chemiluminescent detection (ECL plus, Amersham). To allow comparison between different autoradiographic films, the density of the bands was expressed as a percentage of the average of controls (untreated). The value of ZO-1 protein was normalized to the amount of β -actin immunoreactivity in the same sample and expressed as a percentage of controls.

Patient recruitment

Colonic mucosal biopsies from n=13 IBS patients (4 men; mean age 48.6±14.7) with Rome III criteria (IBS-C, IBS-D, IBS-M) and n=10 HC (7 men; mean age 39.8±9.6), explored for colonic cancer screening, were obtained at the Gastroenterology department of the Hotel Dieu Hospital (Nantes, France). Biopsies were performed using standard forceps in the left colon, between 30 and 40 cm from anal marge. Before performing endoscopy IBS patients and controls gave their informed consent previously validated by the local ethical committee (no. DC-2008-402).

Supernatants collection

Biopsies (n=4) were rapidly placed in 1 ml of Krebs Hepes solution (120 g mol^{-1} of NaH2PO42H2O; 58.44 g mol^{-1} of NaCl; 74.55 g mol^{-1} of KCl; 238.3 g mol^{-1} of Hepes; 180.2 g mol^{-1} of glucose; 147 g mol^{-1} of CaCl2.2H2O; 203.3 g mol^{-1} of MgCl2.6H2O) at 37°C. After 25 min of incubation, the solution was removed and filtered with centrifuge tube filters (0.22 mm, SPIN-X; Corning, New York, USA) 1 min at 11000 g to remove bacterial components. Aliquots of 300 ml of SUP were stored at -80°C until assays.

Statistical analysis

Data resulted from independent experiments performed in duplicate. Statistical significance was evaluated using GraphPad Prism software (GraphPad Software Inc., La Jolla, California, United States). For time and dose-dependent experiments, a two-way ANOVA test followed by a Bonferroni post-hoc test was used. The differences between groups were calculated by a two-tailed Students t-test for nonparametric and unpaired data or by Mann-Whitney U test.

RESULTS

Effects of live, heat inactivated *L. reuteri* or bacterial supernatant upon SLIGRL-induced IEB dysfunction

Treatment of Caco-2 cell monolayers with SLIGRL 100 μM (24 hours) significantly increased paracellular permeability and decreased TER. The pre-incubation with *L. reuteri* $1x10^7$ (Lr 10^7) but not $1x10^6$ (Lr 10^6) prevented the increase of paracellular permeability induced by SLIGRL (Figure 1A). The pre-incubation with Lr 10^7 significantly reversed the decrease of TER induced by SLIGRL treatment (Figure 1B). The pre-incubation with heat inactivated bacteria (Lr 10^7 inact) (Figure 1C) but not with *L. reuteri* SUP prevented the effect of SLIGRL upon paracellular permeability (Lr Sup 10^7), compared to control (Figure 1D).

Effects of live *L. reuteri* upon SLIGRL-activated signalling pathways in intestinal epithelial cells and tight junction expression

The treatment with SLIGRL 100 μM (24 hours) significantly reduced the expression of ZO-1 protein (Figure 2A). Pre-incubation with Lr 10^7 reversed the effect of SLIGRL treatment (Figure 2B).

Effects of *L. reuteri* upon SLIGRL activated signalling pathways in intestinal epithelial cells IL-8 release

The treatment with SLIGRL 100 μM (24 hours) significantly increased the IL-8 release from Caco-2 monolayer cultures (Figure 3A). The increase in IL-8 levels was

prevented by the pre-incubation of Caco-2 monolayers with Lr 10^7 live (Figure 3B) or Lr 10^7 inact bacteria (Figure 3C), but not by the *L. reuteri* SUP (Lr Sup 10^7) (Figure 3D).

Effects of live *L. reuteri* upon intestinal epithelial cells treated with IBS and HC supernatants

Paracellular permeability of Caco-2 cell monolayers treated for 24 hours with SUP of IBS patients was significantly increased compared to the HC-treated Caco-2 monolayers. However, the pre-incubation with Lr 10^7 did not prevent the effect of IBS SUP (Figure 4).

Discussion

This study shows that the pre-incubation of Caco-2 cell monolayers with live or heat-inactivated *L. reuteri* prevented SLIGRL-induced increase in paracellular permeability. We showed that the treatment of Caco-2 cells with SLIGRL reduced ZO-1 expression and that this effect was prevented by *L. reuteri*. The pre-incubation of Caco-2 cells with live or heat inactivated *L. reuteri* prevented the increase of IL-8 levels induced by SLIGRL. Finally, live bacteria were not able to prevent IBS SUP-induced increase in paracellular permeability.

The intestinal epithelial barrier protects from contact with the external environment. Thus, any noxae affecting the epithelial integrity can cause exposure to luminal agents responsible for a number of processes, including immune activation / inflammatory response, which contribute to IBS pathophysiology and symptoms generation (20). In a tight interplay with the epithelial barrier function is the gut microbiota, i.e. the myriad of bacteria and other germs normally resident in the gut lumen, mainly in the colon (21), (22), (23), (24). Changes of the gut microbiota can occur in several conditions (e.g. obesity), including digestive diseases such as inflammatory bowel diseases and functional bowel disorders, i.e. IBS (25), (26), (27).

Receptors of proteases such as PAR-2 are expressed on both apical and basolateral site of enterocytes and their activation increases paracellular permeability through the myosin light chain kinase activation, which, in turn, evoke opening of TJs (28). PAR-2 is activated by mast cell mediators, e.g. tryptases, trypsin and trypsin-like protein by proteolysis. PAR-activating peptides, such as SLIGRL for PAR-2 (corresponding to the amino-acids sequence of tethered ligand), are synthetic peptides able to selectively activate PAR (29). In our study, we applied SLIGRL to Caco-2

monolayers in order to mimic the IEB exposed to noxious agents. This situation was thought reminiscent to that occurring in patients with IBS. In fact, several data showed that the high level of luminal proteases found in colonic contents of IBS patients was able to activate PAR-2 leading to an increase in paracellular permeability (28), (30). Indeed, a selective increase of colonic serine-proteases was found in SUP from stools of IBS-D patients (31). Very interestingly, the effect mediated by SLIGRL on the IEB dysfunction, inflammatory mediators, TER and permeability were reverted by *L. reuteri*.

Our results showed that protease-induced barrier disruption was prevented by *L. reuteri* modulation of TJ proteins, a finding in accordance to Enjoji et al. who demonstrated that the activation of PAR-2 from the basolateral side of epithelial cells leads to the disruption of TJs (32). Commensal bacteria and probiotics have been shown to promote intestinal barrier integrity by preventing myosin phosphorylation mediated by myosin light chain kinase both in vitro and in vivo. Eun et al. demonstrated that *Lactobacillus casei* pre-treatment of IECs reversed the TNF-α and interferon (IFN)-γ-induced epithelial barrier dysfunction, such as an increase of epithelial permeability accompanied by a decrease of TER and ZO-1 expression (33). According with our results, Yang et al. recently showed that *L. reuteri* (I5007) modulated TJ protein expression after LPS stimulation in IECs in vitro and in newborn piglets under normal condition in vivo (34).

Pre-incubation of Caco-2 with live or heat inactivated *L. reuteri* but not bacteria SUP prevented SLIGRL-induced increase in basolateral IL-8 levels. The fact that live or heat-inactivated bacteria have an effect on the paracellular permeability modulation and IL-8 production suggests that, independently of the bacterial viability,

components of bacterial membrane could be responsible of the action of *L. reuteri* on the intestinal immunomodulation. Livingston et al. showed that heat-inactivated *L. reuteri* (100-23) induced the production of anti-inflammatory cytokine IL-10 by bone derived dendritic cells, thus supporting that, *L. reuteri* also regulated the development and recruitment of regulatory T cells to the GI epithelium (35). Ma et al. showed that live *L. reuteri* in contact with the epithelial cells inhibited mRNA up-regulation, cellular accumulation, and secretion of IL-8 induced by TNF-α (36). Our results showed that, in order to prevent SLIGRL-induced increase in basolateral IL-8 levels, we had to incubate Caco-2 monolayer cells with live or heat inactivated *L. reuteri*. These findings are in agreement with the Ma's et al. work and Rachmilewitz's et al. study who reported that the anti-inflammatory effects of *L. reuteri* in a murine model of colitis depend on the action of bacterial DNA on toll-like receptor 9 and that live microorganisms are not required to mediate these effects (37).

Finally, we showed that paracellular permeability of Caco-2 cells treated for 24 hours with SUP of IBS patients was significantly increased compared to those treated with SUP of healthy subjects, although in this setting the pre-incubation with Lr 10^7 did not prevent the damaging effect mediated by IBS SUP. The effects of IBS SUP on paracellular permeability of Caco-2 cell monolayers are in agreement with those described by Piche et al. who observed that, soluble mediators released from colonic biopsies of IBS patients induced a significant increase in the paracellular permeability of Caco-2 cells (4). Nébot-Vivinus et al. showed that a probiotic combination inhibited the increased permeability induced in T84 confluent monolayers by IBS colonic biopsy conditioned medium (38). In our study, it is likely that *L. reuteri* concentration was not adequate for protecting the epithelial barrier

function; another possibility is that other mediators than PAR-2 concur to the lack of effect of *L. reuteri* in preventing the damaging effect mediated by IBS SUP.

In conclusion, our study demonstrates that *L. reuteri* is able to prevent barrier dysfunction induced by PAR-2 agonists in part via a mechanism involving modulation of TJ protein expression. We provided an experimental basis showing that *L. reuteri* can be effective in protecting the IEB from a number of luminal (related or unrelated to gut microbiota changes) that may contribute to symptom generation in IBS. Studies are now eagerly awaited to demonstrate *L. reuteri* efficacy in ad hoc designed clinical trials in IBS patients.

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Figure legends

Figure 1

Effects of *L. reuteri* or bacterial factors involved (ie live or dead bacteria) upon protease-induced barrier dysfunctions. (A) Incubation of Caco-2 monolayers with two different concentrations of *L. reuteri* (1 X 10^6 or 1 X 10^7 bacterial cells 100 μl^{-1}) in absence (- SLIGRL) or presence (+ SLIGRL) of PAR-2 agonist (SLIGRL 100 μM) (n=7). SLIGRL 100 μM significantly increases paracellular permeability in Caco-2 cells monolayers treated for 24 hours (CTL). The pre-incubation of monolayers with *L. reuteri* 10^7 (Lr 10^7) but not 10^6 (Lr 10^6) bacterial cells 100 μl^{-1} prevents SLIGRL-induced increase in paracellular permeability. (B) SLIGRL 100 μM significantly decreases TER in Caco-2 cells monolayers treated for 24 hours (CTL). The pre-incubation of monolayers with *L. reuteri* 10^7 (Lr 10^7) bacterial cells 100 μl^{-1} (but not *L. reuteri* 10^6, data not shown) prevents SLIGRL-induced decrease in TER. (C) Pre-incubation of Caco-2 monolayers with heat inactivated bacteria (Lr 10^7 inact) (n=6) (D) but not with *L. reuteri* SUP (Lr Sup 10^7) (n=5) (E) significantly reversed changes in paracellulalar permeability induced by SLIGRL. Mean ± SEM; *$p<0.05$.

Figure 2

Representative western blot of ZO-1 and β-actin (loading control) proteins from Caco-2 cell monolayers treated with SLIGRL (100 μM) for 24 h after pre-incubation with *L. reuteri* 1 X 10^7 100 μl^{-1} (Lr 10^7). (A) The treatment with SLIGRL 100 μM significantly reduces the expression of ZO-1. Pre-incubation with Lr 10^7 prevents the effect of SLIGRL upon ZO-1 protein expression. (B) Quantitative analyse of ZO-1

protein expression in absence (- *L. reuteri*) or presence (+ *L. reuteri*) of pre-incubation with Lr 10^7 expressed as the ratio of ZO-1 to β-actin (n=5). Mean ± SEM; p<0,05.

Figure 3

Effects of *L. reuteri* upon PAR-2 activated signalling pathways in IECs IL-8 release. The basolateral culture medium of Caco-2 cell monolayers pre-treated or not (A) (CTL) (n=7) with (B) live (L r 10^7) (n=7), (C) inactivated bacteria (Lr 10^7 inact) (n=7) or (D) *L. reuteri* SUP (Lr Sup 10^7) (n=5) were collected after incubation with SLIGRL (100μM) for 24 hours and IL-8 secretion was determined by ELISA. SLIGRL significantly increases IL-8 levels (A). Pre-incubation of Caco-2 cells with Lr 10^7 or Lr 10^7 inact bacteria but not Lr Sup 10^7, prevented SLIGRL-induced increase in basolateral IL-8 levels. Mean ± SEM; p<0,05.

Figure 4

Effects of *L. reuteri* upon Caco-2 cell monolayers treated with IBS (n=13) and HC (n=10) SUP in the absence (- *L. reuteri*) or presence (+ *L. reuteri*) of bacterial pre-incubation for 8 hours. The pre-incubation of monolayers with *L. reuteri* 10^7 (Lr 10^7) bacterial cells 100 μl^{-1} did not prevent IBS SUP-induced increase in paracellular permeability.

Figures

Figure 1

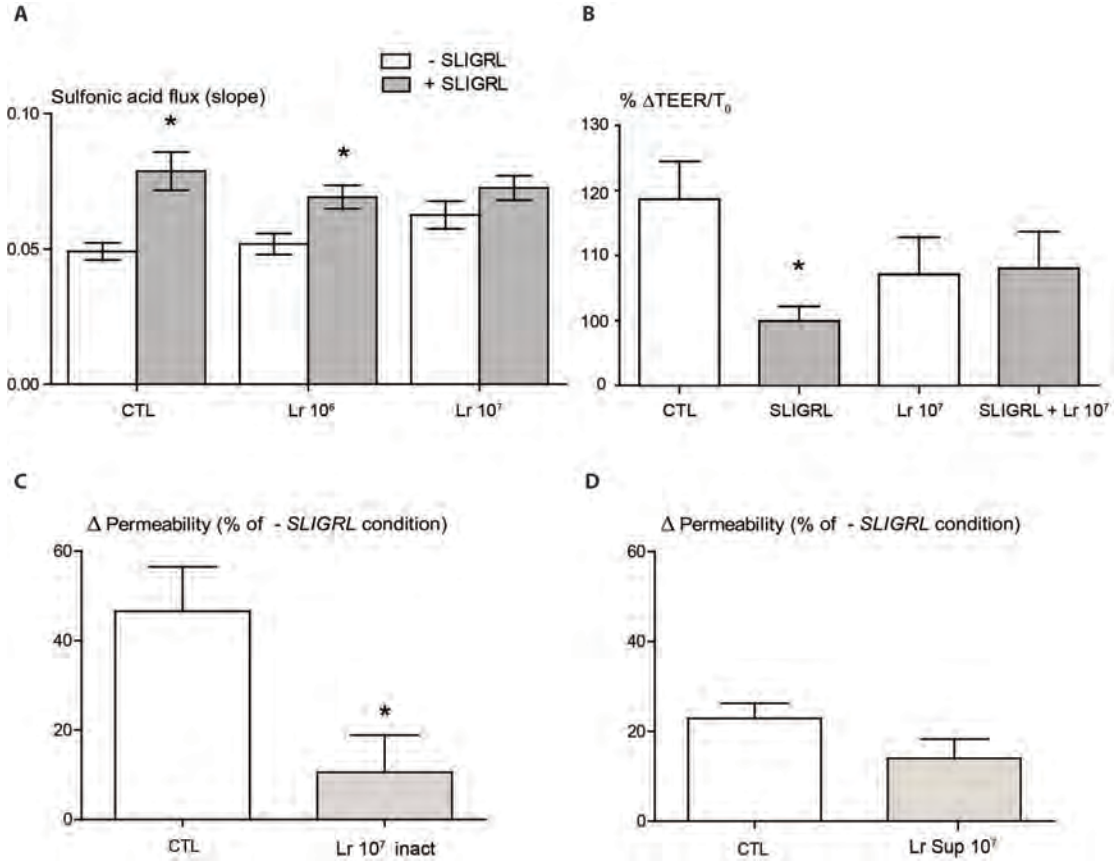

Figure 2

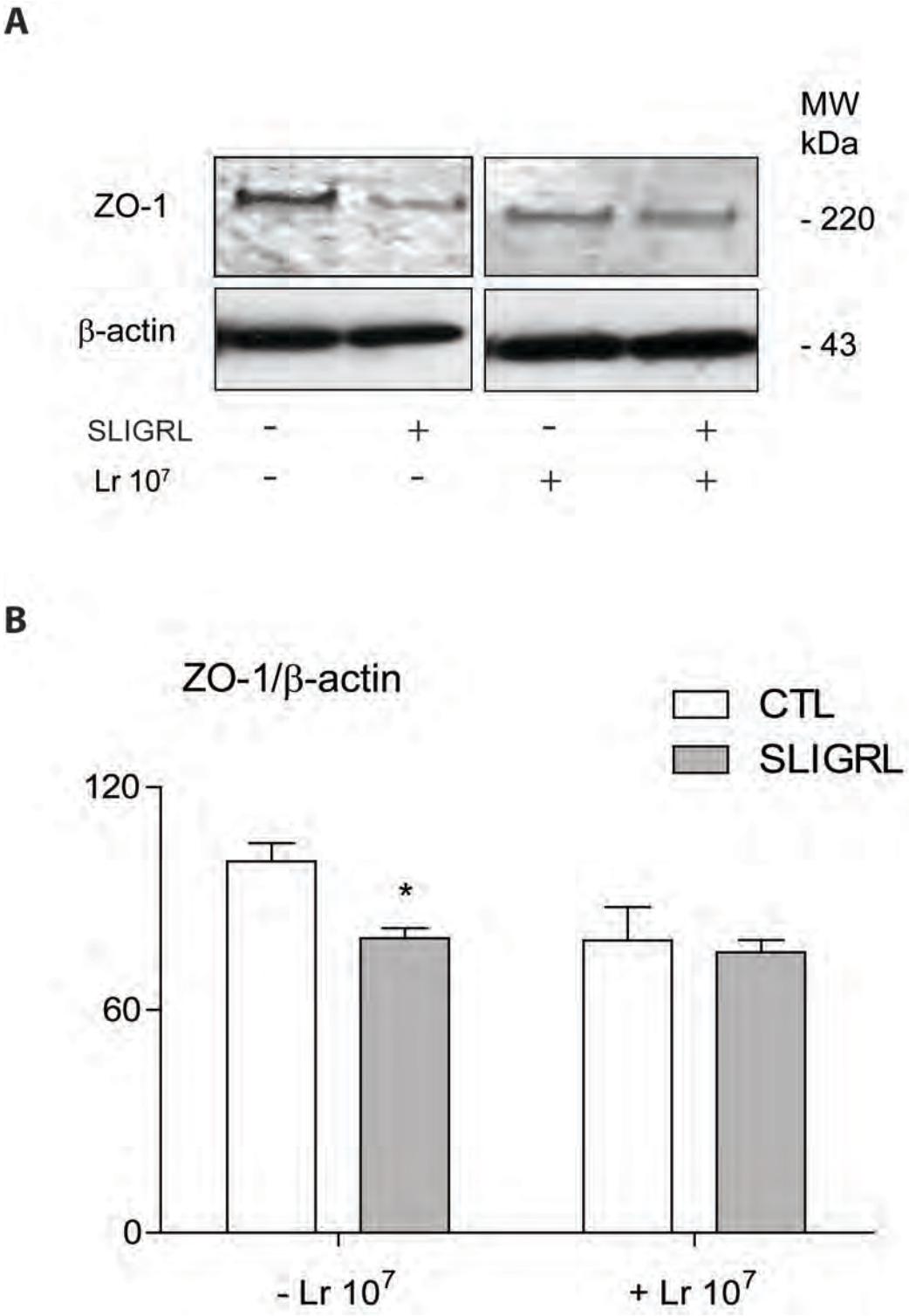
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Figure 3

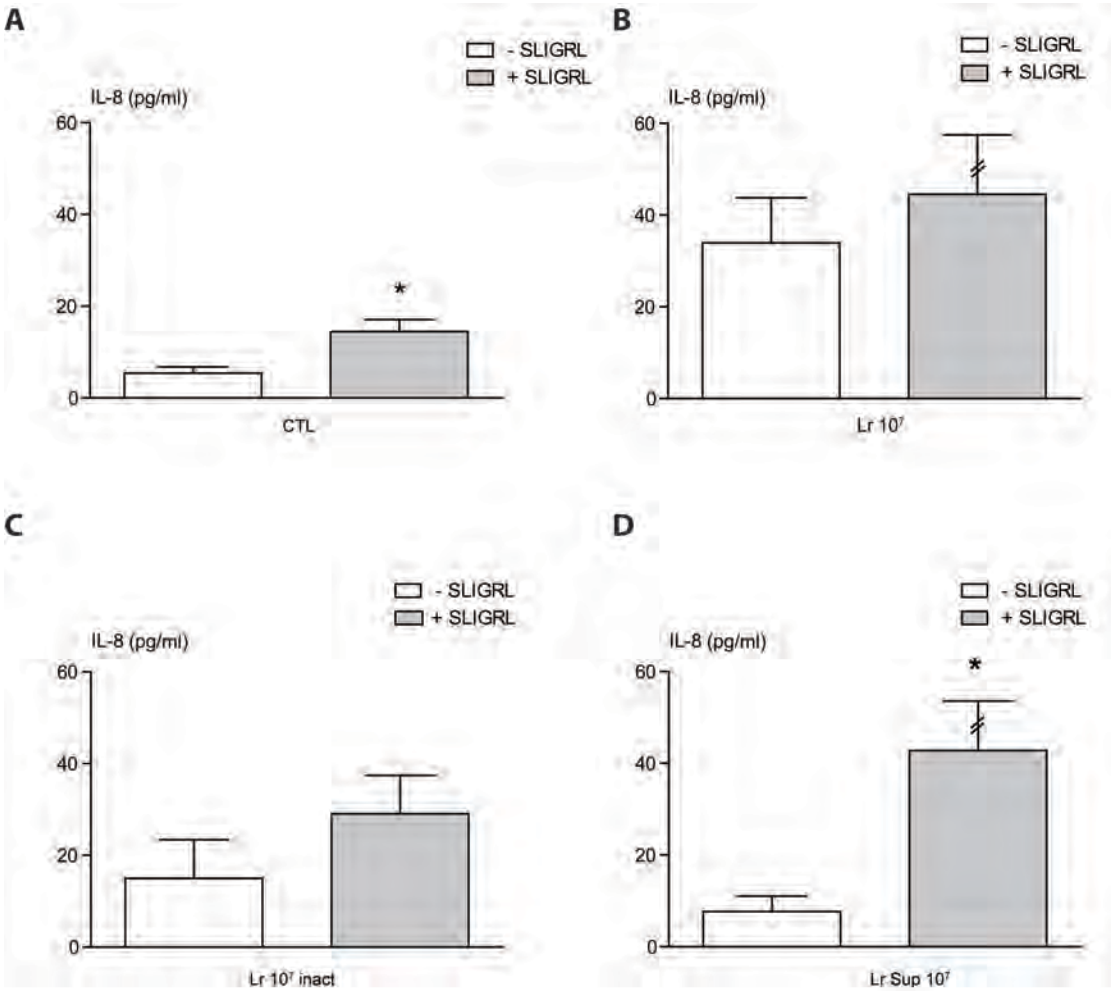
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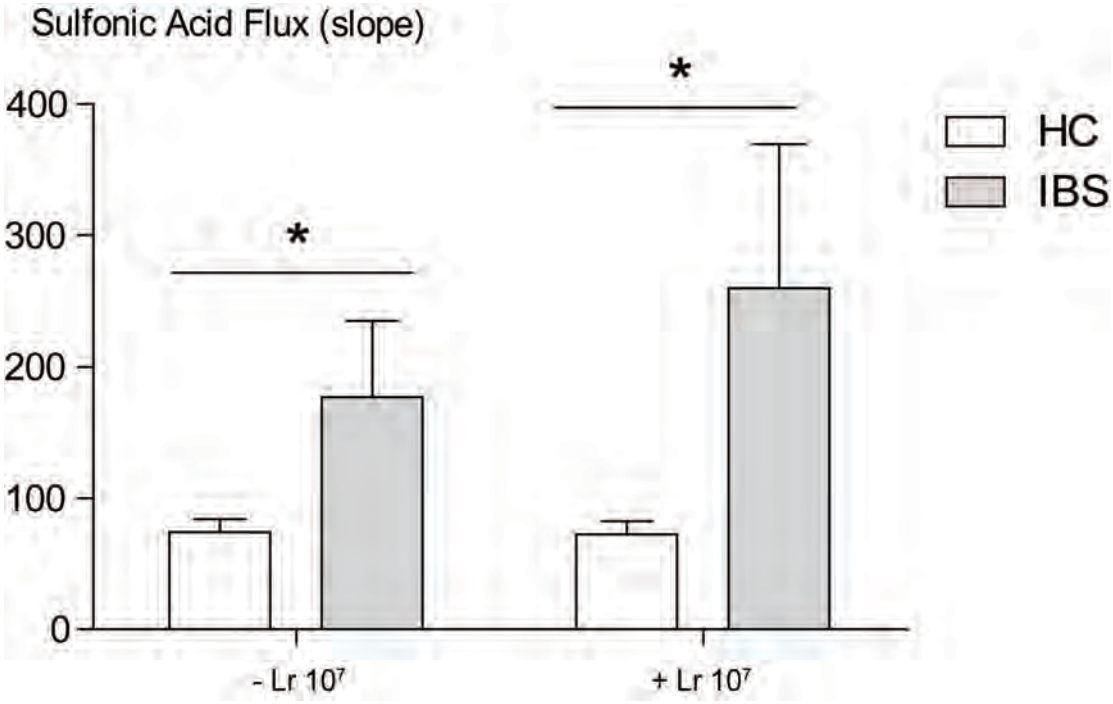
Figure 4



Paper 2: Glio-plastic changes in irritable bowel syndrome

INTRODUCTION PAPER 2

The central role of the enteric nervous system (ENS) during IBS has been highlighted by recent studies showing neuroplastic changes in patients (Dothel et al., 2015; Akbar et al., 2008; Wang et al., 2004). To support the involvement of the ENS in the pathophysiology of IBS, the effect of supernatants from colonic biopsies of IBS patients was observed not only on intestinal permeability but also on the excitability of enteric neurons (Buhner et al., 2012). Enteric glial cells (EGC) support the neuronal component of the ENS (Rühl 2005; Aubé et al., 2006; Fletcher et al., 2002; Zhou et al., 2000) and regulate several digestive functions, such as motility and intestinal permeability. Although an alteration of these functions is reported during IBS, no data about glioplastic changes are currently available, knowing that loss of EGC is associated with intestinal dysmotility both in human and in animal models of glia ablation. The communication between EGC and neurons through adenosine triphosphate (ATP)-dependent P2Y4/P2Y1 pathways actives the glial network via connexin-43 dependent interactions. Interestingly, a targeted alteration of connexin-43 expression is associated with a reduction of intestinal motility.

In this context, the aim of this study was to characterize putative alterations in EGC in IBS and to identify the soluble factor involved in these changes.

To this end, we characterized EGC phenotype in a bio-collection of colonic biopsies from IBS patients and healthy controls (HC) and determined the effect of IBS biopsies' supernatant (SUP) EGC phenotype and function. Finally we identified soluble factor responsible for EGC alterations.

Results

We showed that the EGC network was altered in colonic biopsies from IBS patients. In addition, SUP from IBS patients significantly reduced the proliferation and the amplitude of Ca^{2+} response induced by ATP. Finally, we found that histamine was responsible for the changes induced by SUP via activation of H1 receptor (H1R).

Our results show functional changes of EGC in part involved in the pathophysiology of IBS and a pivotal role of histamine as likely soluble factor implicated.

PAPER 2

Glioplastic changes in irritable bowel syndrome

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Abbreviations used in this paper:

ATP, adenosine triphosphate; **DMEM,** Dulbecco's modified Eagle medium; **EGC,** enteric glial cells; **ENS,** enteric nervous system, **HC,** healthy control; **H1R,** histamine H1 receptor; **IBS,** irritable bowel syndrome; **IBS-C,** constipation-predominant IBS; **IBS-D,** diarrhoea-predominant IBS; **IBS-M,** mixed-IBS; **IEB,** intestinal epithelial barrier; **IR,** immunoreactive; **qPCR,** quantitative PCR; **SUP,** supernatant; **WB**, western blot.

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Results

BACKGROUND & AIMS: Irritable bowel syndrome (IBS) is a functional intestinal disease characterized by a remodeling of cellular components of the mucosal microenvironment such as immune cells or enteric neurons. However, whether changes in enteric glial cells (EGC) occur in IBS is unknown. We investigated glioplastic changes and factors involved during IBS.

METHODS: Mucosal biopsies of IBS (13 IBS-C; 10 IBS-D; 11 IBS-M) and asymptomatic healthy controls (HC; 24) were analyzed. Expression of S100β and GFAP was measured by immunohistochemistry and western blot (WB). Non-transformed EGC cell line was incubated with supernatants (SUP) from mucosal biopsies and their proliferation and electrophysiological properties were analysed by flow cytometry and Ca^{2+} imaging. In addition, immunohistochemical and pharmacological approaches were used to determine the involvement of histamine and H1R in SUP effects upon EGC.

RESULTS: Compared to controls, mucosal area immunoreactive (IR) for S100β was significantly reduced in biopsies of IBS patients, independently of the subtype of IBS. SUP of IBS reduced EGC proliferation only for IBS-C. Furthermore, SUP of IBS-D and M reduced ATP induced-Ca^{2+} response in EGC. We showed that EGC expressed H1R and that SUP effects upon ATP-induced Ca^{2+} response in EGC was blocked by pyrilamine and reproduced by histamine, via H1R, but not PAR-2 agonist or serotonin. SUP of IBS reduced mRNA expression of connexin-43.

CONCLUSION: Glioplastic changes occur in IBS and mucosal soluble factors are involved. Histamine, via activation of H1R dependent pathways in EGC, mediate altered glial response to ATP.

Key words: Ca^{2+} response; enteric glial cells; histamine.

Introduction

Irritable bowel syndrome (IBS) is a functional intestinal disease, which is characterized by chronic abdominal pain or discomfort and altered bowel habits with a world prevalence around 11%[1]. Several subtypes of IBS have been defined by the predominant bowel habit disorder: IBS-C for constipation, IBS-D for diarrhea, IBS-M for mixed bowel habits and IBS-U for undetermined IBS[2]. Although precise pathophysiological mechanisms remain still unknown major advances have been achieved over past years enabling a better understanding of symptoms in IBS. In particular, a remodeling of the gut mucosal cellular microenvironment has been reported in IBS, such as the presence of micro-inflammation with an increased amount of mast cells[3], of T cells and the detection of pro inflammatory cytokines by colonic biopsies supernatants (SUP)[3, 4]. In addition, altered intestinal epithelial cells functions, such as increased paracellular permeability[5] and altered enteroendocrine cell density[6] were observed. Among other constituents of the mucosa microenvironment that undergoes important remodeling in IBS is the enteric nervous system (ENS). In particular, recent studies have described neuroplastic changes in ENS such as increased neuronal fiber outgrowth[7], increased density of substance P[8] or vanilloid receptor 1 (TRPV1)[9]. In addition, in IBS, soluble mediators of mucosal biopsies were shown to increase excitability of enteric neurons[10]. Interestingly, neuroplastic changes in the ENS have been shown to be mediated by mediators such as histamine, serotonin, protease or nerve growth factor[11].

Besides enteric neurons, the ENS is also composed of enteric glial cells (EGC) that outnumber them[12]. Although EGC are central regulator of neuronal, intestinal epithelial barrier (IEB) and gut functions, such as motility[13], reported to be altered in IBS[14], currently no data has, to the best of our knowledge, studied putative enteric glio-plastic changes during IBS, especially in patients. Indeed, EGC regulate neuronal processes such as neuronal

survival[15] and neuro-mediator expression[12, 16, 17, 18, 19]. Conversely, enteric neurons can also communicate to EGC, in particular via adenosine triphosphate (ATP) dependent P2Y4/P2Y1 pathways[20, 21]. Activation of EGC by such pathways leads to glio-glio communication via connexin-43 dependent signalling[22]. Interestingly, glial specific ablation of connexin-43 leads to reduce ATP induced glio-glio Ca^{2+}dependent communication and ultimately reduced intestinal motility[23]. Consistently, loss of EGC has also been reported to be associated with intestinal dysmotility both in human and in animal models of glia ablation[24, 25, 26]. Besides controlling neuronal/motor functions, enteric glia also control intestinal barrier function, such as paracellular permeability[16, 27, 28] functions that are also altered in IBS[5].

In this context, the objective of this study was to characterize putative EGC alterations in IBS and to identify mediators involved, in particular produced by the colonic mucosal microenvironment. We therefore aimed at 1) characterizing EGC phenotype in a bio-collection of colonic biopsies from IBS patients and healthy controls (HC), 2) determining whether IBS biopsies' SUP could induce EGC phenotypic and functional alterations, 3) identify soluble factor responsible for these changes.

Materials and methods

Patients and biopsies

Patients referred for colonoscopy, either for colonic cancer screening or digestive symptoms suggestive of IBS, were considered for enrolment in the study. After giving their informed consent according to the guidelines of the French Ethics Committee for Research on Humans and registered under the no. DC-2008-402, they completed a Rome III questionnaire in order to confirm the presence or absence of IBS criteria, and determine the IBS subtype. The inclusion criteria comprehended discomfort or abdominal pain at least 3 days per month during the last three months, discomfort or pain for at least six months and at least 2 criteria on the following 3: improvement of symptoms by the exemption and/or increase in symptoms by altering stool frequency and consistency. If the patient claimed to have hard stools or bead form over 25% of the time he was ranked in the subtype IBS-C; if the stool was loose or watery over 25% of the time he was ranked in the subtype IBS-D and if these 2 types of stools were present over 25% of the time he was ranked in the IBS-M subgroup. The severity and frequency of abdominal pain and bloating were assessed on a scale from 0 to 4, adapted from previous studies on IBS performed in collaboration with our group (unpublished data). HC answered the same questionnaire and should not have special digestive complaints Clinical data were stored in an electronic database (*Integralis*). Biopsies (n=12/patients) were performed using standard biopsy forceps in the left colon, between 30 and 40 cm from anal marge during the colonoscopy. The biopsies were then placed into physiological serum and immediately transferred to the laboratory.

Microdissection and immunohistochemistry

Micro-dissection was performed as previously described[29] in three out of the twelve colonic biopsies taken per patient. The result of dissection consists of mucosa and sub-mucosa obtained from a single biopsy and containing the internal/intermediary submucosal plexus. Mucosa was used to analyse glial network, submucosa to study the histamine H1 receptor (H1R) expression in IBS patients and HC. Each whole-mount preparation was fixed in paraformaldehyde 4% for 3 hours, washed 3 times with PBS 1X and then stored at 4°C for later immunohistochemistry. Specimens of mucosa and sub-mucosa were permeabilized for 3 hours in PBS/NaN3 containing 1% (v/v) Triton X-100 and 10% (v/v) horse serum and then incubated over night with primary antibodies diluted in permeabilization solution. Whole mount preparations were then rinsed 3 times with PBS for 30 min and secondary antibodies diluted in PBS/NaN3 containing 10% (v/v) horse serum were addedfor 3 hours. Primary and secondary antibodies used in all experiments are listed in Table 1.Whole specimens of mucosa and submucosa were viewed under an Axio Zoom.V16 stereomicroscope (Zeiss, Marly Le Roi, France). The percentage of area occupied by EGC in colonic mucosa from IBS patients and HC was quantified as the S100β protein stained area on the area of field of observation. The results are expressed as the mean of three fields observed for biopsy.

Western blot analysis

Two biopsies per patient and cultures of EGC treated with SUP from biopsies or with drugs were recovered in RA1 lysis buffer to separate RNA and proteins. Samples were processed for electrophoresis using the SDS-PAGE buffer kit (InvitrogenSaint-Aubin, France) and separated on 4%-12% BisTris gel (Invitrogen). Proteins were transferred to nitrocellulosa membranes with the iBlot system (Life Thecnologies). After blocking with Tris-bufferedsaline (TBS), 0.1% Tween 20 and 5% nonfat dry milk for one hour, blots were

incubated overnight at 4°C with primary antibodies diluted in TBS and 5% nonfat dry milk for rabbit anti-GFAP (Dako, Les Ulis, France, 1/2000) and rabbit anti-S100β (Dako, 1/500). Immunoblots were probed with the appropriate horseradish peroxidase conjugated secondary antibodies (Life Technologies, Carlsbad, CA) and visualized by chemiluminescence (Clarity Western ECL Substrate; Bio-Rad, Hercules, CA) using a Gel-Doc imager and the Image Lab Software (Bio-Rad). The value of total protein immunoreactivity was normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) immunoreactivity and expressed as a percentage of the average of controls.

Quantitative PCR

RNA from biopsies or cultures of EGC treated with SUP from biopsies or with drugs was isolated using the Nucleo Spin RNA Triprep Kit or Clean up (Macherey-Nagel, Düren, Germany) respectively, according to manufacturer's instructions. Potential genomic DNA contamination was removed by treatment with Turbo™ DNase (Ambion Inc., Austin, TX, USA) and RNA was quantified using an ND-1000 UV-Vis spectrophotometer (NanodropTechnologies, Wilmington, DE, USA). cDNA was synthesized from 1 µg total RNA using the Super Script III Reverse Transcriptase System kit (Invitrogen) and diluted to a final concentration of 8 ng eq RNA/µl. qPCR was performed using StepOne Plus (Life Technologies) detection system with Fast SYBR Green (Life Technologies) master mix. The PCR signal was normalized against S6 as reference gene to control for variability in the amount and quality of the RNA. The sense and anti-sense oligonucleotide primers used in this study are shown in Table 1.

Biopsies SUP production

Four biopsies were rapidly weighted and then immersed in hard plastic tubes containing 1 ml of Krebs Hepes continuously oxygenated (95% O_2/5% CO_2) at 37°C. After 20 min

incubation, the solution was removed and centrifuged at 11000 RCF (relative centrifugal force) for 1 min before being filtered with centrifuge tube filters (0.22 mm, SPIN-X; Corning, New York, USA) to remove bacterial components. Aliquots of 300 ml of SUP were stored at 80°C until assays. Biopsies were blotted and weighed after collecting the SUP. For culture cell experiments, aliquots of SUP were thawed on ice then heated in water bath before SUP was added to culture.

Cell cultures

Embryonic EGC of rat (cell-lineage JUG2 *Inserm U913*) were isolated from ENS primary culture derived from rat embryonic intestine (E15)[30] according to the procedure previously described[31]. Briefly, after 13 days of culture, primary cultures were trypsinized and seeded in serum-containing media after differential centrifugation. Following 7 days of culture, isolated areas of morphological glial cells-like were trypsinized using cloning cylinder and seeded in culture flask in serum-containing media. After 1month, the cells were assessed for glial, neuronal, and myofibroblast markers by immunohistochemistry. They were immunoreactive for GFAP, Sox10, and S-100β, all glial markers, but not for Tuj-III, PGP9.5, neuronal markers, and-smooth muscle actin, a myofibroblast marker.

EGC were cultivated in 25 cm^2 flasks in Dulbecco's modified Eagle medium (DMEM) Glutamax medium (4.5 g/l glucose; Invitrogen) or DMEM medium (4.5 g/l glucose; Invitrogen) supplemented with 10% heat-inactivated foetal calf serum (FCS) (Abcys, Paris, France), 2mmol/l glutamine (Invitrogen) and 50 IU/ml penicillin, and 50 mg/ml streptomycin (Invitrogen). For WB analyses EGC were seeded at a density of 30000 cells/well in 100 µL of medium on a 96 well plate and incubation with 20µl of drugs or SUP was performed at day 1 and day 2.

Flow Cytometry analysis

EGC were seeded at a density of 10000 cells/well in 200 µL of medium on a 96 well plate. Addition of 20µl of SUP of IBS or HC was performed at day 1, 3 and 5. At day 7 cell culture supernatants were recovered and transferred in a 96-well conical bottom plate and then centrifuged for 2 minutes at 2000 x g. EGC were washed with PBS 1X and trypsinized with 0.25% of trypsin EDTA (Life Technologies) for 5 minutes at 37°C. The trypsin reaction was stopped by adding medium containing 10% fetal calf serum and then EGC were recovered and placed in the 96-well conical bottom plate in the place of supernatants previously removed after the first centrifugation. The 96-well conical bottom plate was centrifuged for 2 minutes at 2000 x g and then PBS/EDTA was added after SUP removal. EGC were re-suspended with 10 µg/ml of 7-Aminoactinomycin D (Sigma) and then fluorescent labelling was measured using a FACS LSR II (BD Biosciences, Le Pont de Claix, France) and analysed with BD Facs Diva software.

Calcium flux assays

EGC were seeded at a density of 4000 cells/well in 200 µL of medium on a 96 well plate. The incubation of EGC culture with 20µl of SUP or drug/antagonists was performed at day 1 and day 2. After 48h of incubation, EGC were washed with PBS and incubated at 37°C in fresh warmed HBSS containing 0.5 µM fluo-4 AM (wavelength of absorption: 494 nm; wavelength of emission: 516 nm) for 60 min. After washing with HBSS, 96 well plate was placed under Olympus IX 50 inverted microscope with a Olympus DP 50 digital imaging system and Ca^{2+} flux analysis was performed for every well at a time after the addition of ATP (final concentration of 100 µM). For each well a sufficiently dense field of cells was selected to analyse Ca^{2+} flux of 30 cells. The acquisition was fixed with an exposure time of 1 second movies were recorded for 120 sec (25 images/sec) using Cell B software.

Ca^{2+} imaging analyse

Movies were converted in a sequence of images (JPEG format) with VirtualDub software and analysed using the ImageJ software with which the outline of thirty individualized cells was traced. Change in fluorescence (F) (calculated as [$\Delta F/F = [F_{MAX}-F_{MIN}]/F_{MAX}$), the area under the curve (AUC) and the fluorescence duration at 50% of maximal amplitude were estimated. All values were expressed as a percentage of the average of control wells for each separate experiment.

Immunohistochemicalstudies

EGC were seeded at a density of 4000 cells/well in 200 µL of medium on a 96 well plate and cultured during 48h. EGC were subsequently fixed in paraformaldehyde 4% for 10 minutes washed 3 times with PBS, incubated 30 minutes with PBS/NaN3 containing 10% (v/v) horse serum and, subsequently, 1 hour with primary antibodies. EGC were rinsed 3 times with PBS for 5 min and secondary antibodies diluted in PBS/NaN3 containing 10% (v/v) horse serum were added for 30 minutes. EGC were subsequently re-fixed in paraformaldehyde 4% (as listed above) and permeabilized for 1 hour in PBS/NaN3 containing 1% (v/v) Triton X-100 and 10% (v/v) horse serum and then incubated over night with primary antibody. EGC were rinsed 3 times with PBS for 5 min and secondary antibodies diluted in PBS/NaN3 containing 10% (v/v) horse serum were added for 30 minutes. Nuclei were stained with 4-6-diamidino-2-phenylindole (*Dapi*) (1:5000, Sigma). Primary and secondary antibodies used in all experiments are listed in Table 1. Fluorescently-labeled EGC were viewed under an Axio Zoom.V16 stereomicroscope (Zeiss, Marly Le Roi, France).

Pharmacological studies

All drugs were diluted and reconstituted in culture medium. PAR-2 agonist (SLIGRL; Biochem) was used at final concentrations from 100 µM to 10^{-8} µM. Histamine (Sigma) and Serotonin (Sigma) were used at final concentrations of 10^{-2}, 1 and 100µM. *Pyrilammine* (Sigma) were used at final concentration of 1 µM.

Statistical analysis

All graphs and statistical significance evaluations were performed using GraphPad Prism Software (GraphPad Software, Inc., La Jolla, California, United States). A Fisher's exact was used to analyse clinical variables expressed in percentage. Differences between groups were calculated by a two-tailed Student's t-test for nonparametric and unpaired data or Mann-Whitney U test and Kruskal-Wallis nonparametric ANOVA test followed by Dunn's *post-test*. For the H1R antagonist effect experiments, a two-way ANOVA test followed by a Bonferroni *post-hoc* test was used. The relation between quantitative variables and clinical data was calculated using a Pearson's correlation coefficient test. Values of $p \leq 0.05$ were considered statistically significant.

Results

Clinical data

From December 2013 to March 2015, 34 patients with IBS and 24 healthy subjects were included and underwent colonoscopy with biopsies. Patients were mostly women (73.5 %) with a mean age of 51 years for the IBS patients and 45.1 years for the healthy controls (HC). The clinical characteristics of the patients according to different IBS-subtypes are described in Table 3. While more patients had recent depression in IBS-M group (50%) as compared to controls (0%), no difference was noted with other groups. All IBS patients reported a higher intensity and frequency of abdominal pain as compared to HC, without any difference between subtypes.

Characterization of enteric glial network in colonic biopsies from healthy controls and IBS patients

The glial network was identified in the mucosa from biopsies of HC and IBS patients with immunohistochemistry using an antibody against S100β protein. The area occupied by EGC was different in biopsies of IBS patients compared with HC (Figure 1A). Indeed, quantitative analysis showed that the S100β staining area was significantly reduced by 27% in biopsies from IBS patients as compared to HC (Figure 1B). Thus, a similar reduction in S100β staining was showed in all subtype of IBS patients (Figure 1C). To verify that the difference observed depended on the change of glial intensity and not on the modification of the mucosal architecture, the number of crypts for field of observation was counted and no difference between IBS patients and HC was detected (data not shown). Interestingly, negative correlations were found between the percentage of S100β stained area and the frequency of

pain or the intensity of pain and bloating (Table 4). We next showed that no difference in the expression of S100β was detected in WB analysis (Figure 1D). Similarly, no changes in GFAP expression could be found (Figure 1E).

Effect of SUP from mucosal colonic biopsies on phenotype and function of EGC

To determine whether biopsies produced soluble factors are able to induce modification of EGC function and phenotype, we first investigated the effect of SUP from biopsies on EGC proliferation. Following treatment of EGC cultures (6 days) with SUP from IBS patients and HC, EGC cell number was evaluated (Figure 2A). The number of EGC was significantly reduced by 9% in cultures treated with SUP from biopsies of IBS-C as compared to control. In contrast, no difference was found in the number of EGC cultured with SUP of IBS-D and IBS-M patients (Figure 2B). Interestingly, a negative correlation between the number of EGC cultured with SUP from biopsies of IBS patients and HC and the frequency of pain was found (Table 4). Treatment of EGC with SUP of IBS did not change the expression of S100β or GFAP proteins (Figure 2C, D).

Effects of supernatant from mucosal colonic biopsies on Ca^{2+} response to ATP of EGC

We next determined whether incubation of EGC (48h) with SUP from biopsies of IBS could modify its electrophysiological properties, and in particular its ATP-induced Ca^{2+} response. The amplitude of Ca^{2+} response induced by ATP was significantly decreased as compared to control (Figure 3A, B). These changes in ATP induced Ca^{2+} response were dependent on IBS subtype. Indeed, the mean amplitude of Ca^{2+}flux was significantly reduced in EGC cultured

with SUP from IBS-D and IBS-M as compared to control but not in IBS-C (Figure 3B). Interestingly, ATP induced Ca^{2+}amplitude was negatively correlated with pain intensity and with frequency of pain (Table 4). As connexin-43 has been identified to regulated ATP induced Ca^{2+}response in EGCs[23] we determined whether incubation of EGC with SUP from IBS patients and HC could modulate the expression of connexin-43. Connexin-43 mRNA expression was significantly increased by 16% in EGC cultured with SUP of IBS patients compared to control. IBS subgroup analysis demonstrated that connexin-43 expression was significantly increased in EGC incubated with SUP from IBS-C and IBS-D patients as compared to control (Figure 3C).

Identification of histamine as a mediator involved in SUP effect upon ATP-induced Ca^{2+}response in EGC

We next aimed at identifying putative candidate involved in SUP effects upon ATP-induced Ca^{2+} response in EGC. Previous studies identified mediators such as histamine, serotonin and proteases as SUP mediators that could modulate ENS functions, and in particular neuronal excitability[10]. Based on these findings, we tested the impact of EGC incubation with histamine, serotonin and protease on ATP-induced Ca^{2+} response in EGC. We found that only histamine was able to reduce ATP-induced Ca^{2+} response as compared to control. Indeed, 48h incubation of EGC with histamine (1µM) reduced ATP-induced Ca^{2+} amplitude by 21% (Figure 4A, B).

Next, we aimed at identifying the expression of histamine receptor on EGC using immunohistochemical methods. We first assessed expression of H1R in submucosal plexus of biopsies from HC subjects and IBS patients. Using confocal microscopy, we found that H1R

was co-localized with S100β immunoreactive (IR) structures and appeared to be localized at the surface membrane of EGC (Figure 5 A-C). Using quantitative immunohistochemistry, we showed that levels of H1R-IR were also identical in EGC of HC as compared to IBS patients (data not shown). We also showed that EGC expressed H1R and that its expression co-localized with the one of NGFR, suggesting membrane localization (Figure 5 D-I).

We next aimed at determining whether the glioplastic effects of histamine and SUP of IBS upon ATP-induced Ca^{2+} responses were mediated via activation of H1R using pharmacological approaches. First, we showed that pre-treatment of EGC with pyrilamine (1μM) significantly reversed the changes in ATP-induced Ca^{2+} response caused by histamine. Moreover, pyrilamine did not modify Ca^{2+} response induced by ATP in controls (Figure 6A). Finally, we showed that the effects of SUP of IBS patients upon ATP-induced Ca^{2+} responses in EGC were prevented following incubation of EGC with SUP in presence of pyrilamine (Figure 6B). To verify that Ca^{2+} flux decrease in EGC cultured with histamine (1μM) was not due to a modification in the expression of ATP receptors, we performed a time-course experiment to study the expression of mRNA of P2Y4 in EGC treated with histamine (1μM) during 48h, 24h, 12h and 6h. No difference was observed in the expression of mRNA of P2Y4 in EGC treated with histamine at any time compared to control (Figure 4C). Finally, histamine also did not modify the expression of connexin-43 and nor proliferation of EGC (data not shown).

Discussion

This study highlights the occurrence of glioplastic changes during IBS and identifies histamine as a putative candidate involved in part of these changes. We first showed a reduced mucosal labelling of S100β glial cells in biopsies of IBS patients as compared to control, independent of IBS subtypes. Next, we showed that SUP of biopsies of IBS-C patients reduced EGC cell proliferation and that SUP of IBS-D and M patients reduced ATP-induced Ca^{2+} response in EGC. We next showed that these latter changes were blocked by H1R antagonist and reproduced by histamine. Our study reveals for the first time glioplastic changes in IBS that are dependent of IBS subtypes suggesting that EGC could be novel contributors to IBS pathophysiological processes.

A first finding of our study was the identification of morphological changes of EGC in the mucosa of IBS patients. In particular, we showed that S100β staining was significantly reduced in IBS as compared to control. However, we did not measure any changes in protein expression in S100β using WB techniques. This could be due to the combination of interindividual variabilities and variability in the proportion of different cell types contained in different biopsies (based on the depth, size…). To the best of our knowledge, no study has previously characterized EGC in IBS. Only a preliminary study has examined glial morphology in human intestinal biopsy samples from IBS patients, without observing any changes[32]. A recent study performed in an IBS animal model (maternal separation followed by acute stress) reported structural changes in EGC, such as elongation and/or bulbous terminal swelling of process[14]. Alterations of glial markers have been reported in other diseases such as inflammatory bowel diseases (IBD). In IBD, increased expression of S100β and GFAP has been reported in ulcerative colitis in inflamed areas as compared to controls[33]. In contrast, in Crohn's diseases (CD) reduced GFAP expression has been reported in mucosa

of non-inflamed areas but no change in inflamed areas as compared to control[33]. Interestingly, in EGC isolated from CD, we also reported no change in S100β or GFAP expression but altered functional changes, in particular reduced ability to produce arachidonic acids metabolites[34]. These results suggest that EGC in IBS could have altered functional response, hypothesis that was further tested using *in vitro* methods.

Using validated model of adoptive transfer of disease, we next showed that long term (ie 48h) incubation of EGC with SUP from biopsies of IBS patients did not alter expression of S100β and GFAP in EGC. However, SUP of IBS-C reduced cell proliferation and SUP of IBS-D and IBS-M significantly reduced ATP induced Ca^{2+} response in EGC as compared to controls. In contrast to other models of adoptive transfer that characterize in general the acute impact of SUP upon ENS functions, we aimed to use a model allowing to observe the remodeling of EGC in response to chronic exposition of biopsies-derived soluble factors. As recent studies have shown that EGC Ca^{2+} signalling is critically involved in the control of GI functions such as motility and secretion, one could suggest that altered EGC functions might be involved in GI dysfunctions observed in IBS. Interestingly, SUP-induced Ca^{2+} response was negatively correlated to pain further reinforcing the hypothesis of a functional link between EGC and GI dysfunctions. In order to determine molecular factors potentially involved in altered ATP-induced Ca^{2+} response, we first aimed to determine whether changes in EGC expression of ATP receptors (P2Y) could be involved. However, incubation of EGC with SUP did not modify P2Y4 mRNA expression. Next we aimed to determine whether connexin-43 expression, which has been shown to mediate glio-glio transmission, was altered. Surprisingly we showed an increased expression of connexin-43 mRNA in EGC incubated with SUP from IBS patients. Interestingly, *McClain et al.* also reported reduced expression of connexin-43 associated with reduced glial Ca^{2+} responses and altered motility in aging mice[23].

There

fore, as ATP has been shown to be a key mediator in the neuro-glio communication in the ENS, altered ATP induced Ca^{2+} signalling in EGC could contribute to GI dysfunctions observed in IBS.

An important finding of our study was the identification of histamine as a key soluble factor of the mucosal microenvironment involved in SUP-induced EGC dysfunctions. Previous studies have reported increased concentration of histamine in SUP from IBS patients[35] and mRNA for H1R and H2R up-regulation in patients with GI diseases, including IBS[36]. Histamine is mainly secreted from mast cells[37], although a wide range of histamine-releasing foods and enteric bacteria are known. In a recent work, 58 % of the IBS patients studied reported GI symptoms from histamine-releasing food items[38]. Histamine from the enteric microbiota might exert immunoregulatory effects[39, 40] but whether these mechanisms are protective or pathological remains to be determined. The involvement of histamine in the pathophysiology of IBS is already known[41] and our results are part of this path. However, the evidence that histamine could play a role in the pathogenesis of IBS is also confirmed by the improvement of IBS symptoms induced by H1R antagonists in humans and animal models[42, 43, 44].

Histamine impacts also the ENS, as demonstrated by several previous studies showing the increased enteric neuronal activity induced in part by histamine during IBS [35, 45, 46]. Histamine involving was also confirmed by the inhibition of enteric neuron activation by H1R-H3R antagonists (pyrilamine, ranitidine and clobenpropit, respectively)[35]. To date, no effects of histamine upon EGC are known except *Kimball et al.*, which reported that EGC responded with Ca^{2+} signalling to acute exposure of several neurotransmitters, including histamine[47]. Conversely, our work showed that a chronic incubation with histamine decreased ATP-induced Ca^{2+} signalling in EGC. Although mechanisms remain to be determined, this

path

way probably is not mediated by the reduction of expression of purinergic receptors. However, whether histamine modulates ATP-induced Ca^{2+} responses modulating Ca^{2+} release from intracellular stores or extracellular Ca^{2+} entry remains to be determined. Interestingly, in a recent study *Ostertag et al.,* showed decreased neuronal activity in the submucous plexus of IBS patients after application of a IBS-cocktail containing several mediators, including histamine[48]. In our study, the involvement of histamine in Ca^{2+} signalling of EGC was confirmed by the completely reversing of its effect by a pre-treatment with H1R antagonist, pyrilamine. However, no differences between SUP-treated and pyrilamine + SUP-treated EGC were found, suggesting that other soluble factors are involved in SUP effects upon ATP-induced Ca^{2+} responses.

These results extend our knowledge on the mechanisms involved in the pathophysiology of IBS emphasizing glioplastic changes and identify histamine as a soluble mediator involved in these modifications. Our study showed the ability of SUP from IBS biopsies to modify electrophysiological properties of EGC, particularly ATP-induced Ca^{2+} response. We found the presence of H1R upon EGC and the blockage with a specific antagonist prevented SUP- and histamine-induced reduction of glial Ca^{2+} response to ATP. In conclusion, we showed, for the first time, the presence of EGC functional alterations in IBS and also identified histamine as potential factors. The consequences of these alterations remain to be determined both in humans and in animal models in the perspective of developing specific drugs or nutritional interventions to help managing IBS patients.

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Author names in bold designate shared co-first authorship

Table and figure legends

Table 1. Details of primary and secondary antibodies used.

Table 2. Oligonucleotides sequences.

Table 3. Characteristics of the study population

Table 4. Correlations between quantitative variables and clinical data

Figure 1

Characterization of enteric glial network in colonic biopsies from healthy control and IBS patients. (**A)** Representative images of glial network in biopsies from a healthy control (HC) and IBS patient (IBS). (**B)** The percentage of S100β stained area was significantly reduced in biopsies from IBS patients compared to control, (**C**) whatever the IBS subtype. The percentage of surface area occupied by EGC was measured as ratio of S100β staining on the total area of field of observation (mean of 3 fields for biopsy observed). Data are expressed as mean values ± SEM of n=15 HC and n= 26 IBS patients (n= 10 IBS-C; n=8 IBS-D; n=8 IBS-M). ***p<0.0001; *p<0.05*. S100β (**D**) and GFAP (**E**) expression were measured by western blotting from biopsies lysates of IBS patients and HC and no difference in protein expressions was observed between IBS patients and HC. Data are expressed as mean values ± SEM of n=16 HC and n=26 IBS patients (n= 10 IBS-C; n= 7 IBS-D; n= 9 IBS-M) for S100β and of n=6 HC and n=15 IBS patients (n= 4 IBS-C; n= 4 IBS-D; n=7 IBS-M) for GFAP.

Figure 2

Effect of supernatant from biopsies on EGC functions and phenotype (**A**) The number of EGC treated (6 days) with SUP from IBS patients was similar to control. (**B**) The treatment with SUP from IBS-C patients significantly reduced EGC number compared to control. Data are expressed as mean values ± SEM of n= 23 HC and n= 29 IBS patients (n= 12 IBS-C; n= 8 IBS-D; n= 9 IBS-M) *$p<0.05$. S100β (**C**) and GFAP (**D**) expression were measured by western blotting from EGC cultured with SUP from IBS patients compared to control and no difference was observed between IBS patients and HC. Data are expressed as mean values ± SEM of n= 9 HC and n= 19 IBS patients (n= 5 IBS-C; n= 7 IBS-D; n= 7 IBS-M).

Figure 3

ATP-induced intracellular Ca^{2+} response in EGC cultured with supernatant from biopsies of IBS patients and healthy controls. (**A**) Graphics representatives of one EGC ATP-induced Ca^{2+} response after treatment (48h) with SUP from an HC and an IBS patient (**B**) The amplitude of fluo-4 fluorescence intensity in EGC cultured with SUP from IBS patients was significantly decreased compared to control, particularly with SUP of IBS-D and IBS-M patients. Data are expressed as mean values ± SEM of n= 11 HC and n= 20 IBS (n= 6 IBS-C; n= 7 IBS-D; n= 7 IBS-M). *$p\leq0.05$ (**C**). mRNA expression of connexin-43 was significantly increased in EGC cultured with SUP of IBS patients (48h) compared to control, especially in EGC cultured with SUP from IBS-C and IBS-D patients. Data are expressed as mean values ± SEM of n= 7 HC and n= 17 IBS (n= 5 IBS-C; n= 5 IBS-D; n= 7 IBS-M). *$p\leq0.05$

Figure 4

Identification of histamine as mediator involved in modification of Ca^{2+} response to ATP of EGC. (A) Graphics representatives of one EGC ATP-induced Ca^{2+} response in control and after treatment (48h) with histamine (1μM). (**B**) The amplitude of fluo-4 fluorescence intensity in EGC cultured (48h) with histamine (1 μM) was decreased compared to control. Data are expressed as mean values ± SEM of n= 11 independent experiments. *$p<0.05$. (**C**) Time-course analysis of P2Y4 mRNA expression in EGC treated (48h, 24h, 12h and 6h) with histamine (1 μM). No difference was observed at any time compared to control. Data are expressed as mean values ± SEM of n= 3 independent experiments.

Figure 5

Characterization of H1R expression in biopsies of IBS patients and healthy controls and in EGC cultures. (**A**-**C**) Representative images of submucosal EGC expression of S100β (**A**; *green*) and H1R (**B**; *red*) proteins in colonic biopsy obtained using merged fluorescence of entire z-stack with orthogonal views (magnification 320X). (**D**-**I**) Immunofluorescent images showing 4-6-diamidino-2-phenylindole-labeled nuclei (**D**; *blue*), S100β (**E**; *magenta*), NGFR (**F**; *green*) and H1R (**G**; *red*) protein expression in EGC cultures. Scale bar = 100 μm. (**H**) Overlay of ***D***-***G*** images. (**I**) Enlarged view of boxed areas in (***H***). Scale bar = 20 μm (Magnification 180X).

Figure 6

Effect of on Ca^{2+} response to ATP of EGC treated with histamine or supernatant from IBS patients. (**A**) The pre-treatment with pyrilamine (1 μM) reversed the significant decrease of Ca^{2+} response to ATP in EGC treated (48h) with histamine (1 μM) compared to control. Data are expressed as mean values ± SEM of n= 6 independent experiments. *$p<0.05$. (**B**) The pre-treatment with pyrilamine (1 μM) reversed the significant decrease of Ca^{2+} response to ATP in EGC cultured (48h) with SUP from IBS patients compared to control. Data are expressed as mean values ± SEM of n= 7 independent experiments. *$p<0.05$.

Tables and figures

Table 1

Table 1.		
Antibody	Source	Dilution
Primary antibodies		
Polyclonal rabbit anti S100β	WAKO, Osaka, Japan	1:1000
Polyclonal rabbit anti histamine H1 receptor	Acris Antibodies, Herford, Germany	1:1000 for sub-mucosa 1:500 for rEGCs
Monoclonal mouse anti NGF receptor p75	Millipore, Île de France, France	1:500
Secondary antibodies		
Donkey anti rabbit IgG Probes Fluor 488	Interchim Innovation, Montluçon, France	1:200
Donkey anti rabbitIgGAffiniPure Cy™3 conjugated	Jackson ImmunoResearch Laboratories Inc, Baltimore, USA	1:500
Goat anti rabbit IgGAffiniPure Cy™5 conjugated	Jackson ImmunoResearch Laboratories Inc, Baltimore, USA	1:500

Table 2

Table 2.		
Gene	**Forward primer**	**Reverse primer**
Rat connexin-43	5’-CTCACGTCCCACGGAGAAAA-3’	5’- CGCGATCCTTAACGCCTTTG-3’
Rat P2Yr4	5’- GTCATCTTCTCGGCTCCGTT-3’	5’-AGGGGTCGAGTCACCTTGTA-3’
Human S6	5’-AAGCACCCAAGATTCAGCGT-3’	5’-TAGCCTCCTTCATTCTCTTGGC-3’
Rat RPS6	5’-GCATTGTGGATGCCAACCTG-3’	5’-GTCCTGGGCTTCTTACCTTCTT-3’

Table 3

Table 3.

	Healthy controls (n=24)	IBS-C (n=13)	IBS-D (n=10)	IBS-M (n=11)	Total
Age	45.17±13.25	46.77±13.61 (p=0.644)	55.10±14.34 (p=0.045)	52.18±18.81 (p=0.328)	48.57±14.83 (p=0.239) (p=0.357)
Sex (% male)	54%	23% (p=0.091)	20% (p=0.128)	36% (p=0.471)	38% (p=0.175) (p=0.701)
Depression (%)	0%	15% (p=0.124)	11% (p=0.281)	50% (p=0.002)	13% (p=0.004) (p=0.192)
Pain intensity	0.08±0.41	1.62±0.77 (p<0.001)	2.10±0.74 (p<0.001)	1.80±0.92 (p<0.001)	1.09±1.09 (p<0.001) (p=0.317)
Bloating intensity	0.42±0.58	1.62±0.87 (p<0.001)	1.50±0.85 (p=0.001)	1.50±0.85 (p=0.001)	1.07±0.92 (p<0.001) (p=0.993)
Pain frequency	0.13±0.61	2.31±0.95 (p<0.001)	2.50±0.97 (p<0.001)	2.20±1.32 (p<0.001)	1.40±1.41 (p<0.001) (p=0.837)
Bloating frequency	0.75±1.26	2.46±1.20 (p<0.001)	2.70±1.42 (p=0.001)	2.30±1.42 (p=0.003)	1.75±1.54 (p<0.001) (p=0.767)

Table 4

Table 4:			
	% of S100β stained area for field	**Ca^{2+} flux amplitude**	**EGC number**
Pain intensity	r= - 0.48; p=0.0017	r= - 0.53; p=0.003	-
Bloating intensity	r= - 0.37; p=0.017	-	-
Pain frequency	r= - 0.42; p=0.0068	r= - 0.40; p=0.03	r= - 0.27; p=0.049

Figures

Figure 1

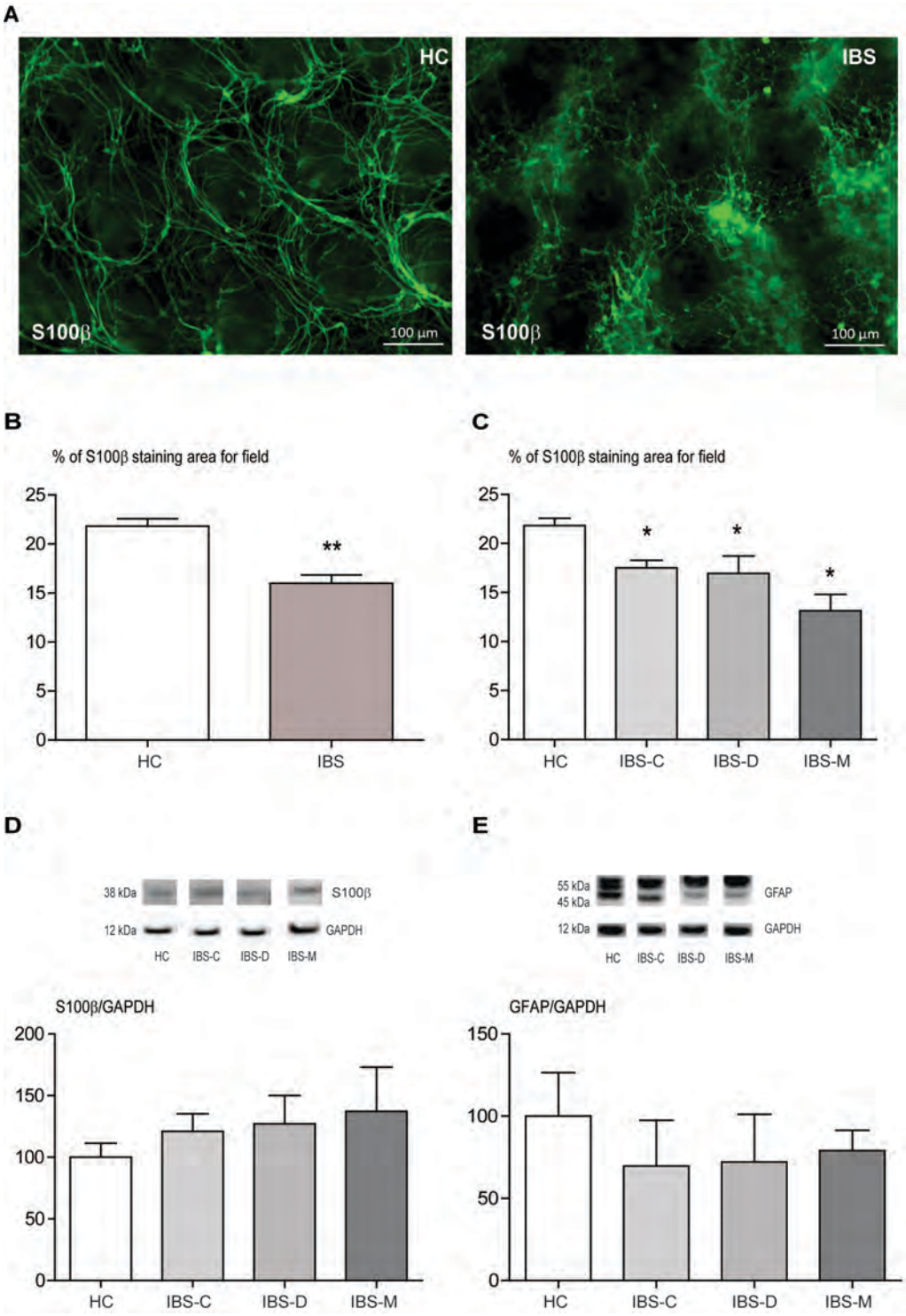
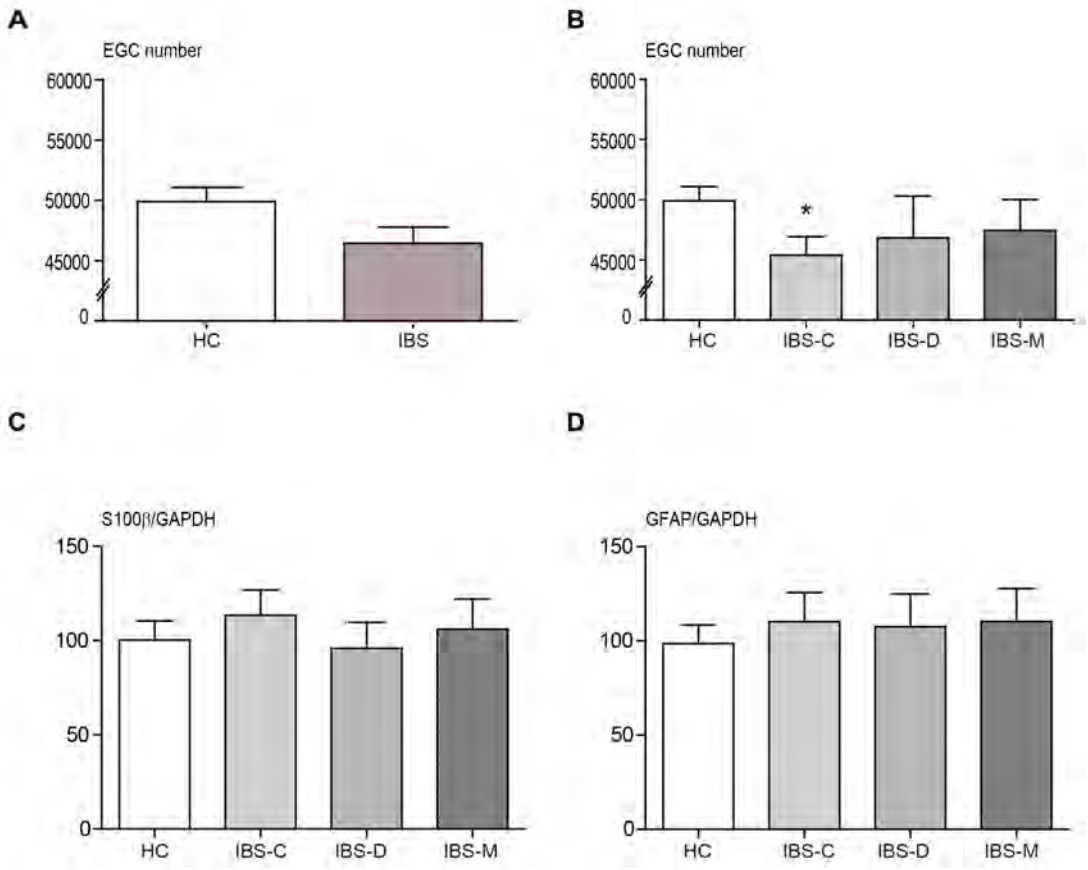


Figure 2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60000
55000
50000
45000
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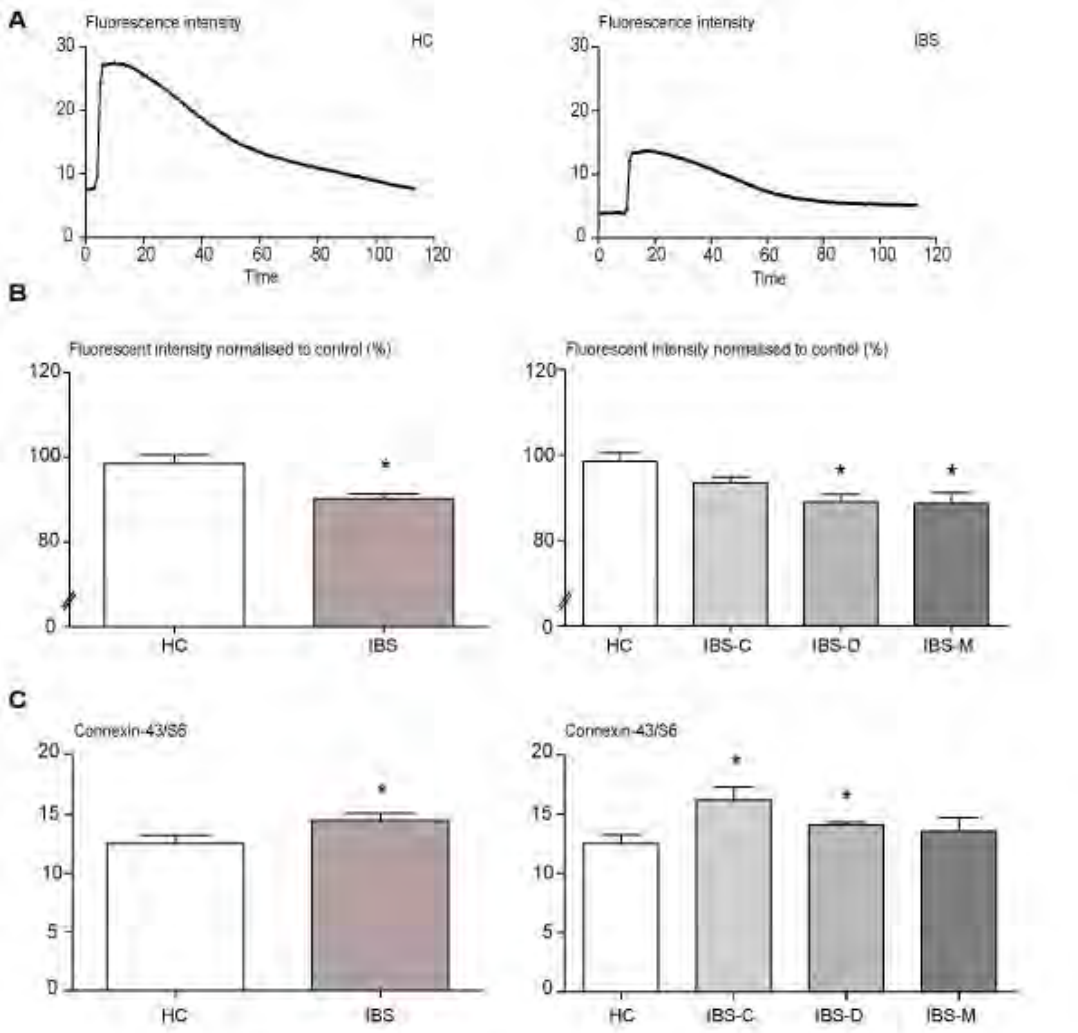
HC
IBS
B
EGC number
60000
55000
50000
45000
0
*
HC
IBS-C
IBS-D
IBS-M
C
S100β/GAPDH
150
100
50
0
HC
IBS-C
IBS-D
IBS-M
D
GFAP/GAPDH
150
100
50
0
HC
IBS-C
IBS-D
IBS-M

Figure 3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Fluorescence intensity
HC
Time
Fluorescence intensity
IBS
Time
B
Fluorescent intensity normalised to control (%)
HC
IBS
Fluorescent intensity normalised to control (%)
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IBS-C
IBS-D
IBS-M
C
Connexin-43/S6
HC
IBS
Connexin-43/S6
HC
IBS-C
IBS-D
IBS-M

Figure 4

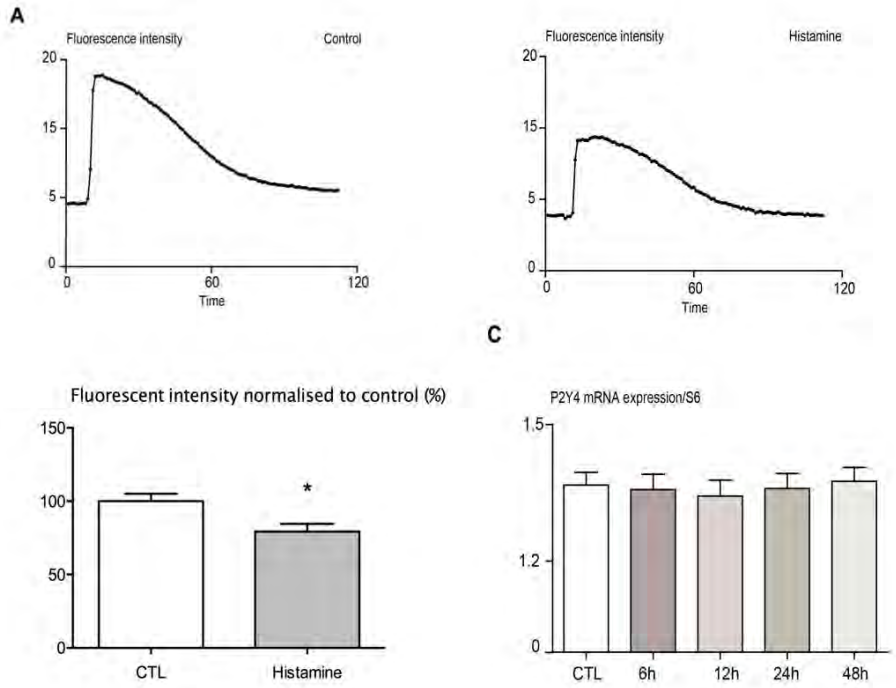
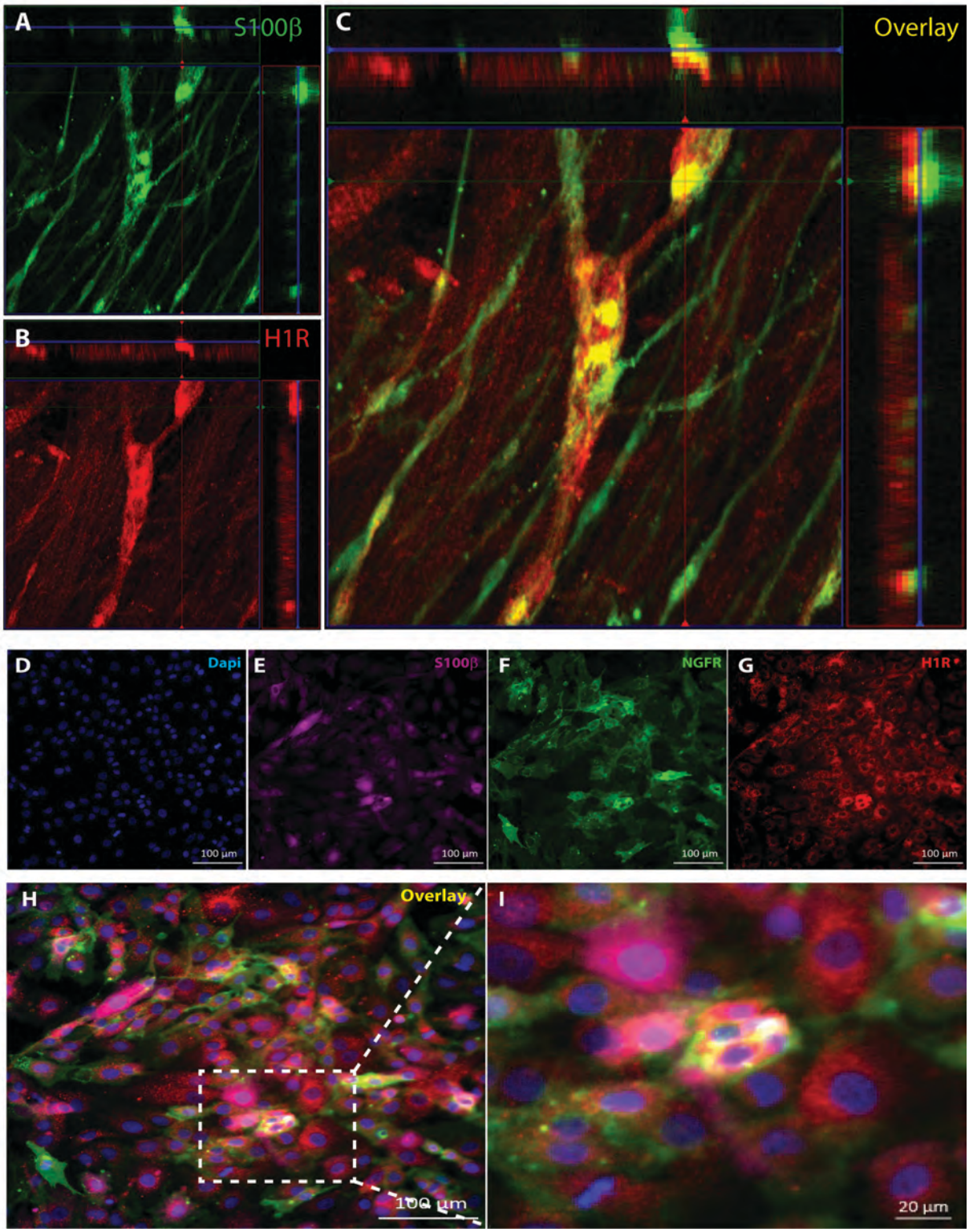


Figure 5


A
S100β
B
H1R
C
Overlay
D
Dapi
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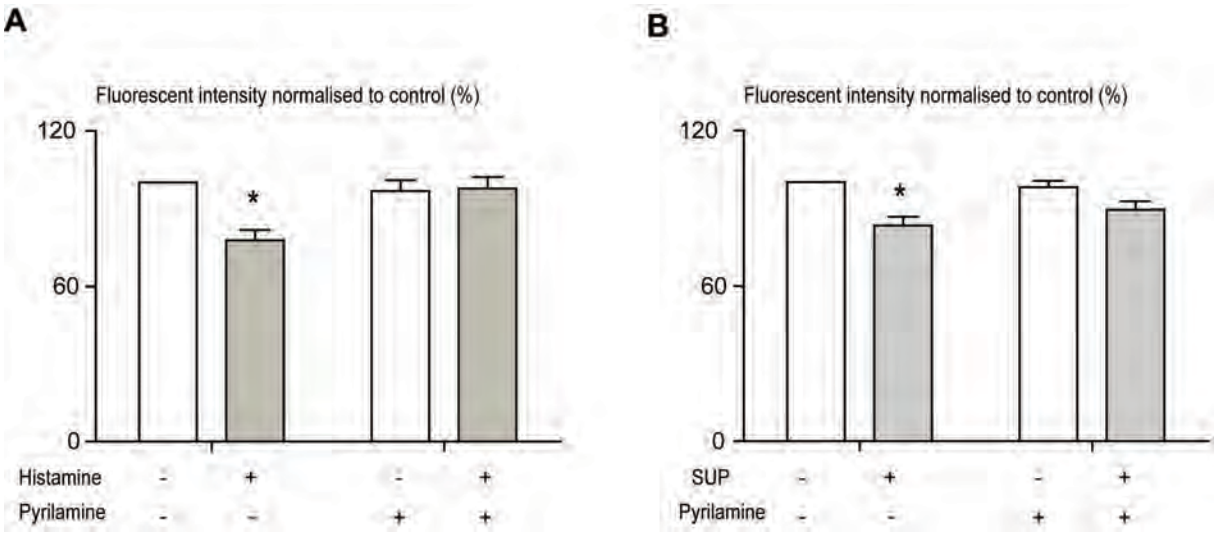
S100β
F
NGFR
G
H1R
100 µm
H
Overlay
100 µm
I
20 µm

Figure 6


A
Fluorescent intensity normalised to control (%)
120
60
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*
Histamine - + - +
Pyrilamine - - + +
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Fluorescent intensity normalised to control (%)
120
60
0
*
SUP - + - +
Pyrilamine - - + +

DISCUSSION

This work has led to analyse the **neuro-glio-epithelial unit** in the irritable bowel syndrome (IBS). The IBS is a multifactorial disease for which the pathophysiological mechanisms have not yet been well defined. What is known is that both intestinal epithelial barrier (IEB) and the enteric nervous system (ENS) play a key role in the development of IBS. A recurring element in the genesis of IBS is the increase of intestinal permeability observed both *in vivo* (lactulose/mannitol ratio in the patient's urine) and *ex vivo* (measure of permeability of colonic biopsies from IBS patients in Ussing's chambers). Animal studies have demonstrated that stress, lipopolysaccharides (LPS), bile salts, mucosal micro-inflammation or bacterial translocation enhance colonic permeability. In this context, an important breakthrough in the understanding of the key mechanisms of IBS was the discovery of the role played by microenvironment of the IEB. Soluble factors contained in faecal homogenates from IBS patients with predominance of diarrhoea (IBS-D) are able to increase paracellular permeability after acute administration in mice (Gecse et al., 2008). Similarly, supernatant (SUP) from colonic biopsies of IBS patients, incubated in a physiological solution for a set time and temperature, increases the paracellular permeability of intestinal epithelial cell (IEC) monolayers *in vitro* after chronic administration. Molecular mechanisms associated with the effects of IBS SUP include the alteration of the IEB components, as demonstrated by the reduction of ZO-1 protein expression in Caco-2 cells incubated with SUP (Piche et al., 2009). All these evidences suggest that soluble factors are involved in the alteration of the IEB during IBS. In this context, mast cell infiltration with increased release of inflammatory mediators, such as trypsin, tryptase and histamine, was reported in the mucosa of IBS

patients (Barbara 2004). Indeed, the gastrointestinal (GI) tract is exposed to several proteolytic enzymes. In addition to their digestive role in protein degradation, proteases are signalling molecules that regulate their functions through the protease-activated receptors (PAR), a family of G protein-coupled receptors activated by N-terminal cleavage. The variety of proteases activating PAR includes digestive enzymes (such as trypsin and trypsinogen), mast cells and neutrophils-released proteases and proteases of the coagulation cascade. These enzymes can be degraded by bacteria, which in turn, are able to release serine-proteases into the lumen. Intestinal epithelial cells express the PAR-2 and its activation is associated with an increase in paracellular permeability by modulating the cytoskeleton contraction, as demonstrated by the intra-colonic administration of PAR-2 agonists in mice (Cenac et al., 2004). In addition, several studies report an increase of serine proteases in IBS suggesting the involvement of PAR-2 activation (Roka et al., 2007; Macfarlane et al., 1988; Gecse et al., 2007; Eutamene et al., 2007; Pienkowski et al., 1989). The reduction in basal colonic permeability and the down-regulation of PAR-2 after antibiotic treatments in the rats have allowed understanding the impact of microflora on the colonic luminal levels of proteases (Roka et al., 2007). In accordance with all these evidences, in our first study we obtained a significant increase of paracellular permeability in Caco-2 cell monolayers after 24 hours of incubation with a PAR-2 agonist (SLIGRL), leading to achieve an *in vitro* model of IEB stress and mimicking lesions of IBS.

Gut microbiota are in constant interaction with the IEB and alteration in its composition is often associated to IBS (König et al., 2014; Frank et al., 2007). One of the recent therapeutic strategies in IBS involves the use of probiotics, to help restore the balance between “good” and pathogenic bacteria. Many different activities have

been ascribed to probiotics, such as enhanced innate and acquired immunity (Gill et al., 2000), increased anti-inflammatory cytokine production (IL-10) (Pessi et al., 2000), and reduced intestinal permeability (Madsen et al., 2001). One of the most relevant results of the first work shows the protective effect of the probiotic *L. reuteri* (DSM17938) in a model of impaired intestinal barrier. Indeed, pre-incubation with live or heat inactivated (but not bacterial SUP) *L. reuteri* were able to prevent the increase of intestinal permeability induced by 24 hours of incubation with SLIGRL. These results suggest that effect of *L. reuteri* is independent from bacterial viability and probably mediated by a bacterial cell wall component. This is the first *in vitro* study proving the protective effect of this *L. reuteri* strain on impaired IEB. Our data are consistent with a recent study showing that *Lactobacillus casei* was able to prevent the alteration of IECs (increase of paracellular permeability and decrease of ZO-1 protein expression) induced by inflammatory cytokines (INF-γ and TNF-α) in Caco-2 monolayers (Eun et al., 2010). The mechanisms by which probiotics exert beneficial effects on epithelial barrier functions remain unclear. In our study *L. reuteri* effect on intestinal paracellular permeability is probably mediated by the regulation of TJ proteins expression. In fact, the pre-incubation with live *L. reuteri* reversed the reduction of ZO-1 protein expression in Caco-2 monolayers. In accordance with our results, *Yang et al*., have recently demonstrated that *L. reuteri* (I5007) improved the intestinal mucosal barrier function of newborn piglets up-regulating the expression of jejunal and ileal epithelial claudin-1, occludin and ZO-1 (Yang et al., 2015). In a study, *Patel et al*., showed that also *Lactobacillus rhamnosus* accelerated maturation of the intestinal mucosal barrier in mice via up-regulating the protein level of claudin-3 (Patel et al., 2012). All these results confirm that one of the effects of probiotics is

to promote the integrity of the IEB by affecting the expression and structure of TJ proteins.

In addition, *L. reuteri* showed an anti-inflammatory effect in its living or heat-inactivated form, contrarily to the bacterial SUP, confirming the results obtained for the paracellular permeability alteration. *Livingston et al*., yet reported the anti-inflammatory effect of heat inactivated *L. reuteri* in human immune cells. When bone-marrow-derived DCs were treated with the strain *L. reuteri* 100-23 and incubated with splenic T cells from ovalbumin T-cell receptor transgenic mice, the production of TGF-β increased (Livingston et al., 2010). In accordance with our results *Ma et al.* demonstrated that the pre-incubation with live *L. reuteri* inhibited mRNA up-regulation, cellular accumulation, and secretion of IL-8 induced by TNF-α in intestinal cell cultures (Ma et al., 2004). In contrast, *Liu* and its collaborators tested the anti-inflammatory effect of four different *L. reuteri* strains. They showed that even though *L. reuteri* strain DSM 17938 did not inhibit LPS-induced IL-8 production in intestinal cell cultures, all strains significantly reduced the intestinal levels of IL-8 and IFN-γ when newborn rat pups were fed with LPS and/or *L. reuteri*. Moreover, intestinal histological damage produced by LPS was significantly reduced by all four strains (Liu et al., 2010). All these results suggest that *L. reuteri* have an anti-inflammatory effect strongly dependent on different experimental conditions including strains and animal models.

Finally, to investigate the possible protective role of *L. reuteri* in the IBS we used SUP from colonic biopsies of IBS patients to induce IEB dysfunctions, as has already been shown by *Piche et al*. Indeed, the incubation of IEC cultures with IBS SUP significantly increased the intestinal paracellular permeability decreasing ZO-1

expression (Piche et al., 2009). In accordance with these results, our study shows the increase of paracellular permeability in IEC cultures incubated with IBS SUP. However, *L. reuteri* did not prevent this effect. In a recent work it has been shown that a mix of probiotics was able to prevent the disruption of the IEB induced by LPS and IBS SUP *in vivo* and *in vitro* (Nébot-Vivinus et al., 2014) possibly via bacteria-derived metabolites or SUP soluble mediators. Additional studies have to definitely establish that the lack of effect observed with *L. reuteri* on the SUP-induced increase of permeability could not be due to our experimental conditions. It is know that lactobacilli exert an anti-protease activity, suggesting a possible degradation or inhibition of proteases present in IBS SUP. However, IBS SUP is a mix of soluble mediators and the trigger factors involved in the increased intestinal permeability include not only proteases but also stress, food allergy and gluten-associated factors (Vanuytse et al., 2013; Vivinus-Nébot et al., 2012; Vazquez-Roque et al., 2013). These evidences suggest that many mechanisms mediate IBS alterations and a mixture of probiotic could have a greater protective effect and prevent the increase of paracellular permeability.

It is now know that soluble factors from mucosal biopsy samples of IBS patients have an effect not only on the IEB but also on the ENS, particularly on enteric neurons. In this manner, an altered signalling from the mucosa to the ENS may be a relevant factor in the pathophysiology of IBS. Several studies have shown that the activation of enteric neurons induced by IBS SUP was often associated to visceral hypersensitivity. As reported by *Barbara et al.*, mast cells in the mucosa of IBS patients can release specific mediators that excite afferent neurons and may thereby cause visceral hypersensitivity (Barbara et al., 2007). More specifically it has been shown that IBS SUP-evoked excitation was mediated by proteases, histamine

and serotonin. Interestingly, IBS patients with visceral hypersensitivity evoke stronger responses in enteric submucous and dorsal root ganglion neurons than samples from normosensitive IBS patients (Buhner et al., 2009). However the role of enteric glia during IBS is still unknown.

One of the aims of this thesis has been to bring out glioplastic changes in IBS identifying the putative soluble mediator responsible for these lesions. For this purpose, using colonic biopsies from IBS patients and healthy controls (HC) (composing a bio-collection of patients referred for colonoscopy for digestive symptoms suggestive of IBS or colonic cancer screening, respectively), we showed a reduced mucosal labelling of S100β glial cells in biopsies of IBS patients as compared to HC. However, any changes in glial markers expression (S100β and GFAP) were observed using either western blot or quantitative PCR techniques, indicating that the heterogeneity in the population of IBS patients analyzed together with the variability of different cellular types and morphology of biopsies may have influenced these results. The only data available in literature related to the expression of enteric glial markers in IBS were obtained in an animal model of IBS showing structural changes in EGC, such as elongation and/or bulbous terminal swelling of process (Fujikawa et al, 2015) reinforcing the hypothesis that glial alterations actively participate in the pathophysiology of IBS.

Are these glioplastic changes affecting glial functional changes?

The use of IBS SUP upon EGC cultures allows studying the impact of microenvironment on glial function, particularly the adenosine triphosphate (ATP)-induced Ca^{2+} signalling. In the ENS purines released from neurons recruit Ca^{2+} responses in the neighboring EGC (Gulbransen et al., 2009). Although the

significance of glial Ca^{2+} responses is still debated, several evidences (such as experimental models of glial ablation) show the necessity of glial Ca^{2+} signalling in the GI physiology (Bush et al., 1998; Cornet et al., 2001). Recently, *McClain et al* carried out the study of Ca^{2+} responses propagation among EGC through cell surface hemichannel of connexin-43, demonstrating the involvement of this pathway in gut motility and intestinal transit (McClain et al, 2014). In our study we showed that IBS SUP significantly reduced ATP-induced Ca^{2+} responses in EGC *in vitro* suggesting the involvement of EGC altered functions in GI dysfunctions reported in IBS. Recently, ATP was identified as an ENS key mediator, both in neuro-glia communication and in EGC-mediated control of digestive motility (Gomes et al., 2009; Gulbransen et al., 2009). Modifications of response to ATP could thereby lead to modifications observed in IBS such as neuronal excitability dysfunction (Buhner et al., 2009), as well as motility disorders or increased permeability. Interestingly, we observed a negative correlation between Ca^{2+} flux intensity and abdominal pain, suggesting that the functional alteration of EGC may be associated with the severity of the pain in IBS patients. To date, mechanisms responsible for decreased Ca^{2+} response to ATP in EGC remain to be determined. On one hand, SUP could modify the ability of Ca^{2+} intracellular stock (reticulum, mitochondria) to release Ca^{2+} in response to ATP. Being identified as regulator of Ca^{2+} signalization in response to ATP, the role of connexin-43 has been suggested. We reported an increased expression of connexin-43 mRNA in EGC incubated with SUP from IBS patients. In astrocytes, connexin-43 expression (Rouach et al. 2002) and function (Karpuk et al., 2011) are regulated by inflammation (Hamby et al., 2012; Bennet et al., 2012) depending on type of duration and distance from inflammatory stimulus (Giaume et al., 2010). In parallel, the increased connexin-43 mRNA expression could be an

adaptive response due to the internalization and degradation of protein induced by inflammatory mediators composing IBS SUP.

One major goal of our study was to identify a potential soluble factor responsible for the effects of IBS SUP in EGC. Like central nervous system (CNS), enteric glia possess signal transduction system that can be stimulated by neuroactives substances, such as histamine, serotonin and proteases, able to induce Ca^{2+} responses (Kimball et al. 1996; Garrido et al., 2002). Although it has been shown that these mediators are able to modify enteric neuron activity, the effect upon enteric glial remains unknown (Barbara et al., 2007; Cenac et al., 2007; Buhner et al., 2009). Our results suggest histamine as a possible soluble mediator implicated in the effects of SUP from IBS patients. However, a specific antagonist of histamine 1 receptor (H1R), *pyrilamine*, reversed the increase of ATP-induced Ca^{2+} response of EGC incubated with histamine confirming this mediator as the possible soluble factor involved in IBS mechanisms via its H1R. Histamine is an important component among soluble mediators released in the SUP from IBS patients, able to increase enteric neuronal activity in IBS (Buhner et al., 2009; Barbara et al., 2007; Breunig et al., 2007). The main storage sites of histamine are mast cells, playing an important role in the regulation of GI functions. The close proximity between mast cells and enteric nerves forms an anatomical basis of neuroimmune interaction in the gut (Stead et al, 1989). Changes in mast cell density and the stimulation-dependent mediator release profile of human intestinal mast cells indicate the involvement of mast cells in allergic reactions, infections, intestinal bowel disease (IBD) and IBS (He et al., 2004; Barbara et al., 2006). However, mast cells are not the only cellular sources of histamine. Some foods, other host cells and an altered microbiome may also contribute to increased histamine levels. For example, IBS patients frequently report postprandial worsening

of their symptoms, and patients typically avoid certain foods to reduce symptoms (Smolinska et al., 2014). In a recent study of *Böhn et al.*, more than 50% of IBS patients observed experienced GI symptoms from histamine-releasing food items and foods rich in biogenic amines (Böhn et al., 2013). Interestingly, it has also been shown the beneficial use of spherical carbon absorbent, which adsorbs molecules such as histamine from the gut lumen for some patients (Tack et al., 2011). The role of histamine has been widely investigated in the CNS where it is produced by mast cells and neurons for regulated release, acting as a neurotransmitter in the mammalian brain. In the astrocytes, histamine exerts several functions involved in different pathways such as growth and proliferation (Rodriguez et al., 1989), neurotrophic factors production (Lipnik-Stangelj et al., 2004; Jurič et al., 2011) induced by H1R-mediated Ca^{2+} signalling (Jung et al., 2000; Jou et al., 1996; Barajas et al., 2008), gliotransmitters release (Shelton et al., 2000) and control of glycogenolysis mediated by H1 and H2 receptors (Arbonés et al., 1990). However, the impact of histamine upon EGC remains to be explored. In the GI tract histamine is principally involved in the GI motility (Bertaccini et al., 1995), immune function (Jutel et al., 2002), ions secretion and gastric acid production (Wang et al., 1990; Tanaka et al., 2002). *Sander et al.*, showed that H1R, H2R and H4R, but not H3R, are expressed in the human GI tract. Interestingly, they also found that H1R and H2R are up-regulated in patients with dysfunctions associated to food allergies or IBS (Sander et al., 2006). These evidences suggest the involvement of histamine in processes that, when altered, induce clinical symptoms related to IBS, such as diarrhoea or abdominal pain.

The Ca^{2+} response to ATP in the EGC is mediated through purinergic receptors P2Y1-4 (Fields et al., 2006; Gomes et al., 2009) coupled to a membrane protein phospholipase C β -type which induces an increase in the cellular

concentration of inositol triphosphate (IP3) and causes intracellular Ca^{2+} release from the endoplasmic reticulum (Zhang et al., 2003). IP3 spread to neighboring EGC via connexin-43 allowing the propagation of the Ca^{2+} wave (McClain et al., 2014). We found that the modification of ATP-induced Ca^{2+} response in the EGC was not due to changes in the expression of purinergic receptor P2Y4, the major functional receptor underlying the ATP response in enteric glia (Gulbransen et al., 2009). The role of these Ca^{2+} waves is still poorly understood but they have a part in the regulation of pre- and post-synaptic transmission of neurons in ENS (Gulbransen et al., 2009). Abnormalities in the regulation of Ca^{2+} waves in enteric glia could lead to abnormalities in the neuro-glial homeostasis possibly related to the increase in the density of the neurite network in IBS patients (Dothel et al., 2015) or disorders of neuronal excitability (Buhner et al., 2009). In astrocytes, Ca^{2+} responses are linked to the modulation of pre- and post-synaptic neurons activity, including release of gliotransmitters and neuronal synchronization (Pascual et al., 2005; Takano et al., 2006; Carmignoto et al., 2006). The alteration of the synaptic activity in response to increased neuronal activation induced by histamine during pathological conditions could therefore results from a functional loss of enteric glia.

The involvement of histamine in the pathophysiology of IBS is already known (Hattori et al., 2010), as demonstrated by the use of H1R antagonists in improving IBS symptoms (Klooker et al., 2010; Tack et al., 2011; Stanisor et al, 2013). *Buhner et al.*, showed that mediators (including histamine) released from mucosal biopsies of IBS patients activated human submucosal neurons and this activation was reversed by pyrilamine, ranitidine and clobenpropit (H1R, H2R and H3R antagonists, respectively) (Buhner et al., 2009). In a recent study, *Ostertag et al.*, using a cocktail containing histamine, serotonin, TNF-α and tryptase (so-called IBS-cocktail) showed

reduced responses of submucosal neurons from IBS patients, suggesting a desensitization to mediators constantly released by immune cells in the gut wall of IBS patients (Ostertag et al., 2015). Although our results showed that the effect of histamine was completely blocked by pyrilamine, there were no differences between SUP-treated and pyrilamine + SUP-treated EGC, suggesting that, together with histamine, other soluble factors mediated the effect of IBS SUP upon ATP-induced Ca^{2+} responses in EGC.

All these results showed EGC functional alterations in IBS identifying histamine as a potential factor involved. To better understand the consequences of these changes, further studies in human and in animal models are required. To confirm the results obtained *in vitro* using SUP from IBS patients, a subsequent investigation could involve the study of enteric glial Ca^{2+} signalling *ex vivo* in IBS biopsies.

CONCLUSIONS

The physiopathology of irritable bowel syndrome (IBS) remains partially understood although recent research has focused on the role played by a number of factors, including micro-inflammation, *dysbiosis* and changes in enteric neuronal functions evoked by changes in the gut lumen. The increase of intestinal paracellular permeability and modification of mucosal microenvironment exert a central role in IBS, as demonstrated by alterations of epithelial cells and enteric neurons components induced by supernatant (SUP) from colonic biopsies obtained during symptomatic exacerbation from IBS patients.

First, we studied the effect of a probiotic strain upon intestinal epithelial barrier (IEB) dysfunctions. We showed that *Lactobacillus reuteri (L. reuteri)* was able to maintain the integrity of IEB *in vitro* through mechanisms involving, at least in part, the modulation of intestinal tight junctions (TJs). In fact, *L. reuteri* was able to prevent IEB abnormalities induced by protease-activated receptor 2 (PAR-2) agonist (SLIGRL), but not by supernatants (SUP) from IBS patients, highlighting a targeted action of *L. reuteri* likely not enough effective to counteract the cocktail of soluble factors present in the SUP from IBS patients. It is tentative to hypothesize that a synergistic action of several probiotics strains could be more effective in the prevention of the IEB abnormalities induced by SUP related soluble mediators. Thus, my study set the scene to further analysis which will expand mechanisms and efficacy of *L reuteri* in IBS as well as other functional bowel disorders.

In a second research project, we investigated the effect of IBS SUP upon enteric glia, showing, for the first time, the involvement of enteric glial cells (EGC) in

the pathophysiology of IBS. Our findings identified histamine as a putative soluble mediator responsible for the effects of SUP from IBS patients on enteric glia. In our model, SUP from IBS patients reduced ATP-induced Ca^{2+} responses in EGC *in vitro* via the involvement of H1 receptor. These results showed EGC as a novel actor in the pathophysiology of IBS and the role of histamine in the glio-neuro communication in IBS.

IBS related changes of IEB allow the passage of several luminal factors, such as lipopolysaccharides (LPS), antigens and bacterial- and dietary-derived factors (e.g. histamine), which, in turn, would activate both mucosal immune cells and enteric neurons as well as EGC. This mechanism provides a conceptual basis to the neuro-glio-epithelial unit, which plays a major role in gut physiology homeostasis and in pathological conditions (e.g. IBS) when its integrity is altered. As highlighted in Figure 37, the ***neuro-glio-epithelial unit*** altered by soluble noxae, such as factors of mucosal / extramucosal origin, play an important role in symptom generation, including visceral pain, one of the major complaint in patients with IBS.

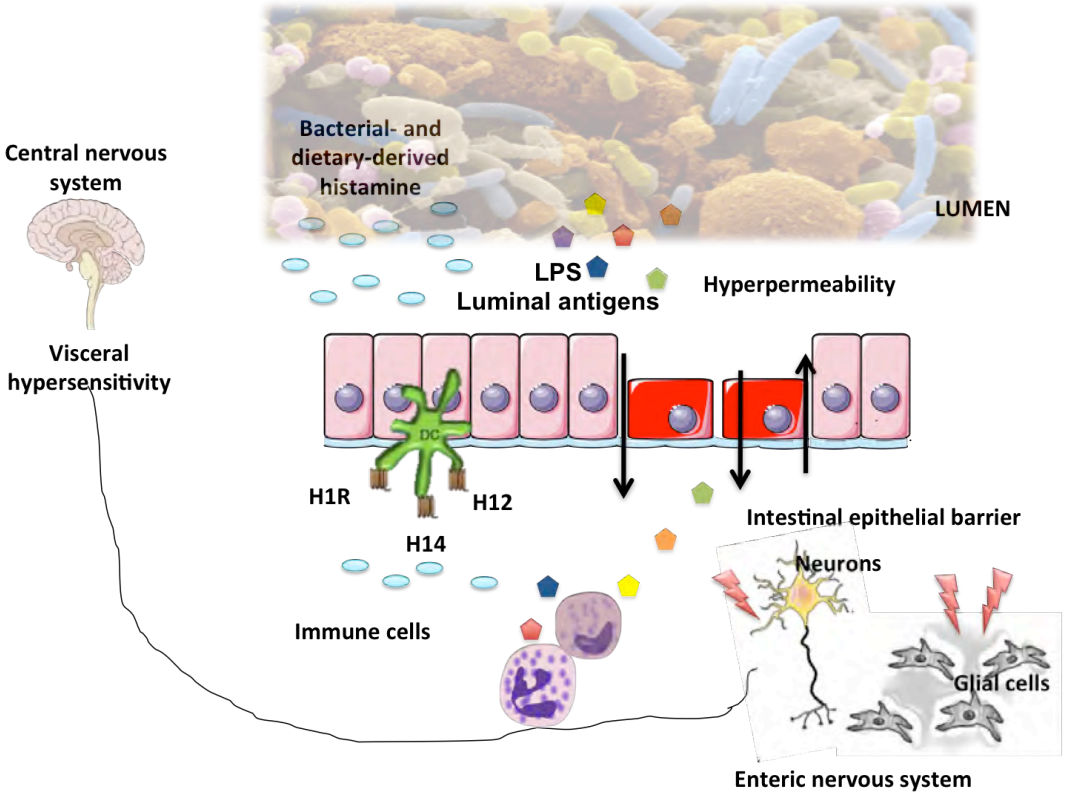


Figure 37: Alterations of neuro-glio-epithelial unit during IBS. *Lesions of the IEB during IBS allow the passage of several luminal factors, such as lipopolysaccharides (LPS), antigens and bacterial-and dietary derived histamine, which would activate both mucosal immune cells and ENS. In this manner, soluble factors play an important role in pain transmission from the periphery to the brain via sensory nerve pathways, contributing to visceral hypersensitivity. LPS, lipopolysaccharides, H1R, histamine 1 receptor; H2R, histamine 2 receptor; H4R, histamine 4 receptor*

ANNEXES

Annex 1: Irritable Bowel Syndrome Questionnaire

Symptom severity

	Absent	No impact on daily activities	Decreasing daily activities	Decreasing strongly daily activities	Confined to bed
Pain/discomfort					
Bloating					
Abnormal bowel movement					

Symptom frequency

	Absent	Rare (1 day per week)	Occasional (2 -3 days per week)	Frequent (4 – 6 days per week)	Very frequent (7 days per week)
Pain/discomfort					
Bloating					
Abnormal bowel movement					

Frequency of bowel movement:

Number of bowel movement per day: .....

Evacuation characteristics:

Evacuation difficulty [ ] Feeling of incomplete evacuation [ ] Urgency [ ] Presence of mucus in stool [ ]

Or per week: .....

GLOBAL EVALUATION OF GENERAL WELL-BEING

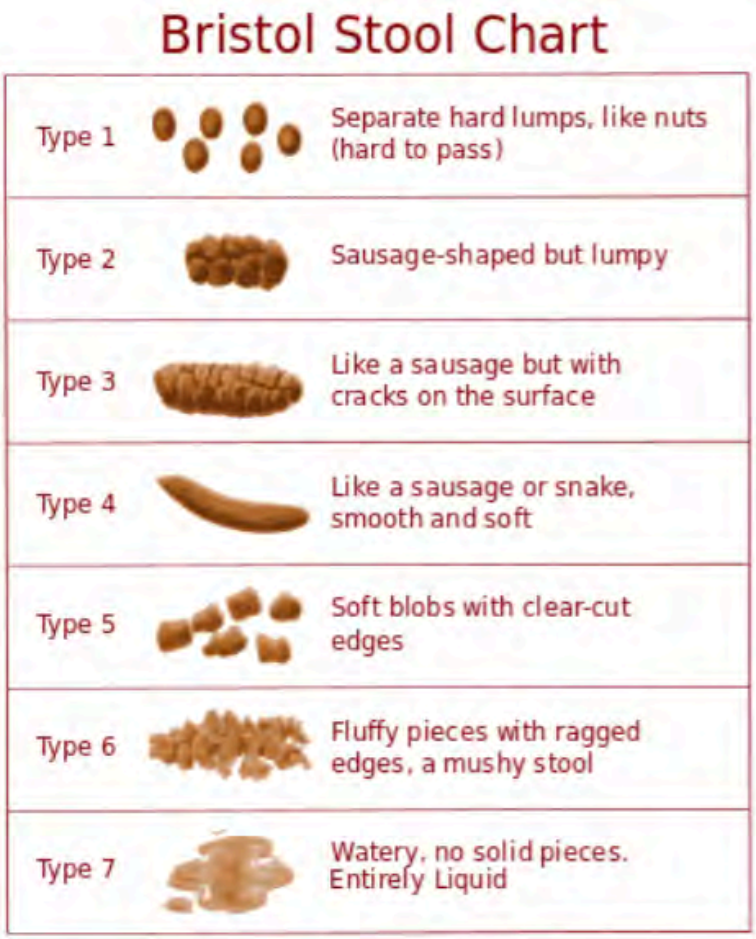
From 0 to 10

HISTORY OF ANXIETY OF DEPRESSION

❑yes, now ❑yes, before ❑no

BRISTOL STOOL CHART

	0-24% of the time	25-49% of the time	50-74% of the time	75-100% of the time
1 or 2				
3 or 4 or 5				
6 or 7				



ROME III QUESTIONNAIRE

1) In the last 3 months, how often did you have discomfort or pain anywhere in your abdomen?

0) Never
1) Less than one day a month
2) One day a month
3) Two to three days a month
4) One day a week
5) More than one day a week
6) Every day

2) For women: Did this discomfort or pain occur only during your menstrual bleeding and not at other times?

0) No
1) Yes
2) Does not apply because I have had the change in life (menopause) or I am a male

3) Have you had this discomfort or pain 6 months or longer?

0) No
1) Yes

4) How often did this discomfort or pain get better or stop after you had a bowel movement?

0) Never or rarely
1) Sometimes
2) Often
3) Most of the time
4)Always

5) When this discomfort or pain started, did you have more frequent bowel movements?

0) Never or rarely
1) Sometimes
2) Often
3) Most of the time
4)Always

6) When this discomfort or pain started, did you have less frequent bowel movements?

0) Never or rarely
1) Sometimes
2) Often
3) Most of the time
4)Always

7) When this discomfort or pain started, were your stools (bowel movements) looser?

0) Never or rarely
1) Sometimes
2) Often
3) Most of the time
4)Always

8) When this discomfort or pain started, how often did you have harder stools?

0) Never or rarely
1) Sometimes
2) Often
3) Most of the time
4)Always

9) In the last 3 months, how often did you have hard or lumpy stools?

0) Never or rarely
1) About 25% of the time
2) About 50% of the time
3) About 75% of the time
4) Always, 100% of the time

10) In the last 3 months, how often did you have loose, mushy or watery stools?

0) Never or rarely
1) About 25% of the time
2) About 50% of the time
3) About 75% of the time
4) Always, 100% of the time

Diagnostic Criteria

Recurrent abdominal pain or discomfort at least 3 days/month in last 3 months associated with two or more of criteria below:

- *Pain or discomfort at least 2-3 days/month (question 1>2)*
- *For women, does pain occur only during menstrual bleeding? (question 2=0 or 2)*
- Improvement with defecation *Pain or discomfort gets better after BM at least sometimes (question 4>0)*

- Onset associated with a change in frequency of stool *Onset of pain or discomfort associated with more stools at least sometimes (question 5>0), OR Onset of pain or discomfort associated with fewer stools at least sometimes (question 6>0)*

- Onset associated with a change in form (appearance) of stool *Onset of pain or discomfort associated with looser stools at least sometimes (question 7>0), OR Onset of pain or discomfort associated wit harder stools at least sometimes (question 8>0)*

- Criteria fulfilled for the last 3 months with symptom onset at least 6 months prior to diagnosis *(question 3=1)*

Criteria for IBS-C

(question 9>0) and (question 10=0)

Criteria for IBS-D

(question 9=0) and (question 10>0)

Criteria for IBS-M

(question 9>0) and (question 10>0)

Criteria for IBS-U

(question 9=0) and (question 10=0)

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UNIVERSITE BRETAGNE LOIRE

UNIVERSITÉ DE NANTES

Thèse de Doctorat

Lésions et ciblage de l'Unité Neuro-Glio-Epitheliale (UNGE) au cours du syndrome de l'intestin irritable".

Lesions and targeting of neuro-glio-epithelial unit during irritable bowel syndrome

Nicoletta Libera LILLI

Résumé

Le syndrome de l'intestin irritable (SII) est une pathologie complexe multifactorielle caractérisée par des douleurs abdominales chroniques et une altération du transit intestinal. Des preuves émergentes suggèrent qu'une augmentation de la perméabilité para-cellulaire pourrait contribuer à la physiopathologie du SII. Par conséquence, les approches thérapeutiques améliorant l'homéostasie de la barrière épithéliale intestinale (BEI) pourraient être de grand intérêt dans la prise en charge du SII. Parmi les acteurs responsables des atteintes de la BEI figure le microbiote intestinal dont des altérations ont été identifiées dans le SII. A ce titre, la capacité des souches bactériennes/probiotiques à réparer la BEI pourrait avoir un intérêt thérapeutique. Parmi d'autres facteurs impliqués dans les altérations des fonctions digestives dans le SII figurent le système nerveux entérique (SNE) et en particulier, les cellules gliales entériques (CGE). Néanmoins, les altérations des CGE dans le SII restent à déterminer. Dans ce contexte, ma thèse a d'abord consisté à identifier la capacité d'une source probiotique à protéger la BEI d'une atteinte par des médiateurs protéolytiques via 1) une augmentation de l'expression des protéines des jonctions serrées 2) une modulation de la production de cytokines par les cellules épithéliales intestinales. D'autres part, mes travaux ont mis en évidence des altérations phénotypiques et fonctionnelles des CGE et identifié l'histamine comme acteur responsable de ces altérations.

Mots clés: syndrome de l'intestin irritable; barrière epithéliale intestinale; système nerveux entérique; cellules gliales entériques; histamine

Abstract

The irritable bowel syndrome (IBS) is a multifactorial complex disorder characterized by chronic abdominal pain and altered bowel habits. Emerging evidences suggest that the increase of the para-cellular permeability may contribute to the pathophysiology of IBS. Consequently, therapeutic approaches to improving the homeostasis of the intestinal epithelial barrier (IEB) could be of great interest in the management of IBS. Among the responsible actors for IEB lesions there are the alterations of gut microbiota, which have been identified in IBS. The ability of bacterial strains/probiotics to repair the IEB could be of therapeutic value. Other factors involved in the alterations of the digestive functions in IBS include the enteric nervous system (ENS) in particular, enteric glial cells (EGC). However, the alterations of EGC in IBS remain to be determined. In this context, my thesis consisted firstly to identify the ability of a probiotic strain to protect the IEB from lesions induced by proteolytic mediators via 1) increase in the expression of tight junction proteins 2) modulation of cytokine production by IEC. On the other hand, my research showed phenotypic and functional alterations of EGC and identified histamine as a responsible actor of these altrations

Key words: irritable bowel syndrome; intestinal epithelial barrier; enteric nervous system; enteric glial cells; histamine