DIFFERENTIAL PHENOTYPE OF JEJUNAL ENTERIC GLIAL CELLS IN DIARRHOEA-PREDOMINANT IRRITABLE BOWEL SYNDROME WITH AND WITHOUT BILE ACID DIARRHOEA. THE IMPACT OF BILE ACIDS ON THE INTESTINAL EPITHELIAL BARRIER AND GLIAL CELL BIOLOGY

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BEATRIZ LOBO ÁLVAREZ, Doctor of Medicine, MARÍA VICARIO PÉREZ, Doctor of Pharmacy and FERNANDO AZPIROZ VIDAUR, Doctor of Medicine,

CERTIFY

That the thesis entitled "Differential phenotype of jejunal enteric glial cells in diarrhoea-predominant irritable bowel syndrome with and without bile acid diarrhoea. The impact of bile acids on the intestinal epithelial barrier and glial cell biology", submitted by **Mercé Albert Bayo** for the degree of Doctor, has been carried out under their supervision, and, considering it completed, authorise its presentation for the defence ahead of the corresponding tribunal.

In witness whereof, they hereby sign this document.

Barcelona, July 2023

Dr. Beatriz Lobo Álvarez Dr. María Vicario Pérez Dr. Fernando Azpiroz Vidaur

The cover is a fluorescence microscope image of enteric glial cells (GFAP marker, green) and tight junction protein 1 (TJP1 marker, red) in the intestinal mucosa of a healthy donor (magnification 200x).

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Somnia fronteres trencant horitzons

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Als meus pares,

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ABBREVIATIONS

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ANS, Autonomic Nervous System

ASBT, Apical Sodium-Dependent Bile Acid Transporter

BA, Bile Acid

BAD, Bile Acid Diarrhoea

BAR, Bile Acid Receptor

BDNF, Brain Derived Neurotrophic Factor

BSH, Bile Salt Hydrolase

CA, Cholic Acid

CDCA, Chenodeoxycholic Acid

CHRM3, Cholinergic Receptor Muscarinic 3

CLDN2, Claudin 2

CM, Conditioned-Medium

CNS, Central Nervous System

CYP, Cytochrome P450

DCA, Deoxycholic Acid

EGC, Enteric Glial Cell

ENS, Enteric Nervous System

FGF19, Fibroblast Growth Factor 19

FXR, Farnesenoid X Receptor

GALT, Gut-Associated Lymphoid Tissue

GDNF, Glial-Derived Neurotrophic Factor

ABBREVIATIONS

GFAP, Glial Fibrillary Acidic protein

GI, Gastrointestinal

GJA1, Gap Junction Protein Alpha 1

GPBAR1, G Protein-coupled Bile Acid Receptor 1

H, Hour

H&E, Haematoxylin & Eosin

HPF, High-Power Field

IBS, Irritable Bowel Syndrome

IBS-C, Constipation-predominant Irritable Bowel Syndrome

IBS-D, Diarrhoea-predominant Irritable Bowel Syndrome

IBS-M, Mixt pattern Irritable Bowel Syndrome

IBS-U, Unclassified Irritable Bowel Syndrome

IECs, Intestinal Epithelial Cells

IELs, Intraepithelial Lymphocytes

LCA, Lithocholic Acid

LDH, Lactate Dehydrogenase

LPLs, Lamina Propria Lymphocytes

NGF, Nerve Growth Factor

NR1H4, Nuclear Receptor subfamily 1 group H member 4

NTCP, Sodium Taurocholate Co-transporting Polypeptide

NTF3, Neurotrophin 3

NTRK1, Neurotrophic Receptor Tyrosine Kinase 1

OCLN, Occludin

P2RX4, Purinergic Receptor P2X 4

P2RX7, Purinergic Receptor P2X 7

PBS, Phosphate-Buffered Saline

PXR, Pregnane X Receptor

RT, Room Temperature

S100β, S100 Calcium Binding Protein β

S1PR2, Sphingosine-1-Phosphate Receptor 2

SeHCAT, Selenium-75-Homocholic Acid Taurine

SOX10, SRY-box 10

TEER, Transepithelial Electrical Resistance

TGR5, Takeda G protein-coupled Receptor 5

TJP, Tight Junction Protein

UCHL1, Ubiquitin C-terminal Hydrolase L1

VDR, Vitamin D Receptor

VILL, Villin

INTRODUCTION

1. STRUCTURE AND FUNCTION OF THE INTESTINE

The intestine is one of the gastrointestinal (GI) tract organs, whose main functions are nutrients digestion and absorption, processing of waste products, as well as defence and immune surveillance. These digestive and defensive functions are possible thanks to the particular anatomy of the intestinal mucosa, which forms a barrier between the lumen and the internal environment, known as "the intestinal barrier function". Epithelial, endocrine, nervous and immune systems interact, being the intestine a crucial element for health and homeostasis maintenance (1). As the longest part of the digestive tract, the intestine is divided into two large regions with specific functions: the small intestine (where most of the digestive process is produced) and the large intestine (where water absorption and elimination of the faecal bolus is mainly performed). Specifically, the small intestine connects the stomach with the large intestine and it is divided into three main segments: the duodenum, the jejunum, and the ileum. The density and size of the villi in the first two parts of the intestine are greater than in the final region, since the small intestine, where most nutrient absorption takes place, is the region most exposed to food and other luminal antigens (2).

1.1. Anatomy of the small intestine

At a structural level, the small intestine is divided in four concentric layers for a proper functioning (**Figure 1**):

-**Mucosa:** it interacts directly with the intestinal lumen, carrying out a large part of the absorptive and barrier function, as it is constantly exposed to a huge diversity of luminal antigens. It is divided in three layers: epithelium, lamina *propria* and muscularis mucosa:

-**Epithelium:** it is a monolayer of several types of intestinal epithelial cells (IECs), 80% of which are enterocytes. To increase the area of exposure and absorption, it forms the villi, folded structures all along the mucosa. At the base of these villi there are the crypts, which contain stem cells from which IECs will develop and differentiate along the crypt-villus axis. With a 4- to 5- day turnover rate, these stem cells generate different IECs, which will respond to stimuli by producing and secreting effector molecules and mediators of different origin: proinflammatory, chemotactic and regulatory mediators (enterocytes), mucus (goblet cells), defensins (Paneth cells) and hormones and neuropeptides (enterochromaffin cells). In

addition, there is also a cell type specialised in antigen transport from the lumen (M cells) (3,4).

-**Lamina *propria*:** basal membrane below the epithelium formed by collagen, reticulin fibers, fibroblasts and a matrix of glycosaminoglycans, through which blood, lymphatic and nervous capillaries flow. A large variety of immune cells can be found: B and T lymphocytes, plasma cells, eosinophils, macrophages, mast cells and dendritic cells. Hence, a big part of the intestinal immune activity takes place in this layer (5). Furthermore, a network of neural cells (including enteric neurons and enteric glial cells (EGCs)) controls digestive functions and intestinal homeostasis through the release of neurotransmitters and, indirectly, neuro-immune interactions.

-*Muscularis mucosae*: It is a thin muscle layer between the lamina *propria* and the submucosa.

-**Submucosa:** a loose connective tissue layer made of lymphatic and blood vessels, ganglia and nerve cells, which constitute the **submucosal plexus** (Meissner's). The submucosal plexus responds to epithelial sensory signals, controlling blood flow and epithelial function and communicates with myenteric neurons (6,7). Its activity is regulated by efferent pathways of the sympathetic system.

-***Muscularis propria*:** It is a muscular layer constituted by two layers of smooth muscle cells, an external longitudinal and an inner circular. Between them, the **myenteric plexus** (Auerbach's) can be found, which coordinates bowel smooth muscle contraction and relaxation. While the submucosal plexus is found only in the small and large bowel, the myenteric plexus has been described from the esophagus to anal sphincter. It is also regulated by efferent pathways of the sympathetic system which inhibit the excitatory effect of myenteric ganglia cells, modulating the intestinal fluid flow. On the other hand, nociceptive, chemoceptive and mechanoceptive signalling takes place through afferent connections from myenteric plexus to thoracolumbar spinal cord and brain stem (8).

-**Serosa:** the outermost layer of the GI wall composed of connective tissue covered by the mesothelium.

1.2. The intestinal barrier function

The anatomical position of the intestinal epithelium defines it as the largest surface area of the body exposed to the external environment, physically separating the intestinal lumen from the internal milieu and representing the main route of entry for pathogens. The gut has therefore developed the intestinal barrier function, a defence system composed of intracellular and extracellular elements that interact to ensure proper epithelial function, immune responses and the acquisition of tolerance to food antigens and the intestinal microbiota, preventing harmful elements from reaching the internal milieu while allowing the selective passage of substances. At the same time, nutrition is provided by active and passive absorption mechanisms in the IECs. The cellular elements of the barrier include the epithelium itself and the immune and neural cells located in the lamina *propria*. Extracellular luminal elements include the mucus layer, gastric, pancreatic and biliary secretions, pH and molecules released by epithelial/immune/neural cells. In addition, peristalsis and secretion of water and chlorine wash out the luminal contents and restrain antigen translocation to the lamina *propria* (9,10). Residing in the lumen, microbiota play an important role in limiting pathogen colonisation, while interacting with epithelial, immune and neural cells to support barrier function through regulation of energy and metabolism, nutrient acquisition and cell proliferation (11,12) (**Figure 1. A**).

The epithelial layer is an essential element of the intestinal barrier and, as described above, is composed of several types of IECs. To ensure an efficient physical barrier, IECs are tightly interconnected by **intercellular junctions** and there are three types of intercellular junctions with different localisation, composition and function (**Figure 1. A.1**) (10). From apical to basal they are:

-**Tight junction proteins (TJPs):** The most apical intercellular junctions, they seal the gaps between the IECs, being essential in the epithelial barrier maintenance and polarity. They limit ion diffusion and luminal antigens translocation to the basolateral part, allowing the selective paracellular passage of ions and solutes through the intestinal epithelium, while preventing the entry of microorganisms, toxins and luminal antigens. They are made up of four transmembrane protein families: occludins, claudins, junctional adhesion molecules, and tricellulins.

The **occludin** is a transmembrane protein closely related to the selective paracellular permeability regulation and essential for a proper epithelium differentiation (13,14). Its main

regulation is mediated by the phosphorylation of its residues, which maintain it in the membrane. Dephosphorylation translates into its redistribution and internalisation into the cytoplasm (15). **Claudins** are a large family of proteins that create paracellular channels of 4Å radius. These small pores regulate electrical resistance by participating in the selective passage of ions (16). Moreover, the ***zonula occludens*** 1, 2 and 3 proteins are a subfamily of peripheral proteins which act as anchor proteins, connecting TJPs with the actin-myosin complex through multiple protein-binding domains. This interaction modulates the tension of the cytoskeleton fibers allowing the opening or closing of the TJPs in a dynamic way (17).

-**Anchoring junctions:** Connect the cell's cytoskeleton with those of neighbouring cells or with the extracellular matrix, ensuring the epithelium structure. They are divided in two subtypes, adherens junctions and desmosomes (18,19).

-**Communicating junctions (GAP junctions):** They are composed of transmembrane proteins (connexins) constituting intercellular channels that connect the cytoplasm of adjacent cells. They allow the rapid exchange of ions, water and small biomolecules, the development, growth and differentiation of IECs, as well as they play an important role in the maintenance of TJPs and adherens junctions (20,21).

Finally, considering the great diversity and quantity of antigens daily faced by the intestine, physical and chemical mechanisms are not enough to face them and the immune system action is required for ensuring a more effective reaction. The **gut-associated lymphoid tissue (GALT)** is the immune component of the intestinal barrier function. GALT is distributed in two compartments, organised GALT, inductor of immunity and tolerance by cells grouped in lymphoid structures, and the diffuse GALT, the effector site through intraepithelial lymphocytes (IELs) and lamina *propria* lymphocytes (LPLs). While IELs are a unique population of T cells, LPLs are constituted by different types of immune cells: plasma cells, T lymphocytes (mainly Th), macrophages, dendritic cells, mast cells and eosinophils (22–24). Both IELs and LPLs are influenced by the commensal microbiota, which also plays a role in the development of the immune system (25). Firstly, the innate immunity supplies an immediate but nonspecific defence, recognizing repetitive and common structures in the pathogens (5) and afterwards, the adaptive immunity is in charge of the specific and effective defence, but not immediate, generating immunologic memory against a precise antigen through the activations of immune cells resident in the lamina *propria* and transporting immunoglobulins across the epithelium (26,27).

1.2.1. Epithelial barrier dysfunction

The ability of the mucosal surface to be penetrated by specific substances is defined as intestinal permeability and is maintained by the structural and functional elements of the barrier function. Small molecules (<600 Da) cross the epithelial layer through the intercellular spaces (paracellular route) while macromolecules through non-selective or receptor-mediated transport by IECs (transcellular route). The paracellular space is controlled by TPJs at the most apical side followed by the other intercellular junctions. Alterations in these proteins can modify the epithelial permeability triggering an abnormal intestinal barrier functioning (28). Moreover, intestinal barrier function is modulated by several factors, including stress, microbiota composition, diet, and drug consumption, and that persistent disruption of this barrier results in increased intestinal permeability. All together enables antigen penetration into the lamina *propria* where immune and nervous cells reside, promoting inflammatory and immune responses as well as enteric nervous system (ENS) and afferent routes to the central nervous system (CNS) activation. Actually, this response may lead to further intestinal permeability alteration and perpetuation of mucosal low-grade inflammation, exacerbating neuro-immune cell communication and neuronal plasticity (1,29) (**Figure 1. B**).

All these barrier function alterations can turn into disease, and, in fact, increased intestinal permeability is associated with digestive (irritable bowel syndrome (IBS), inflammatory bowel disease, microscopic colitis, celiac disease and food allergy) and non-digestive conditions (schizophrenia, diabetes, obesity, and sepsis) evidencing the relevance of the mucosal barrier to human health. However, despite intensive research, whether barrier dysfunction is the cause or consequence of disease and the underlying mechanisms governing transition from health to disease remain unknown. Importantly, all these GI diseases are chronic and share a common clinical outcome (mainly diarrhoea and abdominal pain) that, in many cases, complicate the diagnosis and the therapeutic management, significantly decreasing the quality of life of affected patients, becoming a serious burden to the healthcare system (30,31). Thereby, understanding the molecular mechanisms involved in abnormal intestinal epithelial barrier function is essential to identify therapeutic targets and define better approaches to barrier dysfunction (32).

There are several methods for assessing intestinal permeability, the choice depends on the study approach and the samples available, and knowledge of each technique is necessary to understand the advantages and disadvantages of each. *In vivo* techniques are based on the detection of administered oral probes in the urine, as these molecules are not metabolised but excreted in the urine, and their site of intestinal absorption is known, the time of urine collection shows the part of the intestine crossed by the probes. The most used probes are polyethylene glycol, the saccharides mannitol, lactulose, and sucralose, and chromium-labelled ethylenediaminetetraacetic acid. Intestinal perfusion of an isolated intestinal segment can also be used for measuring intestinal permeability. Between the *ex vivo* approaches, mucosal biopsies are used in an organ bath to measure the passage of a probe across the epithelial layer or in Ussing chambers to quantify the secretory activity and ionic conductivity, assessing both permeability routes. *In vitro* studies are based on intestinal epithelial cell lines (Caco-2, HT-29 or T84) or primary cells and are used to evaluate the structural and functional properties of the epithelium and the transport of molecules across the epithelium, which is commonly used in drug screening of therapeutic molecules. Finally, biopsies can be fixed to study intercellular junction proteins by immunostaining (29,33–35).

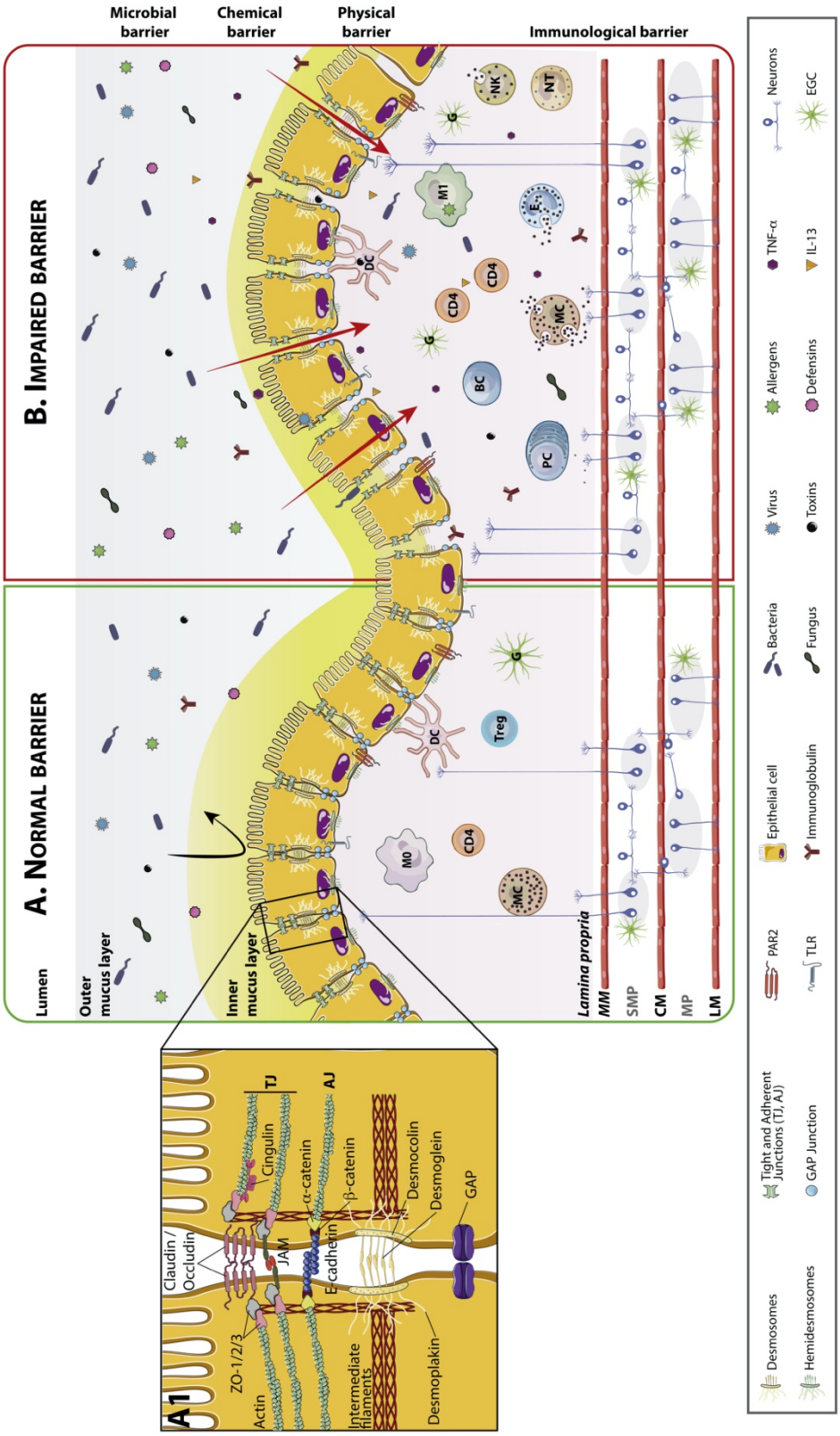

Figure 1 | Intestinal epithelial barrier anatomy and its constituents under proper or altered barrier function. AJ: Adherens junctions; BC: B Cell; CD4: Lymphocyte T helper CD4+; CLDN: Claudin; CM: Circular muscle; D: Desmosomes; DC, Dendritic cell; EGC, Enteric glial cell; ENS, Enteric nervous system; IL-13, Interleukin 13; JAM, Junctional adhesion molecule; LM, Longitudinal muscle; M0, Macrophages type 0; M1, Macrophages type 1; MC, Mast cell; MM, Muscularis mucosae; MP, Myenteric plexus; NK, Natural killer; NT, Neutrophil; OCLN, occludin; PC, Plasma cell; SMP, Submucous plexus; TJ, Tight junctions; TNF-α, Tumor necrosis factor alpha; Treg, T regulatory lymphocyte.

2. THE ENTERIC NERVOUS SYSTEM

The GI tract is characterised by having an intrinsic and widespread nervous system called the ENS. It is the largest and most complex division of the autonomic nervous system (ANS) and is responsible for maintaining proper gastrointestinal function orchestrating the reflex behaviours of the intestine. This system controls very diverse functions, including the intestinal motility, the exchange of fluids, the blood flow and the secretion of intestinal hormones. In addition, it interacts with the immune and endocrine systems, the gut microbiota as well as it is involved in the gut-brain axis and neuro-epithelial interactions (36–40). A remarkable aspect of the ENS is its autonomy from the central nervous system (CNS), reaching 80% of the digestive processes, reason why it is known as the "second brain" or "brain of the gut" (41,42). It has a structure and neurochemistry similar to the CNS, and, in fact, it is more similar in size, complexity and autonomy to the CNS than other elements of the ANS (38,43).

The ENS constitutes an intrinsic neuronal circuit, which receives extrinsic innervation from the CNS and ANS. It contains different types of neurons surrounded by EGCs, all distributed along the GI tract (from esophagus to the rectum) and grouped in ganglia located mainly in two nerve plexuses (Meissner and Auerbach), interconnected with each other. Their prolongations innervate the muscular layers as well as the lamina *propria* and the epithelial basolateral part of the mucosa (**Figure 1**). Neuronal bodies and EGCs situated in the plexuses are close to nerve fibers from the parasympathetic and sympathetic systems, which exert an efferent signalling from ANS. Parasympathetic fibers have an excitatory effect meanwhile sympathetic fibers have an inhibitory one. However, 90% vagal fibers (main innervation from parasympathetic system in small bowel) between the gut and the brain are afferent, highlighting the bidirectional connection with the brain, which seems to be more a receiver than a transmitter (7,8,38,43,44). Thus, the vagal afferent pathways regulate the activity of the hypothalamic–pituitary–adrenal axis coordinating adaptive responses to stressful stimuli of any kind (45,46).

For carrying out its function, the ENS releases a wide variety of neurotransmitters and neuropeptides (47,48) which are able to facilitate the recruitment, proliferation and differentiation of the intestinal immune populations. At the same time, the immune cells can modulate the distribution and function of the mucosal nerve endings, which are also surrounded by blood vessels and fibroblasts, highlighting the importance of **neuro-immune interactions**, particularly at barrier surfaces (49–51).

Neuronal-glial-epithelial interactions are also relevant, as neurons and EGCs interact with epithelial cells to sense chemical and mechanical stimuli from the gut, monitor luminal contents and respond to environmental stimuli by receiving and sending signals to and from the epithelium (52,53).

Therefore, the neural control of bowel function is an integrated system of local reflexes in the gut, the sympathetic and parasympathetic ganglia, and others that go from the gut to the brain and back through the CNS. All this network is traditionally referred to as the **gut-brain axis** (54). In addition, the intestinal microbiota can modulate ENS and CNS development and function through neural, endocrine, immune and humoral signalling. Gut microbiota is able to influence emotional and cognitive centers of the brain as well as the CNS can change microbiota composition probably disturbing the mucosal environment (55,56). Hence, there is a bidirectional communication mediated by microbial molecules and metabolites which defines the so-named **microbiota-gut-brain axis** (57). Its mechanisms are complex and poorly understood since it is affected by a wide variety of factors: diet, genetics, drugs, environment, exercise, cognitive behaviour, stress, social interactions, and fear (58). Functional GI disorders have been re-defined as disorders of gut-brain interaction (59) and emerging evidence shows that alterations in this interaction include gut microbiota (60). In clinical practice, IBS would be the perfect example of disruption of this microbiota-gut-brain communication, switching the way of understanding its pathophysiology (61).

In addition, another relevant concept is the phenomenon of neural plasticity or neuroplasticity at the level of the ENS. This term expresses the adaptive capacity of the nervous system to minimize the effects of injury by modifying its own structural and functional organisation in response to tissue damage (62–64). More recently, the term “gliosis” has been used to describe the reactive EGC phenotype in response to any type of injury (65).

The regulation of intestinal function involves ANS, CNS, hypothalamic–pituitary–adrenal axis and ENS. This wide range of communicated pathways through the microbiota-gut-brain axis allow the brain to react to stimuli and, consequently, to modify ANS and ENS, keeping intestinal permeability. Hence, this bidirectional signalling is regulated at neural, hormonal, and immunological levels and disturbances in these interactions have been associated with a multitude of intestinal inflammatory diseases and functional disorders (**Figure 2**). Nevertheless, the involvement of an impaired ENS in the aetiology of intestinal diseases is not fully understood and could be a consequence of other insults such as inflammation or an

imbalance in its interactions with the intestinal epithelium, immune system and microbiota (44,66).

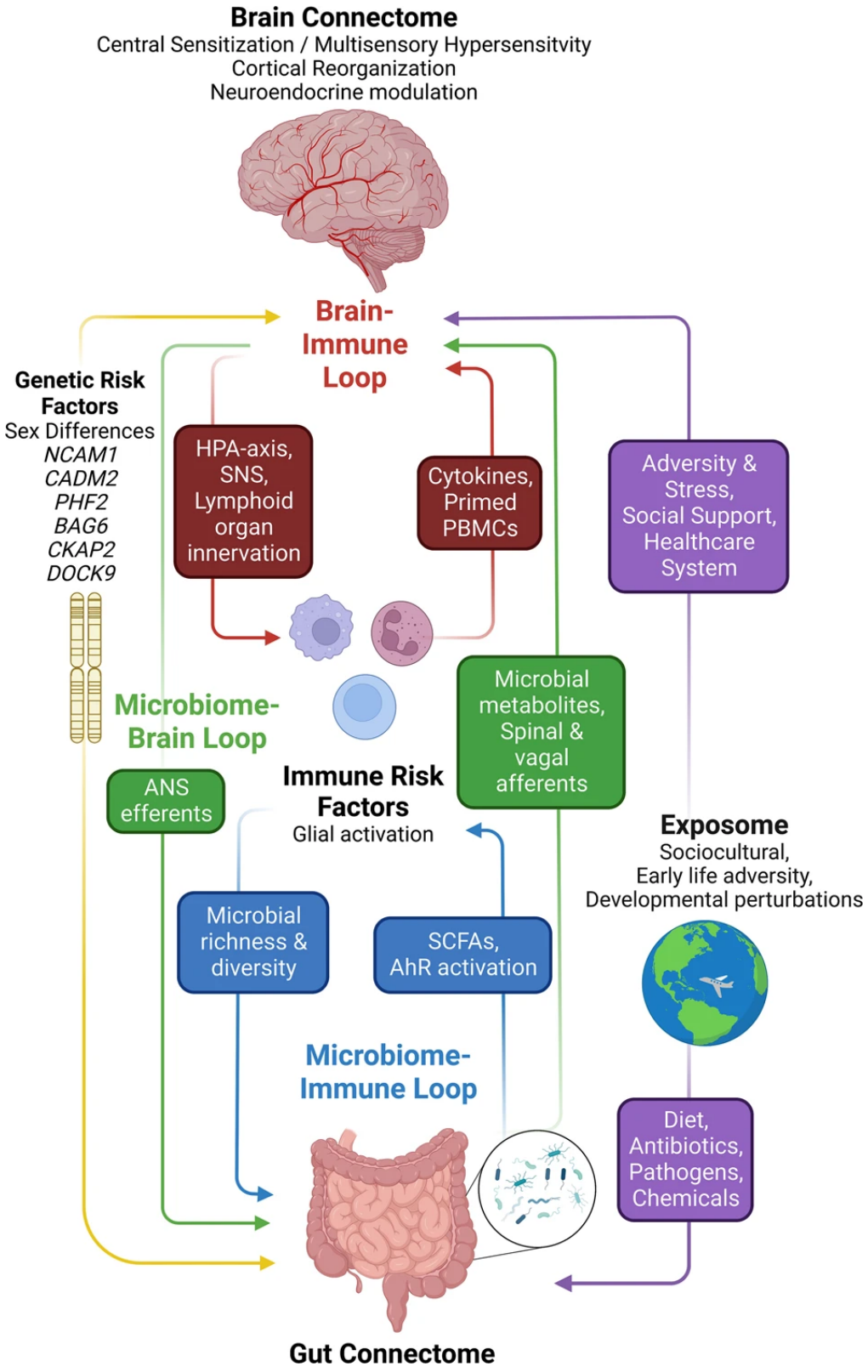

Figure 2 | Microbiota-gut-brain axis. The bi-directional communication between the brain and the gut is an integrated network that includes the ENS, the CNS, the immune system, the gut microbiota and the external environment. ANS: Autonomic nervous system; SNS: Sympathetic Nervous System; PBMCs: Peripheral Blood Mononuclear Cells; SCFAs: Short Chain Fatty Acids; AhR: Aryl hydrocarbon Receptor (67).

2.1. Enteric glial cell

In recent decades, EGCs have emerged as a central element in the field of neurogastroenterology. This cell has traditionally been described as a support cell for enteric neurons, playing its primary role in the structural integrity and feeding of the ENS. However, several studies have challenged this secondary function of EGCs, pointing to this cell as a critical regulator of gastrointestinal function contributing to health and disease, playing a fundamental role in the regulation of the intestinal physiological function, but also in gastrointestinal pathologies (68,69).

The first reference to enteric glia dates from 1899, when Dogiel studied the ganglia in the intestinal plexuses (70), however, due to the lack of methods to study these cells, the concept of enteric glia as a distinct class of cells did not emerge until 1981, when Gabella described them as **EGCs**, a unique population of peripheral glia found in the intestine (71). Morphologically, they share the stellate, branched structure of astrocytes of the CNS, with complex processes and a small cell body that is almost entirely filled by the nucleus, and at the molecular level express several molecules highly restricted to astrocytes (72–74). Functionally, both cells play an essential role in modifying the neuronal environment as well as in the neuron-glia communication. Nevertheless, the origin of these two cells is different, astrocytes develop from neural tube progenitor while EGCs are derived from the neural crest cells, which appear in the embryogenesis from the sacral and vagal neural crest and spread through the GI tract (75). Since EGCs have their own molecular characteristics and form one of the main populations of peripheral glia, they have been classified as a distinct category of peripheral glial cells, apart from Schwann and satellite cells.

2.1.1. Phenotype of enteric glia

There are three widely used molecular markers for EGC: two cytoplasmic proteins, the intermediate filament **glial fibrillary acidic protein (GFAP)** and the **Ca^{+2}-binding protein S100β**, and the **nuclear transcription factor SOX10**. GFAP and S100β are expressed by EGC precursors and differentiated EGCs, whereas SOX10 can be expressed by ENS precursors as well as immature and mature EGCs (76–81). GFAP is widely used for morphological characterisation of EGCs to follow glial processes, although it is not useful for labelling cell bodies or nuclei, so it cannot be used to quantify EGC numbers. For this, Sox10 is the most suitable marker as it labels glial nuclei but does not provide details of glial morphology. On the other hand, S100β detects glial cytoplasm and is an effective marker for glial cell bodies.

However, its expression in the intestine is not exclusive to enteric glia and, depending on the conditions, it can be extruded from glial cells (82).

The phenotype of EGCs is therefore very complex and diverse. These cells are capable of expressing **receptors** for the vast majority of **intestinal neurotransmitters**, particularly those for nucleotides (ADP, ATP, UTP and A2B receptors). The EGC is therefore able to detect extracellular purines in the ENS, as these molecules are essential elements for the function of the EGC. Among other **neuromodulator receptors** expressed by glia are those for norepinephrine (α2a adrenergic receptors), glutamate (mGluR5) and acetylcholine (mAChRs). In addition, EGCs have **receptors for bioactive lipids** (SP1R, LPA1), **endothelin** (ETB receptors), **proteases** (PAR1, PAR2) and **bradikynin** (B2 receptors), although its functional significance is unclear. On the other hand, EGCs can degrade neuroactive compounds through the expression of **neurotransmitter transporters** (peptide transporter, PEPT2, and GABA transporter, GAT2) and **cell-surface enzymes** (ectonucleotidase, eNTPDase2), being remarkable the role of eNTPDase2 hydrolysing ATP to ADP. Furthermore, EGC expresses **cell surface ion channels** (voltage-gated sodium, potassium, connexin-43 and aquaporin-4 channels), **TrkB** (the receptor for the neurotrophins brain-derived neurotrophic factor (BDNF) and NT-4), **toll-like receptors** and the **major histocompatibility complex class II**, showing the interaction of this cell with the ENS and the innate and adaptive immune system. At the same time, EGCs release a wide variety of molecules that regulate neighbouring cells, including **purines**, **nitric oxide**, **antioxidants**, **prostaglandins** and **cytokines**. Thus, depending on the environment, physiological or pathological, this cell has the ability to release beneficial or toxic compounds (82).

2.1.2. Heterogeneity and plasticity of enteric glial cell

The large heterogeneity of the EGC was not assessed properly until 2015 by Boesmans *et al.* using high-resolution genetic labelling (83). They identified four subtypes of EGCs in the myenteric plexus of mice, depending on their morphology and location. Type-I reside within the ganglia of the plexuses, and have multiple, irregularly branched processes connecting neurons. The interganglionic Type-II, present within nerve fiber bundles and are characterised by lanky processes. Type-$III_{MP/SMP}$, form a matrix in the extraganglionic region, interacting with neuronal fibres and small blood vessels. They are typically characterised by four main processes with secondary branches. In the lamina *propria* of the mucosa, Type-III_{MUCOSA} are resident with a similar morphology following either nerve fibers or terminating

at the epithelium (84). Type-IV, associated with nerve fibers that are located within the circular and longitudinal muscle layers.

Depending on the microenvironment in which they reside (part of the GI tract) and the signals they receive from it (physiological context), glia-specific gene expression can vary, demonstrating the functional specialisation of this cell type. Thus, a huge diversity of EGCs expressing combinations of GFAP, S100β and SOX10 has been described, both within the same subtype population and between subtypes. Moreover, differences in the susceptibility to purinergic stimulation have also been described (83). Considering developmental studies have not been able to find different EGCs progenitors but markers point to a common progenitor which express SOX10, it seems EGCs populations have the same origin (40,75,83). Thus, the adaptive capacity of this cell is not only found in the embryonic or postnatal stages, but is maintained throughout adulthood, demonstrating the **high plasticity of the EGC**, which is able to adapt its phenotype and molecular characteristics in response to stimuli. Indeed, when EGCs are isolated from their environment and cultured *in vitro*, the levels of glial expression markers change as a consequence of the loss of and interactions with the environment (83). As the intestine is constantly adapting to the microenvironment of the lumen, the variety of signals to which EGCs are exposed ranges from nutrients, commensal bacteria, pathogenic microbes, mechanical factors to disease.

2.1.3. Functional roles of enteric glial cell

EGCs are important elements for maintaining the intestinal homeostasis. They regulate intestinal motility cooperating with enteric neurons. They also interact with several non-neuronal cell types, notably immune cells and enterocytes, and thus play an essential role in the regulation of intestinal immunity and neuro-immunomodulation, as well as epithelial barrier function. In addition, they can interact with muscle cells, vasculature and enteroendocrine cells, although the physiological implications of these interactions are not yet clear (85). Moreover, it exists a bidirectional interaction between EGCs, particularly those in the mucosa, and the gut microbiota (86,87).

- **Regulation of gut motility and neuro-glia interaction**

EGCs have an active role in enteric reflexes, thereby controlling intestinal motility. There is an interplay between enteric neurons and EGCs based on the release of neurotransmitters/neuromodulators during synaptic communication, with transient increases in intracellular calcium concentration ($[Ca^{+2}]$) being the more studied mechanism

governing EGC excitability (88–91). Not only can EGCs respond to neurons, but EGCs can also signal to neurons, and indeed glial responses alone are sufficient to drive intestinal motility through direct actions on neurons (92–94), pointing to EGCs as an element to consider in motility disorders. However, although it is clear that EGC is involved in the regulation of intestinal motility, how gliotransmission regulates the neuronal control of intestinal motility and its significance is unclear. Furthermore, it is important to consider that EGCs also interact with non-neuronal cells that are important for peristalsis, such as interstitial cells of Cajal and smooth muscle, and they also interact with enteroendocrine cells (95,96). All of these interactions will play a role in gut function, although how they contribute to enteric reflexes is not known.

- **Modulation of epithelial barrier function**

Due to their proximity to IECs, EGCs have been described as essential regulators of physiological processes at the intestinal-mucosal interface. EGCs have been shown to directly control epithelial barrier function, as ablation of EGCs in mice resulted in a significant loss of epithelial barrier function (97–99). Subsequently, several *in vitro* models showed that EGC-derived mediators affect epithelial barrier function by acting on epithelial cells, termed the "**neuronal-glial-epithelial unit**" (100). The list of EGC mediators that communicate with the epithelium is diverse, with different effects on barrier function through different mechanisms. EGC mediators can have either beneficial or detrimental effects on epithelial cells, highlighting the **dual role of EGCs in barrier function** (52). At the same time, the epithelium signals to the lamina *propria*, where EGCs are located (**Figure 3**).

In more detail, **under physiological conditions**, EGC signals to the epithelium to maintain barrier function via the release of glial-derived neurotrophic factor (GDNF), S-nitrosoglutathione, transforming growth factor beta-1, 15-deoxy-delta-12,14-prostaglandin J2, pro-epidermal growth factor and 15-hydroxyeicosatetraenoic acid. In this context, the bidirectional communication between microbiota and EGCs is essential for maintaining intestinal homeostasis modulating the apoptosis, proliferation and differentiation of IECs as well as the release of pro-inflammatory cytokines from IECs through the EGC-derived mediators. **Under pathological conditions**, such as chronic intestinal inflammation, which is normally associated with an increase in intestinal permeability and a change in the composition of the microbiota, luminal antigens are able to reach the lamina *propria* and trigger a pro-inflammatory response that activates the EGC. As a result, EGC further disrupts epithelial barrier function by releasing S100β, nitric oxide, nerve growth factor (NGF), GDNF,

IL-6 and IL-1β. At the same time, this activated EGC undergoes not only functional but also morphological changes, becoming more proliferative and hypertrophic. Thus, unlike the neuron, which "only" releases mediators to the epithelium, the EGC shows a plasticity that could play a role in switching from a protective to a harmful function, making it the most chameleon-like cell in the gut (52,53). Nevertheless, it also exists conflicting literature, as it has been described that EGC is not necessary for the modulation of barrier function after selective removal or alteration of its activity (101,102).

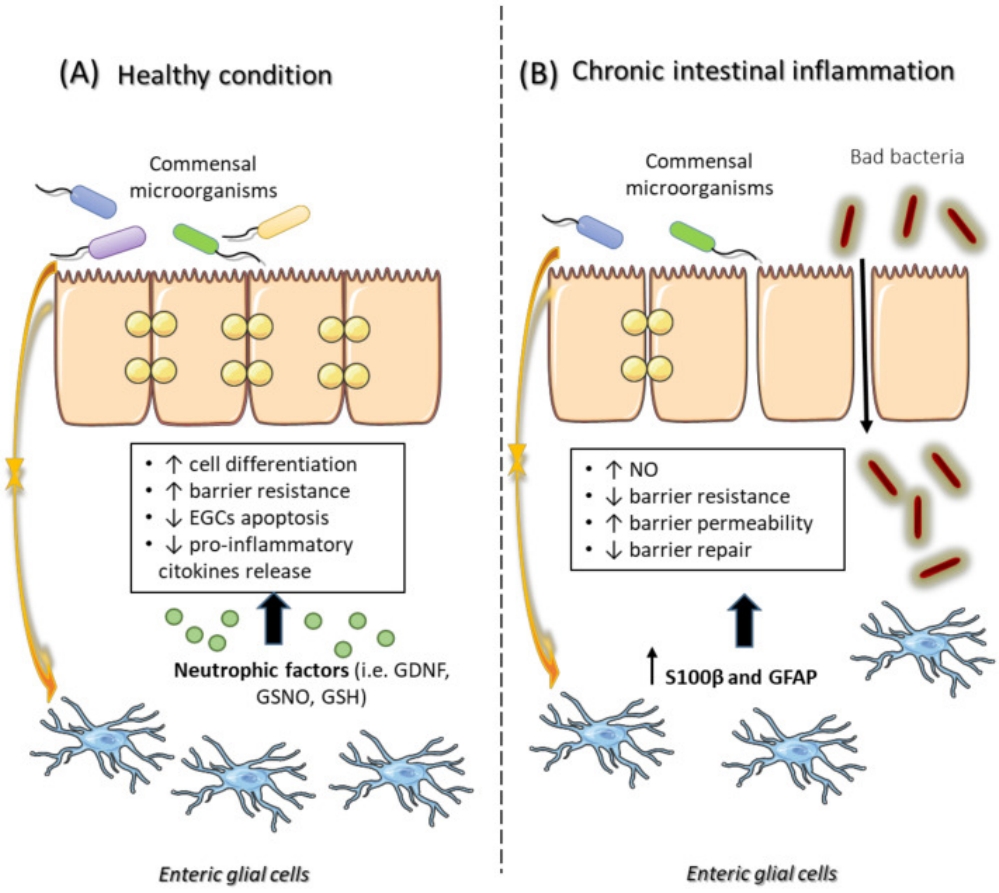

Figure 3 | Dual role of EGCs in intestinal barrier function under healthy or chronic inflammatory conditions. A) Under physiological conditions, EGCs interact with the intestinal microbiota to maintain GI homeostasis and modulate IECs function through various glial mediators to preserve barrier function. **B)** Under pathological conditions, where changes in the intestinal microbiota and an increased permeability are present, translocation of bacteria to the lamina *propria* occurs, triggering an inflammatory response that activates EGCs, which secrete harmful mediators that disrupt epithelial barrier function. EGCs: Enteric Glial Cells; GDNF: Glial cell-Derived Neurotrophic Factor; GFAP: Glial Fibrillary Acidic Protein; GSH: Glutathione; GSNO: Glial-derived S-Nitrosoglutathione; NO: Nitric Oxide (53).

Considering these growing number of studies highlighting the importance of the EGC in epithelial barrier function, characterizing the phenotype and contribution of this cell as well as its derived mediators in pathologies with different grades of epithelial barrier dysfunction and intestinal inflammation may open up great opportunities in the field of neuro-immune gastroenterology in order to find new targets to control barrier function.

- **Immune capacity of EGC**

EGCs actively participate in intestinal immunity, although their role in intestinal immune homeostasis is not fully understood. Although EGCs are non-immune cells, they are capable of secreting and responding to inflammatory mediators. In response to pathogens, EGCs are able to produce several cytokines (IFN-γ, IL-1β, IL-6 and CCL2) (103,104) as they express toll-like receptors (105,106). Recently, they have also been described to regulate the activity of muscularis macrophages secreting colony-stimulating factor or via Cxc110- IFN-γ (107–109). At the same time, EGCs can respond to these cytokines derived from immune cells increasing the inflammation, indeed IL-1β triggers the glia release of IL-6 and CCL2 while IFN-γ+LPS triggers that of IL-1β and IL-18 (110–113). In addition, GDNF release by EGCs can engage type 3 innate lymphoid cells, stimulating the secretion of IL-22 and participating in mucosal homeostasis (114). On the other hand, EGCS also exert a role in the adaptive immune system by presenting antigens to CD4+ T cells via MHC-I and MHC-II (115,116). They also inhibit the proliferation and activity of CD8+ T cells (117) and express the immunosuppressive checkpoint protein CD200 (118). Finally, EGCs may exert an immunomodulatory effect on enteric neurons, as enteric neurons have been described to exert specific immune responses (49,119). In light of all this evidence, it is not surprising that EGCs play a potential role in various immunological intestinal disorders, such as inflammatory bowel disease, celiac disease and autoimmune enteropathy. Nevertheless, the immune capacity of EGCs remains further research to elucidate their mechanisms as well as the significance of their alterations in a disease context (120,121).

In summary, EGCs are able to control intestinal motility by acting directly on neurons, they play a relevant role in the homeostasis of the intestinal barrier through the release of several mediators and their interaction with the microbiota, and they contribute to the intestinal immune response through neuro-immune interactions. It also highlights their role in neuroprotection as well as modulation of synaptic transmission and, most importantly, their capacity for neogenesis of enteric neurons in response to tissue damage (81,122). Given all this, it is not surprising that impaired EGC activity contributes to GI pathophysiology; it has been associated with several GI disorders, but also with other diseases along the gut-brain axis such as Parkinson's disease or multiple sclerosis. Thus, EGCs have been postulated as a critical link between gut health and disease, as well as a target for intestinal disease. However, its involvement in the aetiology of gut disease and the mechanisms contributing to it are not fully understood and may be a consequence of other insults (65,66,69,123). Furthermore, most information on the changes that glia undergo in digestive pathologies is limited to irritable bowel disease, where there is a reactive activation of the inflammatory response (68,124).

Finally, a reactive glial phenotype known as 'gliosis', commonly used to define reactive astrocytes, has recently been used to describe EGCs that respond to any type of injury. It encompasses several dynamic states that are normally associated with upregulation of the inflammatory response and changes in the morphology of EGCs and the expression of their markers. It is not known whether these 'reactive' EGCs play a role in disease pathogenesis, exert beneficial effects to maintain homeostasis, or whether this reactivity is simply a reflection of disease (44,65,69,125,126).

3. BILE ACIDS

In ancient Greece, the theory of the four humours was an important development in medical knowledge. The Greeks believed that the healthy body was a system of four fluids 'humours' in balance: black bile, yellow bile, phlegm and blood. Thus, the role of bile acids (BAs) in human pathophysiology has been postulated since ancient times.

BAs are a large family of atypical steroids, products from cholesterol metabolism, with a peculiar amphipathic structure that gives them detergent properties essential for the digestion and absorption of lipids and fat-soluble vitamins (127). However, research during the last two decades suggest that BAs are signalling molecules, as BA receptors (BARs) have been described in several cell types throughout the body. Thus, beside their function as detergents, these bioactive metabolites exert multiple physiological functions in different organs via their specific BARs, each of which has a specific BA ligand, and they are recognised as mediators of microbiome-gut-brain axis signalling. Molecular studies in the gut have described these receptors on immune and epithelial cells, explaining why BAs act as immunomodulatory factors and have both physiological and pathophysiological roles in the intestinal epithelial barrier. Therefore, BAs regulate several GI functions, and alterations in the composition of the BA pool, as well as impaired BA homeostasis and signalling, have been described to contribute to the pathogenesis of several GI diseases (128–131). Besides, high levels of BAs due to malabsorption leads to diarrhoea (132,133). Given the complexity of the GI tract and the signalling network induced by BAs, the role of BAs on gut function deserves special attention and BAs and their receptors have become an interesting therapeutic target.

3.1. Synthesis and enterohepatic circulation of bile acids

The liver is the primary site for the synthesis of BAs, which enter the enterohepatic circulation, cycling between the liver and intestine via the portal vein 4-12 times per day. Recycling of BAs is very efficient, as only 5% of the total BA pool is lost in the faeces each day, while the remaining 95% is actively reabsorbed in the terminal ileum. Thus, the small amount of BAs excreted in the faeces can be replaced daily by hepatic BA synthesis. The **two primary BAs**, **cholic acid (CA)** and **chenodeoxycholic acid (CDCA)**, are synthesised by human hepatocytes from cholesterol by two different pathways involving several cytochrome P450 (CYP) enzymes: the classical (or neutral) and the alternative (or acidic) pathways (129,134). The majority of total BA synthesis occurs via the classical pathway, where cholesterol is metabolised to 7α-hydroxycholesterol by cholesterol 7α-hydroxylase (CYP7A1), which acts as

the rate-limiting enzyme in this process (135). The 7α-hydroxycholesterol is then metabolised to CA by CYP8B1 or to CDCA by CYP27A1. In the alternative pathway, accounting for 10% of total BA synthesis, CYP27A1 converts cholesterol to 27-hydroxycholesterol, which is then metabolised to CDCA by CYP7B1 (136). Interestingly, in the brain, there is another pathway necessary for neuronal cholesterol clearance, which, after conversion by CYP46A1, is transported to the liver and contributes to CDCA synthesis (137) (**Figure 4**). The final step in both hepatic pathways is the conjugation of primary BAs to the amino acids taurine or glycine by the BA coenzyme A amino acid N-acetyltransferase, followed by biliary excretion. In humans, the conjugation with glycine predominates over taurine in a ratio of approximately 3:1, which can be modified by several factors including diet and intestinal microbiota (138–140). This is an important step as the resulting BAs are impermeable to epithelial cell membranes, thereby retaining their amphipathic properties, which are essential for lipid emulsification in the proximal small intestine (141). Therefore, conjugated BAs require active transport systems to cross cell membranes, such as the apical sodium-dependent BA transporter (ASBT) and the sodium taurocholate co-transporting polypeptide (NTCP) (142), which is mainly present in enterocytes of terminal ileum. BAs are transported from the liver through the biliary tree to the gallbladder for storage. Upon ingestion, they are released into the small intestine to allow for lipid digestion and absorption.

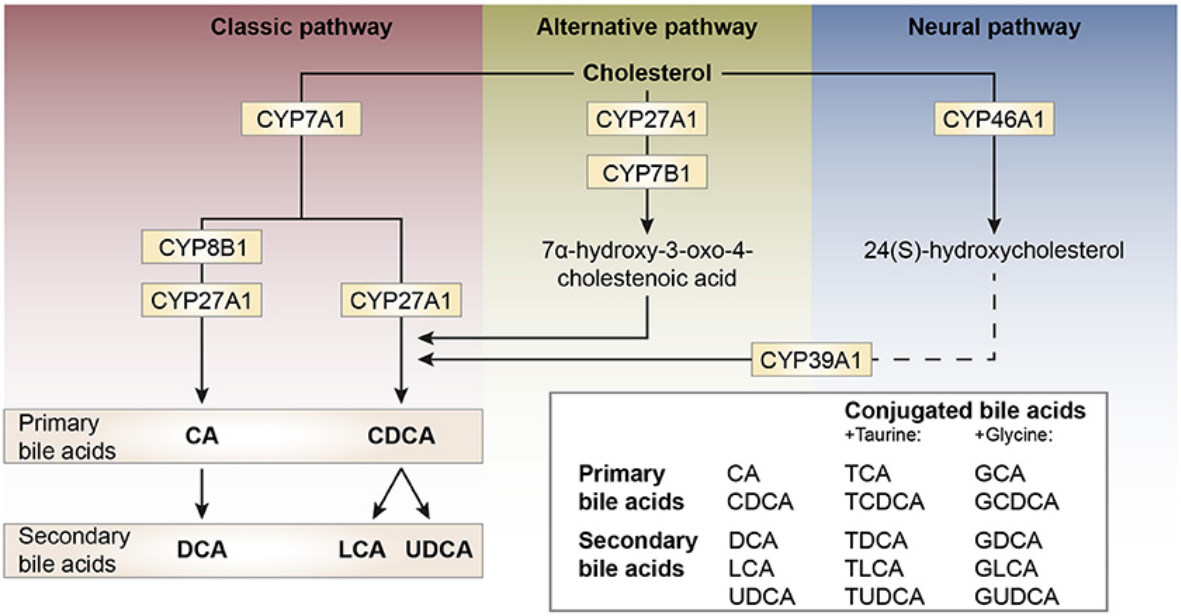

Figure 4 | Human BAs synthesis pathways. CA: Cholic Acid; CDCA: Chenodeoxycholic Acid; CYP7A1: cholesterol 7α-hydroxylase; CYP7B1: 25-hydroxycholesterol 7α-hydroxylase; CYP8B1: sterol 12α-hydroxylase; CYP27A1: sterol 27-hydrolase; CYP39A1: oxysterol 7-α-hydroxylase 2; CYP46A1: cholesterol 24-hydroxylase; DCA: Deoxycholic Acid; G: Glycine; LCA: Lithocholic Acid; T: Taurine; UDCA: Ursodeoxycholic Acid (138).

After the synthesis of CA and CDCA and conjugation with glycine or taurine, they are excreted in the bile and then in the intestine. Along the intestine, the intestinal microbiota carries out various modifications of their structure: deconjugation to remove the amino acids, glycine and taurine, and dihydroxylation to produce secondary BAs, **deoxycholic acid (DCA**) from CA and **lithocholic acid (LCA)** and **ursodeoxycholic acid (UDCA)** from CDCA. Several types of BAs can therefore be found in the GI tract, primary and secondary, unconjugated or conjugated, depending on the segment of the GI tract and the resident microbiota (**Figure 4**) (134,143).

In the ileum, the majority of conjugated BAs are reabsorbed by active transport through ASBT, which is expressed in the brush border of ileal epithelial cells. In the cytoplasm, conjugated BAs bind to the ileal BA-binding protein and are transported via the heterodimeric transporter OST α/β across the basolateral membrane of the enterocyte into the portal venous circulation, and return to the liver where they are taken up by NTCP in a Na^+-dependent active transport mechanism. Unconjugated BAs, deconjugated by small intestinal microbiota, cross the enterocyte throughout the intestine by nonionic passive diffusion and are taken up in the liver in a Na^+-independent manner by organic anion-transporting polypeptides. The rate of BA synthesis is regulated by hormone-like signalling, as in both hepatocytes and enterocytes, BAs bind to the nuclear farnesenoid X receptor (FXR), switching on the expression of the small heterodimer partner and fibroblast growth factor 19 (FGF19), respectively. Thus, there is a direct feedback inhibition of hepatic BA synthesis via hepatic small heterodimer partner-mediated repression of CYP7A1, the rate-limiting enzyme, and via FGF19 secreted from the ileum.

The 5% of primary BAs that are not reabsorbed in the ileum reach the colon where they are metabolised by the resident microbiota to secondary BAs, DCA and LCA. The first step that occurs is the deconjugation of the amino acids glycine or taurine by bile salt hydrolase (BSH), enzymes expressed in bacteria, which dramatically changes the physicochemical properties BAs, making them more lipophilic and partially protonated to facilitate further metabolism by dehydroxylases. Thus, in second place, 7α-dehydroxylation occurs, which transforms BAs into more hydrophobic molecules, reducing their ionisation and allowing BA reabsorption by passive diffusion through colonocytes (129,144,145) (**Figure 5**). It is important to note that although the majority of secondary BAs are produced in the colon, where the microbial density is highest. However, the bacterial enzymes of the microbiota community located in the small intestine, mainly in the distal part, can also be involved in the transformation into secondary BAs.

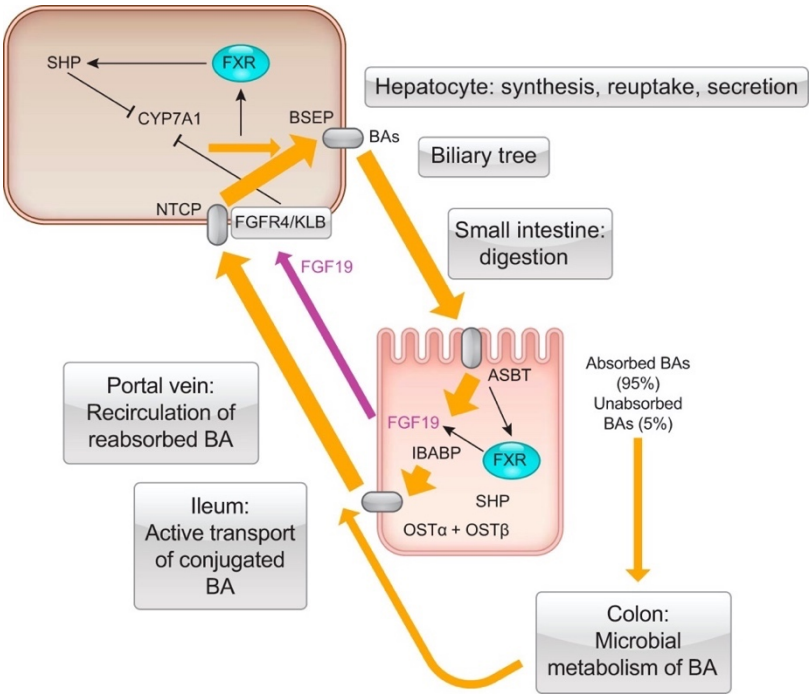

Figure 5 | Enterohepatic circulation of BAs. ASBT: Apical Bile Salt Transporter; BA: Bile Acid; BSEP: Bile Salt Export Pump; FGF19: Fibroblast Growth Factor 19; FGFR4: FGF Receptor 4; FXR: Farnesoid X Receptor; IBABP: Ileal Bile Acid Binding Protein; KLB: Klotho-Beta; NTCP: Na+-taurocholate polypeptide; SHP: Short Heterodimer Partner-1 (144).

3.2. Bile acids and microbiota

The human gut harbours a complex microbial community consisting of trillions of bacteria that convert primary BAs to secondary BAs in the colon through deconjugation and dihydroxylation, making them more hydrophobic molecules that can passively diffuse through colonocytes, as there is no evidence for the presence of BAs transporters on the colonic apical side (146,147). Thus, BAs are biotransformed by the resident microbiota which determines their diversity with different bioactivity and bioavailability of the individual BAs. Microbial deconjugation is carried out by BSH, enzymes present in all major anaerobic bacterial divisions and archaea species in the human intestine including *Bacteroides*, *Clostridium*, *Lactobacillus*, and *Bifidobacteria*. The enzyme 7α-dehydroxylase is expressed by *Clostridium* and *Eubacterium* and carries out the 7α-dehydroxylation on ring B to produce secondary BAs (134,143). In addition, other forms of BA result from bacterial metabolism, known as oxo BAs, which account for approximately 20-30% of gut microbiota-derived BA in the colon (148), depending on their profile in relation to intestinal transit time and the composition of the intestinal microbiome. Recently, new forms of conjugated, BAs with amino acids other than glycine and taurine have been found in humans as a product of microbiota conversion with the ability to re-conjugate BAs with other amino acids including leucine, tyrosine, and phenylalanine, in addition to the classical conversion, known as microbially conjugated BAs (149,150).

The microbiome is essential for the host's metabolism, nutrition and immune system, and alterations in the microbiome (dysbiosis) affect host health and may be one of the driving factors in the pathogenesis of a range of diseases, including intestinal and extra-intestinal disorders. (151–153). In addition, the composition of gut microbiota can change from birth and is highly dynamic, as it can be influenced by several factors, including geographical area, sex, age, the mode of delivery at birth (caesarean or vaginal), diet during infancy (breast or formula feeding) and in adulthood, environment, fitness level, psychological state or the use of antibiotics, among others (154–160). Thus, BAs homeostasis is also influenced by these factors, as changes in the microbiome trigger changes in BAs metabolism, further altering the physicochemical properties of the BAs pool (161,162).

Therefore, there is an interaction between the microbiota and BAs that influences intestinal physiology and pathophysiology. The microbiota is responsible for the biotransformation of BAs, but BAs in turn have also antimicrobial activity that shapes the intestinal bacterial profile, as their detergent-like action damages bacterial cell membranes and inhibits bacterial overgrowth (163,164). In addition, it has been described that BAs are also able to regulate the composition and overgrowth of the microbiome through FXR and the takeda G protein-coupled receptor 5 (TGR5), which exert an anti-inflammatory effect in the liver and intestine (165). Hence, the microbial fingerprint determines the BAs pool, and given that BAs are modulators of intestinal barrier function and immune and nervous system activation, dysregulation of microbiota-BAs interactions can contribute to intestinal and metabolic diseases. In addition, disease progression may also affect the microbiota, raising the question of whether changes in the microbiome and BAs signatures are a cause or a consequence of disease (144).

3.3. Bile acid receptors in the intestinal epithelium

BAs activate both nuclear receptors and plasma membrane associated receptors expressed in IECs. Nuclear BARs are: **FXR**, vitamin D receptor (**VDR**), pregnane X receptor (**PXR**), constitutive androstane receptor and RAR-related orphan receptor gamma t. Of these receptors, only BAs are the main ligands for FXR. On the other hand, G-protein-coupled receptors at the plasma membrane are: **TGR5**, the **M3R** muscarinic receptor, and the sphingosine-1-phosphate receptor 2 (**S1PR2**) (128,129). In addition to direct binding to their receptors, BAs can also mediate epithelial responses by recruiting "non-BARs", highlighting the activation of the epidermal growth factor receptor to regulate intestinal cell growth by TGR5 (166), CHRM3 (167), FXR (168) and VDR (169).

Several lines of evidence show how BAs can induce a rapid and acute response in epithelial cells by activating several receptors on the cell membrane (such as TGR5, M3R or epidermal growth factor receptor) or by inducing membrane perturbations. This leads to changes in intracellular second messengers and activation of signalling pathways. These cascades can also cause longer-term changes that activate transcription factors and regulate gene transcription. In addition, these changes in gene and protein expression can be triggered by direct activation of nuclear BARs (FXR, PXR and VDR) (144). Thus, luminal BAs are capable of altering epithelial structure and intestinal permeability, leading to epithelial barrier dysfunction. There is limited and conflicting evidence on the function of BARs in IECs, and most studies of BAs on IECs have described the effect of BAs on intestinal barrier function through inflammatory changes and alterations in TJPs. However, how BAs interact with IECs, their effect through modulation of the expression of BARs and the underlying molecular mechanisms of the interaction are not well understood (130,131,170). Based on the literature, we will focus on FXR, VDR, TGR5 and M3R as the most promising BARs to play a role in intestinal barrier function.

3.3.1. Farnesoid X receptor

FXR also known as nuclear receptor subfamily 1 group H member 4 (NR1H4) was the first BAR described and its activation regulates the canonical pathways triggered by BAs. BAs are its endogenous ligands including (with decreasing affinity): CDCA>DCA>LCA>CA (171,172). FXR is essential for the maintenance of cholesterol, BAs and energy homeostasis. Indeed, intestinal FXR is crucial for the regulation of hepatic BA synthesis via the induction of FGF19 as a negative feedback mechanism. An extensive bibliography describes the role of FXR in innate and adaptive immunity, as well as its involvement in intestinal inflammation of chronic inflammatory diseases of the gut (173–175). It also plays a role in barrier function in both physiological and pathophysiological states. FXR activation prevents histological intestinal mucosal injury by inducing antibacterial effects and maintaining epithelial integrity. Thus, treatment of IECs with FXR agonists has an effect on TJPs and prevents reduction in villus length and decrease in transepithelial electrical resistance (TEER). However, from a functional point of view, there is conflicting literature as to whether it improves or damages epithelial barrier function (176). For all of this, FXR has been considered a therapeutic target for many GI diseases (177).

3.3.2. Vitamin D receptor

BAs are not the main ligands of the nuclear receptor VDR, as it is activated by Calcitriol (1,25-dihydroxyvitamin D3) and by the secondary BA LCA. Activation of VDR has been linked with increasing intestinal BA absorption and suppressing BA synthesis as well as to trigger the expression of enzymes involved in the detoxification of LCA (128). A growing body of literature suggests that VDR signalling plays a key role in several tissue barriers, including the gut, where VDR effects on the intestinal barrier function in health and disease (178,179). The function of VDR in the intestinal epithelium is not fully understood, but it has been shown to regulate TJPs protein expression (such as claudin 2) and mucosal inflammation exerting cytoprotective and anti-inflammatory actions in the intestine (180–184).

3.3.3. The Takeda G protein-coupled receptor 5

TGR5 also known as the G protein-coupled bile acid receptor 1 (**GPBAR1**) is a G-protein coupled receptor, broadly related to energy homeostasis maintenance and insulin secretion stimulation (185). BAs are its main ligands in the following order of affinity: LCA > DCA > CDCA > CA (186). There is a huge amount of literature on GPBAR1 involvement in GI functions, highlighting its role in intestinal electrolyte transport, gut motility and intestinal inflammation in health and disease (128) but its role on intestinal barrier function is not clear. Recently, its activation has been involved in LCA-mediated proliferation of intestinal stem cells and DCA-mediated suppression of wound healing (187,188).

3.3.4. The muscarinic receptor M3R

Earlier studies demonstrated the interaction between BAs (DCA and LCA) and muscarinic receptors on epithelial cells (189,190). Thus, given the similarity in structure of BAs and acetylcholine, muscarinic receptors have a promising role in intestinal motility, and, indeed, the M3R also known as the cholinergic receptor muscarinic 3 (**CHRM3**) has recently been shown to inhibit contractile responses in mouse and human lung tissue (191). However, little is known about its role in modulating GI function. Limited evidence suggests its involvement in inducing IECs proliferation and promoting tumour progression (167,192).

3.4. Bile acid receptors in the nervous system

The modulation of ENS excitability or function can be affected directly or indirectly via microbe- or host-derived components or metabolites (57,58). Between them, BAs deserve

special attention as BAs have the ability to signal to the CNS exerting the effects via both direct and indirect mechanisms, and are therefore considered to be signalling molecules in the liver-gut-brain as well as the gut-brain axis (193). Outside of the enterohepatic circulation, BAs are also synthesised from cholesterol, and conjugated and unconjugated BAs have been detected in both rodent and human brains (194). Emerging evidence has shown that BA signalling might have a critical role in the regulation of physiological and pathological processes of the CNS. Recently, BAs have been implicated as potential factors involved in the development of neurodegenerative and neurological disorders as well as diseases of an immunological nature associated with neuroinflammation (137,195–198). In fact, BARs expression has been described along several CNS cells, including different types of neurons, glial cells and brain endothelial cells. Specifically, intestine-dominant BA cell nuclear and membrane receptors exist in the CNS, including FXR (199), glucocorticoid receptors (200,201), PXR (202), VDR (203,204) , GPBAR1 (205,206), S1PR2 (207,208) and CHRM3 (209,210), which indicates a role for BAs in the regulation of neurological function. However, most of the functions of the BARs in the brain or throughout the CNS are still under investigation and remain unclear.

Since the ENS and CNS share similar structure, function and signalling, the expression of BARs in the ENS deserves special attention. In the gut, the expression of only GPBAR1 and CHRM3 in myenteric neurons has been shown (211,212), while the expression of other BARs and the type of cellular signalling that is triggered remains to be investigated. Activation of these two receptors within the ENS contributes to the physiological regulation of GI motility. Thus, GPBAR1 in neurons triggers cellular signalling pathways that promote or inhibit colonic motility depending on the type of neuron (213), while CHRM3 mediates cholinergic contractions in the GI tract and its colonic regulation contributes to constipation (214–217). CRHM3 is also expressed by EGCs and its activation contributes to cholinergic pathways in the ENS, although the mechanisms that activate them remain to be elucidated (218). In addition, in a novel chemogenetic mouse model, the modified form of human CHRM3 (hM3Dq) under the transcriptional control of the GFAP promoter had an excitatory effect on enteric motor circuits, triggering changes in intestinal motility (92). However, whether enteric neurons or EGCs express more BARs remains unexplored, as does the role that the interaction of BAs and their receptors might play in ENS function.

4. IRRITABLE BOWEL SYNDROME

4.1. Definition, prevalence, symptoms and diagnosis

IBS is a chronic functional GI disorder with an estimated global prevalence of 4-5% (219,220). Establishing a single prevalence is not fully representative, as this data varies between countries and depends on the heterogeneity of studies (221,222). The condition is more common in women than in men, with a female to male ratio of 2-2.5:1 (223), and there is no evidence of an increased risk of mortality in people with IBS (224). IBS is characterised by the presence of abdominal pain and changes in stool consistency and/or frequency that significantly and chronically affect patients' quality of life (225,226). According to the predominant bowel pattern, patients with IBS can be divided into four subtypes: diarrhoea-predominant (IBS-D), constipation-predominant (IBS-C), a mixture of diarrhoea and constipation (IBS-M), and unclassified IBS (IBS-U) in patients with changes in the number of depositions per day but normal stool consistency (59). IBS-D is the most common subtype of IBS (31.5%), accounting for 1.4% of the global prevalence (227).

Despite its high prevalence and the high health and social costs associated with it, there are currently no specific and sensitive biomarkers for this disorder. The diagnosis of IBS is based on symptoms, using the Rome IV criteria and excluding other organic GI diseases with a similar clinical course (mainly celiac disease, inflammatory bowel disease, cancer and food allergy) (59). According to Rome IV, abdominal pain must have been present at least 1 day per week in the previous 3 months, with onset of symptoms at least 6 months before diagnosis, and be associated with two or more of the following features: pain associated with defecation, onset associated with a change in stool frequency, or onset associated with a change in stool appearance (25% of all stools with abnormal consistency) (228). In clinical practice, stool consistency is classified and assessed using the Bristol scale (229).

Despite intensive research, the aetiology and pathophysiology of IBS have not been identified, although our understanding of it has rapidly increased in recent years. The current treatment is targeted to relieve the predominant symptom and not at the factor implicated in the pathophysiology of IBS, which often brings to an ineffective management (230). Non-pharmacological therapeutic options for IBS-D include diet interventions, physical activity, and psychotherapy for a psychosomatic approach (231). Furthermore, treatment with antispasmodic and antidiarrhoeal drugs can help to reduce abdominal pain and to control bowel habits, respectively (232). In addition, a high proportion of patients with IBS also suffer

from other nongastrointestinal symptoms, including somatic disorders such as fibromyalgia and chronic fatigue, and mental disorders such as anxiety and depression, with a higher prevalence of mental disorders than healthy controls (233,234). This, in addition to the reduced effectiveness of available treatments, determines a significant deterioration in the quality of life of these patients and imposes a progressive increase in direct and indirect health care costs, being estimated to be up to €8 billion in Europe (60,226,235–237). In fact, a significant disease burden has been recently attributed to IBS, with 20% of adults estimated to develop IBS symptoms in any given year (238). This supports the need for novel, effective biomarkers and therapies to improve the diagnosis and management of IBS-D.

4.2. Pathophysiological mechanisms of Irritable Bowel Syndrome

Although the aetiology of IBS is not fully understood, it is postulated that the development of this disorder depends on complex interactions between the host and the environment. Host factors include age, gender, epigenetics and genetic predisposition, psychological factors and the circadian rhythm, while environmental factors include diet, GI infections, use of antibiotics, life stress and socio-cultural factors (223,239–243). On the other hand, not only the aetiology but also the pathophysiology of IBS and its mechanisms are not clear. IBS is now considered a multifactorial disorder, as not only functional changes such as visceral hypersensitivity and altered bowel motility, but also GI abnormalities such as immune activation, dysbiosis, nerve sensitisation and altered gene expression profiles have been described in IBS patients (232,244).

Intestinal barrier dysfunction

An altered intestinal barrier function and increased epithelial permeability has been identified as an early factor in IBS, contributing to low-grade immune cell infiltration in the intestinal mucosa. Disruption of TJPs seems to be essential in this barrier dysfunction triggering a loss of functional integrity (29,245,246).

Immune activation

The activation of the immune system in the development of IBS is undeniable, and indeed an enhanced immune response has been reported in about half of patients with IBS (247). Although there are discrepancies in immune cell counts, the number of T lymphocytes and mast cells has been reported to be increased in the gut of IBS patients compared to healthy controls, as well as mast cell-derived mediators, suggesting that this cell is a key element in

inducing and maintaining this low-grade immune activation (248,249). In addition, the involvement of humoral mechanisms in the pathophysiology of IBS-D has been described, with increased levels of B cells, plasma cells and IgG, and a greater proximity between plasma and mast cells (250).

Nervous system sensitisation

The nervous system seems to play an important role in IBS symptoms, in fact, distinct brain signatures (by image analysis) in patients with IBS have been described, as well as functional and structural alterations in task-related brain networks (251). Recently, there has been widespread interest in the role of the ENS in the development of IBS (67). Mucosal mediators from IBS patients excite visceral and somatic pain pathways, triggering abnormal neural signalling and increased nerve fibre outgrowth (252,253). Closer proximity between nerve endings and mast cells and increased nerve innervation have also been described in the colonic mucosa of IBS-D patients (249,254). In addition, the jejunal mucosa of IBS-D patients has been reported to have a greater proximity between nerve endings and plasma cells compared to healthy volunteers, and this shorter distance has been associated with higher levels of stress and depression and a loss of epithelial integrity (241,255,256). On the other hand, glioplasticity has recently been identified in IBS-D, pointing to the EGC as a novel component that may contribute to the pathophysiology and development of this entity (257–259).

In this context, it is important to consider the impact of stress, as IBS is a common **stress-sensitive GI disorder**, psychological stress has been reported as an important factor in the development of IBS, and a high prevalence of psychiatric comorbidities has been described in IBS patients (233,234,239,260). Furthermore, an association between humoral immunity and psychiatric comorbidities, as well as between stress episodes and the onset/exacerbation of functional GI disorders has been reported (250,261). All this evidence highlights the importance of **neuroimmune interactions** in the pathophysiology of IBS and confirms the bidirectional link between the nervous and immune systems.

Given this complex pathophysiology, it is not surprising that IBS has been reclassified as a **disorder of gut-brain interaction** rather than a functional GI disorder, highlighting the importance of neuronal signals in regulating mucosal barrier integrity and immune cells (262). In addition, several studies have reported a decrease in the stability and diversity of the gut microbiome in IBS patients, and a dysregulation of the metabolites they produce,

such as BAs (263–265). Given that the gut microbiota is capable of interacting with the CNS, involving nervous, endocrine and immune signalling mechanisms (266,267), the gut microbiome may also play an important role in the onset and exacerbation of IBS symptoms, which is why IBS has been postulated as a **microbiome-gut-brain interaction disorder** (263,268–270).

Visceral hypersensitivity is a consequence of nervous system sensitisation and it plays a major role in the aetiology of IBS symptoms. A variety of mediators and receptors contribute to visceral pain and discomfort by sensitising afferent nerve pathways from the GI tract. Although the exact pathogenesis is unclear, visceral hypersensitivity is a multifactorial process involving both the peripheral and CNS and is triggered by multiple intrinsic and environmental factors via complex cascades through the microbiota-neuroimmune cross-talk (271–273).

In conclusion, although the chain of events in the IBS pathophysiology is not well understood, it is thought that increased permeability in IBS patients may underlie altered mucosal immune responses, which may modulate the ENS plasticity and alter ENS circuitry and activity, which in turn disrupts the connectivity with the surrounding intestinal resident cells. In addition, immune and non-immune mediators released by different intestinal resident cell types can activate elements of the nervous system that secrete a wide range of neuropeptides, neurotransmitters and cytokines with the capacity to modulate barrier function. At the same time, gut microbiota-derived metabolites and even diet-derived bioactive compounds have regulatory effects on the intestinal epithelial barrier and immune cells as well as on the ENS circuitry. Finally, the ENS can act as an intermediary between the changes within the intestinal lumen and mucosal signalling via vagal afferent nerve endings to the CNS (232,244) (**Figure 6**). Disturbances in the ENS activity and signalling could cause alterations in the gut-brain axis that regulate the intestinal processes. Therefore, these changes may promote the development of heterogeneous symptoms such as those seen in IBS by triggering visceral hypersensitivity and altered GI motility and secretion.

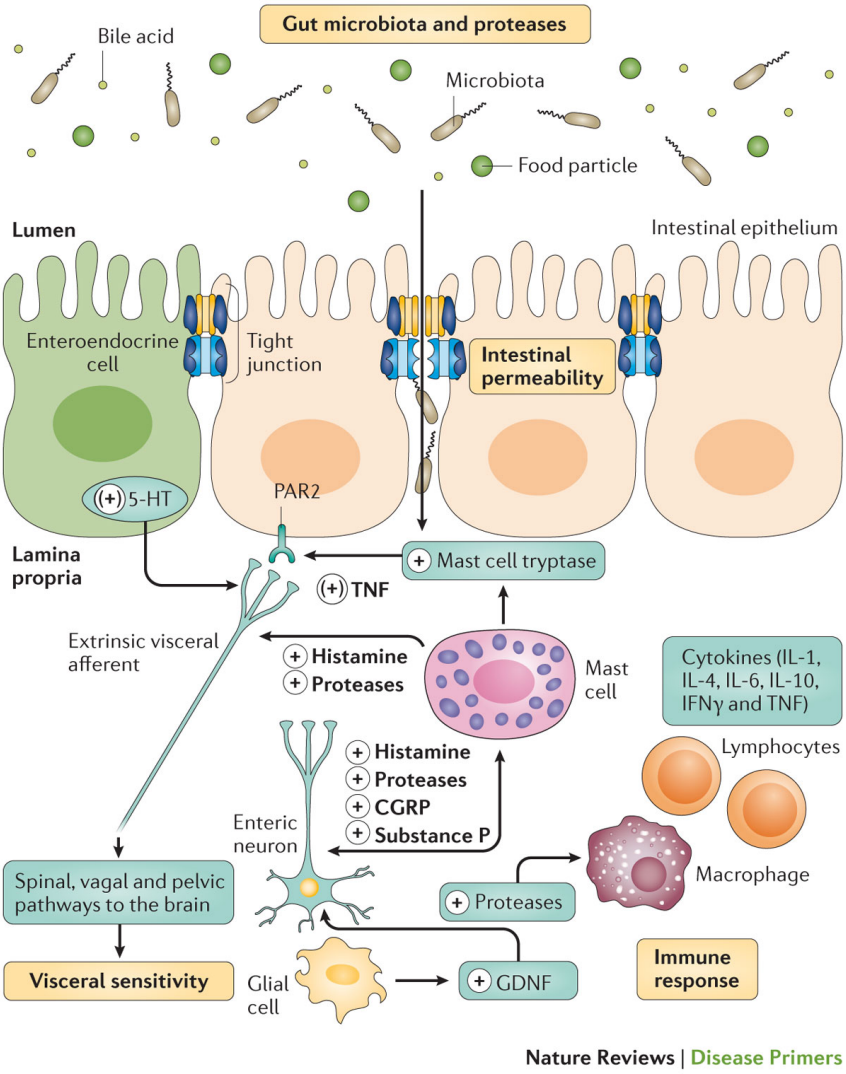

Figure 6 | Pathophysiology of IBS. Gut microbiota, intestinal permeability, immune and nervous system are key factors in IBS aetiology. In this figure the mediators that possibly have a role in IBS pathology are shown. 5-HT: 5-hydroxytryptamine (also known as serotonin); CGRP: calcitonin gene-related peptide; GDNF: glial cell-derived neurotrophic factor; IL: interleukin; PAR2: proteinase activated receptor 2; TNF: tumor necrosis factor (232).

4.2.1 Irritable Bowel Syndrome and Enteric Glial Cell

Alterations in the ENS contribute to the development of IBS, as the ENS is known to play a critical role in intestinal homeostasis. EGCs have been implicated in the regulation of epithelial barrier integrity, immune response, intestinal motility and visceral sensitivity (52,69,92,109), as well as communicating with and responding to signals from the microbiota and the CNS (87). As all these factors are involved in the pathophysiology of IBS, aberrations in EGC function may contribute to the development of IBS. However, the role of EGC in this entity is largely unknown as most studies have been limited to inflammatory bowel disease (120,124,274).

In recent years, some studies have identified glioplasticity in the colon of IBS patients. This has been correlated with abdominal pain (257), and indeed the enteroglial-nervous unit has been shown to be involved in colonic visceral hypersensitivity in IBS (258), and EGCs have been postulated as a key element in chronic abdominal pain mechanisms through the brain-gut axis (275). In addition, a differential alteration in EGC-mast cell interaction has been shown in women with IBS (259), and glial-derived neurotrophic factor has been described to be increased in IBS and to play a role in altered gut homeostasis induced by acute stress (276). In animal models of IBS, EGC has been implicated in stress-induced colonic hypercontraction (277) and in butyrate-induced visceral hypersensitivity via nerve growth factor (278). Previous research has shown that the molecular phenotype of EGCs is often altered in pro-inflammatory conditions (279). However, little is known about the expression of EGC markers in IBS patients compared to healthy individuals, and the few existing studies show conflicting results (257–259). Taken together, these findings point to EGC as a relevant component that may contribute to the pathophysiology and development of IBS, although further research is needed to better understand its role in this entity.

4.2.2. Irritable Bowel Syndrome and Bile Acids

IBS has been postulated as a microbiome-gut-brain interaction disorder (263,268–270). Given that IBS patients have an altered microbial profile (280), the impact of this dysbiosis in the aetiology of IBS may be mediated by metabolites derived from the microbiota, including BAs, molecules that are able to regulate intestinal fluid absorption and secretion, motility and sensitivity, and may therefore contribute to IBS pathophysiology, especially in IBS-D (281,282). In fact, several recent studies have shown IBS-D presents an alteration in the gut microbiome together with differential microbial metabolites, identifying between them BAs (283–286). Thus, IBS studies on BAs levels are variable and they have been focused on faecal samples given BAs are able to induce diarrhoea by triggering colonic chloride secretion (287).

Total faecal BAs have been reported to be increased in IBS-D patients compared to healthy controls in some Asian groups, although other Western studies have not shown these differences (282). More consistently, primary BAs are increased and secondary BAs are decreased in the faeces of IBS patients compared to healthy controls (284,288–293). However, two novel studies point to an increase of conjugated secondary BAs in the stool of these patients (283,286). In most of these studies, these changes correlate with the stool consistency (Bristol stool score) and bowel frequency highlighting their potential contribution to IBS pathophysiology.

The altered BA profile of IBS patients could be due to changes in the number of bacteria involved in BA transformation or reduced deconjugation activity, both of which have been reported in IBS (288,289,294). In fact, 8 microbiome genera reduced in IBS-D were negatively correlated with primary BAs and positively correlated with secondary BAs (290) as well as Clostridia-rich microbiota from IBS-D with elevated faecal BAs correlated positively with total faecal BAs but negatively with serum FGF19 levels (294). However, correlation is not sufficient to demonstrate direct causation and further research is required to confirm this association.

In addition, around one third of patients with IBS-D present BAs malabsorption (295) and a 25 % have and excess of total BA excretion in faeces which was accompanied by decreased FGF19 serum levels and increased diarrhoeal symptoms when comparing with IBS-D patients without BA malabsorption and healthy controls (294). Thus, IBS-D with increased BA faecal excretion or synthesis present greater intestinal permeability and colonic transit as well as higher body mass index and faecal fat than patients with normal BA profile (292,296,297). In addition, two BAs synthesis genes, klotho B and fibroblast growth factor receptor 4 are related with greater small intestinal or colonic transit in IBS-D (298,299).

Therefore, there is a clear relationship between faecal BA excretion, faecal BA profile and colonic transit in IBS-D, but further research is needed to elucidate whether this altered BA profile is due to a dysfunction of microbial transformation induced by gut dysbiosis or to a greater colonic transit that reduces the time for gut microbiota to metabolize BAs in IBS-D.

Regarding BARs expression in IBS-D, only one study has investigated it and they found increased levels of GPBAR1 and no differences for VDR in the colonic mucosa of IBS-D patients and healthy controls by immunohistochemistry (293). Interestingly, GPBAR1 genotype has also been associated with colonic transit and total faecal BAs in IBS (292), as well as a single nucleotide polymorphism in the GPBAR1 gene with altered small bowel and colonic transit in lower functional GI disorders (299), supporting in humans the well-established colonic motor and secretory function of GPBAR1 in animal studies. In addition, this increased levels of the BAR GPBAR1 may contribute to IBS-D visceral hypersensitivity as primary BAs and an increase in TGR5 have been correlated with abdominal pain in IBS-D (289,293). Furthermore, colesevelam treatment, a BA sequestrant, increased colonic mucosal expression of GPBAR1 and decreased NR1H4 expression in an RNA sequencing study performed in IBS-D patients with increased BA synthesis or faecal excretion (300).

Interestingly, patients with IBS-D have symptoms that overlap with those of patients diagnosed with bile acid diarrhoea (BAD). Idiopathic BAD is a common and under-diagnosed condition that causes chronic diarrhoea. Up to a quarter of IBS-D patients show BAD, often leading to a clinical problem of misdiagnosis (133,301). Idiopathic BAD results from overproduction of BAs due to disruption of the negative feedback mechanism that regulates BA synthesis (133,232,302). In Europe, the gold standard for diagnosing BAD is the selenium-75-homocholic acid taurine (SeHCAT) test (303,304). As previously described, BAs are known to cause excessive fluid secretion, increase intestinal permeability and accelerate colonic transit in IBS-D patients with BAD. Thus, it is useful to test patients for BAD because IBS-D patients with BAD have more severe symptoms and a worse quality of life than IBS-D patients without BAD (305).

In addition, BAD and IBS-D differ in terms of therapeutic management. While relief of symptoms caused by BAD can be achieved by the administration of BA sequestrants, there are no curative treatment options for IBS-D and its management consists of a combination of non-pharmacological and pharmacological therapies focused on the relief of cardinal symptoms (133,232). Remarkably, IBS-D patients with severe BAD have a differential faecal metabolome and microbial profile than IBS-D patients with other BAD grades, postulating glycerophospholipids and oligopeptides as predictive metabolites for BAD (285). Therefore, testing for BAD in IBS-D is crucial to adjust the therapeutic approach and to differentiate between $SeHCAT^{+}$ and $SeHCAT^{-}$ patients when assessing the pathophysiology.

Although IBS-D patients present epithelial barrier dysfunction and nervous system activation together with dysbiosis and an altered BA profile, to date there are no studies about the impact of BAs on the expression of the TJPs proteins or the ENS in IBS-D.

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

Bile acids in the intestinal lumen alter the molecular regulation of epithelial barrier function and promote enteric glial cell plasticity, which are actively involved in the physiopathology of diarrhoea-predominant irritable bowel syndrome.

OBJECTIVES

Main objective

To identify the molecular phenotype of enteric glial cells in diarrhoea-predominant irritable bowel syndrome and to define whether bile acids determine specific molecular and clinical features in this disorder, to gain insight into their implication in intestinal barrier dysfunction and glial cell activation.

Specific objectives

1. To differentiate biological and physiological outcomes between patients with diarrhoea-predominant irritable bowel syndrome with and without bile acid diarrhoea by:

    1. Determining the phenotype of enteric glial cells, epithelial cells and bile acid receptors in the jejunal mucosa.

    2. Establishing the association between the observed molecular changes in the jejunal mucosa and the presentation and severity of clinical manifestations.

    3. Identifying the bile acid profile in the jejunal intestinal fluid.

2. To evaluate the effects of short and long-term exposure to bile acids on epithelial barrier function using an *in vitro* approach.

3. To characterise the impact of bile acids on the glial cell by direct and indirect (enterocyte-mediated) exposure to bile acids, using an in *vitro* approach.

CHAPTER 1

Differential phenotype of enteric glial cells, epithelial integrity and luminal bile acid profile in the jejunum of diarrhoea-predominant irritable bowel syndrome patients, with and without bile acid diarrhoea

1. INTRODUCTION

Irritable bowel syndrome (IBS) has traditionally been considered a chronic functional gastrointestinal (GI) disorder. After several decades of progress in understanding its multifactorial underlying pathophysiological mechanisms, a new term, disorder of gut-brain interaction, has emerged to refer to it, in which disturbances in the microbiota-gut-brain interaction have been implicated (1). It is diagnosed on the basis of clinical criteria (Rome IV) by clinical recurrent abdominal pain associated with altered bowel habits, with a global prevalence of 4-5% (2,3). The different subtypes of IBS according Rome IV criteria are defined by the predominant stool pattern, where the most common is the **diarrhoea-predominant IBS (IBS-D)** subtype (31.5%) with a global prevalence of 1.4%, determined by >25% loose stools and >25% hard stools (4). Due to its heterogeneous clinical presentation, current treatment is aimed at alleviating the predominant symptom rather than addressing the different putative mechanisms involved and individualising therapeutic strategies. As a result, IBS is associated with a significant disease burden, a progressive increase in direct and indirect healthcare costs (5,6) and a poor quality of life. Several pathophysiological mechanisms have been identified including visceral hypersensitivity, activation of the mucosal immune response, gut motility and permeability alterations, gut microbiota imbalance and blunted bile acid (BA) metabolism in association with chronic psychosocial stress (7).

Growing evidence support that altered enteric nervous system (ENS) homeostasis may predispose to several GI disorders such as IBS (8,9). Enteric glial cells (EGCs) are one of the elements within the ENS that require particular attention due to their singular biology with a high accomplishment of diverse roles in the GI homeostasis functions, which is explained by the plasticity in response to changes in the gut microenvironment including microbiota-derived metabolites and different types of insult (9). Moreover, the EGCs are a heterogeneous population with a diverse morphological and molecular phenotype, expressing several markers including **glial fibrillary acidic protein (GFAP)**, **S100 calcium-binding protein β (S100β)** and **SRY-box transcription factor 10 (SOX10)**, conditioned by their location and the surrounding microenvironment along the GI tract (9–11).

In IBS-D patients, an altered molecular and ultrastructural basis at the **tight junction proteins (TJPs)** in both the small and the large intestine has been demonstrated, leading to intestinal mucosal barrier dysfunction, with an increased epithelial permeability and subsequently

contributing to low-grade inflammation and ENS activation in the intestinal mucosa (12–14). Notably, ENS plasticity in the context of IBS (8) is gaining further attention, specifically on EGCs. A recent study has reported changes in the expression of several glial markers in the colonic mucosa of all subtypes of IBS patients, and mucosal supernatants from IBS-D patients alter the Ca^{2+} response to ATP in EGCs (15). Furthermore, colonic mucosa of IBS patients exhibits signs of increased reactive EGCs profile and altered EGC-mast cell interaction which allows a greater bacterial translocation through the colonic epithelial barrier (16,17). These findings, together with their glioplasticity, suggest their putative role in IBS pathophysiology and may determine IBS phenotype. Additionally, the gut microbiota, as a relevant factor of the luminal microenvironment, regulates and modulates several features involved in IBS pathogenesis, and an altered the gut microbiome pattern (18) is associated with IBS severity (19). Multi-omics analysis of gut microbiome composition and function has allowed the identification of a differential functional pattern in IBS-D patients compared to IBS-C patients and healthy controls (20). Among other differentially abundant faecal metabolites, the increased concentration of BAs stands out, which contribute to altered intestinal fluid absorption and secretion, as well as impaired intestinal motility and visceral sensitivity, particularly in IBS-D (21), although a direct causal relationship has not yet been established (20,22–25).

Up to a quarter of IBS-D patients show idiopathic bile acid diarrhoea (BAD) (26), exhibiting excessive fluid secretion, increased intestinal permeability, and accelerated colonic transit (27–29). In addition, greater levels of immunoreactivity for a BA receptor (BAR), GPBAR1, have recently been described in IBS-D colonic samples (30), which was previously associated with increased faecal excretion of primary BAs and abdominal pain, and reduced colonic transit and increased total faecal BAs in IBS (27).

IBS-D show epithelial barrier dysfunction together with dysbiosis, an altered BA profile, and altered ENS plasticity and function where EGCs act as a signalling bridge between the gut luminal microenvironment and all type of cells resident within the gut wall. Their central role in the regulation of gut functions together with findings of glioplasticity in colonic mucosa in IBS suggest their putative role in the pathophysiology and may determine the IBS phenotype, although it is not yet clear whether their activation state has a protective or detrimental role in the intestinal barrier function.

Most of the studies on IBS have focused mainly on the colon, in part because it is more accessible for samples collection from the distal areas of the bowel. In addition, most

research on BAs has focused on the ileum, where the majority of BAs are reabsorbed, or in the colon, where they are transformed by the microbiota. Our group has demonstrated that mucosal alterations are present also in the proximal small intestine, highlighting the need of better understanding the role of this segment in health and disease, especially on key physiological functions such as defence, nutrient digestion and absorption (14,31–37). Due to its fundamental role in intestinal physiology, exploring BAs to better understand its role in the jejunum deserves special attention.

A deeper molecular characterisation of IBS is needed, including the identification of EGCs contribution to mucosal disturbances and its potential impact on clinical symptoms. Moreover, to date, no studies have investigated the expression pattern of EGC markers in the jejunal mucosa from IBS-D patients and the role of increased levels of BAs in the intestinal lumen on the EGCs plasticity has not been thoroughly investigated. Therefore, the aim of this study is to characterize the molecular phenotype of EGCs and the epithelial integrity in the jejunal mucosa of IBS-D patients with and without BAD as well as to evaluate their association with the clinical phenotype and severity of symptoms in IBS-D patients.

2. EXPERIMENTAL DESIGN

A retrospective study was designed to:

1. Characterize the molecular phenotype of the ENS, in particular EGCs, the epithelial barrier integrity and BARs in the jejunal mucosa from IBS-D patients, including patients with and without BAD.
2. Identify the disease-associated jejunal BA profile.
3. Investigate the potential clinical-molecular relationship between jejunal mucosal nerve plasticity and barrier integrity.
4. Investigate the potential association of the jejunal BA profile with the plasticity of the EGCs and with molecular changes in markers of jejunal barrier integrity and with clinical features.

Participants were classified into two groups: healthy volunteers (HVs) or IBS-D patients according to their clinical features (see "Participants and clinical assessment" section below). BA malabsorption was assessed by selenium-75-homocholic acid taurine (SeHCAT) test in the IBS-D group, rendering two subgroups of patients: IBS-D-SeHCAT^{+} and IBS-D-SeHCAT^{-}. Demographic characteristics, clinical assessment and biological samples (jejunal mucosal biopsies and intestinal fluid) were obtained from all participants (**Figure 1**). All samples used in this study were codified and blindly analysed by the study researchers.

The experimental design was adapted to the main objectives of the study, as follows:

1) Characterisation of the molecular phenotype of the ENS, in particular EGCs, the epithelial barrier integrity and BARs in the jejunal mucosa from IBS-D patients. To investigate whether there is a distinct molecular profile associated with BAD, a sub-analysis was performed including IBS-D with and without BAD.

    - First, an analysis of the structure of the jejunal mucosa was performed together with mucosal leukocyte counts: the number of intraepithelial lymphocytes (CD3^{+}), mast cells (CD117^{+}) and eosinophils (eosin^{+}) were assessed by immunohistochemistry (IHQ) or haematoxylin & eosin staining.

    - Second, the EGC markers S100β and SOX10 were assessed by IHQ to evaluate the EGC location in the mucosa.

- Third, gene and protein expression of markers of the EGC, neuron, neurotrophic factors, intercellular communication and BARs were determined by real-time qPCR or western blot.

2) Identification of a disease-associated jejunal BA profile. To determine the composition of the BA pool, the amount of conjugated and unconjugated primary and secondary BAs was determined by liquid chromatography-mass spectrometry (LC-MS/MS) from jejunal fluid collected from HVs and IBS-D patients with and without BAD.

3) Investigation of the potential clinical-molecular relationship. A correlation analysis was performed between the mucosal nerve plasticity and barrier integrity markers and clinical variables.

4) Investigation of the potential association between the jejunal BA profile and EGC, epithelial integrity and clinical features. A correlation analysis was performed between the different markers and clinical variables.

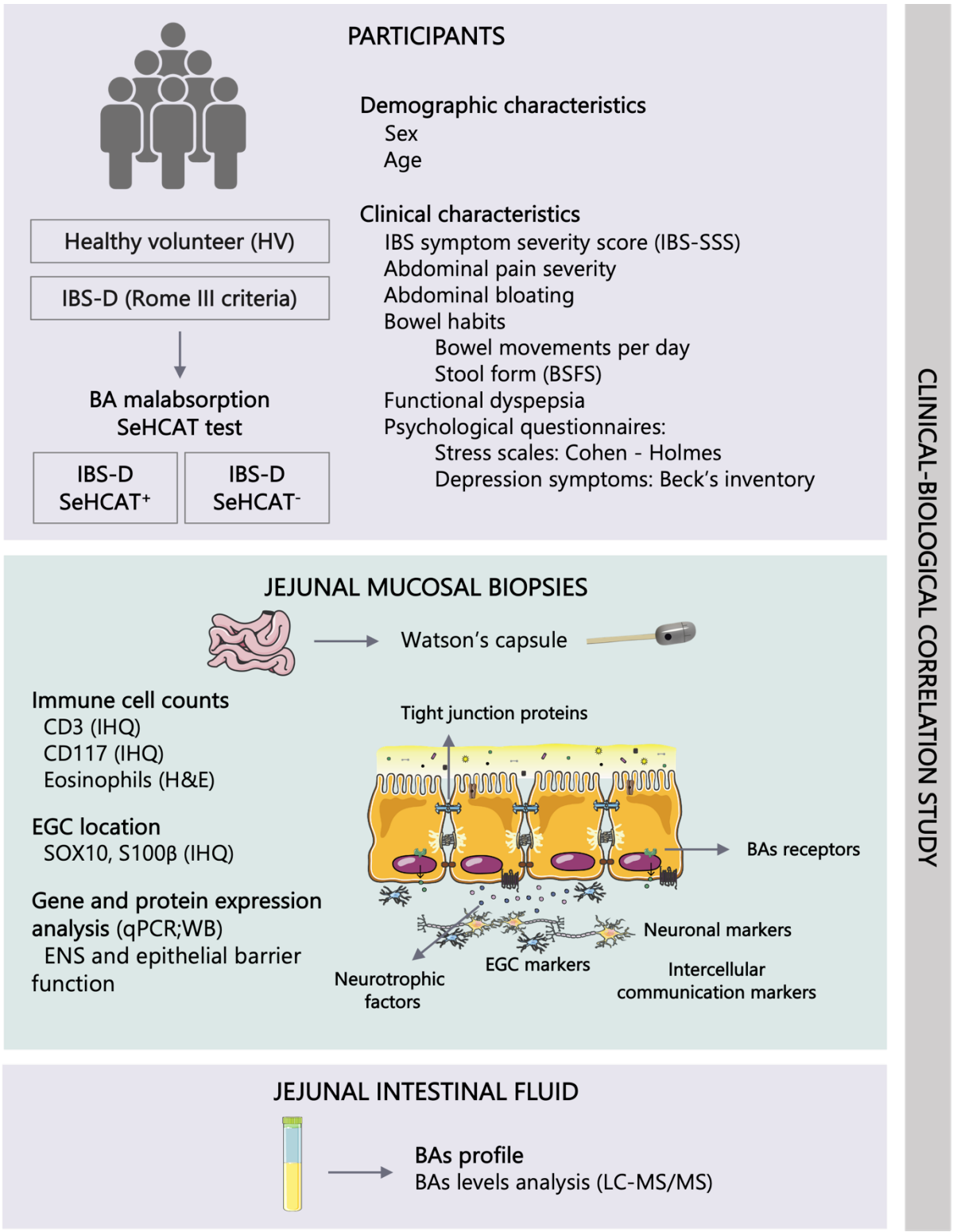

Figure 1 | Experimental design and work-flow. BA: Bile Acid; BSFS: Bristol Stool Form Scale; EGC: Enteric Glial Cell; H&E: Haematoxylin and Eosin; IHQ: Immunohistochemistry; IBS-D: Diarrhoea-predominant Irritable Bowel Syndrome; LC-MS/MS: Liquid Chromatography-Mass Spectrometry; qPCR: quantitative PCR; S100β: S100 Calcium Binding Protein β; SeHCAT: 75-Selenium Homocholic Acid Taurine; SOX10: Transcription Factor SOX-10; WB: Western Blot.

3. METHODS

3.1 Participants and clinical assessment

All participants were evaluated by a gastroenterologist at the Gastroenterology department of the Vall d'Hebrón Barcelona Hospital Campus (Barcelona, Spain). Newly diagnosed diarrhoea-predominant IBS (IBS-D) patients were retrospectively recruited from the outpatient gastroenterology clinic, all of them fulfilling Rome III criteria (38). HVs were recruited from the general population by public advertising. A complete structured clinical questionnaire, evaluating digestive symptoms, was conducted in all candidates prior to entering the study. All participants were aged between 18 and 60 years old. Prior to inclusion in the study, participants were instructed to fill out a Rome criteria-based questionnaire for GI symptoms to characterise digestive symptoms of IBS-D patients and to establish the absence of symptoms in HVs. In all IBS-D patients, to ensure the absence of GI comorbidities other than BAD, participants were subjected to serological and biochemical analyses, including anti-transglutaminase antibodies and thyroid hormones. BA malabsorption was assessed in IBS-D patients using the ^{75}SeHCAT test. Inclusion and exclusion criteria are summarised in supplementary **Table S 1**. Clinical and demographical data of each participant and the different experimental procedures performed are detailed in supplementary **Table S 2** and supplementary **Table S 3**, respectively. The research protocol was approved by the Ethics Committee at Vall d'Hebrón Barcelona Hospital Campus (Barcelona, Spain) [(PR(AG)211/2018)], and was conducted following the principles of the Declaration of Helsinki. All subjects gave written informed consent prior to enrolment.

GI and psychological symptoms were assessed by validated clinical questionnaires completed by all participants during 10 days prior to jejunal fluid and mucosal biopsy collection by all participants. The clinical assessment was carried out as follows:

GI symptoms: The severity of IBS-related symptoms was assessed using the validated Spanish version of the IBS Severity Scoring System (IBS-SSS) (39,40). The IBS-SSS is a prompted visual analogue scale consisting of four questions, each with a maximum score of 100, and it includes the severity of abdominal pain and abdominal bloating, dissatisfaction with bowel habits, and interference of IBS with life in general. In addition, the questionnaire also includes the total number of days with pain in the 10 days prior to biopsy collection (maximum score 100). All questions combined result in a maximum IBS-SSS score of 500 points (39). Data on bowel habits were collected by determining stool consistency and form using the Bristol Stool

Chart (41) and bowel movement frequency, scored as the maximum number of bowel movements per day. Participants were also evaluated for dyspepsia according to Rome III criteria (38).

Psychological evaluation: Data on acute and chronic background psychological stress and depression traits were assessed using validated Spanish versions of the Perceived Stress Scale (PSS) developed by Cohen (42), the Social Readjustment Rating Scale (SRRS) developed by Holmes and Rahe (43), and Beck Depression Inventory (BDI) (44), respectively. The PSS and SRRS report on experienced stress, the former in the last month and the latter in the last year. The BDI measures symptoms of depression during the last week.

BA malabsorption evaluation: All IBS-D patients were tested for the presence of BAD using ^{75}SeHCAT scanning, which allowed dividing the IBS-D group into two subgroups. Those with associated BAD were named IBS-D SeHCAT^{+} and those without associated BAD were name IBS-D SeHCAT^{-}. Following the currently preferred protocol developed by Brydon *et al.* (45), two scans were performed at the Nuclear Medicine Department with an interval of 7 days to calculate the retention rate of a radiolabelled taurine-conjugated BA analogue (^{75}Se) administered orally prior to the first scan. Retention values <15% were considered diagnostic for BAD. Prior to the first day of testing, participants were instructed to discontinue the use of BA sequestrants and anti-diarrhoeal medication to avoid interfering with the test results, and they were asked to fast for at least four hours (h) before taken the SeHCAT capsule (370 kBq).

Allergy symptoms: All candidates underwent a battery of skin prick test for 22 common food allergens and 12 inhalants (Leti SA, Barcelona) (46). Subjects with food allergens positivity or with clinical history consistent with food allergy (digestive and/or extra-digestive symptoms associated with exposure to certain food components) were excluded.

3.2. Collection of jejunal biopsy and intestinal fluid

Participants were orally intubated, previous overnight fasted, and a Watson's capsule with a 3 mm diameter aspiration tube was positioned into the proximal jejunum (5-10 cm distal to the angle of Treitz) under fluoroscopic guidance as previously described (36). Jejunal intestinal fluid was collected (5 mL) by gentle aspiration, snap frozen and stored at -80 °C for BA levels analysis. Afterwards, a single jejunal mucosal biopsy was obtained by suction with a 50 mL syringe. Immediately, the biopsy was divided into equal parts using a sterile scalpel, one piece was sent to the Department of Pathology and Anatomy for histological analysis and

exclusion of other pathologies. The two other fragments were placed in RNA later solution (QIAGEN) for gene/protein analysis or in formalin and embedded in paraffin for further immunohistochemistry evaluation.

3.3. Analytical procedures

3.3.1. Histology and immunohistochemistry

Jejunal mucosal biopsies were fixed in formalin, embedded in paraffin, cut at 4µm with a microtome and transferred to slides for histological analysis. On the one hand, at the pathology department of the Vall d'Hebrón Hospital campus, samples were processed for routine haematoxylin/eosin (H&E) staining to asses epithelial morphology and eosinophilic infiltration. Besides, CD3 and CD117 stainings were processed by validated immunohistochemistry procedures to count the number of intraepithelial lymphocytes (IELs) and mast cells, respectively. All tissue samples were analysed and cells counted blindly by an expert pathologist. The number of mast cells ($CD117^+$) and eosinophils ($eosin^+$) is expressed as per high-power field (HPF) in the lamina *propria* meanwhile the number of IELs T-subtype ($CD3^+$) is expressed as per 100 epithelial cells.

In parallel, at the digestive physiology and pathophysiology laboratory of Vall d'Hebron Institut de Recerca, EGC markers SOX10 and S100β immunohistochemistry stainings were carried out. Tissue specimens were deparaffined in xylene, and gradually rehydrated by using a battery of decreasing ethanol concentrations. Antigenic recovery was performed by heat-induced epitope retrieval (HIER) in tris-EDTA at pH9 buffer or citrate buffer at pH6 (depending on the antibody) during 10 min at 120 ºC in an autoclave. Permeabilisation was carried out with triton and Tween. Endogenous peroxidase activity was inactivated using Peroxidase-Blocking Solution (K4007/K4010, Agilent) and unspecific unions were blocked with a Protein Block Serum-Free (X0909, DAKO). Afterwards, primary antibody was incubated. Incubation conditions, the type of antigen retrieval method, permeabilisation and blocking are listed in **Table 1**. The primary antibody was absent in the negative control. The HRP-labelled polymer method (K4007/K4010, Agilent) was used for revealing the positive staining during 30 min, followed by the staining with the peroxidase substrate 3,3'-diaminobenzidine tetrachloride (K4007/K4010, Agilent). Samples were counterstained with 20% haematoxylin, dehydrated and mounted for being observed under an optical microscope.

Digital scans of slides immunohistochemically stained for SOX10 or S100β were performed in the Department of Anatomy and Pathology. SOX10 and S100β staining was quantified to determine the EGC phenotype. To quantify SOX10 staining, the number of $SOX10^+$ cells per HPF was counted at 400x magnification using a Leica DMLB optical microscope (Leica Microsystems GmbH), and a minimum of five non-overlapping HPFs were analysed per participant. S100β staining was quantified using QuPath bioimage analysis software (version 0.4.1) (47). First, the area of interest was selected: the largest possible area of the lamina *propria* suitable for analysis and holes were excluded. Epithelial tissue located inside the area of interest was selected, after which it was inserted into hierarchy and inverted, excluding it from the area of interest. As a result, the area of interest consisted of only lamina *propria* tissue with a minimum size of 170.000 μm^2. Stained area quantification was then performed by creating a thresholder to detect S100β staining. S100β stained area was expressed as a percentage of the total selected area of lamina *propria*.

Table 1 | Primary antibodies used in the immunohistochemical study.

Antibody	Host and target	Manufacturer and reference	Ag retrieval method	Permeabilisation method	Protein blocking conditions	Dilution and incubation conditions
Anti-Sox10	Mouse anti-human	Proteintech 66786-1-Ig	HIER Citrate	Triton 0.2% + Tween 0.1% in PBS	3 % BSA in Protein Block, 3 h	1/250 in 3% BSA O/N 4 °C
Anti-S100B	Rabbit anti-human	DAKO IS504	HIER EDTA	Triton 0.4% + Tween 0.1% in PBS	Protein Block, 2 h	Ready to use, 2 h

BSA: bovine serum albumin; HIER: heat-induced epitope retrieval; O/N: Over-night; PBS: phosphate-buffered saline; S100β - S100 Calcium Binding Protein β; SOX10: Transcription Factor SOX-10.

3.3.2. RNA isolation

RNA isolation was performed from jejunal mucosal biopsies stored in RNA later solution at -80°C. Sample homogenisation was carried out with FastPrep and lysing matrix D tubes (MP Biomedicals) after adding 1 mL TRIzol Reagent (Invitrogen) to the chopped biopsy. The homogenate was passed through an insulin syringe (27G) 10 times in order to facilitate tissue disruption and increase the RNA yield and it was separated into aqueous and organic phases by chloroform addition and centrifugation. RNA remains exclusively in the aqueous phase, DNA in the interphase and proteins in the organic phase. The interphase and organic phase were stored at -80 °C for future isolation of DNA and protein. The RNA (aqueous phase) was precipitated and bound to the silica membrane of the MirVana™ mRNA Isolation Kit (Ambion) according to manufacturer's instructions. After elution, total RNA concentration

and quality was analysed by capillary electrophoresis (Agilent 2100 Bioanalyzer; Agilent Technologies). Samples with a low RNA integrity number (RIN) (<5) were excluded from the study (supplementary **Table S 4**). 13 HVs and 21 IBS-D patients (9 IBS-D-SeHCAT^{+}; 12 IBS-D-SeHCAT^{-}) were blindly chosen for gene expression analysis**.**

3.3.3. cDNA synthesis and quantitative real time PCR

cDNA synthesis was performed using 1 µg of total RNA using the High Capacity Reverse Transcription Reagents Kit (Thermo Fisher Scientific), following manufacturer's instructions. Quantitative real-time PCR (qPCR) was carried out with the ABI PRISM® 7500 FAST Sequence Detection System (Applied Biosystems). ENS, intestinal barrier and BAs receptors markers were evaluated (**Table 2**) using validated TaqMan Gene Expression Assays (GEA) detailed in supplementary **Table S 5** (Applied Biosystems). RNase-free H_2O was used as negative control. Samples were run in triplicate and their expression was normalised to the house-keeping genes *18S* and *GAPDH*. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method (48); were ΔCt=Ct target gene – Ct endogenous gene and ΔΔCt = ΔCt patient – ΔCt control expressed as fold change.

3.3.4 Protein extraction

Proteins were isolated from the organic phase obtained after lysing jejunal mucosal biopsies with TRIzol Reagent. After DNA precipitation with ethanol 100%, proteins were precipitated from the phenol-ethanol supernatant adding 3 volumes of acetone. Proteins were washed with a solution of 0.3M guanidine hydrochloride in 95% ethanol + 2.5 % glycerol solution. A final wash was performed using ethanol 100% containing 2.5 % glycerol. After briefly air-drying the protein pellet, proteins were solubilised with 7M Urea + 2M Thiourea + 2% CHAPS solution including protease and phosphatase inhibitor cocktail (Thermo Scientific). 9 HVs and 12 IBS-D patients (6 IBS-D-SeHCAT^{+}; 6 IBS-D-SeHCAT^{-}) were blindly chosen for protein expression analysis.

3.3.5. Protein quantification

Protein determination was carried out with the Quick Start™ Bradford Protein Assay (Bio-Rad) following manufacturer instructions. A volume of 5 µL of each sample as well as the standard values were added in duplicate to a microplate. Afterwards, 250 µL of tempered 1X dye reagent was added to each well. After 5 minutes of incubation, absorbance was

measured at 595 nm in an ELx800 spectrophotometer microplate reader (BIO-TEK Instruments, INC).

Table 2 | Experimental procedures performed for each marker evaluated.

Markers	Synonym	qPCR	WB
Enteric Nervous System			
Enteric Glial Cell			
S100β - S100 Calcium Binding Protein β		Yes	Yes
SOX10 - Transcription Factor SOX-10		Yes	Yes
GFAP - Glial Fibrillary Acidic protein		Yes	Yes
Neuron			
UCHL1 - Ubiquitin C-terminal Hydrolase L1	PGP9.5	Yes	Yes
PRPH - Peripherin		No	Yes
Neurotrophic Factors			
GDNF - Glial Derived Neurotrophic Factor		Yes	Yes
BDNF - Brain Derived Neurotrophic Factor		Yes	No
Intercellular Communication			
P2RX7 - Purinergic Receptor P2X 7		Yes	No
GJA1 - Gap Junction Protein Alpha 1	CX43	Yes	No
NGF - Nerve Growth Factor		Yes	No
NTF3 - Neurotrophin 3		Yes	No
NTRK1 - Neurotrophic Receptor Tyrosine Kinase 1	TRKA	Yes	No
Intestinal Barrier Function			
Tight junction proteins			
CLDN2 - Claudin 2		Yes	Yes
OCLN - Occludin		Yes	Yes
TJP1 - Tight Junction Protein 1	ZO-1	Yes	Yes
Enterocyte function.			
VILL – Villin		No	Yes
Bile acids receptors			
NR1H4 - Nuclear Receptor Subfamily 1 Group H Member 4	FXR	Yes	No
GPBAR1 - G Protein-Coupled Bile Acid Receptor 1	TGR5	Yes	No
VDR - Vitamin D Receptor		Yes	No
FGF19 - Fibroblast Growth Factor 19		Yes	No

CX43: Connexin 43; FXR: Farnesoid X Receptor; PGP9.5: Protein Gene Product 9.5; TGR5: Takeda G Protein-Coupled Receptor 5; TRKA: Tropomyosin Receptor Kinase A; ZO-1: Zonula Occludens-1.

3.3.6. Western Blot

Expression of ENS and intestinal barrier-related markers were evaluated **(Table 2**). 40 mg of protein were separated by electrophoresis with NuPAGE™ 4-12% Bis-Tris Protein Gels (Invitrogen) and blotted to a polyvinylidene fluoride (PVDF) membrane (Invitrogen) using the Trans-Blot SD semi-dry transfer cell (Bio-Rad). After blocking with TBS(1X)-Tween(0,05%) + 10% non-fat milk (Bio-Rad) during 2 h, membranes were incubated with primary antibodies. Later, they were washed with TBS(1X)-Tween(0,05%) and incubated with the specific secondary antibody for 1 h at room temperature. Primary and secondary antibodies were diluted in TBS(1X)-Tween(0,05%) + 1% non-fat milk and their experimental conditions are indicated in **Table 3.** Proteins were detected with SuperSignal TM West Femto Maximum Sensitivity Substrate reagent (Thermo scientific). Images were digitised with Odyssey Fc 2800 (LI-COR). Brain lysate (10 mg of protein) and Caco2 cell lysate (40 mg of protein) were used as a positive control for ENS markers and intestinal barrier markers respectively.

3.3.7. Bile acids determination in jejunal intestinal fluid

Intestinal fluid samples were thawed and homogenised by vortexing for 5 min, and the homogenate was sonicated for 10 min. Then 300 uL were filtered using an Amicon Ultra-0.5 Centrifugal Filter Unit, pore size 10 kDa NMWCO (UFC5010, Merck Millipore) at 13000 rpm for 40 min at 4 °C. 5 μL of the filtrated sample was diluted in 1 mL of water and 10 μL of internal standard was added. The internal standards consist of two BA standard mixtures of uncojugated (MSK-BA1-1) and conjugated (MSK-BA2-1) BAs (Cambridge Isotope Laboratories, Inc). After vortexing for 2 min, BAs were extracted by solid-phase extraction using a solid-phase extraction manifold and reversed-phase columns C18-S-500/3 (Interchim, France) (500 mg stationary phase/3 mL sample). After vortexing for 2 min, BAs were extracted by solid phase extraction using a solid phase extraction manifold and reversed phase columns C18-S-500/3 (Interchim, France) (500 mg stationary phase/3 mL sample). The extracts were then reconstituted in 50 μL of methanol and analysed by LC/MS/MS using a triple quadruple mass spectrometer (Agilent™ 6410 BA) and uPLC quaternary (Agilent™ 1260 Infinity). A volume of 5 μL of samples were injected and BAs were eluted (0.3 mL/min flow) and analysed by ion acquisition. Internal standards were optimised and calibration curves were constructed to interpolate the levels of BAs in the jejunal fluid.

Table 3 | Antibodies and experimental conditions used for Western blot.

Antibodies	Host and target	Manufacturer and reference	Incubation conditions
Primary antibodies			
Anti-Actin	Mouse anti-human	Sigma 030M4788	1:10.000 30 min RT
Anti-Claudin 2	Rabbit anti-human	ThermoFisher Scientific 51-6100	1:500 O/N 4 °C
Anti-GDNF	Goat anti-human	R&D Systems AF-212-NA	1:200 O/N 4 °C
Anti-GFAP	Mouse anti-human	Proteintech 60190-1-Ig	1:250 O/N 4 °C
Anti-Occludin	Rabbit anti-human	Invitrogen 71-1500	1:1000 O/N 4 °C
Anti-Peripherin	Rabbit anti-human	Abcam ab4666	1:500 O/N 4 °C
Anti-S100β	Rabbit anti-human	Abcam ab52642	1:200 O/N 4 °C
Anti-Sox10	Mouse anti-human	Proteintech 66786-1-Ig	1:250 O/N 4 °C
Anti-UCHL1	Rabbit anti-human	Proteintech 14730-1-AP	1:500 O/N 4 °C
Anti-Villin	Rabbit anti-human	Abcam ab133510	1:10.000 1 h RT
Anti-TJP1	Mouse anti-human	Invitrogen 339100	1:250 O/N 4 °C
Secondary antibodies			
Secondary Antibody, HRP	Goat anti-rabbit	ThermoFisher Scientific 32460	1:5000 1h RT
Secondary Antibody, HRP	Goat anti-mouse	ThermoFisher Scientific 32430	1:5000 1h RT
Secondary Antibody, HRP	Rabbit anti-goat	Dako P0449	1:5000 1h RT

GDNF: Glial Cell Derived Neurotrophic Factor; GFAP: Glial Fibrillary Acidic Protein; HRP: Horsearadish peroxidase; O/N: Over-night; RT: Room Temperature; S100β: S100 Calcium Binding Protein B; Sox10: Transcription Factor SOX-10; TJP1: Tight Junction Protein 1; UCHL1: Ubiquitin C-terminal Hydrolase L1.

3.3.8. Statistical analysis

Data distribution was tested by the D'Agostino and Pearson, Shapiro-Wilk and Kolmogorov–Smirnov normality tests. Parametric distributed data are expressed as mean ± standard deviation and compared by the unpaired Student's t test (two-tailed) or One-Way Anova test. Non-parametric are expressed as median with interquartile range (IQR) and comparison between groups was analysed by the Mann-Whitney U test or Kruskal-Wallis test with Dunn's multiple comparison pos-hoc test. Frequency and percentage were calculated for qualitative variables and comparison between groups was analysed by the appropriate Fisher's exact or Chi-square test. Relationships between clinical variables and gene and protein expression, as well as the levels of BAs in the jejunal fluid were assessed by Spearman's rho correlation. Values of $p \leq 0.05$ were considered significant. Datasets were analysed using GraphPad Prism 9.01 software (Graphpad Software, San Diego, CA, USA).

4. RESULTS

4.1. Study population

A total of 24 HV and 25 patients fulfilling ROME III criteria for IBS-D were included in this study, among IBS-D patients, 11 subjects had BAD associated. Demographic, clinical and psychological variables according to subgroups are summarised in **Table 4**. Both groups, HV and IBS-D, showed similar age and ratio female: male. Despite not reaching statistical differences, in the comparison between both subsets of IBS-D patients according to SeHCAT, the IBS-D-SeHCAT$^-$ group displayed higher ratio female: male than IBS-D-SeHCAT$^+$ group 64% vs 36%, respectively (p=0.238). As reported in the literature, IBS-D patients scored significantly higher IBS-SSS questionnaire (p<0.0001) and, as expected, reported more bowel movements and lower stool consistency than HVs. Indeed, compared to HVs, both IBS-D SeHCAT$^+$ and IBS-D SeHCAT$^-$ patients scored significantly higher on the IBS-SSS questionnaire and reported higher scores for each individual IBS-SSS domain. In addition, IBS-D SeHCAT$^+$ patients reported a significantly higher maximum number of bowel movements per day and scored significantly higher on the Bristol Stool Chart compared to the control group. In fact, the SeHCAT$^+$ group displayed significantly higher Bristol score than those of the SeHCAT$^-$ group ($p < 0.05$). Regarding the proportion of patients who exhibited overlapping symptoms of functional dyspepsia in the two IBS-D subgroups, it was comparable between groups (IBS-D-SeHCAT$^-$: 27%; IBS-D-SeHCAT$^+$: 36%). Finally, as already reported by our research group, both IBS-D SeHCAT$^+$ and IBS-D SeHCAT$^-$ patients scored significantly higher levels of depression than HV subjects.

Table 4 | Clinical and demographic characteristics of participants. HV and IBS-D were compared using the Mann-Whitney test, study groups were compared using Kruskal-Wallis test and Dunn's multiple comparison test. Data are presented as median (IQR). IBS-D-SeHCAT^{+} vs HV: Ψ; IBS-D-SeHCAT^{-} vs HV: ϕ; IBS-D-SeHCAT^{-} vs IBS-D-SeHCAT^{+}: ¥. 1 symbol p<0.05; 2 symbols p<0.01; 3 symbols p<0.001; 4 symbols p<0.0001.

	HV (n=24)	IBS-D (n=25)	p-value Mann-Whitney	IBS-D SeHCAT^{+} (n=11)	IBS-D-SeHCAT^{-} (n=14)	p-value ANOVA
Gender, F:M	9:15	13:12	0.393	4:7	9:5	0.225
Age, years	28.1 (23.8-39.9)	36.7 (29.9-40.9)	0.112	37.4 (29.5-45.0)	35.1 (28.1-39.6)	0.238
SeHCAT, 7-day % retention	NA	20.1 (8.3-33.7)	-	6.8 (5.0-10.0)	33.3 ¥¥¥¥ (29.8-40.1)	-
IBS-SSS	3.0 (0.0-13.0)	**236.0 (149.5-320.5)**	**<0.0001**	**236.0** ΨΨΨΨ **(159.0-325.0)**	**247.0** ϕϕϕϕ **(137.5-318.3)**	**<0.0001**
Severity of the abdominal pain, score	NA	30.0 (14.0-60.5)	-	37.0 (27.0-58.0)	20.0 (11.0-62.0)	-
Frequency of the abdominal pain, number of days	NA	4.0 (2.0-8.0)	-	5.0 (2.0-9.0)	3.5 (2.0-8.0)	-
Severity of abdominal bloating, score	0.0 (0.0-0.5)	**32.0 (15.3-50.5)**	**<0.0001**	**28.0** ΨΨΨ **(2.0-52.0)**	**33.0** ϕϕϕϕ **(17.8-55.8)**	**<0.0001**
Bowel habit dissatisfaction, score	2.0 (0.0-12.0)	**63.0 (33.5-81.0)**	**<0.0001**	**68.0** ΨΨΨ **(54.0-84.0)**	**52.5** ϕϕϕϕ **(17.3-80.5)**	**<0.0001**
IBS interference with life, score	0.0 (0.0-0.0)	**62.0 (48.0-76.0)**	**<0.0001**	**62.0** ΨΨΨΨ **(49.0-76.0)**	**65.0** ϕϕϕϕ **(29.5-83.8)**	**<0.0001**
Bowel movements, number/day	1.0 (1.0-2.0)	**2.3 (1.3-3.5)**	**0.0005**	**3.5** ΨΨΨ **(2.0-4.0)**	2.0 (1.0-3.5)	**0.0004**
Stool form, Bristol score	4.0 (3.4-4.0)	**5.5 (4.5-6.3)**	**<0.0001**	**6.3** ΨΨΨΨ **(5.5-6.6)**	**4.5** ϕ ¥ **(4.0-5.6)**	**<0.0001**
Functional dyspepsia, yes/no	0	8/17	-	3/8	5/9	-
Depressive symptoms, BDI	0.0 (0.0-1.0)	**8.0 (4.0-14.0)**	**<0.0001**	**4.0** ΨΨΨ **(3.0-14.0)**	**8.5** ϕϕϕϕ **(4.8-13.3)**	**<0.0001**
Perceived acute stress, Cohen scale	16.0 (12.0-21.0)	19.0 (14.0-24.5)	0.142	19.0 (10.0-27.0)	19.0 (16.0-24.3)	0.245
Perceived chronic stress, Holmes scale	90.5 (58.8-140.5)	116.0 (45.3-169.0)	0.563	119.0 (64.8-164.8)	104.5 (37.0-177.5)	0.761

BDI: Beck Depression Inventory; F: Female; HV: Healthy Volunteer; IBS-D: Diarrhoea-predominant Irritable Bowel Syndrome; IBS symptom severity score; M: Male; NA: Not Applicable; SeHCAT: 75-Selenium Homocholic Acid Taurine.

4.2. Jejunal mucosal structure and leucocyte populations

On routine histology, all biopsies disclosed no abnormalities with a proper epithelial architecture and no inflammatory infiltrate or presence of eosinophils, parasites, microbial and viral inclusions. Jejunal mucosa immune populations of all participants were evaluated, including intraepithelial lymphocytes ($CD3^+$), mast cells ($CD117^+$) and eosinophils ($eosin^+$). Specific stainings revealed that cell counts were comparable between IBS-D patients and HVs as well as between both IBS-D-SeHCAT subgroups and HVs (**Figure 2**). Results are summarised in supplementary **Tables S 6 and S 7**.

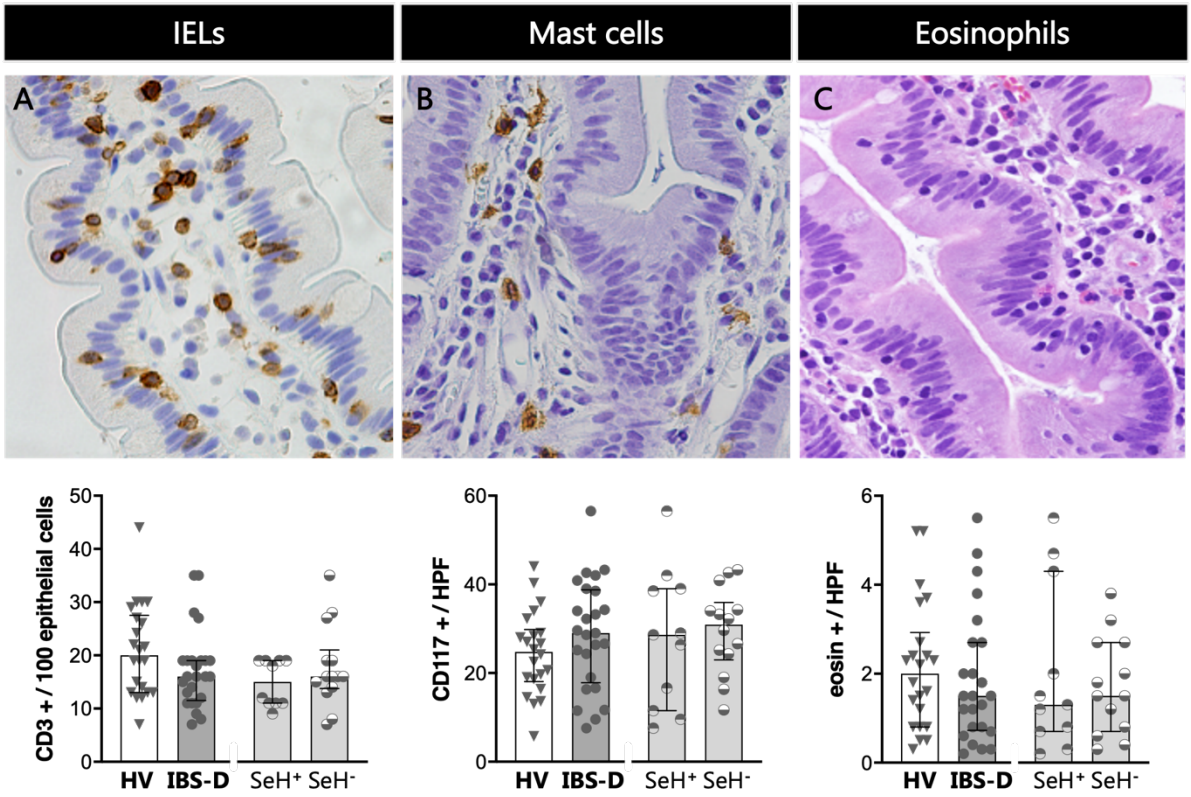

Figure 2 | Mucosal immune populations in the jejunal mucosal biopsies of HV and IBS-D ($SeHCAT^+$/$SeHCAT^-$) patients. Representative stainings (up; magnification 400x) and immune cell counts (down) of IELs (**A**), mast cells (**B**) and eosinophils (**C**). The number of IELs is expressed as per 100 epithelial cells and the number of mast cells and eosinophils is expressed as per HPF in the lamina *propria*. HV and IBS-D were compared using the Mann-Whitney test, study groups were compared using Kruskal-Wallis test and Dunn's multiple comparison test. Data are presented as median (IQR). HPF: High Power Field; HV: Healthy volunteer; IBS-D: Diarrhoea-predominant Irritable Bowel Syndrome; IELs: Intraepithelial lymphocytes; SeH: 75-Selenium Homocholic Acid Taurine.

4.3. Increased S100β stained area in the lamina propria of IBS-D patients

Jejunal mucosal sections from HVs, IBS-D SeHCAT^{+} and IBS-D SeHCAT^{-} patients were immunohistochemical stained for SOX10 and S100β to visualize the presence of EGCs within the lamina *propria* in all groups (**Figure 3. A**). Quantitative analysis of S100β immunoreactivity from IBS-D patients showed a significant increase in the percentage of stained area compared to HV. In fact, the comparison of both IBS-D SeHCAT^{+} and IBS-D SeHCAT^{-} patients with the HV group revealed a trend to be higher in the jejunum from IBS-D SeHCAT^{+} patients (HV: 2.94 [1.58-4.69]; IBS-D SeHCAT^{+}: 5.41 [2.85-6.93]; IBS-D SeHCAT^{-}: 3.81 [2.99-6.45]; % S100β stained area; p=0.09) (**Figure 3. B left**). However, the comparison of the number of SOX10^{+} EGCs per HPF revealed no significant differences between all study groups (HV: 4.57 [3.91-7.42]; IBS-D SeHCAT^{+}: 5.58 [4.33-6.82]; IBS-D SeHCAT^{-}: 5.38 [3.11-7.31]; SOX10+ cells/HPF; p=0.87) (**Figure 3. B right**).

4.4. Differential gene and protein expression analysis in the jejunal mucosa of IBS-D patients with and without BAD.

In order to investigate whether changes in the expression profile of markers related to the jejunal ENS, the intestinal barrier and the BAs signalling may determine the different clinical phenotype in IBS-D patients with and without BAD, a gene and protein expression analysis was performed in jejunal mucosal biopsies from the different study groups. Specifically, related markers to EGC, neuron, neurotrophic factors, cell-cell communication, TJPs, enterocyte function and BARs which experimental procedures performed for each marker are detailed in **Table 2**.

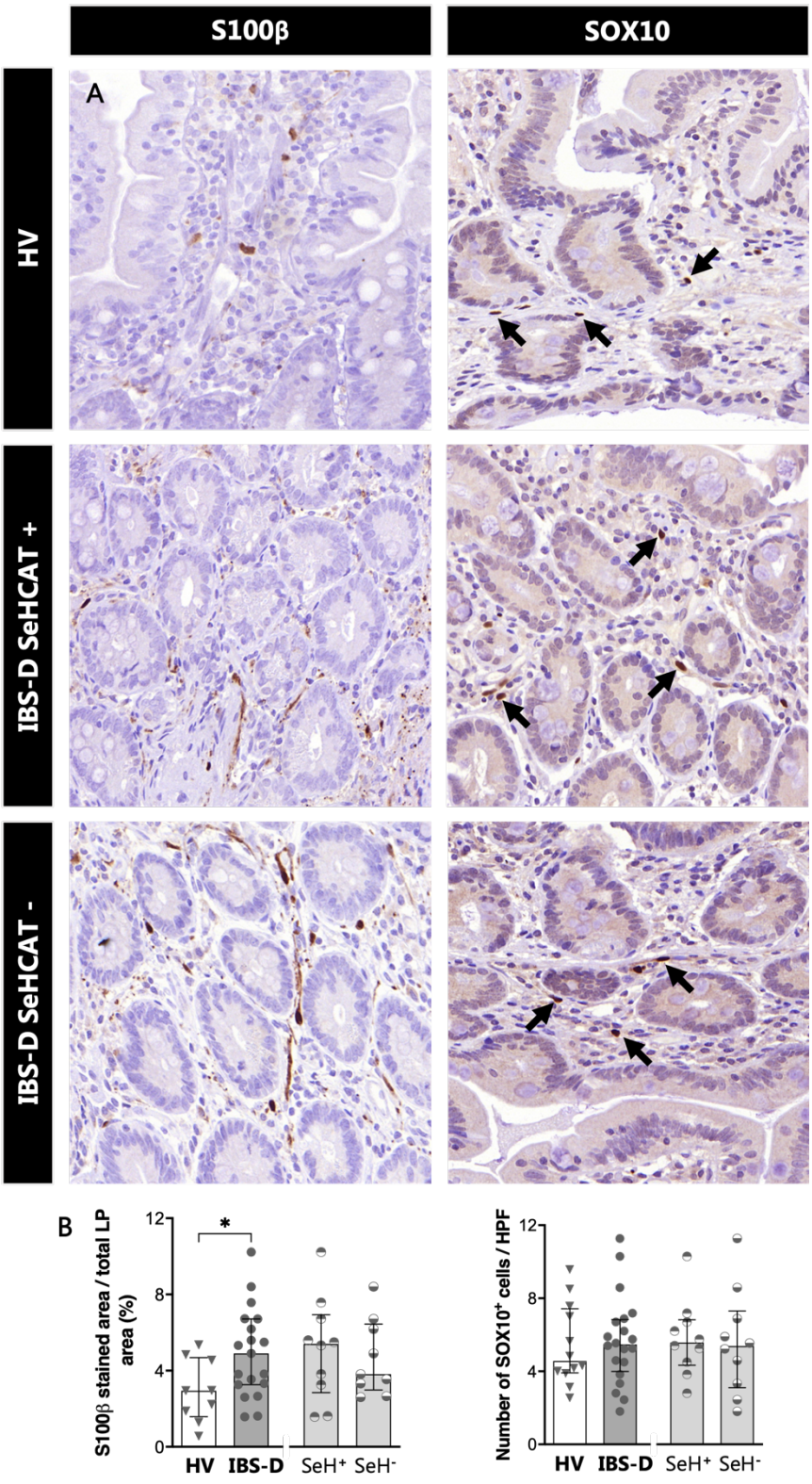

Figure 3 | Profile of the EGC markers S100β (left) and SOX10 (right) in jejunal mucosal sections of HV and IBS-D (SeHCAT$^+$/SeHCAT$^-$) patients. A) Representative images of IHQ staining, magnification: 400x. **B)** Quantification of positive staining in the lamina *propria* (HV=12; IBS-D SeHCAT$^+$=11; IBS-D SeHCAT$^-$= 10). The S100β stained area was calculated as a percentage of the total selected area of the lamina *propria*. The number of SOX10+ cells (arrowheads) per HPF was determined. HV and IBS-D were compared using the Mann-Whitney test, study groups were compared using Kruskal-Wallis test and Dunn's multiple comparison test. Data are presented as median (IQR). * $p \leq 0.05$. HPF: High Power Field; HV: Healthy volunteer; IBS-D: Diarrhoea-predominant Irritable Bowel Syndrome; LP: lamina *propria*; S100β: S100 Calcium Binding Protein β; SeH: 75-Selenium Homocholic Acid Taurine; SOX10: Transcription Factor SOX-10.

4.4.1. Distinct jejunal enteric nerve plasticity in IBS-D patients with and without BAD.

In contrast to the lack of difference in the expression levels of *S100β* mRNA in the jejunal mucosa between IBS-D patients and HVs, a significant down-regulation of *SOX10* mRNA expression was observed in the mucosa of IBS-D patients. Notably, the reduction in *SOX10* mRNA expression was statistically significant only in the $SeHCAT^-$ group when comparing both IBS-D subgroups of patients with HVs (**Figure 4. A-B**). Surprisingly, *GFAP* mRNA expression was not detected in any of the jejunal mucosal samples analysed, regardless of the group.

At the protein level, the changes in jejunal mucosal SOX10 expression followed the same pattern as gene transcription: IBS-D patients displayed a significant down-regulation, and again, the levels were significantly reduced only in the IBS-D-$SeHCAT^-$ group compared to HV (**Figure 4. D**). On the other hand, curiously, the protein expression of GFAP was measurable, and the jejunal mucosa of IBS-D patients displayed a significant up-regulation of GFAP and S100β compared to HV. Moreover, the comparison between the two IBS-D subgroups of patients with HVs showed a significantly increased GFAP expression level only in $SeHCAT^+$ patients, whereas no differences in the expression levels of S100β were observed between all groups studied (**Figure 4. C-E**).

No changes in the expression of the neuronal markers UCHL1 (gene and protein) and PRPH (protein only) were observed between IBS-D or both IBS-D subgroups and HV (**Table 5**).

Mucosal EGCs and IECs contribute to maintaining the gut barrier integrity by producing different neurotrophic factors. Mucosal gene expression of *GDNF* and *BDNF* was also assessed, and mucosa from IBS-D patients exhibited a significant down-regulation of *GDNF* compared to HV. In addition, the analysis of the IBS-D subgroups showed that only IBS-D-$SeHCAT^-$ patients had a significant reduction in the *GDNF* gene expression levels compared to HV. Although at the protein level, only GDNF expression was assessed with no differences in levels between all the groups (**Table 5**).

Within the gut, the components of the ENS interact with different cell types, so we also examined the expression of several genes involved in the intercellular communication in the jejunal mucosa of the different study groups. The *NGF* gene expression was not detected in any of the jejunal mucosal samples analysed, regardless of the group. Among the different genes evaluated, IBS-D samples exhibited a significant down-regulation of *NTRK1* only, and again, IBS-D-$SeHCAT^-$ samples had a significant reduction compared to HV (**Table 5**).

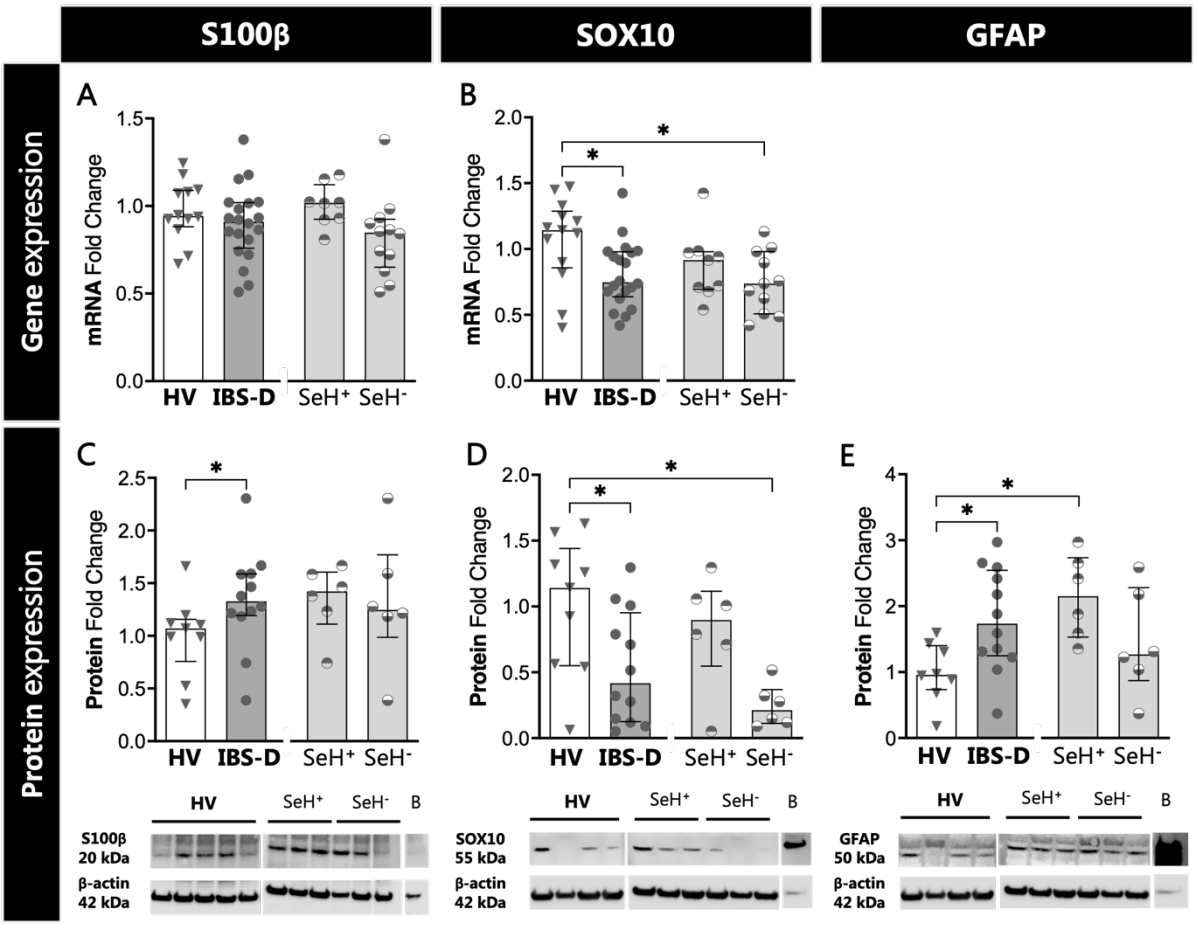

Figure 4 | EGC-specific markers expression in the jejunal mucosa of HV and IBS-D (SeHCAT^{+}/SeHCAT^{-}) patients. Gene expression (HV=13; IBS-D SeHCAT^{+}=9; IBS-D SeHCAT^{-}= 12) of *S100B* (**A**) and *SOX10* (**B**) was assessed by qPCR. Protein expression (HV=9; IBS-D SeHCAT^{+}=6; IBS-D SeHCAT^{-}=6) of S100β (**C**), SOX10 (**D**) and GFAP (**E**) was assessed by WB. HV and IBS-D were compared using the Mann-Whitney test, study groups were compared using Kruskal-Wallis test and Dunn's multiple comparison test. Data are presented as median (IQR). * p≤0.05. B: Brain; Da: Dalton; GFAP: Glial Fibrillary Acidic Protein; HV: Healthy volunteer; IBS-D: Diarrhoea-predominant Irritable Bowel Syndrome; S100β: S100 Calcium Binding Protein β; SeH: 75-Selenium Homocholic Acid Taurine; SOX10: Transcription Factor SOX-10.

Table 5 | Gene and protein expression results of neuron, neurotrophic factors and intercellular communication markers. HV and IBS-D were compared using the Mann-Whitney test, study groups were compared using Kruskal-Wallis test and Dunn's multiple comparison test. Data are presented as median (IQR). IBS-D-SeHCAT$^-$ vs HV: ϕ. 1 symbol p<0.05; 2 symbols p<0.01; 3 symbols p<0.001; 4 symbols p<0.0001.

Marker	HV	IBS-D	p-value Mann-Whitney	IBS-D-SeHCAT$^+$	IBS-D-SeHCAT$^-$	p-value ANOVA
GENE EXPRESSION						
Neuron						
UCHL1	0.86 (0.69-0.99)	0.98 (0.77-1.36)	0.168	1.04 (0.94-1.62)	0.92 (0.62-1.20)	*0.093*
Neurotrophic factors						
GDNF	1.04 (0.76-1.42)	**0.66 (0.45-0.73)**	**<0.001**	0.73 (0.60-0.77)	**0.49 ϕϕϕ (0.39-0.70)**	**0.001**
BDNF	0.99 (0.80-1.25)	0.92 (0.75-1.20)	0.456	1.02 (0.88-1.28)	0.91 (0.70-1.07)	0.472
Intercellular communication						
P2RX7	0.95 (0.74-1.38)	0.81 (0.67-1.10)	0.181	0.92 (0.70-1.23)	0.77 (0.65-1.03)	0.224
GJA1	1.10 (0.77-1.26)	0.87 (0.79-1.16)	0.361	0.98 (0.82-1.27)	0.82 (0.77-0.97)	0.146
NTF3	1.01 (0.82-1.10)	0.98 (0.88-1.09)	0.917	0.96 (0.88-1.03)	1.01 (0.82-1.20)	0.941
NTRK1	1.02 (0.84-1.24)	**0.65 (0.48-0.86)**	**0.0023**	0.74 (0.44-1.13)	**0.57 ϕϕ (0.46-0.66)**	**0.003**
PROTEIN EXPRESSION						
Neuron						
UCHL1	1.00 (0.88-1.11)	1.06 (0.92-1.13)	0.412	1.04 (0.87-1.17)	1.12 (1.00-1.47)	0.261
PRPH	0.96 (0.90-1.16)	1.03 (0.88-1.20)	0.808	1.03 (0.85-1.12)	1.07 (0.88-1.56)	0.741
Neurotrophic Factors						
GDNF	1.01 (0.80-1.17)	1.09 (0.94-1.72)	0.384	1.17 (0.94-1.75)	1.01 (0.83-1.84)	0.599

BDNF: Brain Derived Neurotrophic Factor; GDNF: Glial-Derived Neurotrophic Factor; GJA1: Gap Junction Protein Alpha 1; HV: Healthy Volunteer; IBS-D: Diarrhoea-predominant Irritable Bowel Syndrome; NTF3: Neurotrophin 3; NTRK1: Neurotrophic Receptor Tyrosine Kinase 1; PRPH: Peripherin; P2RX7: Purinergic Receptor P2X 7; SeHCAT: 75-Selenium Homocholic Acid Taurine; UCHL1: Ubiquitin C-terminal Hydrolase L1.

4.4.2. Differential expression of tight junction proteins in IBS-D patients with and without BAD.

In the IBS-D group, a trend towards an increase in *CLDN2* gene expression was observed when compared with the HV group. Interestingly, the analysis of the IBS-D subgroups showed that SeHCAT^{+} patients had a significant increase in *CLDN2* expression in contrast to HV (**Figure 5. A**). Gene expression of *OCLN* and *TJP1* were similar between all groups (**Figure 5. B-C**).

At the protein level, changes in jejunal mucosal CLDN2 expression followed a similar pattern as the gene transcription. Here, the analysis showed a significantly increased expression of CLDN2 in the IBS-D group besides that SeHCAT^{+} patients also had an increment of this TJP compared to HVs (**Figure 5. D**). On the contrary, OCLN expression was significantly decreased in IBS-D patients as well as in the SeHCAT^{-} subgroup compared to HVs (**Figure 5. E**). For TJP1, no differences were found between any of the groups studied (**Figure 5. F**).

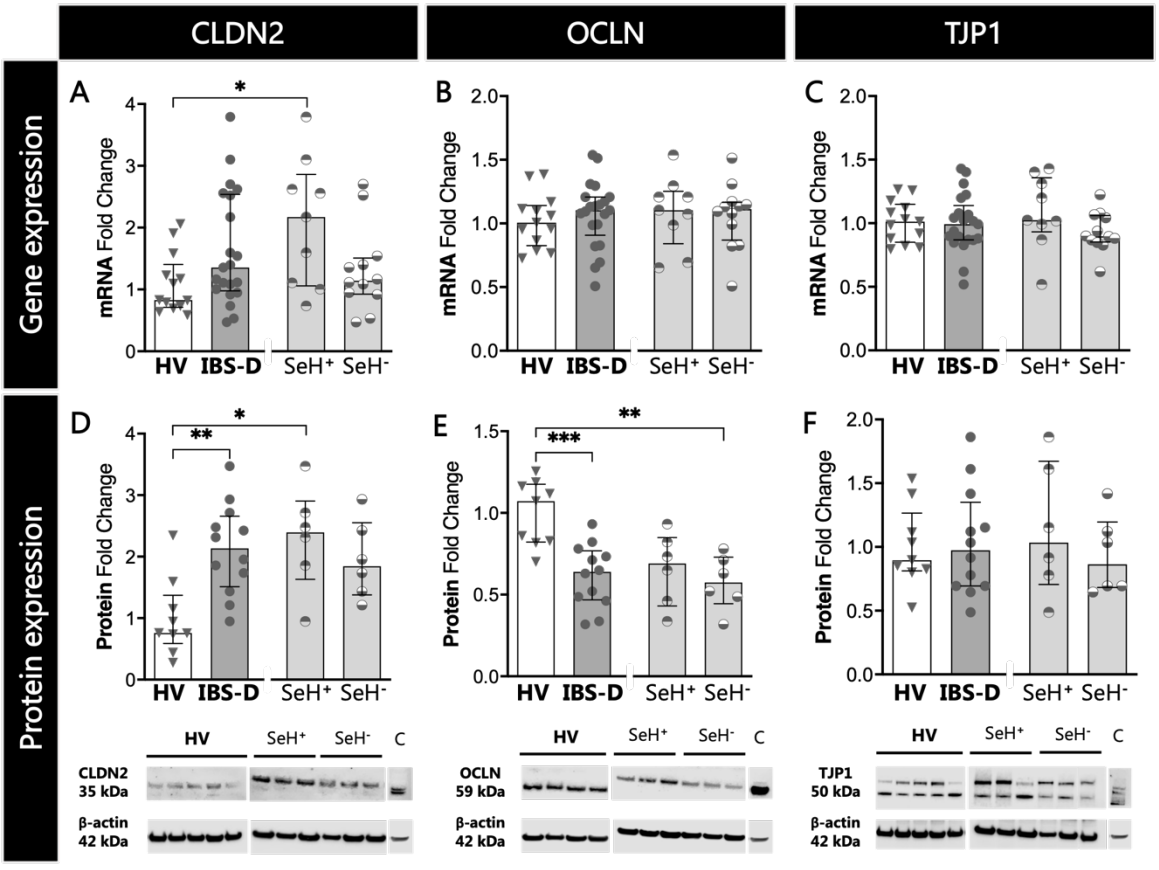

Figure 5 | Tight junction proteins expression in the jejunal mucosa of HV and IBS-D (SeHCAT^{+}/SeHCAT^{-}) patients. Gene expression (HV=13; IBS-D SeHCAT^{+}=9; IBS-D SeHCAT^{-}= 12) of *CLDN2* (**A**), *OCLN* (**B**) and *TJP1* (**C**) was assessed by qPCR.Protein expression (HV=9; IBS-D SeHCAT^{+}=6; IBS-D SeHCAT^{-}=6) of CLDN2 (**D**), OCLN (**E**) and TJP1 (**F**) was assessed by WB. HV and IBS-D were compared using the Mann-Whitney test, study groups were compared using Kruskal-Wallis test and Dunn's multiple comparison test. Data are presented as median (IQR). * p≤0.05, **p ≤0.01, ***p ≤0.001. C: Caco-2 cells; CLDN2: Claudin 2; Da: Dalton; HV: Healthy volunteer; IBS-D: Diarrhoea-predominant Irritable Bowel Syndrome; OCLN: Occludin; SeH: 75-Selenium Homocholic Acid Taurine; TJP1: Tight Junction Protein 1.

4.4.3. Reduced protein expression of Villin in IBS-D patients

The protein expression of VILL, a protein found in the intestinal brush border, was lower in the mucosa of IBS-D patients compared to HVs, despite not reaching statistical significance. Interestingly, the analysis of the IBS-D subgroups showed that SeHCAT$^-$ patients also had a trend towards reduced expression compared to HVs (**Figure 6**).

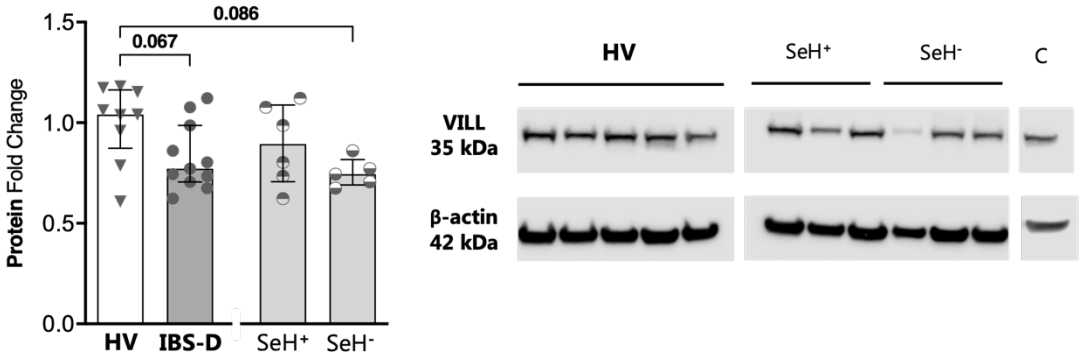

Figure 6 | Villin expression in the jejunal mucosa of HV and IBS-D (SeHCAT$^+$/SeHCAT$^-$) patients. Protein expression (HV=9; IBS-D SeHCAT$^+$=6; IBS-D SeHCAT$^-$=6) of CLDN2 was assessed by WB. HV and IBS-D were compared using the Mann-Whitney test, study groups were compared using Kruskal-Wallis test and Dunn's multiple comparison test. Data are presented as median (IQR). C: Caco-2 cells; Da: Dalton; HV: Healthy volunteer; IBS-D: Diarrhoea-predominant Irritable Bowel Syndrome; SeH: 75-Selenium Homocholic Acid Taurine; VILL: Villin.

4.4.4. Bile acid receptors expression in the jejunal mucosa of IBS-D patients

Differences in the gene profile related to EGCs and gut barrier integrity between the groups suggest that BAs play a role in IBS-D pathophysiology. We next analysed the expression profile of the genes of several BARs in the jejunal mucosa of the participants in this study. The expression levels of GPBAR1 were higher in IBS-D patients (p=0.08; borderline value) compared to HV (**Figure 7. B)**. However, no differences in the expression levels of the BARs NR1H4 and VDR were found between patients and controls (**Figure 7. A-C**). Likewise, the analysis of the IBS-D subgroups showed no differences in the expression levels for the different genes among the different groups. In addition, the FGF19 gene expression was not detected in any of the jejunal mucosa analysed, regardless of the group.

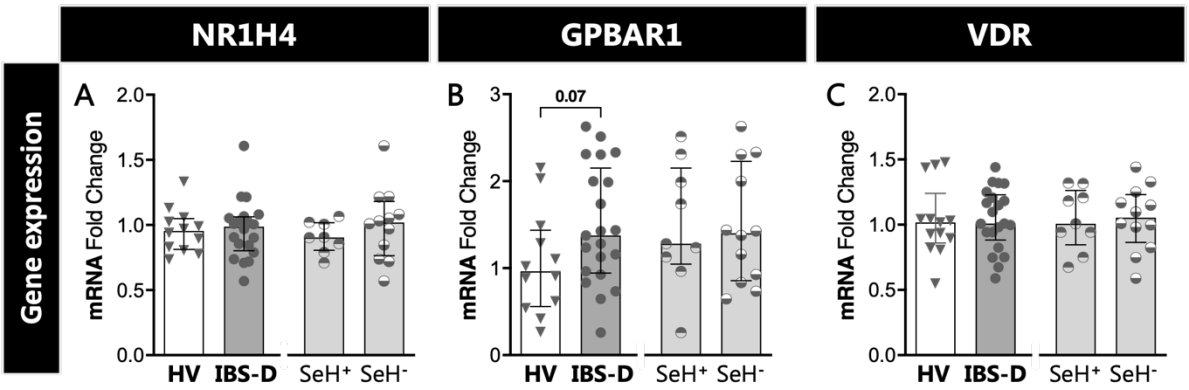

Figure 7 | BARs expression in the jejunal mucosa of HV and IBS-D (SeHCAT$^+$/SeHCAT$^-$) patients. Gene expression (HV=13; IBS-D SeHCAT$^+$=9; IBS-D SeHCAT$^-$= 12) of *NR1H4* (**A**), *GPBAR1* (**B**) and *VDR* (**C**) was assessed by qPCR. HV and IBS-D were compared using the Mann-Whitney test, study groups were compared using Kruskal-Wallis test and Dunn's multiple comparison test. Data are presented as median (IQR). GPBAR1: G Protein-Coupled Bile Acid Receptor 1; HV: Healthy volunteer; IBS-D: Diarrhoea-predominant Irritable Bowel Syndrome; NR1H4: Nuclear Receptor Subfamily 1 Group H Member 4; SeH: 75-Selenium Homocholic Acid Taurine; VDR: Vitamin D Receptor.

4.5. Distinct clinical-biological association in IBS-D subgroups

We investigated whether the IBS-D subgroup-specific changes in EGCs markers in the jejunal mucosa were associated with those in the epithelial barrier. Interestingly, in the SeHCAT$^+$ group, the S100β expression correlated positively with those of CLDN2 (r*s*=0.59; p=0.02), GFAP expression correlated positively with those of OCLN (r*s*=-0.56; p=0.04) and TJP1 (r*s*=0.68; p=0.01), and finally SOX10 expression correlated positively with those of VILL (r*s*=0.59; p=0.02). On the other hand, in the SeCHAT$^-$ group, the S100β and SOX10 expression correlated positively with those of CLDN2 (r*s*=0.68; p<0.01) and VILL (r*s*=0.81; p<0.001), respectively.

In view of the different plasticity and TJP expression profile within the jejunal mucosa between the different study groups, we next investigated the potential association between the expression of the different proteins determined and the clinical features recorded in this study.

First, including all the patients in the IBS-D group, regardless of the SeHCAT result, we found a correlation between the expression of those proteins considered markers of EGCs and symptoms. Thus, the expression of S100β and GFAP correlated positively with bowel habits, specifically with bowel movements (r*s*=0.58; p=0.006) and stool form (r*s*=0.58; p=0.008), respectively, whereas the level of SOX10 correlated negatively with the severity of abdominal bloating (r*s*=-0.54; p=0.011) and the presence of depressive symptoms (r*s*=-0.66; p=0.001).

On the other hand, there was also an association between protein expression of TJPs and symptoms. Among these, CLDN2 and OCLN were found to be the most associated. Thus, higher expression of CLDN2 and OCLN correlated inversely with the Bristol score (r*s*=0.72; p<0.001 and r*s*=-0.50; p=0.021, respectively) and the presence of depressive symptoms (r*s*=0.46; p=0.04 and r*s*=-0.45; p=0.040, respectively). In addition, CLDN2 also correlated with the number of bowel movements (r*s*=0.50; p=0.021) and negatively with the frequency of abdominal pain (r*s*=-0.57; p=0.048) while OCLN correlated negatively with the severity of the disease (r*s*=-0.58; p=0.006) and the abdominal bloating (r*s*=-0.62; p=0.003). TJP1 expression did not correlate with any of the clinical features. Finally, VILL correlated negatively with the abdominal bloating (r*s*=-0.45; p=0.047) and depressive symptoms (r*s*=-0.74; p<0.001).

To identify whether the differential profile observed between the two independent IBS-D subgroups, SeHCAT$^+$ and SeHCAT$^-$ was associated with their symptom characteristics, we performed a second variable correlation analysis.

Interestingly, when only the SeHCAT$^+$ group was analysed together with HVs (**Table 6**), the higher expression of GFAP in SeHCAT$^+$ patients correlated positively with bowel habits and also with the severity of IBS. The higher expression of CLDN2, apart from the bowel habits, correlated positively with the intensity of abdominal bloating. In addition, the expression of S100β correlated positively with bowel movements, OCLN negatively with the IBS severity and stool form, and VILL negatively with the presence of depressive symptoms.

When only the SeHCAT$^-$ group was analysed together with HVs (**Table 7**) a completely different pattern was found. Most of the correlations found between the expression of SOX10, VILL, and OCLN in the SeHCAT$^-$ patients and the intensity of abdominal bloating, the intensity of abdominal pain, the severity of IBS, as well to the presence of depressive symptoms were strongly negative. Furthermore, CLDN2 expression correlated positively with abdominal bloating and stool form, while TJP1 with the chronic stress score, S100β with the perceived stress score, and GFAP negatively with the frequency of abdominal pain.

The results in the correlation analysis suggest different mechanisms involved in the pathogenesis of symptoms, with molecular changes in EGCs-markers and tight junctions being differentially associated. In SeHCAT$^+$ patients these were associated with worse bowel habits, whereas in SeHCAT$^-$patients they were negatively associated with features of visceral hypersensitivity such as intensity of abdominal pain and abdominal bloating and the presence of depressive symptoms.

Table 6 | Correlations of EGC and epithelial barrier markers protein expression with clinical characteristics in HVs and IBS-D SeHCAT$^+$ patients.

	EGC						INTESTINAL BARRIER FUNCTION							
	S100β		SOX10		GFAP		CLDN2		OCLN		TJP1		VIL1	
	rs	p	*rs*	p	*rs*	p	*rs*	p	*rs*	p	*rs*	p	*rs*	p
SeHCAT	-0.31	0.56	0.31	0.56	-0.26	0.66	-0.66	0.18	0.20	0.71	-0.43	0.42	0.14	0.81
IBS-SSS	*0.45*	*0.09*	0.02	0.95	**0.71**	**0.01**	*0.45*	*0.09*	**-0.59**	**0.02**	0.14	0.62	-0.20	0.48
Severity of the abdominal pain	-0.26	0.66	-0.26	0.66	-0.49	0.36	-0.26	0.66	0.26	0.66	-0.54	0.30	-0.31	0.56
Frequency of the abdominal pain	-0.12	0.84	0.03	0.99	-0.49	0.34	-0.46	0.39	0.06	0.94	-0.55	0.27	-0.03	0.99
Severity of abdominal bloating	0.41	0.13	-0.38	0.16	0.38	0.18	**0.54**	**0.04**	-0.44	0.10	-0.05	0.85	-0.27	0.34
Bowel movements	**0.63**	**0.01**	-0.21	0.44	**0.62**	**0.02**	**0.81**	**<0.001**	*-0.44*	*0.09*	0.02	0.94	0.02	0.95
Stool form	*0.48*	*0.07*	-0.36	0.18	**0.62**	**0.02**	**0.78**	**<0.001**	**-0.53**	**0.05**	0.11	0.69	-0.35	0.20
Becks Depression Inventory	0.23	0.40	-0.44	0.10	0.41	0.14	0.38	0.17	-0.42	0.12	-0.04	0.88	**-0.65**	**0.01**
Cohen Scale	0.36	0.19	0.04	0.89	0.28	0.34	0.22	0.42	0.04	0.87	0.10	0.72	-0.35	0.20
Holmes-Rahe Scale	0.06	0.84	-0.05	0.86	0.24	0.41	-0.03	0.93	-0.35	0.20	-0.12	0.67	*-0.46*	*0.09*

Comparisons were performed using the Spearman's rho correlation. Data are represented as: r*s* (Spearman's rank correlation coefficient) and p (p value). Values of p of ≤0.05 were considered significant and it is shown in bold in the table.

Table 7 | Correlations of EGC and epithelial barrier markers protein expression with clinical characteristics in HVs and IBS-D SeHCAT$^-$ patients.

	EGC						INTESTINAL BARRIER FUNCTION							
	S100β		SOX10		GFAP		CLDN2		OCLN		TJP1		VIL1	
	rs	p	*rs*	p	*rs*	p	*rs*	p	*rs*	p	*rs*	p	*rs*	p
SeHCAT	0.14	0.80	-0.37	0.50	0.43	0.42	0.49	0.36	-0.37	0.50	-0.60	0.24	-0.70	0.23
IBS-SSS	0.29	0.29	-0.40	0.14	0.30	0.30	*0,45*	*0.09*	**-0.70**	**<0.01**	-0.03	0.91	-0.45	0.11
Severity of the abdominal pain	0.49	0.36	**-0.94**	**0.02**	-0.77	0.10	-0.60	0.24	0.09	0.92	-0.09	0.92	**-1.00**	**0.02**
Frequency of the abdominal pain	0.21	0.73	-0.76	0.12	**-0.88**	**0.05**	-0.76	0.12	0.33	0.53	0.27	0.62	-0.67	0.30
Severity of abdominal bloating	0.39	0.15	**-0.63**	**0.01**	0.15	0.62	**0.63**	**0.01**	**-0.71**	**<0.01**	-0.18	0.52	**-0.56**	**0.04**
Bowel movements	0.41	0.12	-0.16	0.57	-0.08	0.78	0.37	0.18	-0.13	0.64	0.01	0.99	0.21	0.47
Stool form	0.26	0.35	-0.44	0.10	0.24	0.40	**0.57**	**0.03**	*-0.47*	*0,07*	0.08	0.79	-0.39	0.17
Becks Depression Inventory	0.25	0.37	**-0.74**	**<0.01**	0.05	0.88	0.37	0.18	**-0.64**	**0.01**	-0.20	0.47	**-0.80**	**<0.01**
Cohen Scale	**0.63**	**0.01**	0.04	0.88	0.36	0.21	*0.45*	*0.09*	-0.30	0.28	0.31	0.27	-0.11	0.71
Holmes-Rahe Scale	0.08	0.79	0.36	0.18	0.27	0.35	-0.08	0.77	-0.15	0.60	**0.54**	**0.04**	0.13	0.66

Comparisons were performed using the Spearman's rho correlation. Data are represented as: r*s* (Spearman's rank correlation coefficient) and p (p value). Values of p of ≤0.05 were considered significant and it is shown in bold in the table.

4.6. Distinctive profile of BAs in the jejunal fluid of IBS-D patients

These previous results suggest the presence of an IBS-D subgroup-specific molecular profile in the jejunum, which could be explained by a different composition of the jejunal BA pool. Next, we performed a quantitative analysis of the content of BAs in the intestinal fluid of the jejunum of a subset of the participants included in this study.

First, only conjugated BAs and not unconjugated BAs were identified in the jejunal fluid of HVs and both IBS-D subgroups. In fact, all types of BAs conjugated with glycine (GCA, GCDCA, GDCA, GLCA and GUDCA) were detected, but only three types of BAs conjugated with taurine (TCA, TCDCA and TUDCA). Thus, all the groups in the study presented a greater proportion of BAs conjugated with glycine than with taurine (**Table 8**).

Interestingly, both IBS-D SeHCAT subgroups had significantly higher content of total combined conjugated BAs compared to HVs (HV: 11.0 (0.0-60.3); IBS-D SeHCAT^{+}: 52.3 (2.9-170.7), p=0.007; IBS-D SeHCAT^{-}: 69.9 (15.5-290.4), p<0.0001). Second, the total secondary to primary BA ratio was similar in HV and SeHCAT^{-} subjects, whereas the SeHCAT^{+} subgroup showed an increase in the amount of secondary BAs (**Figure 8. A**). Third, the comparative analysis of the type of amino acid conjugating the BAs between the two IBS-D subgroups and HV showed a remarkable and significant increase in taurine-conjugated BA levels in the SeHCAT^{-} group (HV: 0.1 (0.0-18.0); IBS-D SeHCAT^{+}: 1.9 (0.0-49.4), p>0.999; IBS-D SeHCAT^{-}: 30.9 (5.6-113.8), p=0.008) (**Figure 8. B**).

According the composition of the BA pool in the different study groups, it is noteworthy that HVs had a higher content of the primary BA, GCA, in jejunal fluid than IBS-D patients. The IBS-D SeHCAT^{+} subgroup had a higher content of the secondary BA GLCA than the other two groups. In addition, IBS-D SeHCAT^{-} patients had more total combined taurine-conjugated BAs than the other groups (**Figure 8. C**).

Glycine-conjugated forms were significantly increased in IBS-D vs. HV. Regarding the taurine-conjugated forms of BAs, only TUDCA was significantly increased in IBS-D compared to HV. In contrast, when the comparative analysis of the different BAs was performed by stratifying the IBS-D group into SeHCAT$^{+/-}$, the GDCA concentration showed a trend towards an increase only in SeHCAT^{+} patients compared to HV (p=0.08, borderline value), whereas no difference was found in the SeHCAT^{-} group. In addition, the levels of all the taurine-conjugated BA forms were significantly increased only in the SeHCAT^{-} patients (**Table 8**).

Finally, we investigated whether there was a possible relationship between the composition of the BA content in the jejunal fluid and the protein expression of the neuroepithelial markers studied previously. Interestingly, the content of secondary glycine-conjugated BAs, GDCA and GLCA, correlated positively with GFAP expression (r*s*=0.71; p=0.028 and r*s*=0.72; p=0.024) and negatively with OCLN expression (r*s*=-0.73; p=0.021 and r*s*=-0.77; p=0.01). In addition, the content of the two conjugated forms of the primary BA CA, GCA and TCA, also correlated negatively with OCLN expression (r*s*=-0.87 p=0.002 – r*s*=-0.85; p=0.003). On the other hand, from the study related to the association between the content of BAs in the intestinal fluid and the clinical features of all participants, it is noteworthy the positive correlation found between the conjugated secondary BAs, GDCA, GLCA and GUDCA, and bowel habits together with abdominal bloating (**Table 9**).

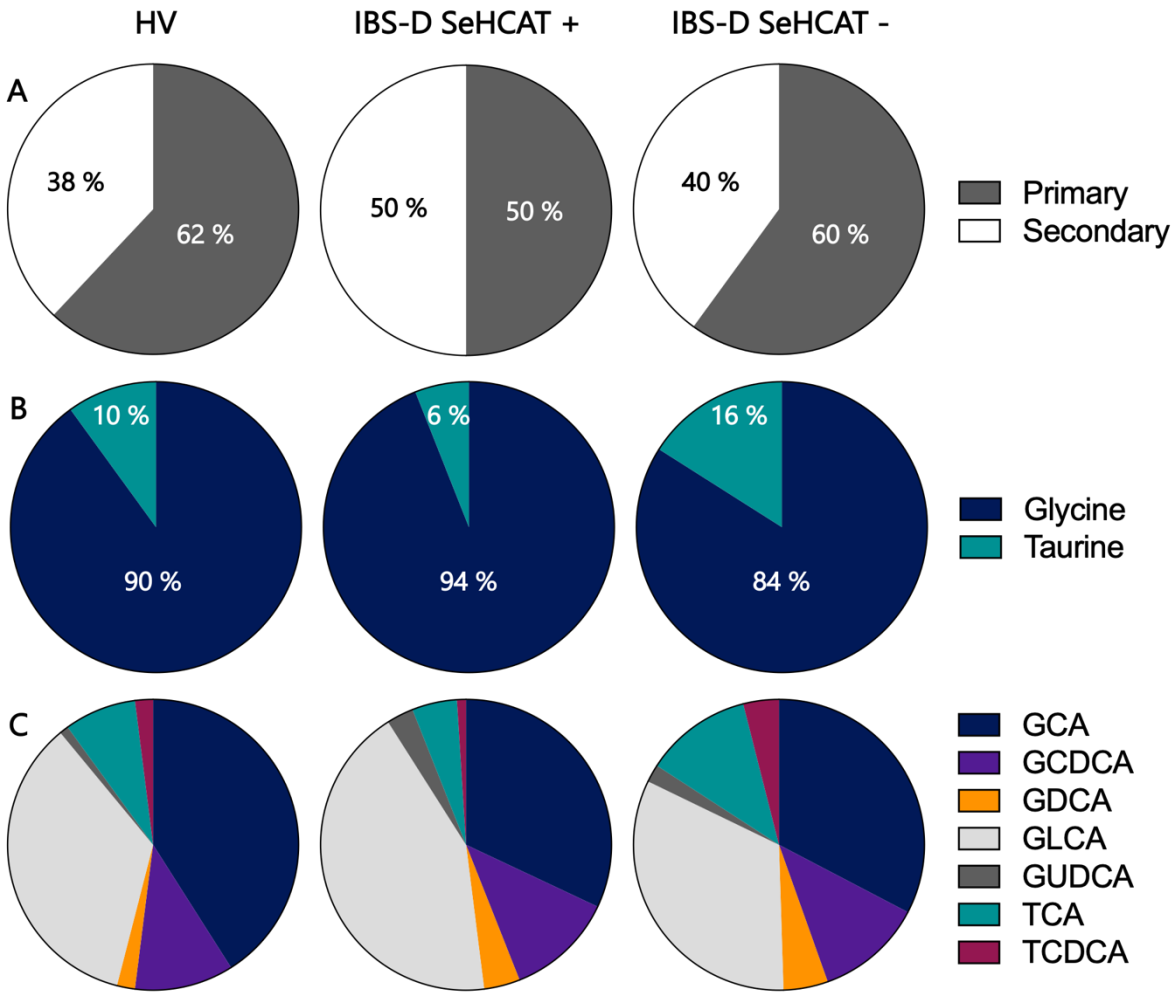

Figure 8 | Proportion of BAs in the participants' jejunal intestinal fluid. Proportion of primary and secondary BAs (**A**), glycine and taurine conjugation (**B**) and types of BAs (**C**) in HV, IBS-D SeHCAT^{+} and IBS-D SeHCAT^{-} patients. GCA: Glyco-Cholic Acid; GCDCA: Glyco-Chenodeoxycholic Acid; GDCA: Glyco-Deoxycholic Acid; GLCA: Glyco-Lithocholic Acid; GUDCA: Glyco-Ursodeoxycholic Acid; HV: Healthy Volunteer; IBS-D: Diarrhoea-predominant Irritable Bowel Syndrome; SeHCAT: 75-Selenium Homocholic Acid Taurine; TCA: Tauro-Cholic Acid; TCDCA: Tauro-Chenodeoxycholic Acid.

Table 8 | BAs concentration in the jejunal intestinal fluid in the different groups. HV and IBS-D were compared using the Mann-Whitney test, study groups were compared using Kruskal-Wallis test and Dunn's multiple comparison test. All values in the table are in μM. Data are presented as median (IQR).

BAs	HV	IBS-D	p-value Mann-Whitney	IBS-D $SeHCAT^+$	IBS-D $SeHCAT^-$	p-value ANOVA
GLYCINE CONJUGATED PRIMARY BAs						
GCA	166 (85-396)	445 (179-657)	**0.044**	422 (166-470)	564 (205-739)	0.098
GCDCA	29 (11-71)	177 (77-222)	**0.001**	**108 (82-217)**	**184 (60-292)**	**0.006**
GLYCINE CONJUGATED SECONDARY BAs						
GDCA	7 (2-36)	43 (8-83)	**0.022**	*46 (13-83)*	41 (3-70)	0.064
GLCA	299 (0-385)	496 (429-557)	**<0.001**	**494 (441-536)**	**498 (373-703)**	**0.002**
GUDCA	1 (0-5)	21 (11-53)	**<0.001**	**35 (9-56)**	**21 (12-46)**	**0.002**
TAURINE CONJUGATED PRIMARY BAs						
TCA	22 (8-75)	63 (44-114)	0.101	59 (42-84)	**143 (56-290)**	**0.053**
TCDCA	0 (0-17)	19 (0-60)	0.093	0 (0-31)	**33 (10-123)**	**0.040**
TAURINE CONJUGATED SECONDARY BAs						
TUDCA	0 (0-0)	1 (0-6)	**0.015**	0 (0-3)	**6 (0-7)**	**0.026**

BA: Bile Acid; GCA: Glyco-Cholic Acid; GCDCA: Glyco-Chenodeoxycholic Acid; GDCA: Glyco-Deoxycholic Acid; GLCA: Glyco-Lithocholic Acid; GUDCA: Glyco-Ursodeoxycholic Acid; HV: Healthy Volunteer; IBS-D: Diarrhoea-predominant Irritable Bowel Syndrome; SeHCAT: 75-Selenium Homocholic Acid Taurine; TCA: Tauro-Cholic Acid; TCDCA: Tauro-Chenodeoxycholic Acid; TUDCA: Tauro-Ursodeoxycholic Acid.

Table 9 | Correlation of glycine conjugated secondary BAs concentration in the intestinal fluid and bowel habits and abdominal bloating.

	GDCA		GLCA		GUDCA	
	r*s*	p	r*s*	p	r*s*	p
Bowel movements	0.52	0.133	**0.38**	**0.048**	**0.433**	**0.024**
Stool form	**0.59**	**0.002**	**0.574**	**0.002**	**0.52**	**0.006**
Severity of abdominal bloating	0.29	0.147	**0.48**	**0.012**	**0.40**	**0.037**

Data are represented as: r*s* (Spearman's rank correlation coefficient) and p (p value). Values of p of ≤0.05 were considered significant and it is shown in bold in the table.

5. DISCUSSION

In this study, we have identified a distinct molecular plasticity of EGCs in the jejunal mucosa of IBS-D patients in a step-wise approach. First, we found jejunal molecular changes in the phenotype of EGCs in IBS-D patients. Second, we found a differential expression of TJPs and the phenotype of EGCs in the jejunal mucosa of patients with IBS-D with or without BAD that are associated with clinical features. Third, we found a different composition of the BA content in the jejunal fluid of IBS-D patients, highlighting the differences in the proportion of conjugated secondary and primary as well as of glycine- or taurine-conjugated forms of BAs between IBS patients with and without BAD and HV. Interestingly, in patients with BAD, the EGC phenotype is "reactive", which is associated with dysregulation of TJPs, where secondary BAs could be involved. Remarkably, these molecular features are associated with bowel habits.

BAs are amphipathic molecules of increasing interest due to their contribution to the regulation of gut health by modulating different physiological mechanisms, mainly those related to epithelial ion and fluid transport in addition to their role in intestinal lipids digestion and absorption. It is also worth noting the high prevalence of BAD in IBS-D patients with overlapping symptoms (26), and in those patients with BAD, BAs have been shown to cause excessive fluid secretion, increase intestinal permeability and accelerate colonic transit (27–29). Indeed, changes in the composition of the intestinal luminal BA pool through activation of different receptors are associated with impaired intestinal barrier integrity and with the modulation of the intestinal mucosal immune response, promoting the development of several GI disorders (49). In this line, increasing evidence suggests that BAs, through their role as signalling hormones, act as a bridge in gut-brain communication and, therefore, alterations in the BA pool are gaining interest as a putative mechanism underlying conditions with dysregulation in the gut-brain axis, such as IBS (50).

Previous data from our group have demonstrated a transcriptional signature of genes specific to the jejunum in IBS-D that is associated with dysregulation of the TJPs and the activation of mast cells (32). Evidence from the literature supports that low-grade inflammation with an immune activation in the intestinal mucosa, closely associated with nerve endings, combined with an abnormal neural signalling, as well as alterations in EGC interaction with mast cells (16) may underpin the pathophysiology of IBS (31,51–55).

For all these reasons, our aim was to first investigate the molecular ENS phenotype in the jejunum of IBS-D patients, focusing mainly on the EGCs, and whether alterations in EGC biology are linked with dysfunction of the molecular profile of TJPs. Furthermore, since a subset of IBS-D patients have associated BAD, we wanted to explore whether the presence or absence of idiopathic BAD in IBS-D defines a different signature in the jejunal mucosa in terms of intestinal neuroplasticity and barrier integrity, at gene and protein expression level.

Enteric nerve plasticity in the jejunal mucosa of IBS-D patients

Since EGC populations are known to be heterogeneous and able to differentially express EGC markers under surrounding microenvironmental triggers (9) , we determined different specific protein markers. First, in contrast to the similar gene expression levels of S100β found between IBS-D and HVs, at protein level, a significant and elevated S100β immunoreactivity confirmed by Western blot was found in the jejunal mucosa of both subgroups of IBS-D patients compared to HVs. However, the levels of S100β protein expression were slightly higher but non-significant in the $SeHCAT^{+}$ group than HV. Our data show higher expression at the protein level in the jejunal mucosa by two techniques. This is in contrast previous results from the colonic mucosa of IBS patients, where Lilli *et al.* (15) found no differences in the protein expression of S100β, while in the lamina *propria* showed a significantly lower staining area of S100β compared to healthy controls, and the exposure of EGCs to mucosal supernatants of these patients induced a reduced Ca^{+2} response. Conversely, Meira de-Faria *et al*. (16) observed similar immunofluorescence as well as mucosal S100β protein expression (assessed by WB and ELISA) in the colon of female IBS patients and healthy controls. Interestingly, they, as well as Wang *et al*., reported that the colonic mucosal specimens of IBS patients exhibited gliosis, as indicated by significantly increased GFAP protein expression (16,17). Wang *et al.* also observed this feature in the colon of mice after intracolonic infusion of faecal supernatants from IBS-D patients. Here, our data also show significantly increased GFAP protein expression in the jejunal mucosa of IBS-D patients, being remarkably higher in those patients with a positive SeHCAT than in HV. Increased expression of both EGC makers, especially GFAP, is indicative of gliosis and of glial activation, and several lines of evidence, mostly conducted in inflammatory bowel disease (56), involve GFAP-positive EGCs in the regulation of the inflammatory response as well as intestinal epithelial integrity (57–60). Thus, increased GFAP has been observed in the colon in neurological diseases such as Parkinson's disease (61). In fact, different densities of GFAP-positive cells overlapping with neurons have also been described in the colonic mucosa and

submucosa of IBS-like animal models (62) as well as greater co-expression with the nerve growth factor assessed by immunofluorescence staining (63).

In addition, in this study, we also analysed the expression of another specific EGC marker, SOX10, a transcription factor required for early ENS development and, in the adult gut, for EGC differentiation and survival (11,64). Our data show a significant gene and protein downregulation of SOX10 expression in the jejunal mucosa of IBS-D patients, especially in the IBS-D SeHCAT$^-$ subgroup, compared to HVs. To date, no studies have focused on SOX10 expression in IBS-D patients, although observations in other GI disorders, including ulcerative colitis, colorectal, gastric and esophageal tumors (65,66), have described a downregulation of SOX10. However, our data do not show any significant differences in the number of SOX10$^+$ cells per HPF in the jejunum between the different groups. The discrepancy observed between the results obtained using protein and immunohistochemical analysis may result from the method used, which only assesses the number of SOX10-positive EGCs without considering other features such as the size of these cells and the staining intensity. Furthermore, in the present study, the results of the immunohistochemical analysis reflect the number of SOX10-positive cells in the jejunal lamina *propria*, whereas the results of protein analysis reflect the expression by the entire jejunal mucosa (epithelium and lamina *propria*). However, SOX10 is known to be an EGC marker and there is a lack of research on SOX10 expression by other intestinal cells.

On the other hand, EGCs are a source of neurotrophic factors, such as GDNF and BDNF, which exert beneficial effects on neurons and epithelial cells. Thus, GDNF has been suggested to protect enterocytes from cytokine-induced apoptosis (67–69) and to promote neuronal survival (70). Although we found a significant downregulation of GDNF gene expression in the jejunum of IBS-D patients, in particular in the SeHCAT$^-$ group, in comparison to HVs, we did not confirm this at the protein level. With regard to GDNF, there are conflicting results in the literature regarding its expression in the colonic mucosa of IBS patients; Lee *et al.* (71) showed only changes in GDNF mRNA in IBS-C patients, with no difference in IBS-D patients compared to controls. Two other studies reported a variable pattern of protein expression, with decreased release of GDNF levels in colonic samples from IBS patients analysed by ELISA (16) and increased GDNF protein levels were found in IBS-D patients compared to healthy subjects (72). The variability of these results may be due to the fact that GDNF expression in the gut is not EGC-specific, as other cell types within the mucosa also express GDNF (72,73). Regarding BDNF, several studies have associated methylation changes in the BDNF gene with

early life stress and somatic symptoms. Our data showed no differences in jejunal BDNF mRNA between the different groups, in agreement with results from Konturek *et al.* (74). However, previous findings have reported elevated expression in the colonic mucosa of IBS (17,74–77). In addition, since neutrophins have been implicated in the pathogenesis of IBS, we also examined the gene expression of NT3 and NGF as well as NTRK1 (receptor of NGF). While our results showed no significant differences for NTF3 expression and we were unable to detect the expression of NGF, we observed a significant downregulation of NTRK1 expression in IBS-D patients, in particular in the $SeHCAT^-$ group, in comparison to healthy controls. In contrast, Dothel *et al.* (53) and Mola *et al.* (78) described an increase in colonic levels of NGF and NTRK1 in IBS and inflammatory bowel disease, respectively, compared to controls.

Since we observed an alteration of glial markers expression in the jejunum of IBS patients, we next investigated whether these changes would affect neuronal density. In contrast to previous findings of increased density of mucosal nerve fibers and nerve outgrowth in the colonic mucosa of IBS patients (53,55), in our study, we found no differences in the expression of the panneuronal marker UCHL1 in the jejunal mucosa of IBS-D patients, neither in the protein expression of another neuronal marker, PRPH. Despite these similar levels of neuronal markers, we cannot exclude alterations in glial-neuronal interaction and neuronal circuits with differential neurotransmitter release, highlighting the importance of proper characterisation of the ENS in the jejunal mucosa of IBS-D patients. Indeed, we evaluated whether there is consequently a dysregulation of the intercellular communication within the jejunal mucosa in IBS-D and whether it differs between those with and without BAD, given the enormous diversity of signalling through the microbiota-gut-brain axis described in IBS (8). Despite the importance of the P2X7-dependent neuronal-glial pathway and the glial purinergic-connexin-43 pathway (79,80), we found no differences in the gene expression of P2X7 and GJA1 (connexin-43) in the jejunum of IBS-D patients and HVs however without functional studies we cannot conclude these pathways are not involved in IBS-D.

Intestinal barrier function in the jejunal mucosa of IBS-D

Alterations in TJPs expression, together with increased intestinal permeability, are well-recognised within the etiopathogenesis of IBS-D, but whether BAs mediate these alterations remains unexplored. The human jejunum is a poorly studied intestinal region in the IBS context. As previously shown by our group (14,33), we observed an increased protein expression of CLDN2 in the jejunum of IBS-D patients, which was significantly upregulated in the IBS-D SeHCAT^{+} subset, in comparison to healthy controls. Indeed, *in vitro* and preclinical studies have described an increased CLDN2 expression of Caco-2 cells after exposure to DCA (81) and excessive upregulation of CLDN2 expression along the small intestine in a rat model of chronic cholestasis after bile duct ligation (82), respectively. Taken together with our results, this evidence suggests that it is promising to investigate the effect of BAs on intestinal dysfunction through their effect on CLDN2.

We also observed a significant downregulation of OCLN by protein expression but not by mRNA expression in the jejunum of IBS-D patients as well as in the SeHCAT^{-} subgroup, both compared to HVs. Interestingly, studies performed in the colonic mucosa of IBS patients found the same pattern as we did, decreased OCLN protein expression but no differences in OCLN mRNA levels between IBS-D patients and controls (71,83–85). Our group is the only one so far to have studied the levels of the phosphorylated form of OCLN in the jejunal mucosa together with its internalisation from the membrane into the cytoplasm of epithelial cells, a fact related to an increased permeability (33). Although in our previous study we did not find differences in the global expression of OCLN protein, in the present study we show a reduced global expression of OCLN protein in the jejunum of IBS-D, especially in those without BAD associated, a result that is in agreement with previous findings in the colon (71,83–85).

BAs profile in the jejunal fluid of IBS-D patients

Jejunal BAs in IBS-D were evaluated for the first time in this study. Firstly, we were able to quantify only conjugated BAs in jejunal fluid, all in the mM range, and no unconjugated BAs, as described by Humbert *et al.* (86), and in agreement with previous findings in the duodenal content in other GI diseases (86–88). We were able to quantify all glycine-conjugated forms of BAs (primary and secondary), whereas for the taurine-conjugated forms of BAs, we were only able to find the primary forms and the secondary BA TUDCA. This finding could be explained by the fact that the biotransformation of glycine conjugates to unconjugated forms

by intestinal microbiota is preferred and faster than taurine conjugated forms (89). Thus, the ratio of glycine to taurine conjugated BAs in humans is approximately 3:1, as we have observed, with only a few variations along the three study groups. Similarly, the most abundant BA in all the participants is the primary BA GCA, as previously described (87,90). Consistent with previous reports in IBS-D, although with some discrepancies, our results show a significant increase in the total amount of different BAs in jejunal fluid from both IBS-D subgroups compared to HV (25). These findings highlight that not only the SeHCAT^{+} group, but all IBS-D patients have an elevated concentration of BAs excreted to the jejunum with an altered BA profile, which could be due to variations in the deconjugation process, alterations in the feedback mechanism including the BA absorption step (89). Unexpected, however, we did not find an increase in FXR and we were unable to detect the hormone FGF19, so we cannot confirm that these patients have altered BAs signalling, and therefore an impairment in the biofeedback regulation of synthesis.

Furthermore, the BAs profile found was different between the SeHCAT subgroups. On the one hand, SeHCAT^{+} patients showed higher amount of secondary BAs than the other two groups, suggesting that these patients with BAD may differ in the composition of the gut microbiota population, which has higher BSH-deconjugation activity compared to the other groups, which could be influenced by the type of diet. Similar findings of increased levels of faecal conjugated secondary BAs in IBS patients compared to healthy control women were reported by Kamp *et al.* (22), although they did not find an association with clinical features. Notably, in our study, in the comparative analysis between IBS-D subgroups according to SeHCAT$^{+/-}$ with HVs, we also found a trend towards higher concentration of GDCA only in the SeHCAT^{+} group compared to HVs. Recent findings have also described higher stool content of glycodeoxycholate, the sodium salt of GDCA, in IBS patients (20). On the other hand, SeHCAT^{-} patients have more taurine-conjugated BAs than the other two groups. Interestingly, taurine conjugated forms have special properties as they are less toxic (91) and more potent agonists for GPBAR1 (92) than the glycine ones, more and their metabolism is modified by the diet (93), which could be a differential factor between our SeHCAT cohorts.

Furthermore, our results show a positive relationship between the concentration of some of the individual BAs in jejunal fluid and GFAP expression, and a negative relationship with OCLN expression, suggesting their potential role in the differential phenotype of EGC and TJPs observed in the jejunal mucosa. On the other hand, an alteration of the gut microbiome has been reported in IBS-D, besides the significant increase of primary BAs in the stool compared

to HV. In addition, higher levels of the primary BAs were also present when individuals experienced a worsening of symptoms compared to baseline, highlighting their potential contribution to IBS pathophysiology (23). We therefore investigated whether there was a possible relationship between the BA composition of the jejunal fluid and the clinical symptoms. Surprisingly, our results show that the levels of different conjugated secondary BAs correlate with bowel habits and distension suggesting that alterations in the jejunal BAs pool may have an impact on IBS-D symptoms. Therefore, we have described an altered and differential BA profile in the jejunal fluid of IBS-D SeHCAT subgroups suggesting that may have an impact on the pathophysiology of IBS-D.

Several studies have shown an alteration of the gut microbiome together with differential microbial metabolites at the colonic level, but the participation of the jejunum is unexplored. However, it is not yet known whether over-exposure to BAs is a cause or a consequence of a distinctive microbiota, as there is a complex interplay between BAs, the microbiome and other environmental factors such as diet. In addition, other modification of BAs such as sulfation (94), the effect of other microbiota-derived metabolites on BAs (95) and the involvement of BAs transporters (96) should be evaluated.

BA-activated receptors in the jejunal mucosa of IBS-D

Taking all these results together, we therefore decided to investigate the expression of receptors whose ligands are the BAs in the jejunal mucosa of IBS-D patients. There is only one study that has examined the expression of these receptors in IBS-D, but in the colon. Wei *et al*. found increased GPBAR1 immunorreactivity in the colonic mucosa of IBS-D patients compared to healthy controls, with not differences in VDR levels (30). We also found an increased expression of GPBAR1, but at the gene level, in the jejunum of IBS-D patients ($p=0.07$, borderline value), which genotype has interestingly been associated with colonic transit and total faecal BAs in IBS (27). In addition, increased primary BAs along with GPBAR1 expression have been correlated with abdominal pain in IBS-D suggesting a role for this BAR in IBS-D visceral hypersensitivity (30,97). However, we found no differences between the two SeHCAT subgroups and HVs. The lack of differences in BARs expression could be due to the fact that we evaluated the whole jejunal mucosa biopsy and these receptors are expressed in different intestinal cells. Future studies focusing on the epithelium or lamina *propria* cells would be useful to delimit the variability and understand the site where BAs exert their effects.

Clinical-biological correlation study

Using the SeHCAT tool we show here, for the first time, that BAs have an effect on specific IBS-D GI symptoms associated with changes in the expression of EGCs and TJPs. We found remarkable relationships according to the SeHCAT subgroup where the different pattern of protein expression found between the different SeHCAT subsets and HVs also had a differential association with the clinical characteristics of these patients. Thus, the markers upregulated in IBS-D patients with a $SeHCAT^{+}$ test (GFAP and CLDN2) correlated with the worse bowel habit that had compared to HV. On the other hand, the markers downregulated in IBS-D patients with a $SeHCAT^{-}$ test (SOX10, OCLN and VILL) correlated negatively with the severity of abdominal pain, bloating and discomfort, as well as the presence of depressive symptoms. Most of the molecular dysregulation observed in the jejunum reinforces the role of the small intestine in the pathophysiology of IBS-D and the pathogenesis of symptoms, including in patients with BAD. Therefore, we hypothesise that the physiopathological mechanisms of IBS-D differ between the SeHCAT subgroups, whereas $SeHCAT^{+}$ patients have altered bowel habits and a more diarrhoeal phenotype as a consequence of over-exposure to BAs, the mechanisms in the $SeHCAT^{-}$ group may be more related to visceral hypersensitivity and explained by alterations in the brain-gut axis. However, it is important to be cautious as correlation does not imply causation and further research is needed to elucidate the functionality of the changes in the expression of EGC and TJPs markers in IBS-D as well as the differences between the SeHCAT groups. Therefore, the use of the SeHCAT test to stratify our study groups, gives this work an exceptional value and a completely new point of view in the study of the pathophysiology of IBS-D, as it is the first study to evaluate the expression of several markers in the intestinal mucosa, differentiating between patients with and without BAD.

The main limitation of the present study is the small number of participants that were included. This reduces the statistical power, and therefore the results should be interpreted with caution, although they are novel and promising. It would also be interesting to perform a better characterisation of the jejunal EGC populations by a triple immunofluorescence staining using the same markers studied here. This would allow us to determine whether there is a different expression pattern by EGCs in IBS-D depending on the subgroup. The lack of study of the jejunal microbiota is another limitation, as we cannot to distinguish whether there is a distinctive microbiota that contributes to increase the content of BAs or whether over-exposure to BAs alters the microbiota. Additionally, future studies could focus on characterizing the molecular phenotype of EGC in the colon of IBS-D patients in order to evaluate the phenotype of this cell through the GI tract.

The main strength of this study is that it is the first to present a characterisation of the EGC molecular phenotype in the jejunal mucosal tissue from IBS-D patients. Our findings contribute to the understanding of the EGCs plasticity in the complex pathophysiology of IBS. However, to further elucidate its role, additional studies must be conducted. Moreover, this is the first study differentiating between IBS-D patients with and without BAD by the SeHCAT test and the first to evaluate the BAs concentration in the intestinal fluid. The differential expression of markers and BAs concentration suggests that IBS-D patients with and without BAD could have different physiopatohological mechanisms and reinforces the need to test BAD in IBS-D patients. These are needed because IBS-D causes a substantial burden of disease, and while IBS-D current treatment options are only effective in alleviation of symptoms. Relief of symptoms caused by BAD can be achieved by administering BA sequestrants in those patients with $SeHCAT^{+}$ (98,99).

In conclusion, patients with IBS-D exhibit a different phenotype at the level of the EGC and TJPs of the jejunal mucosa as well as the profile of BAs in the intestinal fluid in relation to the presence or absence of BAD, which are associated with clinical characteristics, suggesting their involvement in the IBS-D pathophysiology. However, additional research should be conducted to gain a comprehensive understanding of the pathophysiology of this disorder, which should be designed to address the identification of biomarkers and the development of therapeutic targets.

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CHAPTER 2

In vitro assessment of the effect of bile acids on epithelial barrier function

1. INTRODUCTION

The intestinal lumen contains a large amount of microbes, metabolites, host-secreted molecules and food-derived compounds that continuously interact with the epithelium and the underlying mucosal immune system and enteric nervous system (ENS). To avoid the access of potential antigens to the internal *milieu*, while allowing the absorption of nutrients, an efficient barrier in the gastrointestinal (GI) tract is essential to protect the host (1,2). Beyond this balancing function, two barriers of defence are necessary: the physico-chemical barrier and the immunological barrier. The main components of the physico-chemical barrier are the mucus, produced by Goblet cells, and the epithelium, composed by intestinal epithelial cells (IECs) which form a polarised monolayer that is highly dynamic and balance barrier functions with vectorial transport of water, ions and nutrients. This dynamic and tight regulation is accomplished in part by the intercellular junctions comprising protein complexes, which are on the apical side of the IECs that form semi-permeable contacts between cells. The most apical are the tight junction proteins (TJPs), composed of four transmembrane protein families: **occludins (OCLNs)**, **claudins (CLDNs)**, **junctional adhesion molecules** and **tricellulins** (3), which are essential for maintaining the polarity and integrity of the barrier. In addition, the ***zonula occludens*** (also known as tight junction protein, **TJP**) 1, 2 and 3 are a subfamily of peripheral proteins that confer dynamic properties, by regulating the intercellular space in an adaptive way (4). Dysregulation of these proteins by pathophysiological factors, including stress, altered microbiota composition, diet and drug use, modulates the tightness and the integrity of the intestinal barrier. These changes are associated with an increased intestinal permeability and uncontrolled antigen flux to the lamina *propria* which, subsequently, trigger inflammatory responses, ENS activation, potentially increasing neuro-immune interactions and signalling to the central nervous system (1,5), interactions that may facilitate the promotion of GI and systemic diseases. Nevertheless, despite intensive research, whether barrier dysfunction is cause or consequence of disease and the underlying mechanisms governing the transition from health to disease remain unknown (6,7).

Among the variety of metabolites present in the intestinal lumen, bile acids (BAs) deserve special attention, due to their ability to modulate the integrity of the intestinal barrier and, consequently, to alter the intestinal permeability. The primary BAs, **cholic acid (CA)** and **chenodeoxycholic acid (CDCA)**, are synthesised mainly by the enzymatic oxidation of cholesterol and are conjugated in the liver and then secreted into the small intestine, where

they contribute to the digestion and absorption of dietary fat. Later, these primary BAs are biotransformed by intestinal bacterial enzymes into the secondary BAs **deoxycholic acid (DCA)** from CA and **lithocholic acid (LCA)** from CDCA. Both types of BAs are either taken up from the gut lumen by active transport, which mainly occurs in the distal ileum. BAs are also passively absorbed along the entire intestine, entering the enterohepatic circulation and returning to the liver, and the non-absorbed fraction is excreted in faeces (5%) (8,9). The gut microbiota is critical in shaping the metabolic profile of the BA pool and in modulating its impact on host physiology (10,11). In order to maintain the balance of the BAs pool and to avoid hepatic accumulation and cytotoxicity, there are complex negative feedback mechanisms that inhibit BAs synthesis through BAs-dependent signalling pathways. BAs are the natural ligands of several receptors expressed in the distal small intestine and hepatocytes; by activating these BA receptors (BARs), BAs regulate not only their own synthesis but also lipid, glucose and energy balance. When BAs are absorbed in the ileum, they activate the **Farsenoid-X-receptor (FXR)** expression on enterocytes, inducing the expression of **hormone-like protein fibroblast growth factor 19 (FGF19)**, which is secreted into the portal blood and reaches the liver where represses the synthesis of BAs and regulates BAs homeostasis (12).

Several lines of evidence have demonstrated the presence of BARs in numerous organ systems, including the GI tract, supporting the crucial role of BAs as versatile signalling molecules that regulate various physiological processes throughout the body by interacting with these receptors. Notably, disruption of BA homeostasis and signalling is involved in the pathogenesis of several GI diseases, such as irritable bowel syndrome (IBS), inflammatory bowel disease or colorectal and oesophageal cancer (13,14). The major BARs expressed by enterocytes are the cell membrane receptor **Takeda G-protein-coupled receptor 5 (TGR5)**, also known as G protein-coupled bile acid receptor 1 **(GPBAR1)**, and two nuclear receptors: **FXR**, also known as nuclear receptor subfamily 1 group H member 4 (**NR1H4**), and **vitamin D receptor (VDR)** (15,16)**.** Besides their traditional function in digestion and absorption of lipids and fat-soluble vitamins, luminal BAs act as inflammatory mediators and modulators of intestinal permeability by interacting with immune and IEC receptors, resulting in modulation of intestinal permeability, especially in enterocytes, by activating or inhibiting different cell signalling pathways. There are, at least, two mechanisms by which intestinal BAs affect enterocyte biology. First, BAs act as detergent molecules causing imbalances in water and electrolyte secretion (17–19). The hydrophilic-hydrophobic balance of the individual BAs is an important factor in determining their absorption, binding affinity to their different

receptors and therefore their physiological properties, with primary BAs being more hydrophilic than secondary BAs. Furthermore, the cytotoxicity of BA positively correlates with their hydrophobic index, as they are able to disrupt cell membranes, and therefore high concentrations of those BAs with the highest hydrophobic index can be detrimental as they have proapoptotic, proinflammatory and toxic effects. Second, BAs are receptor ligands/agonists expressed by IECs, although the underlying molecular mechanisms of interaction are not yet fully understood. FXR is a nuclear transcription factor present in the intestine with a differential expression gradient between small and large bowel, whose natural ligands are the conjugated primary BAs. The ligand binding domain of FXR contains a hydrophobic pocket that accommodates lipophilic BAs, thus their decreasing potency in activating FXR is ranked as: CDCA > DCA > LCA > CA (20). FXR plays an essential role in barrier function, protecting the epithelium by preventing bacterial overgrowth and improving mucosal integrity and its response to oxidative stress, as well as limiting the uptake of BAs by the GI epithelium. Although there are conflicting data on the effects of CDCA-mediated activation of FXR on IECs, it has been shown that treatment with FXR agonists induces TJPs expression, prevents reduction in villus length and decreases transepithelial electrical resistance (TEER) (21). GPBAR1 is a G-protein coupled receptor, whose activation has been involved in LCA-mediated proliferation of intestinal stem cells and DCA-mediated suppression of wound healing (22). In addition to FXR and GPBAR1, the **vitamin D receptor (VDR)** has a critical role in maintaining barrier function by influencing the expression of TJPs such as CLDN-2 and inhibiting inflammation (18). In the distal small intestine, this receptor is activated by the secondary BA LCA, leading to IEC signalling that protects against inflammation (23,24).

Several lines of evidence show how BAs modulate epithelial barrier, although most of the studies have described the effect of BAs on intestinal barrier function in animal models of intestinal inflammation. Unfortunately, results are not always consistent and the large variability described seem to depend on the type of BA and its concentration (18,19,25). Despite previous research, the exact mechanism by which BAs modulate the epithelial integrity has yet to be elucidated.

Thus, our hypothesis is that luminal BAs modulate BARs expression and reduce intestinal epithelial barrier integrity by modulating the expression of TJPs structure. The aim of this chapter is to characterise the effects of short and long-term exposure to BAs on epithelial barrier function based on an *in vitro* study in Caco-2 epithelial cells.

2. EXPERIMENTAL DESIGN

A set of *in vitro* experiments using the epithelial cell line Caco-2 in transwell inserts were designed in order to (**Figure 1**):

1) Establish a polarised intestinal epithelial barrier model.

2) Study the effects on Caco-2 monolayer after short and long-term exposure to BAs.

1) EPITHELIAL CELL LINE CHARACTERISATION

The human epithelial cell line Caco-2 was phenotypically and functionally characterised before any treatment as follows:

- Epithelial polarised monolayer integrity was assessed by measuring the TEER.
- The epithelial barrier structure was analysed by haematoxylin & eosin (H&E) staining, TJP-1 immunofluorescence and determination of TJPs gene expression levels (*CLDN2*, *OLCN*, *TJP-1*) by real-time qPCR.
- BARs expression was evaluated by identification of *GPBAR1*, *NR1H4*, *VDR* and *FGF19* by real-time qPCR.

2) EPITHELIAL RESPONSE TO BILE ACIDS

An *in vitro* study in Caco-2 cells in transwell inserts were treated **for 2 and 24 hour (h) with** two **unconjugated primary BAs (CDCA and CA) and the unconjugated secondary BA (DCA) at 200 µM - 500 µM** to characterise their impact on epithelial barrier integrity and function**.** The epithelial response was assessed as follows:

- Cell viability was measured using the lactate dehydrogenase (LDH) cytotoxicity assay kit to measure the activity of LDH released from the cytoplasm of the dead and damaged Caco-2 cells into the supernatant at the highest concentration of BAs (500 µM).
- Integrity of the epithelial barrier by measuring the TEER to evaluate barrier function at both times, **2 and 24 h,** and the paracellular permeability by the 4 kDa fluorescein isothiocyanate (FITC)-dextran assay.
- Gene expression of TJPs and BARs was quantified by real-time qPCR.

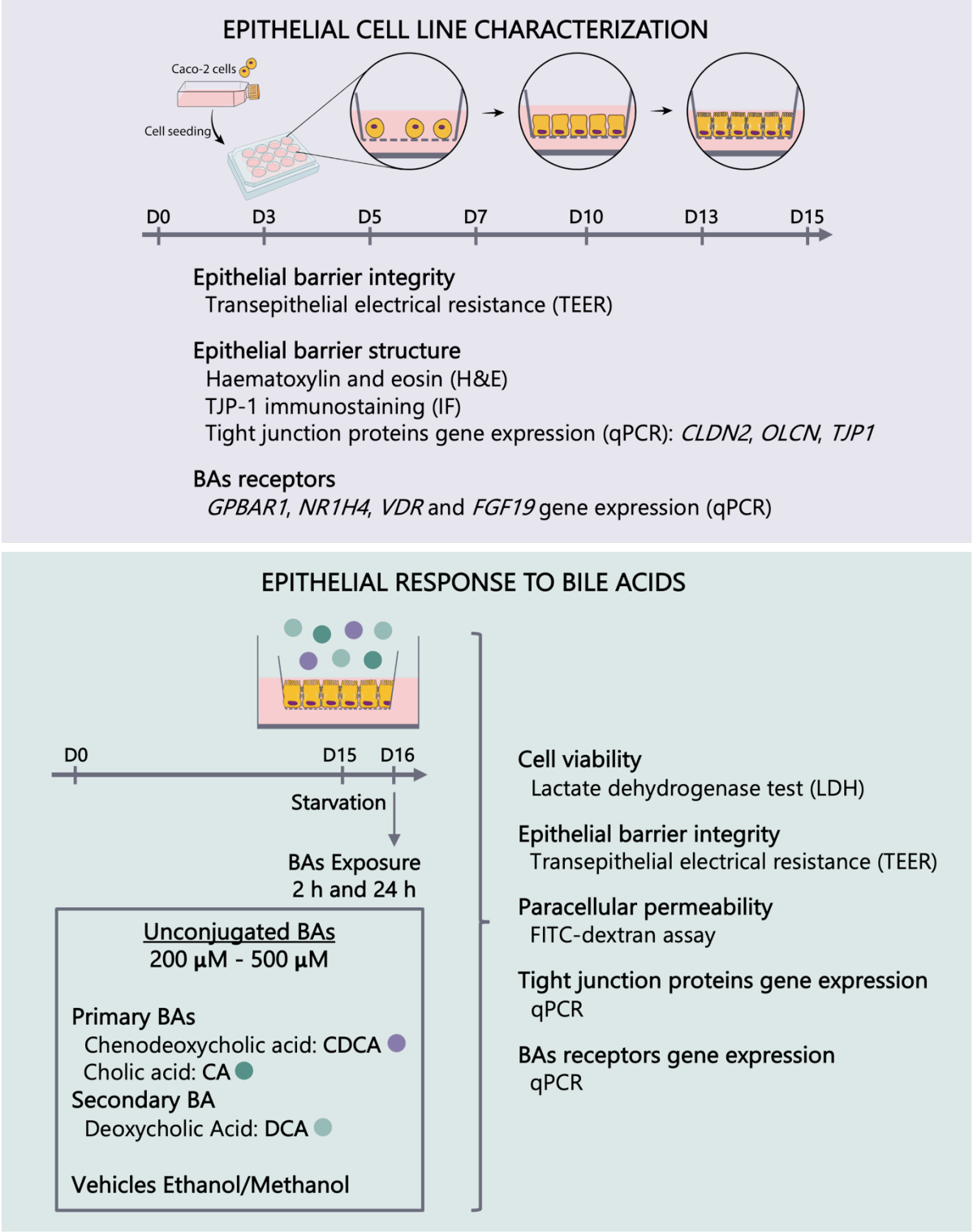

Figure 1 | Chapter 2 illustration of the experimental design. BA: Bile Acid; CLDN2: Claudin 2; D: Day; FGF19: Fibroblast Growth Factor 19; FITC: Fluorescein Isothiocyanate; GPBAR1: G Protein-Coupled Bile Acid Receptor 1; NR1H4: Nuclear Receptor Subfamily 1 Group H Member 4; OCLN: Occludin; qPCR: quantitative PCR; TJP1: Tight Junction Protein 1; VDR: Vitamin D Receptor.

3. METHODS

3.1. Caco-2 cell culture

The epithelial cell line Caco-2 isolated from human colon was purchased from the American Type Culture Collection (HTB-37, Manassas, VA, USA). Cells were grown in culture flasks in Dulbecco's modified Eagle's medium (DMEM) / GlutaMAX™ supplemented with 10% fetal bovine serum, 1% Penicillin-Streptomycin and 1% non-essential amino acids under conditions of 5% CO2 at 37 °C (all reagents purchased from Gibco). Cells were grown until 80-90% confluence and trypsinised using 0.05% Trypsin-EDTA (1X) (Gibco). For each new passage, cells were split and seeded in T75 cm^2 culture flasks for maintenance (Sarsted). When cells were at passage number range between 10 and 20, they were used for performing the experiments. To evaluate the epithelial barrier function, Caco-2 cells were seeded onto the apical surface of 12 mm culture inserts (transwell 3460, polyester membrane and 0.4 µm pore size, Corning Costar) at 1×10^5 cells/cm^2 in each insert. Standard growth medium (500 µL apical part – 1.5 mL basolateral part) was added to each well and replaced every 2 days.

3.2. Haematoxylin & eosin staining

Differentiated Caco-2 cells grown in transwell chambers formed a polarised monolayer that was stained to visualize the structure of the monolayer. Cells were washed with phosphate-buffered saline (PBS) (Gibco), fixed with 4 % paraformaldehyde for 15 minutes and washed again with PBS. The 10 µm thick transwell membrane was extracted from the device and cut into strips which were embedded in paraffin with the cells facing upwards. Samples were cut at 4 µm, stained with H&E (Casa Álvarez) and mounted onto a glass. Samples were observed under an optical Olympus BX61 microscope, an OLYMPUS DP26 video camera and the computer program CellSens Standard 1.7.

3.3. Immunofluorescence staining

Cells grown in transwell were fixed with 4% paraformaldehyde in PBS at 37 ºC for 15 min and washed with cold PBS. Samples were incubated for 15 min with protein blocking solution (1X PBS/1% BSA/0.5% Triton X-100) at room temperature (RT). To visualize cell-to-cell adhesion of the monolayer, cells were incubated with primary antibody TJP-1 (33-9100, Invitrogen), diluted in the antibody dilution buffer (1X PBS/ 1%BSA) at a 1:100 dilution, for 3 h in a wet chamber at RT. Afterwards, cells were washed with PBS and incubated with the secondary

antibody, diluted in the antibody dilution buffer, for 1 h in a wet chamber in the dark at RT. After extensive washings with PBS the coverslips were mounted with DAPI (1:1000) 10 minutes at RT to counterstain nuclei. Finally, the cell membrane was cut from the transwell and mounted onto a glass with the cells facing upwards.

3.4. Transepithelial Electrical Resistance Determination

To check the integrity of Caco-2 cells monolayer, the TEER was measured under culture conditions with an EVOM epithelial voltmeter (WPI) and a MERSSTX03 electrode (Merck), which was cleaned before each measurement. To avoid the influence of temperature variability on TEER measurements, the plates were removed from the incubator and the temperature stabilised on a 37 °C warming plate (SP Bel-Art) for 15 min. For TEER determination, the electrode was immersed at a 90° angle with one tip in the basolateral chamber and the other in the apical chamber, avoiding electrode contact with the monolayer. Measurements were performed in triplicate, averaged and recorded for each monolayer. An insert with media without cells was used as a blank and its average resistance subtracted from all samples. The final TEER values are shown in $\Omega \cdot cm^2$ and calculated as: $R_{cell\ layer} = (R_{sample} - R_{blank})$ x MA, being R the resistance in Ω and MA the membrane area in cm^2. TEER was recorded every 2-3 days to assess the development of barrier function over time until constant values were obtained, indicating a tight barrier is achieved. After exposure to unconjugated BAs (see next section 3.5), TEER values were obtained before and after all treatments, with an additional and intermediate TEER recording at 20 min for the 2 h condition.

3.5. Bile acids preparation and exposure to Caco-2 cells

The primary BAs, CDCA and CA, and the secondary BA, DCA were purchased from Sigma-Aldrich. After dissolving each BA in ethanol or methanol (depending on BA solubility), the stock solution was prepared in PBS freshly solubilised. The information and preparation details are listed in **Table 1**. After overnight starvation in standard serum-free growth medium, the apical side of differentiated Caco-2 cells in transwell was then treated with diluted stocks of BAs (200 µM and 500 µM) in this serum-free growth medium for 2 h and 24 h. Every condition was performed and assessed in triplicate and repeated in 3 different days. In every experiment, cells were exposed to the same concentration of serum-free growth medium with ethanol or methanol without BAs (vehicles). Non-treated wells were maintained as control.

Table 1 | Detailed preparation conditions and dilutions of CA, CDCA and DCA for *in vitro* stimulations.

Bile acid	Initials	Manufacturer and catalogue number	Solubility	Stock solution in PBS
Chenodeoxycholic acid	CDCA	Sigma-Aldrich, C9377	50 mg/mL, ethanol	1:50 1 mg/mL
Cholic acid	CA	Sigma-Aldrich, C1129	50 mg/mL, methanol	1:25 2 mg/mL
Deoxycholic acid	DCA	Sigma-Aldrich, D2510	50 mg/mL, ethanol	1:50 1 mg/mL

3.6. Cytotoxicity Assay

The release of the mitochondrial enzyme LDH into the medium occurs during cell lysis and is indicative of cellular toxicity. The toxicity of BAs on Caco-2 cells was evaluated in the apical supernatants with a LDH cytotoxicity assay kit (K6330400, BioChain) by measuring the activity of LDH released from the dead and damaged Caco-2 cells after treatment with the highest BAs concentration used, 500 µM. Briefly, after exposure, the apical culture supernatant was collected, centrifuged and incubated with the assay mixture (catalyst solution: dye solution, 1:2) for 30 min at RT, protected from the light. Afterwards, stop solution was added and absorbance was measured at 492 nm with the Multiskan EX microplate reader (Thermo Scientific). Assay medium was added as background control and its absorbance value was subtracted from all other absorbance values. Caco-2 cells in 1x lysis solution (provided in the kit) were used as high toxicity control while untreated Caco-2 cells were used as low toxicity control. Measurements were performed per triplicate in three different days. The % of cytotoxicity was calculated as: ((exposure value – low control) / (high control – low control)) x 100. Finally, the % of viability was obtained as: 100 - % cytotoxicity.

3.7. Paracellular permeability assay

Paracellular permeability after the treatment with BAs was assayed by measuring the passive transport of 4 kDa FITC–dextran (FD-4, Merck) across cells after 120 minutes. The cells were carefully washed with HBSS and FITC-dextran (1 mg/mL diluted in HBSS) was added to the apical side of the insert, while the basolateral side was filled with HBSS solution. After 2 h, the amount of FD-4 in the basolateral sample was collected and determined by fluorescence using a 96-well plate reader Varioskan LUX (Thermo Fisher). The excitation and emission wavelengths were 498 nm and 517 nm, respectively. Assay medium was added as background control and its fluorescence was subtracted from all wells. Measurements were made in

triplicate wells per condition on three different days. Results are expressed as percentage of FITC-dextran flux relative to vehicle.

3.8. RNA isolation and quantitative real time PCR

Total RNA was extracted from cell pellets harvested, after each treatment, in RLT buffer + β-mercaptoethanol (100:1) and frozen at -80 °C. Triplicates of each condition from 3 independent experiments were pooled into a single pellet. Samples were passed through an insulin syringe (27G) 10 times in order to facilitate cell lysis and increase the RNA yield, followed by elution in the RNeasy mini columns (QIAGEN), according to manufacturer instructions. Finally, RNA was eluted in 50 µL of RNase-free water and quantified using NanodropTM (Nanodrop ND-1000, Nanodrop Products). Samples were stored at -80 ºC until processed for cDNA synthesis. Samples exposed to CDCA and DCA together with their respective vehicles were chosen for gene expression analysis.

cDNA synthesis was performed using 1 µg of total RNA using the High Capacity Reverse Transcription Reagents Kit (Thermo Fisher Scientific), following manufacturer's instructions. Quantitative real-time PCR was carried out with the ABI PRISM® 7500 FAST Sequence Detection System (Applied Biosystems). TJPs and BARs markers were evaluated using validated TaqMan Gene Expression Assays detailed in **Table 2** (Applied Biosystems). RNase-free H_2O was used as negative control. Samples were run in triplicate and their expression was normalised to the house-keeping genes 18S and GAPDH. Relative gene expression was calculated by the 2-ΔΔCt method (26); were ΔCt=Ct target gene – Ct endogenous gene and ΔΔCt = ΔCt patient – ΔCt control expressed as fold change.

Table 2. Gene expression assay probes used for qPCR analysis.

Gene symbol	Gene name	TaqMan assay
	Tight junction proteins	
CLDN2	Claudin 2	Hs00252666_s1
TJP1	Tight Junction Protein 1	Hs00268480_m1
OCLN	Occludin	Hs00170162_m1
	Bile acid receptors	
FGF19	Fibroblast Growth Factor 19	Hs00192780_m1
GPBAR1	G Protein-Coupled Bile Acid Receptor 1	Hs01937849_s1
NR1H4	Nuclear Receptor Subfamily 1 Group H Member 4	Hs01026590_m1
VDR	Vitamin D (1,25- Dihydroxyvitamin D3) Receptor	Hs01045843_m1
	Endogenous genes	
18S	Eukaryotic 18S rRNA	Hs99999901_s1
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	Hs02758991_g1

3.9. Statistical analysis

Experiments were conducted in triplicate in 3 different days for each treatment, vehicle and control groups. Data distribution was tested by the D'Agostino and Pearson, Shapiro-Wilk and Kolmogorov–Smirnov normality tests. Due to the small sample size, the data were considered as non-normally distributed and are expressed as median (interquartile range). Given the experimental design, the effect of treatment, doses and timing was studied by a three-way ANOVA followed by multiple comparisons. Comparison between treatments and their respective vehicles was performed using the Mann-Whitney U test. The association between TEER increment and FITC-dextran flux was tested by Spearman's rho correlation. Values of $p \leq 0.05$ were considered significant. The statistical analyses were performed using GraphPad Prism 9.01 software (Graphpad Software, San Diego, CA, USA).

4. RESULTS

4.1. Epithelial cell line characterisation

Over a period of 2 weeks of cell culture, the increase in TEER values was progressive during the differentiation period of 15 days, until reaching a stabilisation at 1.500 Ω/cm^2 (**Figure 2.A**). Thus, it was considered that the monolayer of Caco-2 cells had a proper stability after 16 days of cell culture, in which all the treatments with BAs were carried out (next section). A proper epithelial monolayer on the transwell membrane was confirmed by H&E staining (**Figure 2.B**) and the location of the cell contacts for TJP-1 of Caco-2 monolayers by immunofluorescence (**Figure 2.C**) after 15 days of culture.

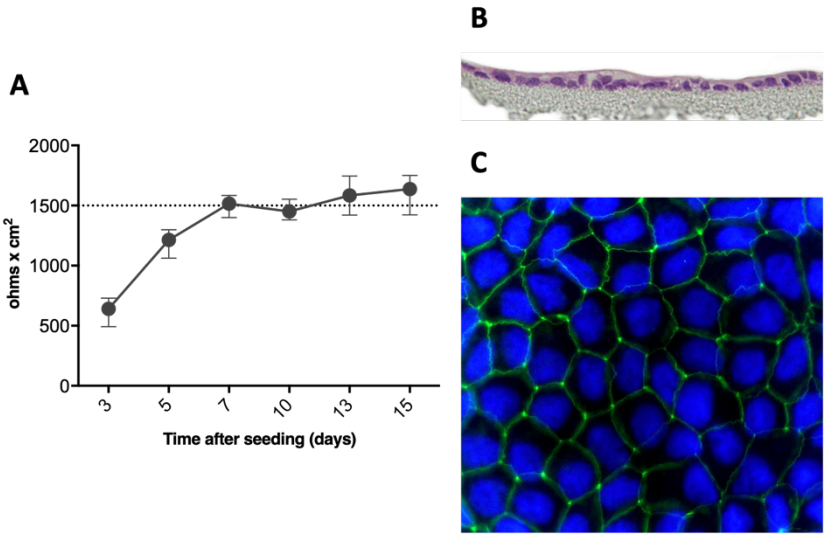

Figure 2 | Functional and morphological assessment of Caco-2 cells. A. Representation of TEER measurements after seeding and during differentiation period of the cell monolayer. n=6. Dotted line represents TEER stabilisation. **B.** Representative H&E staining of Caco-2 cells at day 15 after seeding. Image magnification: 100X. **C.** Representative TJP-1 immunofluorescence staining of Caco-2 cells at day 15 after seeding. Caco-2 monolayer stained for TJP-1 (green) and nuclei (blue). Image magnification: 400X.

4.2. Bile acids have an impact on epithelial barrier function through changes in TEER measurements and paracellular permeability

Once the Caco-2 monolayers were differentiated, to study the effect of BAs on epithelial integrity, an incubation with two concentrations (200 µM and 500 µM) of different unconjugated BAs (CDCA, CA, DCA) for 2 and 24 h was carried out.

No changes in the activity of LDH released from the Caco-2 cytoplasm, and therefore in cell viability, were observed after 24 h incubation with the different BAs at the two concentrations tested (**Figure 3**). This result excludes toxic effects of the tested BAs on the monolayer, and suggests that the response of Caco-2 to BA treatment can be considered specific without any effect on cell death.

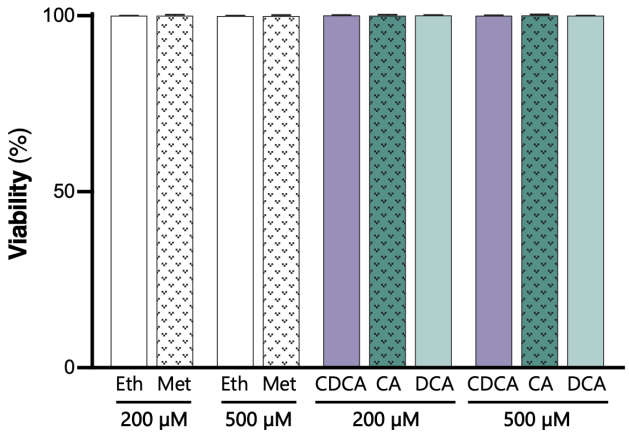

Figure 3 | Cell viability of Caco-2 cell line after exposure to different concentrations of BAs and their respective vehicles (200-500 µM) for 24 h. Graphs represent data from 3 independent experiments. Data are expressed as median (IQR). CA: Cholic Acid; CDCA: Chenodeoxycholic Acid; DCA: Deoxycholic Acid. Eth: Ethanol; Met: Methanol.

Treatment with the primary BA, CDCA at 500 µM induced a significant drop in TEER, which increased over time, although normal values were not reached yet after 24h (treatment x dose x time; F[2,91]=12.26, *P*<0.0001), when paracellular permeability (apical to basal direction) significantly increased (treatment x time F[1,62]=9.354, *P*=0.0033)(**Figure 4.A**). Treatment with CA, at 200 µM caused a greater reduction in TEER than at 500 µM at all time points, the latter was only significant after 2 h when compared to its vehicle, without modifying FITC-dextran flux across the monolayer (**Figure 4.B**). Treatment with DCA induced a significant decrease in TEER at 500 µM at all time points (treatment x dose x time; F[2,95]=4.242, *P*=0.0172) and a significant increment in FITC-dextran flux across monolayer (treatment x time F[1,63]= 5.584, P=0.0212) compared to its vehicle, although no differences were found in the multiple comparisons analysis (**Figure 4.C**).

No correlation was observed between TEER and FITC-dextran flux values, suggesting that all BAs tested act differently on epithelial barrier function, affecting permeability to ions more than to large molecules.

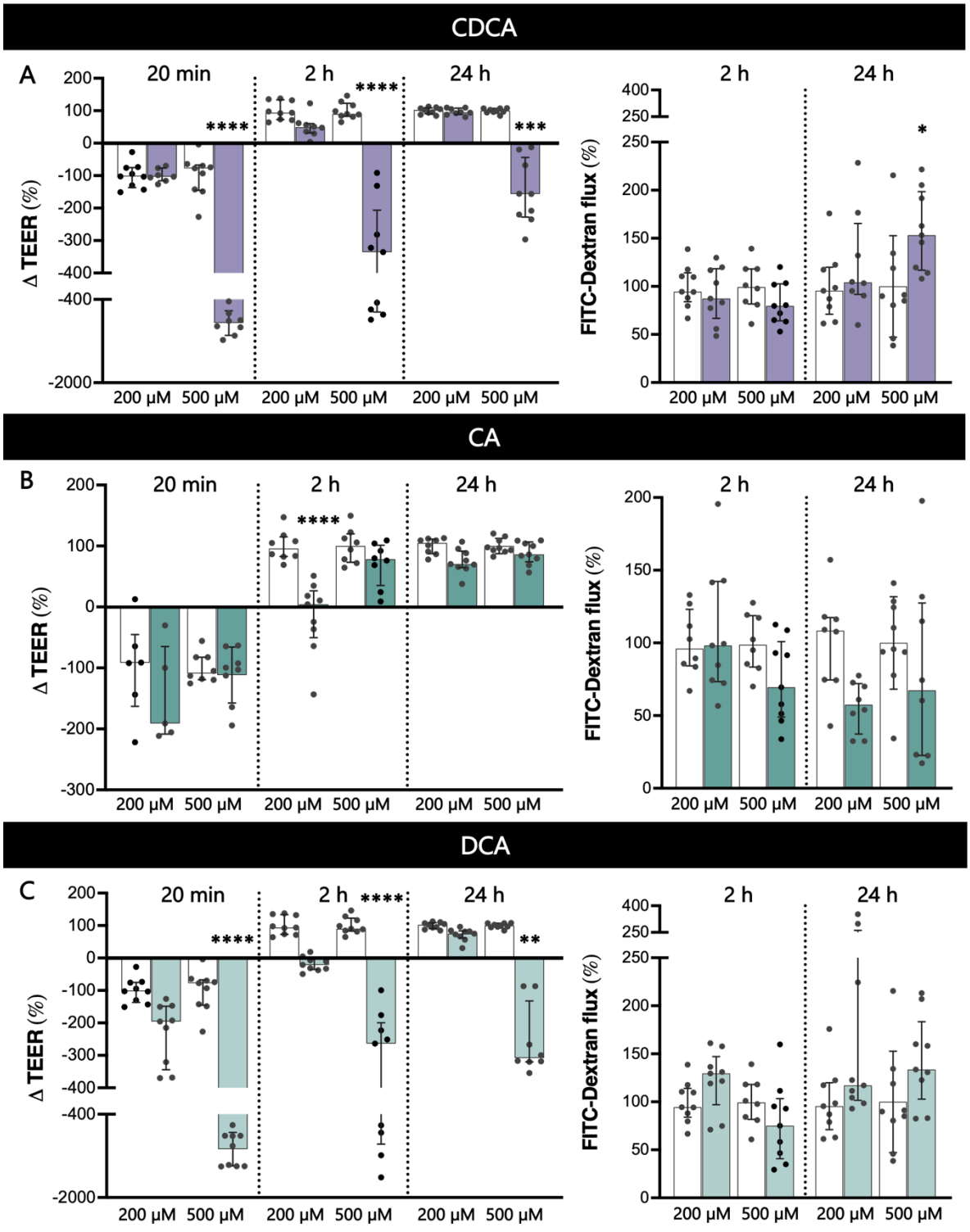

Figure 4 | TEER (left) and FITC-Dextran flux (right) after exposing Caco-2 cells to different concentrations of BAs (200-500 µM) for 2 and 24 h. A. CDCA stimulation. **B.** CA stimulation. **C.** DCA stimulation. TEER values at time zero were subtracted from the TEER values at each time point. TEER and FITC-Dextran flux were expressed as the increment (percentage) relative to the vehicle average for each time point. Graphs represent data from 3 independent experiments, expressed as a number of observations. Data are expressed as median (IQR). Comparison between BAs and their respective vehicles was performed using the multiple comparisons test of the three-way ANOVA. * p≤0.05, **p ≤0.01, *** p≤0.001, **** p≤0.0001. CA: Cholic Acid; CDCA: Chenodeoxycholic Acid; DCA: Deoxycholic Acid; FITC: Fluorescein Isothiocyanate; h: Hour; min: minute; TEER: Transepithelial electrical resistance.

4.3. Effect of bile acids on tight junction proteins and bile acids receptors' expression

To investigate the underlying molecular changes induced by the exposure of Caco-2 cell monolayer to CDCA and DCA (the BAs with greater effect on epithelial barrier integrity and paracellular permeability), we next evaluated the impact of BAs on TJPs. Thus, the gene expression of *CLDN2*, *OCLN*, and *TJP1* was measured after exposure to both BAs at 200 and 500 µM for 2 and 24 h.

Exposure to both BAs modified TJPs gene expression without statistical significance (**Figure 5**). The highest increase in *CLDN2*, *OCLN* and *TJP1* was induced by CDCA at 500 µM at both time points, and only at 24 h by DCA. The lack of statistical significance in TJPs expression is probably explained by the limited number of *in vitro* experiments performed.

In parallel, gene expression of several BARs, including *GPBAR1*, *NR1H4* and *VDR*, was examined under the same conditions. Overall, exposure to both BAs for 24 h induced a reduction in the expression of the genes tested, although none of them were statistically significant. In addition, exposure to both, DCA and CDCA, at 500 µM for 2 h induced an increase in *GPBAR1* expression. Finally, to study the effect of BAs on the regulation in the BA feedback pathway, the gene expression of *FGF19* was also evaluated. After exposure to both BAs for 24 h an increase of *FGF19* levels was observed with the highest concentration (500 µM) (**Figure 6**).

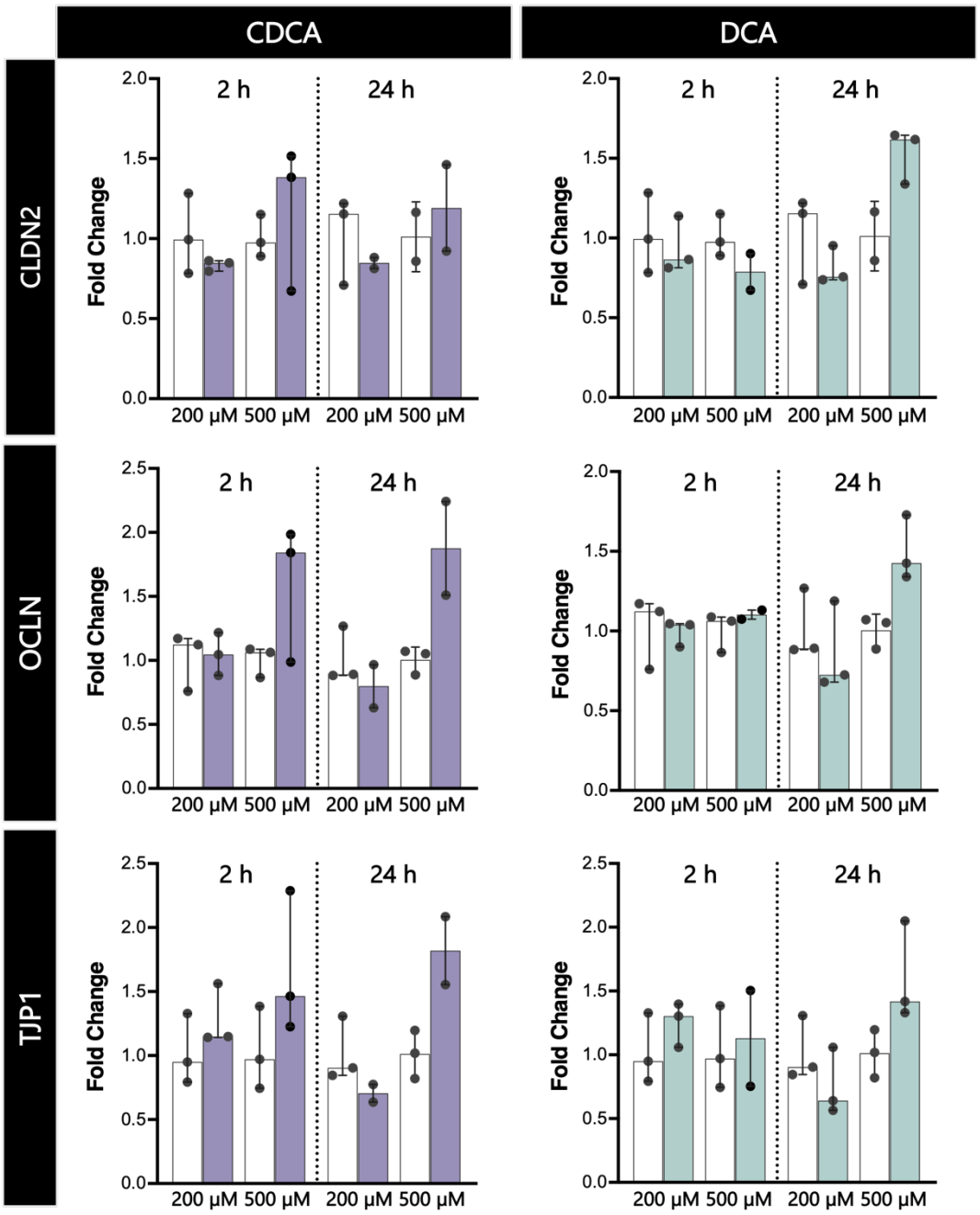

Figure 5 | TJPs expression of Caco-2 cells after exposure to 200 or 500 µM CDCA and DCA for 2 and 24 h. mRNA expression of CLDN2, OCLN and TJP1 was assessed by qPCR. To obtain the fold-change value for each sample the ratio between target gene and the reference gene (18S and GAPDH) was calculated for each sample and then normalised to the average of the vehicle group (white bars). Graphs represent data of 3 independent experiments. Data are expressed as median (IQR). Comparison between BAs and their respective vehicles was performed using the Mann-Whitney U test. CDCA: Chenodeoxycholic Acid; CLDN2: Claudin 2; DCA: Deoxycholic Acid; h: hour; OCLN: Occludin; TJP1: Tight Junction Protein 1.

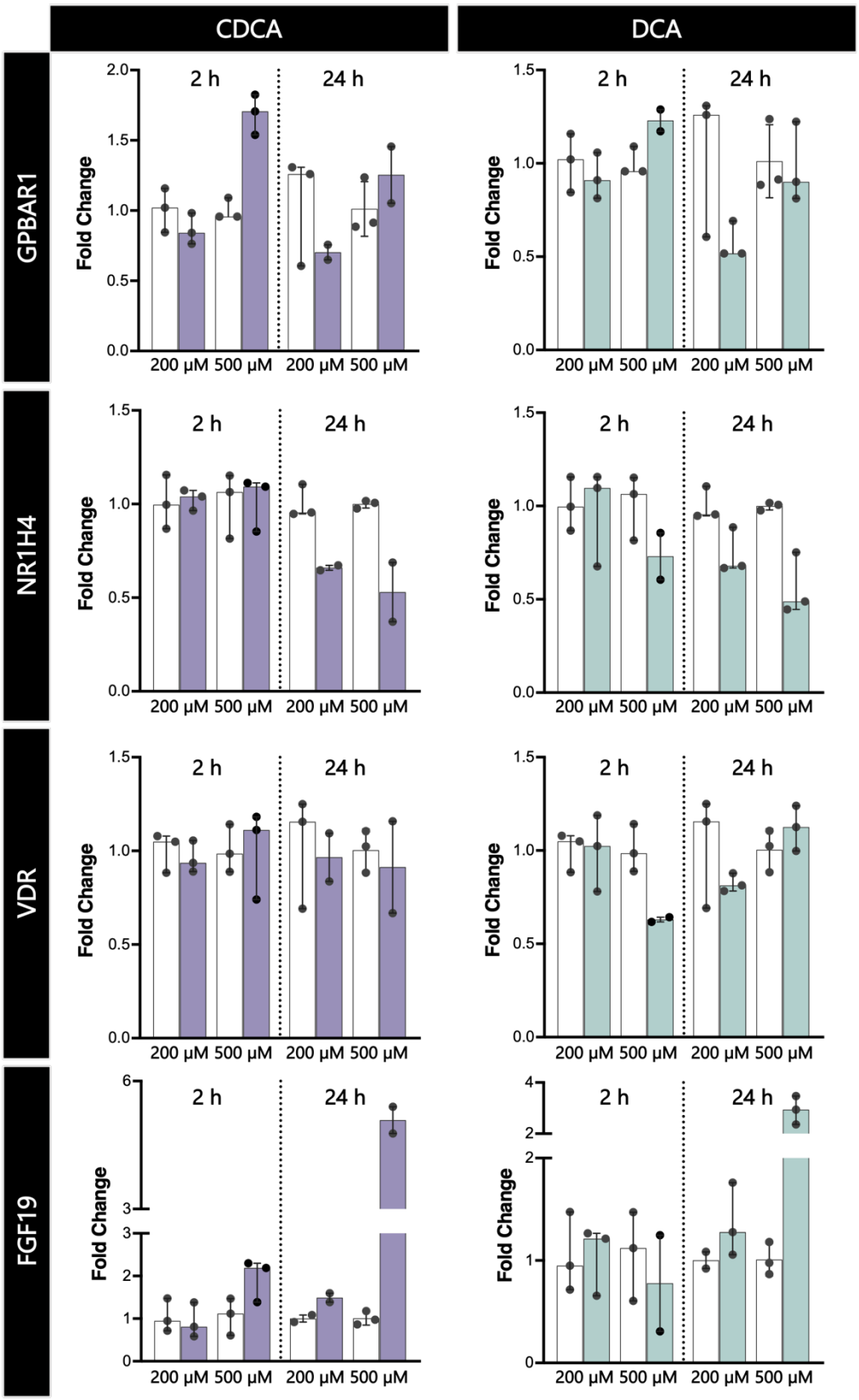

Figure 6 | BARs and FGF19 expression in Caco-2 cells after exposure to 200 and 500 µM CDCA and DCA for 2 and 24 h. mRNA expression of GPBAR1, NR1H4, VDR and FGF19 was assessed by qPCR. To obtain the fold-change value for each sample the ratio between target gene and the reference gene (18S and GAPDH) was calculated for each sample and then normalised to the average of the vehicle group (white bars). Graphs represent data of 3 independent experiments. Data are expressed as median (IQR). Comparison between BAs and their respective vehicles was performed using the Mann-Whitney U test. CDCA: Chenodeoxycholic Acid; DCA: Deoxycholic Acid; FGF19: Fibroblast Growth Factor 19; GPBAR1; G Protein-Coupled Bile Acid Receptor 1; h: hour; NR1H4: Nuclear Receptor Subfamily 1 Group H Member 4; VDR: Vitamin D Receptor.

5. DISCUSSION

This study investigated the effects of the short and long-term exposure to unconjugated BAs on the epithelial barrier in an *in vitro* epithelial cell model. After characterizing the Caco-2 cell model by assessing the morphology and integrity of the epithelial monolayer, we showed that the unconjugated BAs, CDCA and DCA, had an impact on the integrity and the paracellular permeability of the monolayer, especially at the highest concentration tested (500 µM) and after 24 h of exposure. These two BAs also induced an increase in the gene expression of the TJPs *CLDN2*, *OCLN* and *TJP1*, and a decrease of the BARs, although without significant differences.

Alterations in the BAs pool profile could be involved in the pathophysiology of those GI disorders characterised by chronic diarrhoea and in the pathogenesis of different type of disorders characterised by chronic intestinal inflammation (27,28). In fact, an altered faecal BA profile with an increased concentration of primary BAs has been observed in individuals with IBS-D, together with an association with cardinal clinical manifestations as stool consistency and frequency (29,30). In this line, previous reports have shown that a subgroup of patients with IBS-D have greater excretion of BAs in the stool, together with increased intestinal permeability (31). Therefore, in a recent large cohort of patients with and without bile acid diarrhoea (BAD), an increased colonic permeability was reported only in those patients with BAD (32). Luminal BAs are capable of disrupting epithelial barrier function by altering the physical properties of the epithelium, thereby increasing the intestinal permeability. However, these effects vary depending on the type of BA (structure, hydrophobicity and conjugation), concentration, and the model used to test, animal or *in vitro* (33,34). For example, opposite effects on barrier function have been described for different BARs (35), by interacting with IECs and affecting their molecular processes (apoptosis, cytokine secretion), and activating different receptors that lead to downstream pathways that reduce TEER and increase permeability of intestinal epithelial monolayers. However, the role of BARs activation in the molecular mechanisms underlying TJPs regulation by BAs is not well understood. Therefore, this study investigated the effect of unconjugated BA exposure on Caco-2 cells to assess its effect on epithelial barrier function and to elucidate to what extent this BA effect on the epithelium might be mediated by BARs.

Here, after 16 days of culture, Caco-2 monolayers achieved appropriate polarity and were exposed to the two primary and one secondary unconjugated BAs that are most abundant in

the BA pool size in the human enterohepatic circulation (36) and faeces from individuals with IBS-D (37): CDCA, CA and DCA. Previous similar *in vitro* models used concentrations ranging from 10-500 µM (35,38–45). Therefore, we chose to treat the monolayers with individual BAs at 200 µM and 500 µM for 2 and 24 h. In order to investigate the effects of short- and long-term exposure to each BA on intestinal epithelial barrier integrity an additional measurement of TEER was also performed 20 min after the start of treatment (46). In this study, a serum-free medium was used and Caco-2 cells were starved overnight prior to exposure to BAs in order to have more reproducible test conditions and to improve barrier formation (47). In addition, cytotoxicity induced by BAs in the colon has been described in a cell- and BA-specific manner (48,49). However, in our case, the exposure to the highest concentration of BAs tested (500 µM), which is below the physiological amount of BAs in the intestinal lumen in the mM range (50,51), did not increase the amount of LDH released, excluding the toxic effects on Caco-2 cells and ensuring a specific response without any effect on cell death.

Short- and long-term treatment with CDCA and DCA had similar effects, with a decrease in TEER values at the highest concentration (500 µM). In the case of 24 h of exposure to CDCA, this was accompanied by an increase in paracellular permeability. The primary BA CA had a less pronounced effect than the other BAs tested at the lower concentration and for shorter periods of time.

Interestingly, the greatest reduction in TEER was observed after 20 min of treatment with each individual BA, which then progressively increased over time with a recovery of TEER values after 2 h in the case of exposure to CA. Interestingly, acute exposure to CDCA and DCA at 500 µM alters epithelial barrier integrity, which is consistent with previous findings reported by Raimondi *et al.* where they found greater changes in epithelial barrier function after exposure to CDCA and DCA compared to CA. In contrast to our results, they also found few but significant changes effects after exposure to CA for 20 min. Treatment with CA and DCA for 2 h at 50 µM also caused a reduction in TEER within the first 20 min, but this effect did not last for 2 h (39). Our data show a more sustained effect on epithelial integrity, which may be due to the exposure of the monolayer to higher concentrations. It should be noted that the 20-minute recordings observed in the vehicle-treated monolayers were also slightly reduced, which could be explained by the short time after stimulation, which may not be enough for the medium to stabilise.

In addition to the pro-secretory effect of the unconjugated forms of dihydroxy BAs, CDCA and DCA, these BAs also have detergent-like properties that induce the following effects on the

intestinal mucosa: mucus denudation, mucin secretion and mucosal damage (52–55). In this study, similarly to a previous report in which T84 cells were treated with CDCA at 500 µM (35), we showed only an increase in dextran flux with a concomitant decrease in TEER values after treatment with the highest concentration of CDCA for 24 h. The no variation for the lower concentration is in agreement with other studies (38,39). Similar to the effects of CDCA on the integrity and permeability of the Caco-2 monolayer, we found a reduction in TEER values without altering the FITC dextran flux after 2 and 24 h of treatment with DCA at 500 µM. In contrast to previous reports in which incubation with DCA at various concentrations up to 250 µM for 6, 12 and 24 h impaired the integrity of the Caco-2 monolayer along with an increase in permeability (38,43,44). Again, as with CDCA, exposure to lower concentrations of DCA (50 µM) had no effect on neither the integrity nor the permeability of the epithelial barrier (39).

On the other hand, incubation with CA at 200 µM, in contrast to CDCA and DCA, had a greater effect than at 500 µM for all the time points on epithelial barrier integrity, although it was only significant for the 2h incubation condition. These results showing no changes in TEER or in FITC dextran flux after the incubation with CA at the highest concentration at any time point are similar to most of the previous findings in the literature, where treatment with CA generally shows a lower effect on epithelial monolayer integrity than treatment with CDCA and DCA (38,39), and some have shown no effect on TEER after 24-h of exposure to CA (44). This is supported by previous research showing that CA does not alter neither intestinal ion secretion nor the absorptive function of the epithelium (33).

Furthermore, we describe for the first time changes in TEER as early as 2 h after exposure, although high concentrations are required. The data from our model suggest that CDCA and DCA induce changes in epithelial barrier function by inducing a TEER decrement and an increase in epithelial permeability, indicating alterations in the pore and the leak function, respectively. Therefore, the expression of TJPs related genes involved in the regulation of the pore function (56), such as CLDN-2, and the leak function regulated by OCLN and TJP1 were examined. Indeed, our group has demonstrated dysfunctional epithelial barrier in the jejunal mucosa of IBS-D patients with alterations in TJPs signalling pathways (57,58). BAs are known to modulate epithelial barrier function, but the results are highly variable and the exact mechanism and effect on TJPs expression remains to be elucidated.

Overall, we found that in addition to the changes induced by treatment with CDCA and DCA at 500 µM for 24 h in TEER and the increased permeability of the Caco-2 monolayer, these

exposures also modified all the genes studied, in particular CLDN2 expression was increased after DCA treatment, which is consistent with previous results of protein expression also in Caco-2 cells after 24 h of exposure (43). However, they also showed upregulation of CLDN2 protein expression in DCA-treated Caco-2 cells at low concentrations such as 20 µM at 8 and 24 h post-treatment, in contrast to our observations where lower time and concentration of exposure did not induce an increase. However, these molecular changes are not comparable because one refers to gene while the other to protein expression, where time and concentration of exposure may play a role in the results.

It should be noted that the gene expression of OCLN and TJP1 was increased after 2h- and 24h-exposure to the two BAs at 500 µM, although without statistical significance compared to the monolayers treated with vehicle. With regard to the effects of exposure to DCA and CDCA on OCLN protein expression, Hughes *et al.* found a significant increase in OCLN protein expression after 12 h of incubation with DCA (250 µM) and none with CDCA (38). On the contrary, Raimondi *et al.* 2008 showed that exposure to both BAs (50 µM) for a maximum of 120 min induced a rapid and transient dephosphorylation of OCLN protein in Caco-2 cells, with a peak at 20 min, an effect similar to the decrement in TEER and the increase in epithelial permeability (39). CDCA, in particular, also induced an intracellular reallocation of this protein. In addition, treatment with DCA at concentrations up to 250 µM induced a downregulation of gene expression for OCLN and TPJ1 (44). It is important to be cautious in the comparison between the different existing data as the results are quite variable depending on the type of BA, the concentration and the time of exposure. It could be that the molecular changes that we observed in the expression of TJPs after 2 and 24 h stimulation do not explain the effects on TEER and permeability, we could be overlooking those that occurred within the first 20 min that may better justify these changes. We cannot exclude that the increase in gene expression of OCLN and TJP1 could be compensatory mechanism for the impairment in the epithelial barrier function.

In our experiments, no cytotoxic effect was observed in the epithelial monolayer at the different concentrations used, therefore, it is unlikely that the DCA- and CDCA-induced changes in epithelial monolayer integrity and permeability are due to their detergent properties. Therefore, in addition to transcriptional changes in CLDN2, other mechanisms may underlie these features in the epithelial barrier, such as activation of BARs (8,59). BAs are considered to be signalling molecules that act on IECs through the interaction with various BARs, the most studied being GPBAR1, NR1H4 and VDR. Most approaches focus on the role

of BAs via their receptors in the regulation of the immune response, and in the context of an inflammatory trigger. Nevertheless, few studies have focused on how BAs interact with IECs and the resulting transcriptional changes in BARs and their downstream pathways following BAs exposure in the absence of an inflammatory trigger (18,19,25). Only Pi *et al.* have investigated the molecular changes in the expression of BARs from colon epithelial cells after exposure to BAs and, in contrast to us, they showed that DCA at 100 µM increased NR1H4 gene expression and did not alter GPBAR1 expression after 24 h exposure in Caco-2 cells.

Overall, our results show a non-statistically significant decrease of most of the genes examined after 24 h exposure to DCA and CDCA at 200 and 500 µM, except that CDCA at 500 µM induced a slight increase in GPBAR1 gene expression. GPBAR1 has a greater affinity for DCA and NR1H4 for CDCA (20,60), but from our results we cannot conclude that the changes in expression are greater for one BA than the other. Surprisingly, our results show a greater effect on GPBAR1 gene expression after 2 h of exposure to CDCA than to DCA at 500 µM, both inducing an increase in GPBAR1 expression, where a TEER decrement was also observed. Indeed, it is known that chloride secretion is stimulated by CDCA and DCA in T84 cells (61), although activation of GPBAR1 does not appear to be involved (41,62), which may be explained by the activation of the cystic fibrosis transmembrane conductance regulator. Various smaller effects on VDR expression were also observed, which could be explained by the fact that the only BA described as ligand is LCA (20) although, here, it also seems to respond to CDCA and DCA. Finally, stimulation with DCA and CDCA at the highest concentration for 24 h showed a non-statistically significant reduction in NR1H4 gene expression, associated with an increase in FGF19 gene expression in the treated monolayers. Activation of this BAR is involved in the regulation of epithelial transport and exerts an anti-secretory effect by modulating the expression of cystic fibrosis transmembrane conductance regulator. It is also responsible for controlling BA biosynthesis in the liver, where the increased expression of FGF19 is important to maintain BA synthesis homeostasis (12).

We are aware of the limitations of this study, being the small number of experiments performed the first one to address. Additional experiments are needed to confirm the trend in gene expression observed in some of the conditions. Importantly, protein expression analysis will also be mandatory to verify the functional relevance of the gene expression results obtained. Also, the slight reduction in TEER recordings in the vehicle-treated monolayer within the first 20 min as a consequence of the non-stabilisation of the medium, could be a possible methodological limitation. As for the observed effects, we did not use

BARs antagonists or assessed downstream signalling pathways to more precisely confirm that the quantified changes in the epithelium are induced by the interaction BA-BAR.

In addition, the basolateral exposure of BAs has not been addressed here, but could also bring new insights into BAs-mediated effect on epithelial cells as may mimic the passive diffusion of secondary BAs in those pathological situations with increased permeability, which allow the passage of luminal components, including BAs, to the lamina *propria*.

In conclusion, this study describes the effect of three different BAs on the integrity and functionality of the intestinal epithelial barrier and that changes in the expression of TJPs and BARs, may underlie alterations in the barrier function. This preliminary study warrants further analysis to elucidate the effects of BAs on barrier function and their action through BARs.

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CHAPTER 3

Enteric glial cell responsiveness to bile acids through changes in its plasticity and alteration in the expression of bile acid receptors

1. INTRODUCTION

The gut contains a plethora of bioactive functional metabolites involved in gut-brain and brain-gut signalling. Advances in the field of neurogastroenterology support the existence of an exquisitely organised and bidirectional interplay between the central nervous system (CNS) and the intestinal lumen via the enteric nervous system (ENS) (1). The ENS is a complex neuronal network embedded in the gastrointestinal (GI) wall, estimated to contain 170 million of neurons (2), in which enteric glial cells (EGCs) and neurons play an essential role in the regulation of all GI physiological reflexes (3,4). In addition, increasing evidence highlights the critical role of ENS in regulating intestinal epithelial barrier function and the luminal environment (5,6). Furthermore, modulation of ENS excitability or function may be directly or indirectly influenced by microbe- or host-derived components (7,8).

The EGCs are the largest population of glia outside the brain and the most abundant cell type within the ENS. EGCs are located along the different layers of the intestinal wall, mainly in the two plexuses, the myenteric and the submucosal. Traditionally, EGCs have been described as the component of the ENS solely responsible for supporting and feeding enteric neurons. However, research conducted over the last decade further supports their role in maintaining intestinal homeostasis through their interaction with non-neuronal cells such as epithelial and immune cells, explaining their involvement in multiple intestinal functions (motility, epithelial secretion and absorption, mucosal integrity, immune response, neuroprotection, synaptic transmission, perception and adult neurogenesis (9–14). The cross-talk between the EGCs and the gut microbiota must not be forgotten, given their proximity. The microbiota is able to shape the activity of EGCs and, consequently, their signals can alter the gut function (15,16).

Three molecular markers are widely used to identify EGCs: two cytoplasmic proteins, the intermediate filament **glial fibrillary acidic protein (GFAP)** and the **Ca^{+2}-binding protein S100β**, and the **nuclear transcription factor SOX10** (14,17–21). EGCs also express **nestin** in their intermediate filaments, although its specific function is unknown (22,23). Our knowledge of EGCs has increased over the last decade and new insights into their local and functional heterogeneity have been linked to a molecular profile of specific markers (GFAP, S100β and SOX10) that may be conditioned by the environment, suggesting their ability to adapt to changes in response to local stimuli (3,24). The subsequent effects of EGCs activation are thought to be beneficial and aimed at maintaining homeostasis. Under conditions of

physiological perturbation, EGCs are able to respond by adopting a reactive phenotype called gliosis, which is a dynamic phenomenon that depends on the environment and the disease, although its consequences remain unknown (9). Given all this, it is not surprising that EGCs have been implicated in several GI diseases as a critical link between gut health and disease (9,25–27).

On the other hand, recent data point to bile acids (BAs) as a relevant, recognised mediators of microbiome-gut-brain axis signalling, among others. BAs are a large family of atypical steroids that are essential for the digestion and absorption of lipids and fat-soluble vitamins due to their detergent properties conferred by their amphipathic structure (28). The first BAs synthesised from cholesterol by hepatocytes are the primary BAs, **cholic acid (CA)** and **chenodeoxycholic acid (CDCA),** which undergo further conjugation and are released into the intestinal lumen. Most of the BAs are reabsorbed in the ileal region and are transported to the liver. The non-reabsorbed BAs reach the colon and are metabolised by the gut microbiota resulting in their respective secondary BAs, **deoxycholic acid (DCA)** and **lithocholic acid (LCA)** (29). These bioactive metabolites exert multiple physiological functions beyond the gut via their specific receptors, each of which has a specific BA ligand. Molecular studies of gut specimens have identified these receptors as being expressed on immune and epithelial cells, helping to explain why BAs are understood as immunomodulatory factors and have both physiological and pathophysiological roles in the control of the intestinal epithelial barrier (30–32). The two best-characterised BA receptors (BARs) are the **Farsenoid-X-receptor (FXR)**, also known as nuclear receptor subfamily 1 group H member 4 (**NR1H4**), and the **Takeda G-protein-coupled receptor 5 (TGR5)**, also known as G protein-coupled bile acid receptor 1 **(GPBAR1)**. Intestinal FXR activation prevents histological damage to the intestinal mucosal by inducing antibacterial effects and maintaining epithelial integrity (33) and GPBAR1 plays a role in intestinal electrolyte transport, motility and inflammation (30). Each type of BA has a specific and different potency for activating these receptors; it has been estimated that the differential potency of BAs is CDCA>DCA>LCA>CA for the FXR receptor and LCA > DCA > CDCA > CA for TGR5. Another nuclear receptor to which BAs also bind and activate is the **vitamin D receptor (VDR)** but, unlike NR1H4, BAs are not its major ligands (34,35). In fact, the primary BAs do not activate it, only calcitriol (1,25-dihydroxyvitamin D3) and LCA do, and the downstream effect resulting from the LCA-VDR interaction is the promotion of epithelial barrier homeostasis (36–38). The BAs DCA and LCA conjugates, share structural properties with acetylcholine and can activate other BARs such as muscarinic acetylcholine receptors, that modify cholinergic signalling involved in intestinal motility (39–

42). The **cholinergic muscarinic receptor 3 (CHRM3)** is of particular interest because there is limited evidence that it is involved in intestinal epithelial cell proliferation and in promoting tumour progression (43–45). However, little is known about its role in modulating GI function.

Taken together, this evidence highlights the importance of the interaction between BAs and their receptors in the underlying pathophysiological mechanisms of GI disease. At the level of the ENS, the expression of BARs remains unexplored. To date, the expression of GPBAR1 and CHRM3 expression has been described in enteric neurons (46,47) and only CHRM3 in EGCs (48), but whether EGCs express additional BARs and the consequences of their activation on ENS function remains unknown.

Therefore, we hypothesised that intestinal BAs may modulate the EGCs towards a reactive phenotype. The aim of this study is to characterise the phenotype of EGCs in response to BAs. To address the potential direct and indirect (enterocyte-mediated) effects of intestinal BAs on the EGCs signalling, the project relies on *in vitro* studies in rat myenteric glial cells as well as in mouse ENS-derived cells.

2. EXPERIMENTAL DESIGN

A series of *in vitro* experiments were designed to determine the effect of BAs on EGCs:

(1) to first define the phenotype and the presence of BARs in EGCs.

(2) to determine the direct and indirect ability of BAs to promote glioplasticity (**Figure 1**).

1) ENTERIC GLIAL CELL MODELS CHARACTERISATION

Two myenteric-derived glial cells, a rat EGC cell line (CRL-2690) and primary mouse EGCs, were used as models of EGCs to study:

- Gene expression of the EGC markers *Gfap*, *S100β*, *Sox10* and *Nes*
- Gene expression of the BARs *Chrm3*, *Gpbar1*, *Nr1h4* and *Vdr*
- Protein expression of the EGC markers Gfap, S100β, and Sox10 by immunofluorescence to visualise their distribution and localisation in the cell.

2) EFFECT OF BILE ACIDS ON ENTERIC GLIAL CELLS

This study relies on two experimental approaches to uncover the potential role of direct and enterocyte-mediated effects of BAs on the EGCs phenotype based on the evaluation of the expression of various glial and BAs-related genes.

APPROACH 1: DIRECT BILE ACID SIGNALLING

A preliminary BAs experiment was performed on CRL-2690. The cell monolayer was exposed for 24 hour (h) to four BAs (primary/secondary), CDCA, CA, LCA and DCA at three different concentrations (10 µM - 50 µM - 100 µM). The responsiveness of EGCs was assessed as follows:

- Cell proliferation was monitored using a live cell imaging system (IncuCyte) to determine the effect of BAs on the proliferation rate to confluence.
- Cell viability was measured using the MTT assay to determine the total number of living cells at the highest concentration of BAs.

- Gene expression analysis of canonical EGC markers and neuron-glia signalling markers (*Gja1*, *P2rx4*, *P2rx7*) genes were determined by real-time qPCR to evaluate EGC phenotype in response to BAs concentrations.

According to the preliminary study carried out on the glial cell line, murine primary EGCs were also exposed to the same four BAs at a single concentration (100 µM) for 24 h. Their response was evaluated by quantifying EGC markers, BARs and glial inflammatory derived cytokine genes (*Il-1β*, *Il-6*, *Tnf-α*) by real-time qPCR.

APPROACH 2: ENTEROCYTE-MEDIATED BILE ACID SIGNALLING

In order to investigate the enterocyte-mediated effect on EGCs of BAs, the EGC cell line was treated with the Caco-2-conditioned medium (CM) collected after incubation of Caco-2 cells with the secondary BA DCA (as described in Chapter 2) at a concentration of 500 µM for 2 and 24 h. The choice of this condition was based on its greater effect on transepithelial electrical resistance (TEER) as a marker of epithelial barrier disruption (see Chapter 2). Both time points were chosen in order to compare the effect of short-term and long-term exposure to BA on the epithelium. CRL-2690 cells were exposed to this supernatant for 6 h. Cell viability was then measured using the MTT assay to verify the lack of toxicity of the supernatants. Gene expression analysis of EGC markers and BARs was performed to assess the EGC response to the epithelial exposure to BAs.

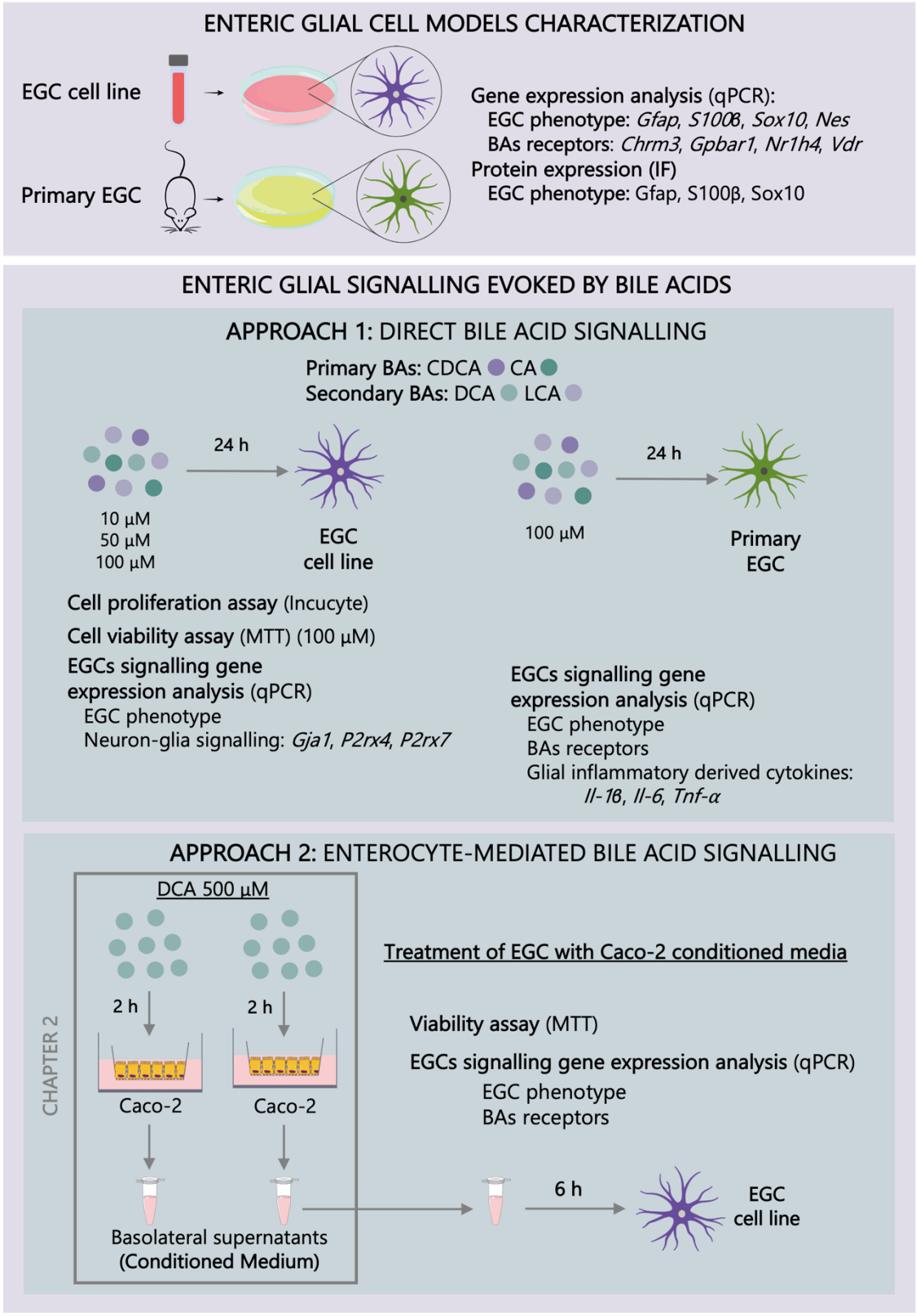

Figure 1 | Chapter 3 experimental design and work-flow. BA: Bile Acid; CA: Cholic Acid; CDCA: Chenodeoxycholic Acid; Chrm3: Cholinergic Receptor Muscarinic 3; DCA: Deoxycholic Acid; EGC: Enteric Glial Cell; Gfap: Glial Fibrillary Acidic Protein; Gja1: Gap Junction Protein Alpha 1; Gpbar1: G Protein-Coupled Bile Acid Receptor 1; IF: Immunofluorescence; Il-1β: Interleukin 1 Beta; Il-6: Interleukin 6; LCA: Lithocholic Acid; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; Nr1h4: Nuclear Receptor Subfamily 1 Group H Member 4; P2rx4: Purinergic Receptor P2X 4; P2rx7: Purinergic Receptor P2X 7; qPCR: quantitative PCR; S100β: S100 Calcium Binding Protein Beta; Sox10: SRY-box 10; Tnf-α: Tumor Necrosis Factor-Alpha; Nes: Nestin; Vdr: Vitamin D Receptor.

3. METHODS

3.1. Enteric glial cell line

A stable enteroglial cell line, CRL-2690, derived from rat jejunal myenteric plexus (49), was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbeccos modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Biowest) and 0.5% penicillin-streptomycin (P/S) (Sigma-Aldrich). CRL-2690 were incubated in a humid atmosphere under conditions of 5% CO2 and 95% air at 37 °C. Cells were trypsinised twice a week using trypsin-EDTA solution 1X (Sigma-Aldrich) and grown until they achieved confluence. Cells at each passage were split and seeded into 75 cm^2 cell culture flasks for maintenance and used for study in passages 65-75. Cells were seeded at a density of 50.000 or 75.000 cells/well in 12-well plates (Sarsted) for gene expression and Caco-2-CM experiments, respectively. For viability and proliferation assays cells were seeded at a density of 2.000 cells/well in 96-well plates (Sarsted).

3.2. Primary enteric glial cell culture

Primary myenteric EGCs were isolated from the small intestine of two adult male mice (*Mus musculus*, C57BL/6JEnvigo, 8-12 weeks old age range). These animals were housed in the animal facility (BIOMED, University of Hasselt, Belgium) and handled according to the principles and procedures outlined in the Council Directive 86/609/EEC. Mice had ad libitum access to water and standard chow diet (Ssniff, Germany) and were maintained on a 12h light/dark cycle in a humidity and temperature-controlled room. All studies were conducted in accordance with the institutional guidelines and approved by the Ethical Committee for Animal Experiments of University of Hasselt.

To obtain cells, the intestine was removed, cleaned with phosphate buffered saline (PBS) and cut into 1 cm pieces. The longitudinal muscle myenteric plexus (LMMP) was separated with a moist cotton swab and washed extensively in dissection media: DMEM/F12 + GlutaMAX (Thermo Scientific) containing 12.5 mM HEPES (Thermo Scientific), 2 % FBS (Biowest) and 1 % P/S (Sigma-Aldrich). After plexus isolation, LMMP samples were minced with scissors and then the tissues were incubated for 35 minutes on a shaker at 37 °C in 30 mL of a digestion solution consisting of dissection media supplemented with collagenase II (327 U/mL; Thermo Scientific) and 1 mg/mL bovine serum albumin (BSA; Sigma-Aldrich). The digested LMMPs were then mechanically triturated with a 1000 µL tip, washed twice with dissection media

and seeded into 6-well plates coated with fibronectin-bovine plasma (1:50) (Sigma-Aldrich) in DMEM/F12 + GlutaMAX containing 12.5 mM HEPES, 10 % FBS and 1 % P/S under conditions of 5% CO2 at 37 °C. After 24 h, the medium was replaced with DMEM/F12 + GlutaMAX containing 12.5 mM HEPES, 1% P/S, 1% N2 supplement (Thermo Scientific), 1% G5 supplement (Thermo Scientific) and 50 ng/mL nerve growth factor-7s (Alomone Labs), a specific medium to promote glial growth. On the third day of culture, the same medium was half renewed. On the fourth day, when the cells were proliferating at the highest rate, they were trypsinised with trypsin-EDTA solution 1X (Sigma-Aldrich), passed through a 40 µm filter in order to obtain a single cell suspension and discard any residual tissue clumps, and seeded into 12-well plates (50.000 cells/well). According to previous studies, a confluent layer of mature primary EGCs is formed and ready for experimental procedures at day 6 after-seeding.

3.3. Preparation of bile acids for in vitro stimulation

The two models of EGCs, CRL-2690 cells and primary cultures, were treated with both the primary BAs CDCA and CA, and the secondary BAs LCA and DCA. Detailed preparation conditions and dilutions of CDCA, CA and DCA are described in the methods of Chapter 2. In this chapter, LCA (L6250; Sigma-Aldrich) was also tested (solubility in ethanol: 50 mg/mL; stock solution in PBS: 1:40 - 0.5 mg/mL). The stock solutions of the BAs were diluted with the standard growth medium of the respective model. A series of experiments were performed using three different concentrations of the four BAs in both EGC models. In each experiment, cells were simultaneously exposed to the same concentrations of ethanol/methanol without BAs (vehicles). Untreated wells were kept as control. All the stimulations were carried out one day after their seeding and in 3 to 4 independent experiments.

3.4. Incubation of EGC with Caco-2-derived conditioned medium

The Caco-2-CM after treatment with 500 µM DCA was selected for incubation with the EGC line, as it showed the greatest reduction in TEER among the conditions tested in Chapter 2. CM were collected after 2 and 24 h of 500 µM DCA or vehicle exposure (see methods of Chapter 2), frozen and stored at -80 °C. The supernatants obtained after treatment (2 and 24 h) were used to stimulate EGC following short and long-term exposure of the enterocyte to a secondary BA (DCA). For each condition, triplicates were pooled to assess whether there was a different effect between treated and untreated Caco-2 supernatants. The collected CM was centrifuged at 800 xg for 10 min and filtered through 0.45 µm filters to remove dead cells and large debris. One day after seeding, the CRL-2690 cell medium was replaced with Caco-2-

derived CM (80% total volume) and fresh growth medium (20% total volume) for 6 h. It is important to note that this 80% of the volume comes from a serum-starved medium (see Methods in Chapter 2). Three independent experiments were performed in duplicate.

3.5. Cell viability assay

One day after seeding, CRL-2690 were exposed to 100 µM BAs/vehicles or Caco-2-derived CM for 24 h or 6 h, respectively. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (M6494, InvitrogenM) assay was then used to confirm the non-cytotoxicity of these stimulations. Briefly, after treatment, MTT solution (0.5 mg/mL) was added to each well and incubated at 37°C for 4 h. Active cells convert the water-soluble MTT to an insoluble purple formazan, in which crystals are solubilised by adding of 100 µL DMSO (Fisher) to each well and incubated on a shaker at room temperature (RT) for 30 min. Formazan concentration was determined by measuring the absorbance (OD) at 595 nm with background subtraction at 655 nm using a CLARIOstar^{Plus} microplate reader (BMG LABTECH). The total number of live cells was calculated in comparison to non-treated cells, representing 100 % of viability. Three independent experiments were performed in duplicate.

3.6. Cell proliferation assay

On the day after seeding, and after stimulating the cells with three increasing concentrations (10 µM, 50 µM and 100 µM) of each of the BAs tested, the cells were placed in an IncuCyte® S3 live cell imaging system (Essen BioScience) in a humidified atmosphere (5% CO_2 and 95% air) at 37 °C and were monitored for 24 h. Phase contrast images of all wells were acquired every 2 h with a tenfold lens (four images per well). Cell confluence in each well was determined for all time points using the IncuCyte S3 2021B software and normalised with respect to its time zero. Three independent experiments were performed in triplicate.

3.7. RNA isolation and quantitative real time PCR

After stimulation of CRL-2690 cells with 10 µM, 50 µM or 100 µM of BAs during 24 h or with Caco-2-CM for 6 h, and primary EGCs with 100 µM concentration of each of the four BAs for 24 h, the cells were washed twice with PBS, harvested in RLT buffer + β-mercaptoethanol (100:1) and stored at -80 °C. RNA was then isolated by first homogenizing the samples by passing them through a 19G (1.1 mm) needle 10 times. After disruption, they were eluted in RNeasy mini columns (QIAGEN) according to the manufacturer´s instructions. Finally, RNA

was eluted in 50 µL of RNase-free water and quantified using Nanodrop™ (Nanodrop ND-1000, Nanodrop Products, Maarssen, The Netherlands). Samples were immediately snap frozen and stored at -80 °C until processed for cDNA synthesis. A total of 1 µg RNA was employed for cDNA synthesis using the qScript cDNA SuperMix (Quantabio), following manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed using the QuantStudio™ 3 Real-Time PCR System (Applied Biosystems). Specific primer sequences for rat (CRL-2690 cells) and for mouse (primary EGCs) were designed for EGC phenotype, BARs, neuron-glia signalling and inflammatory cytokines (supplementary **Table S 1**). As most of the BARs of interest are highly expressed in the liver and the intestine, both tissues were used as positive controls: small intestine for CRL-2690 and primary cells and liver only for CRL-2690 cells. Fast SYBR™ Green Master Mix (Applied Biosystems) was used to run the qPCR reaction and RNase-free H_2O was used as negative control. The relative value of each gene by qPCR is a measure of its expression intensity, which is expressed in cycles to threshold (Ct). Because of the variability between cell lines and tissues, we compared the normalised Ct values to analyse the differential expression of the genes of interest. Gene expression was normalised to those of the housekeeping gene within each EGC model, *Actb* (rat) and *Gapdh* (mouse), and were presented as the delta Ct value (ΔCt=Ct of gene – Ct endogenous gene), which is inversely correlated to the gene expression level. Relative gene expression was calculated by the 2-ΔΔCt method (50); were ΔΔCt = ΔCt sample – ΔCt control expressed as fold change. All gene expression data are presented as the median and interquartile range (IQR) of the number of observations of 3-4 independent experiments performed in duplicate.

3.8. Immunofluorescence staining

CRL-2690 cells were cultured on coverslips (prepared with 1M HCl and stored in 100% ethanol) in 12 well plates (50.000 cells/well). After the treatment, cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min and washed again with PBS. To avoid non-specific signals, samples were incubated with the protein blocking solution (10% donkey serum + 0.3% Triton-X100 in PBS) for 2 h at RT with shaking. The cells were then incubated with primary antibodies overnight in a humidified container at 4°C with shaking. The next day, after washing, samples were incubated with the appropriate secondary antibody for 90 min in the dark at RT in a humidified container. After incubation, cells were washed and incubated with DAPI (1/1000) at RT to counterstain nuclei. The cells were then washed and the coverslip was placed on the glass with mounting medium. Control experiments were

performed by omitting the primary antibodies. Primary and secondary antibodies were diluted in 1% donkey serum + 0.3% Triton-X100 in PBS solution (**Table 1)**.

Table 1 | Antibodies and experimental conditions used for immunofluorescence.

Reactives	Host and target	Manufacturer (catalogue)	Working dilution and incubation
Primary antibodies			
Polyclonal anti-GFAP	Chicken anti-rat	Abcam (ab4674)	1:400 O/N 4 °C
Polyclonal anti-S100β	Rabbit anti-rat	Proteintech (15146-1-AP)	1:400 O/N 4 °C
Polyclonal anti-Sox10	Rabbit anti-rat	Proteintech (10422-1-AP)	1:400 O/N 4 °C
Secondary antibodies			
Alexa Fluor 488	Donkey anti-chicken	ThermoFisher Scientific (A78948)	1:500 90 min RT
Alexa Fluor 647	Donkey anti-rabbit	ThermoFisher Scientific (A31573)	1:500 90 min RT
Alexa Fluor 488	Donkey anti-rabbit	ThermoFisher Scientific (A21206)	1:1000 90 min RT

O/N: Over-night; RT: Room Temperature.

3.9. Statistical analysis

Data distribution was tested by the D'Agostino and Pearson, Shapiro-Wilk and Kolmogorov–Smirnov normality tests. Due to the small sample size, the data were considered as non-normally distributed, are expressed as median (interquartile range) and compared using the Mann-Whitney U test. Relationships between gene expression of markers of interest were tested by Spearmans rho correlation. Values of $p \leq 0.05$ were considered significant. For correlation studies, the Benjamini and Hochberg method was used to test for multiple comparisons, and a false discovery rate (FDR) of ≤0.05 was considered significant. Statistical analyses were performed using GraphPad Prism 9.01 software (Graphpad Software, San Diego, CA, USA).

4. RESULTS

4.1. Enteric glial cell models do express canonical glial markers and bile acid receptors

The canonical EGC markers *Gfap*, *S100β*, and *Sox10* were constitutively expressed with low delta cycle threshold (ΔCt) values by both of the two EGC models. The marker with the highest expression in both models was *S100β*, whereas primary EGCs expressed more *Gfap* and less *Sox10* compared to CRL-2690 cells (**Table 2**). Representative images of the appearance of the cells and the structure of these markers by immunofluorescence (protein expression) are shown in **Figure 2**.

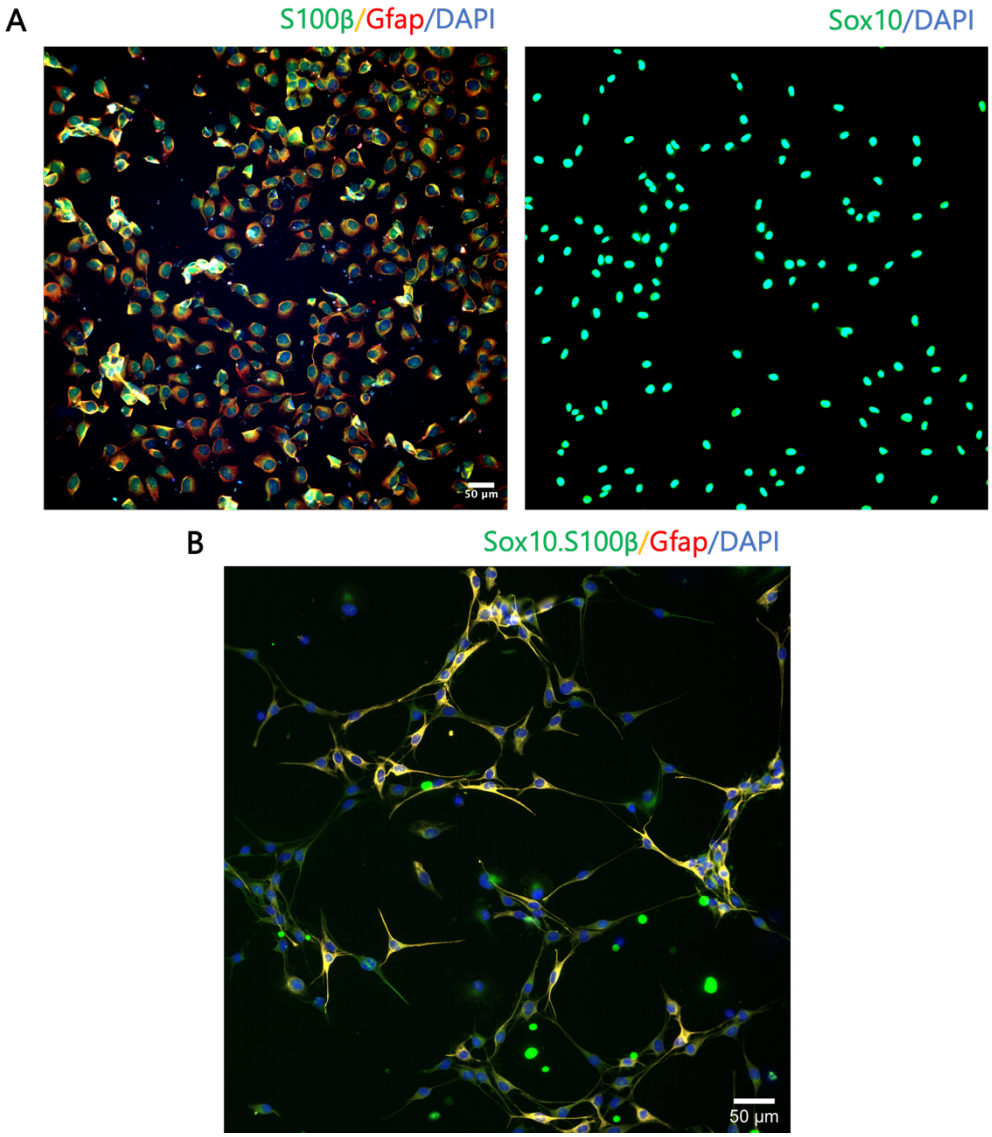

Figure 2 | Representative images of enteric glial cell markers staining in CRL-290 cell line (A) and primary EGC culture (B). Magnification: 200x.

Table 2 | Gene expression of EGC markers in CRL-290 cell line and primary EGC culture.

EGC markers	CRL-2690	Primary EGC
Gfap ΔCt	**12.8** (12.5-13.4)	**9.3** (8.3-10.4)
S100β ΔCt	**4.3** (4.0-4.9)	**6.5** (6.0-7.2)
Sox10 ΔCt	**7.4** (7.0-8.2)	**10.9** (10.1-11.9)

Relative expression was analysed by qPCR. Sample expression was normalised to the house-keeping genes *Actb* (CRL-2690) and *Gapdh* (primary EGC) being ΔCt=Ct target gene – Ct endogenous gene. Data are expressed as median (IQR).

Both EGCs models showed high expression of most of the BARs genes *(Chrm3, Gpbar1, Nr1h4, Vdr*) with low ΔCt values and comparable to other tissues used as positive controls (**Table 3**). The genes *Chrm3*, *Gpbar1* and *Vdr* were expressed at similar levels in both EGC models, while *Nr1h4* was expressed but not quantified by CRL-2690 cells.

Table 3 | Gene expression of BA receptors in CRL-290 and primary EGC culture. Liver and small intestine were used as positive controls.

Bile Acid Receptor	RAT			MICE	
	CRL-2690	Liver	Small Intestine	Primary EGC	Small intestine
Chrm3 ΔCt	**13.2** (12.8-13.5)	**13.2** (12.3-14.0)	Not detected	**12.1** (10.5-12.3)	**10.8** (10.6-10.9)
Gpbar1 ΔCt	**10.1** (9.6-11.8)	**12.5** (11.3-13.7)	**4.2** (3.8-4.7)	**8.5** (8.1-8.9)	**12.3** (12.0-12.6)
Nr1h4 ΔCt	–	**2.2** (2.1-2.2)	**6.7** (6.7-6.7)	**12.7** (12.6-12.7)	**6.1** (6.1-6.1)
Vdr ΔCt	**9.6** (9.5-9.6)	Not detected	**6.2** (5.7-6.6)	**9.1** (8.4-9.4)	**2.3** (2.3-2.3)

Relative expression was analysed by qPCR. Samples expression was normalised to the house-keeping genes *Actb* (CRL-2690) and *Gapdh* (primary EGC) being ΔCt=Ct target gene – Ct endogenous gene. Data are expressed as median (IQR).

4.2. Effect of bile acids on enteric glial cell proliferation, viability, phenotype and signalling in CRL-2690 cells.

4.2.1. Bile acid treatment does not affect enteric glial cell proliferation and viability.

The effect of BAs on proliferation and viability was evaluated to test any potential toxicity. The EGCs treated for 24 h with the different concentrations of the different BAs and their respective vehicles did not show any changes in the confluence ratio over time, and did not alter or affect the rate of cell proliferation (**Figure 3.A**). Notably, there was no difference in the number of viable cells, demonstrating the absence of toxicity or metabolic changes of the EGCs when exposed to the highest concentration of 100 µM (**Figure 3.B**).

4.2.2. Bile acids modify enteric glial phenotype and purinergic signalling

Chapter 1 described a different phenotype of EGCs depending on the presence or absence of malabsorption of BAs in patients with diarrhoea-predominant irritable bowel syndrome (IBS-D), suggesting that BAs may have an effect on EGC activation. Considering that EGCs express a distinct molecular profile of specific markers conditioned by the environment (24), in which BAs act as signalling molecules, we evaluated the impact of BAs on the EGC phenotype (*Gfap*, *S100β*, *Sox10* and *Nes*) and the possible mediation through the ATP signalling pathway (*Gja1, P2rx4* and *P2rx7*) by gene expression analysis.

All BAs had an effect on EGC markers in the 10-100 µM range, significantly altering their gene expression (**Table 4**). The changes were not dose dependent and were more pronounced at 10 and 100 µM concentrations. However, the only concentration that had an effect on the gene expression of all EGC markers gene expression was 100 µM, which induced the greatest changes in expression towards a reactive EGC phenotype. On the other hand, CRL-2690 showed changes in purinergic receptor gene expression in response to treatment with secondary BAs (LCA and DCA) at concentrations of 10 and 50 µM (**Table 5**). Interestingly, lower concentrations of DCA alter the expression of both S100β and purinergic receptors. However, none of the changes in purinergic signalling were associated with changes in the expression of EGC markers, suggesting that the BA-modulated EGC phenotype may not be due to ATP-dependent genes.

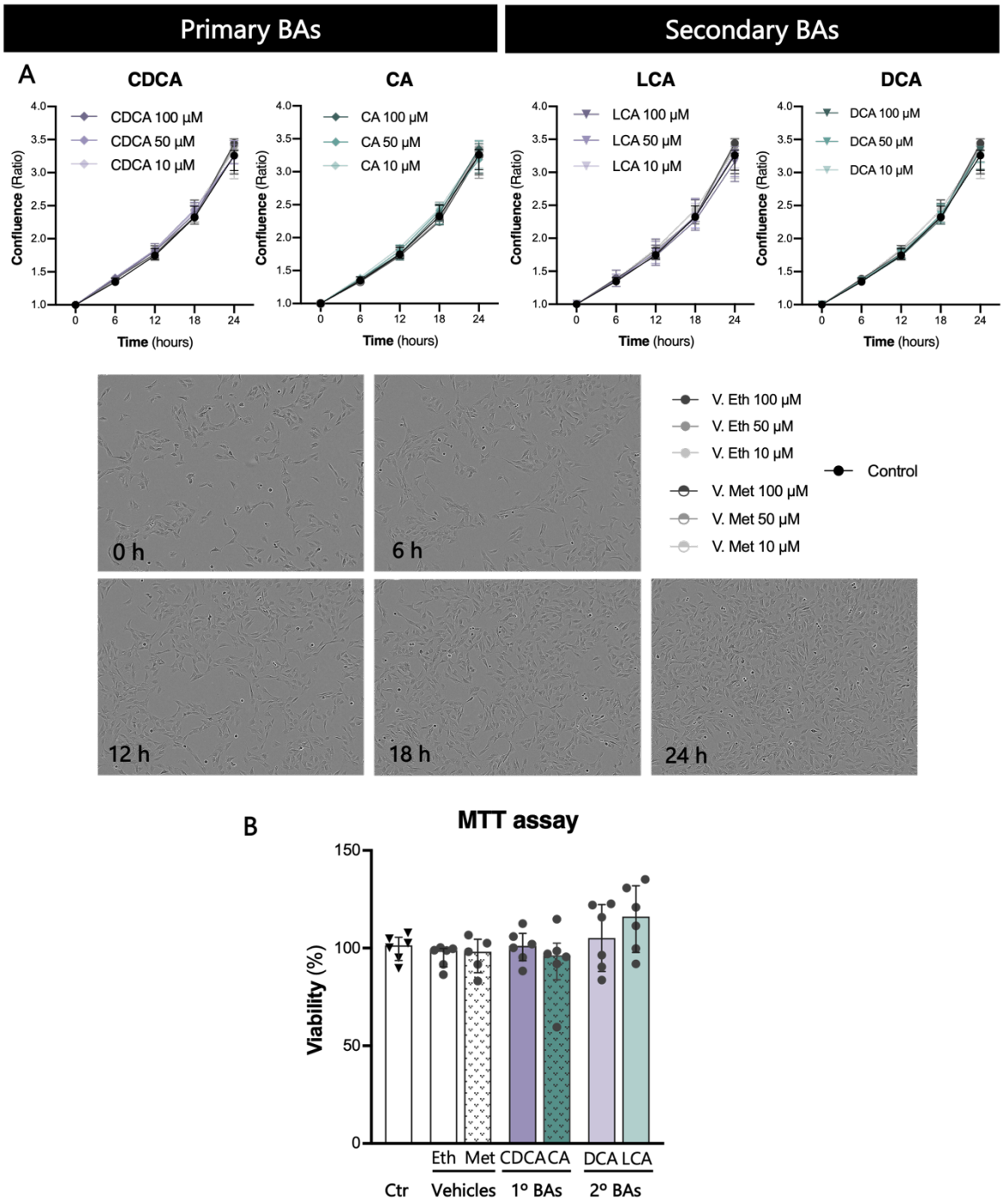

Figure 3 | Cell proliferation and viability of EGC cell line (CRL-2690) after exposure to BAs during 24 hours. A) EGC proliferation was evaluated after exposing CRL-2690 to 3 increasing concentrations (10 µM, 50 µM and 100 µM) of the primary (CDCA and CA) and secondary (LCA and DCA) BAs during 24 h. Phase contrast images were acquired every 2 hours with a ten-fold lens by IncuCyte® S3 live-cell imaging system. Cell confluence in each well was established for all time points and normalised with respect to its time zero (ratio). Graphs represent data of 3 independent experiments performed in triplicate. **B)** EGC viability was assessed by the MTT assay after exposing CRL-2690 to 100 µM BAs during 24 h. The total amount of healthy cells was calculated in comparison to non-treated cells, representing 100 % of viability. Graphs represent data of 3 independent experiments, expressed as number of observations. Data are expressed as median (IQR). Comparison between BAs and their respective vehicles was performed using the Mann-Whitney U test. Eth: Ethanol; Met: Methanol; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromde.

Table 4 | EGC markers gene expression analysis after dose-dependent BAs exposure. CRL-2690 cells were stimulated with four bile acids (CDCA, CA, LCA, DCA) at three increasing concentrations (10 µM - 50 µM - 100 µM) during 24 hours. Relative expression of EGC markers was analysed by qPCR.

Bile acid	Concentration	*Gfap*	*Gfap* vs Vehicle P value	*S100β*	*S100β* vs Vehicle P value	*Sox10*	*Sox10* vs Vehicle P value	*Nes*	*Nes* vs Vehicle P value
CDCA	10 µM	1.62 (1.46-1.75)	0.056	1.11 (0.82-1.50)	0.329	**0.73 (0.57-0.85)**	**0.007**	1.10 (0.59-1.57)	>0.999
	50 µM	0.94 (0.57-1.27)	0.721	**0.66 (0.60-0.74)**	**0.001**	0.73 (0.62-1.05)	0.328	1.26 (0.97-1.45)	0.181
	100 µM	**1.32 (1.16-1.70)**	**0.038**	**0.65 (0.61-0.77)**	**0.009**	0.93 (0.58-1.42)	0.645	1.15 (0.86-1.30)	0.228
CA	10 µM	0.90 (0.55-1.08)	0.228	1.01 (0.57-1.31)	0.959	0.99 (0.48-1.40)	0.852	1.10 (0.65-1.46)	0.645
	50 µM	1.44 (1.26-1.64)	0.130	0.94 (0.87-1.14)	0.721	1.04 (0.69-1.36)	0.852	1.09 (0.90-1.19)	0.310
	100 µM	0.97 (0.72-1.46)	>0.999	1.19 (0.99-1.40)	0.279	**1.38 (1.13-1.87)**	**0.043**	*0.82 (0.53-0.98)*	*0.083*
LCA	10 µM	**1.57 (1.45-1.88)**	**0.021**	1.56 (0.91-2.42)	0.161	0.99 (0.79-1.32)	0.965	**1.38 (1.31-1.47)**	**0.005**
	50 µM	0.92 (0.56-1.10)	0.195	**0.74 (0.64-0.97)**	**0.028**	0.90 (0.58-1.32)	0.798	*1.43 (1.12-2.10)*	*0.081*
	100 µM	1.15 (0.92-1.43)	0.161	**0.65 (0.58-0.70)**	**0.004**	0.91 (0.59-1.58)	0.879	1.21 (0.95-1.34)	0.142
DCA	10 µM	1.49 (0.93-2.11)	0.173	**2.02 (1.75-2.20)**	**<0.001**	0.77 (0.43-0.93)	0.056	1.53 (0.96-1.92)	0.232
	50 µM	0.99 (0.75-1.29)	0.959	**0.67 (0.52-0.76)**	**<0.001**	0.85 (0.73-0.92)	0.345	1.61 (1.16-2.16)	0.059
	100 µM	**1.44 (1.27-2.28)**	**0.001**	1.15 (1.04-1.24)	0.589	0.99 (0.64-1.53)	0.798	1.18 (0.66-1.34)	0.345
Vehicle Ethanol	10 µM	1.08 (0.69-1.46)		0.95 (0.90-1.04)		0.94 (0.85-1.26)		1.01 (0.91-1.06)	
	50 µM	1.03 (0.84-1.19)		0.96 (0.84-1.22)		1.01 (0.60-1.65)		1.03 (0.76-1.34)	
	100 µM	1.04 (0.83-1.12)		1.00 (0.80-1.27)		1.11 (0.61-1.58)		1.02 (0.92-1.07)	
Vehicle Methanol	10 µM	1.15 (0.60-1.39)		1.07 (0.79-1.20)		1.00 (0.91-1.08)		1.01 (0.83-1.20)	
	50 µM	0.97 (0.71-1.44)		0.93 (0.69-1.47)		0.98 (0.91-1.13)		1.02 (0.93-1.08)	
	100 µM	0.96 (0.81-1.32)		1.00 (0.84-1.43)		1.01 (0.77-1.25)		1.08 (0.72-1.55)	

Sample expression was normalised to the house-keeping gene *Actb* being ΔCt=Ct target gene – Ct endogenous gene. Fold change with respect to the vehicle is shown and expressed as median (IQR). Each condition was compared with its respective vehicle using the Mann-Whitney test. P values ≤0.05 were considered significant and are shown in bold in the table.

Table 5 | Neuron-glia signalling markers gene expression analysis after dose-dependent BAs exposure. CRL-2690 cells were stimulated with four bile acids (CDCA, CA, LCA, DCA) at three increasing concentrations (10 µM - 50 µM - 100 µM) during 24 hours. Relative expression of *Gja1* and purinergic receptors *P2rx7* and *P2rx4* was analysed by qPCR.

Bile acid	Concentration	*Gja1*	*Gja1* vs Vehicle P value	*P2rx7*	*P2rx7* vs Vehicle P value	*P2rx4*	*P2rx4* vs Vehicle P value
CDCA	10 µM	1.02 (0.80-1.29)	0.888	1.10 (0.80-1.62)	0.673	0.96 (0.91-1.11)	0.328
	50 µM	0.88 (0.70-1.21)	0.414	0.92 (0.61-1.00)	0.281	1.02 (0.87-1.15)	0.818
	100 µM	0.92 (0.85-1.10)	0.879	1.10 (0.92-1.19)	0.382	1.08 (1.00-1.19)	0.328
CA	10 µM	0.93 (0.88-1.08)	0.240	0.97 (0.85-1.24)	0.937	1.05 (0.90-1.45)	0.699
	50 µM	0.89 (0.70-1.55)	0.694	0.97 (0.82-1.03)	0.414	0.87 (0.73-1.02)	0.142
	100 µM	1.02 (0.93-1.19)	0.721	0.92 (0.82-1.02)	0.235	0.96 (0.88-1.07)	0.798
LCA	10 µM	0.87 (0.78-0.97)	0.470	1.44 (0.65-1.56)	0.351	**0.80 (0.75-0.88)**	**0.016**
	50 µM	0.98 (0.85-1.6)	>0.999	0.98 (0.80-1.34)	>0.999	**1.21 (1.12-1.58)**	**0.008**
	100 µM	0.86 (0.78-1.07)	0.442	1.17 (1.04-1.45)	0.050	0.96 (0.80-1.05)	0.234
DCA	10 µM	1.03 (0.79-1.34)	0.918	**1.31 (1.18-1.42)**	**0.042**	0.97 (0.86-1.10)	0.758
	50 µM	1.06 (0.85-1.41)	>0.999	**0.78 (0.69-0.81)**	**0.040**	**1.24 (1.14-1.40)**	**0.003**
	100 µM	0.99 (0.88-1.31)	0.505	1.18 (1.03-1.33)	0.108	1.06 (0.74-1.23)	0.721
Vehicle Ethanol	10 µM	1.15 (0.79-1.24)		1.03 (0.82-1.26)		1.03 (0.85-1.18)	
	50 µM	1.02 (0.93-1.07)		1.07 (0.79-1.16)		0.98 (0.94-1.06)	
	100 µM	0.99 (0.82-1.24)		1.01 (0.87-1.10)		1.03 (0.91-1.13)	
Vehicle Methanol	10 µM	1.03 (0.95-1.13)		1.00 (0.85-1.28)		0.95 (0.93-1.21)	
	50 µM	0.97 (0.82-1.27)		1.04 (0.83-1.22)		1.00 (0.87-1.13)	
	100 µM	1.02 (0.85-1.15)		1.06 (0.77-1.24)		1.00 (0.87-1.13)	

Sample expression was normalised to the house-keeping gene *Actb* being ΔCt=Ct target gene – Ct endogenous gene. Fold change with respect to the vehicle is shown and expressed as median (IQR). Each condition was compared with its respective vehicle using the Mann-Whitney test. P values ≤0.05 were considered significant and are shown in bold in the table.

4.3. Effects of bile acids on the expression of enteric glial cell markers, bile acid receptors and glial inflammatory cytokine production in primary enteric glial cells.

In a second step, in order to better understand the mechanisms by which EGCs change their phenotype in response to BAs, we exposed primary EGC cultures for 24 h to the same BAs tested in the first part of this study at the concentration that had the greatest effect on molecular changes: 100 µM. The two ENS cell models were used to study the direct effects of BAs, by assessing molecular changes in **EGC phenotype markers** (*Gfap, S100β, Sox10* and *Nes*), **BARs** (*Chrm3*, *Gpbar1*, *Nr1h4* and *Vdr*) and **glial inflammatory derived cytokines** (*Il-1β*, *Il-6*, *Tnf-α*). Here, due to the lack of molecular changes related to purinergic receptors with the previously studied BAs we did not check their expression in primary EGCs.

4.3.1. Bile acids alter the phenotype of enteric glial cells.

At the transcriptional level, DCA treatment induced a significant upregulation of *Gfap* in both EGC cultures, whereas CDCA upregulated *Gfap* in the cell line and downregulated it in primary cell cultures. A similar response was observed with LCA exposure in primary EGCs (**Figure 4.A**). Treatment with CDCA and LCA induced a downregulation of *S100β* expression in both models, although more profound in the primary cell culture. DCA also induced a significant downregulation of *S100β* in primary cell cultures in contrast to CRL-2690 cells (**Figure 4.B**). Regarding *Sox10* expression, treatment with CDCA and the secondary BAs (LCA and DCA) induced a significant downregulation in primary EGCs, whereas CA treatment induced a significant upregulation in CRL-2690 cultures (**Figure 4.C**). Finally, *Nes* expression remained unchanged in the cell line and was reduced by CDCA in primary cell cultures (**Figure 4.D**). In summary, these result show that EGCs have a reactive phenotype in response to primary and secondary BAs, although the data in both models were not always consistent.

Most of these molecular changes induced by the treatment with the different BAs were positively correlated with each other in primary cell cultures. However, the treatment with DCA showed a significant and negative correlation between the expression of *S100β* and *Gfap* **(Table S 2**).

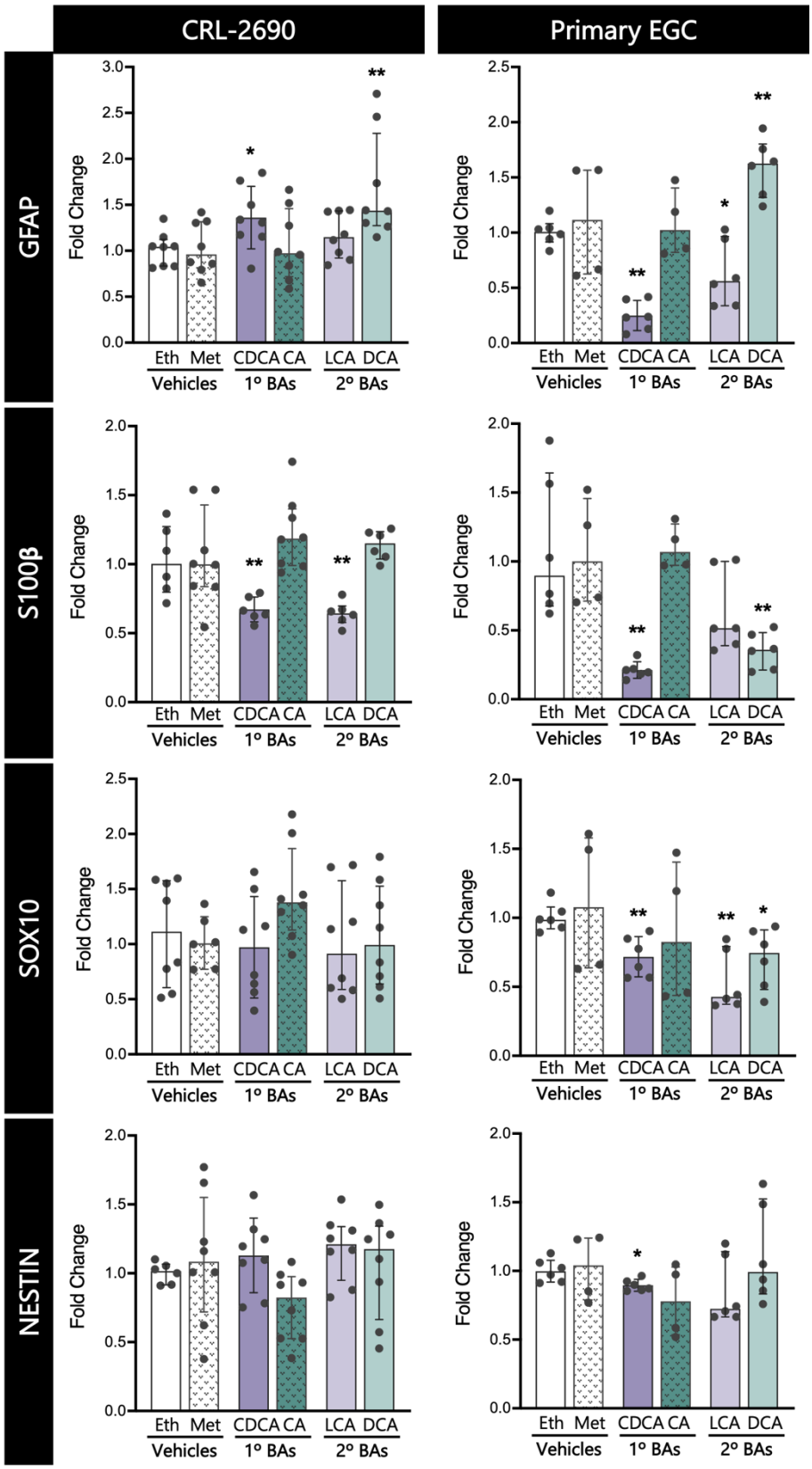

Figure 4 | EGC-specific markers expression after exposure to 100 µM BAs in the EGC cell line CRL-2690 and primary EGC cultures during 24 h. mRNA expression of *Gfap* (**A**), *S100β* (**B**), *Sox10* (**C**) and *Nes* (**D**) was assessed by qPCR. To obtain the fold-change value for each sample the ratio between target gene and the reference gene (*Actb*: CRL-2690; *Gapdh*: Primary EGC) was calculated for each sample and then normalised to the average of the vehicle group. Graphs represent data of 4 independent experiments, expressed as number of observations. Data are expressed as median (IQR). Comparison between BAs and their respective vehicles was performed using the Mann-Whitney U test. * p≤0.05, **p ≤0.01. BA: Bile Acid; Eth: Ethanol; Met: Methanol.

4.3.2. Bile acids reduce the expression of bile acid receptors on enteric glial cells.

Next, we investigated whether BAs modulate the expression of BARs in EGCs. We first performed gene expression analysis in primary EGC cell cultures of *Chrm3*, *Gpbar1*, *Nr1h4*, and *Vdr*. Treatment with both primary BAs CDCA and CA, as well as the secondary BA DCA induced a downregulation of *Chrm3* expression, although did not reach statistical significance due to the variability found in the respective vehicles. *Gpbar1* expression remained unchanged with the treatment of the different BAs. It is noteworthy that treatment with the primary BA CDCA, and the two secondary BAs, LCA and DCA, induced a significant downregulation of *Nr1h4* and *Vdr* expression on EGCs (**Figure 5**). Interestingly, the only BA that did not alter the expression of any of the BARs studied on EGCs was the primary BA, CA, as already observed above in relation to the EGC-specific markers.

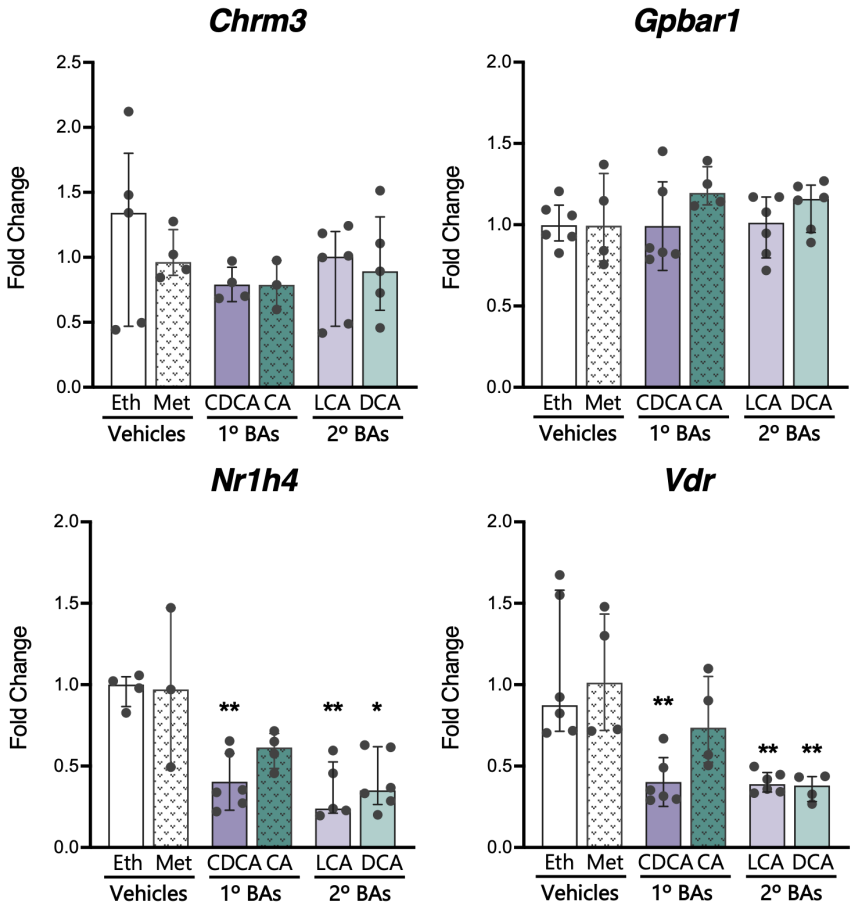

Figure 5. Profile of BAs receptors expression after exposure to 100 µM BAs in primary EGC cultures during 24 h. mRNA expression of *Chrm3*, *Gpbar1*, *Nr1h4* and *Vdr* was assessed by qPCR. To obtain the fold-change value for each sample the ratio between target gene and the reference gene (*Gapdh*) was calculated for each sample and then normalised to the average of the vehicle group. Graphs represent data of 4 independent experiments, expressed as number of observations. Data are expressed as median (IQR). Comparison between BAs and their respective vehicles was performed using the Mann-Whitney U test. * p≤0.05, **p ≤0.01. BA: Bile Acid; Eth: Ethanol; Met: Methanol.

4.3.3. Bile acids modulate the phenotype of enteric glial cell via the nuclear bile acid receptors Nr1h4 and Vdr.

To gain further insight into the reactive EGC phenotype induced by BAs, we performed an association analysis between the gene expression of EGC-specific markers and BARs in the primary EGC cell culture. Remarkably, most of the molecular changes in the markers of EGCs induced by the treatment with CDCA, LCA and DCA were highly and positively correlated with the expression of *Nr1h4* and *Vdr* (**Table 6**). No significant association was observed between *Chrm3* or *Gpbar1* gene expression, while a negative association was found between *Gfap* and *Vdr* expression following DCA treatment (r*s*=-0.85; FDR=0.027). These findings suggest that the BARs, *Nr1h4* and *Vdr*, may contribute to the reactive EGC phenotype following exposure to BAs.

Table 6 | Correlation analysis of EGC markers and BARs. Analysis of the correlations between the gene expression of EGC markers and BARs after 100 μM BAs stimulations on primary EGCs.

		Chrm3			*Gpbar1*			*Nr1h4*			*Vdr*		
		rs	p	FDR	rs	p	FDR	rs	p	FDR	rs	p	FDR
CDCA	*Gfap*	0.05	0.91	1.02	0.03	0.94	1.01	**0.92**	**<0.01**	**0.02**	**0.79**	**<0.01**	**0.016**
	S100β	-0.23	0.55	0.77	-0.01	0.97	1.01	**0.83**	**0.01**	**0.02**	*0.66*	**0.02**	*0.061*
	Sox10	0.12	0.12	0.94	0.13	0.13	0.89	0.48	0.48	0.31	*0.67*	**0.02**	*0.063*
	Nes	-0.25	0.52	0.77	-0.23	0.47	0.73	*0.70*	**0.03**	*0.07*	0.51	0.09	0.187
CA	*Gfap*	-0.57	0.20	0.47	0.48	0.24	0.52	0.11	0.84	1.02	0.76	**0.04**	0.15
	S100β	-0.43	0.35	0.62	0.45	0.27	0.53	0.07	0.91	1.02	0.71	*0.059*	0.18
	Sox10	-0.29	0.56	0.87	0	1.00	1.00	0.32	0.50	0.82	**0.93**	**<0.01**	**0.03**
	Nes	-0.25	0.60	0.83	-0.10	0.84	0.98	0.179	0.71	0.95	**0.93**	**<0.01**	**0.02**
LCA	*Gfap*	0.17	0.62	0.72	-0.16	0.62	0.70	**0.87**	**<0.01**	**0.03**	*0.62*	**0.04**	*0.08*
	S100β	-0.32	0.34	0.46	-0.07	0.84	0.87	**0.80**	**0.01**	**0.05**	0.49	0.11	0.21
	Sox10	0.25	0.47	0.57	0.01	0.97	0.97	**0.87**	**<0.01**	**0.04**	**0.87**	**<0.01**	**0.01**
	Nes	-0.39	0.24	0.37	-0.56	0.06	0.14	*0.73*	**0.03**	*0.08*	0.45	0.14	0.25
DCA	*Gfap*	0.07	0.87	0.97	0.44	0.15	0.360	-0.56	0.10	0.25	**-0.85**	**<0.01**	**0.03**
	S100β	-0.20	0.59	0.74	-0.54	0.08	0.27	**0.81**	**<0.01**	**0.04**	0.61	*0.07*	0.27
	Sox10	0.10	0.79	0.96	-0.28	0.38	0.56	**0.82**	**<0.01**	**0.04**	*0.87*	**<0.01**	*0.06*
	Nes	0.03	0.95	0.98	0.01	0.99	0.99	0.42	0.23	0.43	0.09	0.81	0.95

Comparisons were performed using the Spearman's rho correlation and the obtained p values were adjusted by the Benjamin and Hochberg method. Data are represented as: r*s* (spearman's rank correlation coefficient), p (p value) and FDR (false discovery rate). FDR of ≤0.05 was considered significant and it is shown in bold in the table.

4.3.4. Bile acids induce an inflammatory response in enteric glial cells.

To elucidate whether reactive EGC phenotype could lead to an inflammatory response, cytokine gene expression was assessed. Treatment with CDCA and DCA significantly upregulated *Il-1β* and *Il-6* and expression, respectively, while *Tnf-α* remained unchanged (**Figure 6**).

The association analysis between the expression of inflammatory cytokines derived from EGCs and EGC-specific markers showed that only under DCA treatment *Il-6* correlated positively with *Gfap* (r*s*=0.74; FDR:0.055) and negatively with *S100β* (r*s*=-0.90; FDR=0.004). Interestingly, *Gfap* expression was negatively correlated with *Il-1β*, although this association did not reach the statistical significance (r*s*=-0.83; FDR:0.065) (**Table S 3**). In addition, no association was found between BARs and the expression of inflammatory-derived cytokines in EGCs.

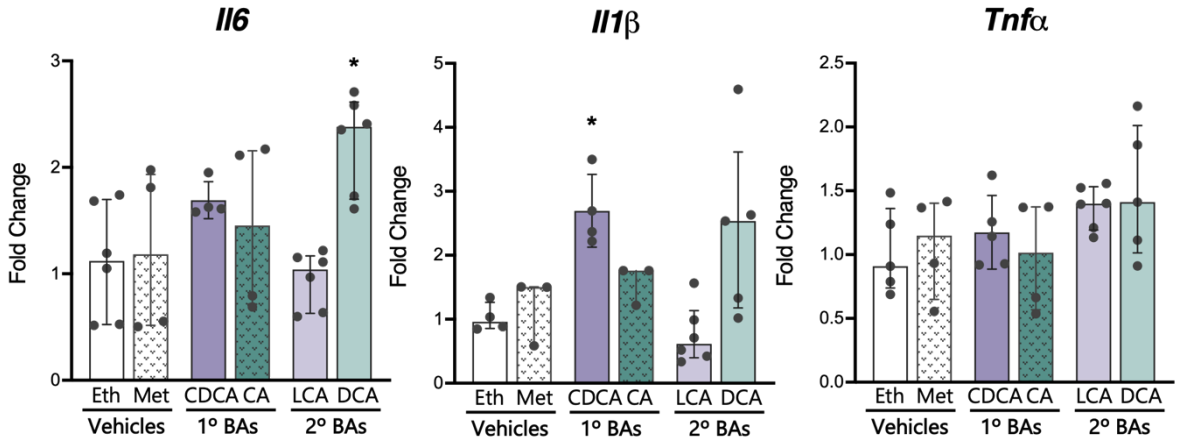

Figure 6 | Expression of inflammatory cytokines of primary EGC cultures after exposure to 100 µM BAs during 24 h. mRNA expression of *Il6*, *Il1β* and *Tnfα* was assessed by qPCR. To obtain the fold-change value for each sample the ratio between target gene and the reference gene (*Gapdh*) was calculated for each sample and then normalised to the average of the vehicle group. Graphs represent data of 4 independent experiments, expressed as number of observations. Data are expressed as median (IQR). Comparison between BAs and their respective vehicles was performed using the Mann-Whitney U test. * p≤0.05. BA: Bile Acid; Eth: Ethanol; Met: Methanol.

4.4. The enteric glial cell is capable of responding to bile acid-mediated signalling through the enterocyte.

To examine the enterocyte-mediated BA signalling on EGC, we performed another experimental design (approach 2), in which CRL-2690 cell cultures were incubated with Caco-2-CM after treatment with DCA. Cell viability was preserved as no differences were observed between each condition, being the median percentages of cell viability higher than 85% (data not shown).

A gene expression analysis was performed in the CRL-2690 cultures for those genes that were previously studied and involved in the reactive EGC phenotype. We first compared the vehicle-treated CM CRL-2690 cells with the untreated-CRL-2690 cells, with no statistical differences between them. Therefore, all results of the gene expression analysis presented here result from the comparison between DCA-treated CM (2 and 24h) and vehicle-treated CM (2 and 24h) CRL-2690 cells, respectively. CRL-2690 cell cultures incubated with CM treated with DCA or vehicle for 2 h did not show any significant change for EGC-specific markers. However, those incubated with CM treated with DCA or vehicle for 24 h showed a downregulation of *S100β* and *Sox10* expression, which was significant only for *Sox10* expression. Taken together, these results suggest that glia respond only to epithelial signalling pathways induced by long-term DCA exposure conditions, and not to those induced by acute DCA exposure (**Figure 7**).

Regarding the expression of all BARs, DCA-treated CMs for 24 h showed a trend towards an increase in *Vdr* expression (p=0.064) compared with those vehicle-treated CMs. No changes in BARs expression were observed when DCA-treated CMs were compared with vehicle-treated CMs for 2 h (**Table 7**).

The association analysis between gene expression of EGC-specific markers and BARs showed no significance.

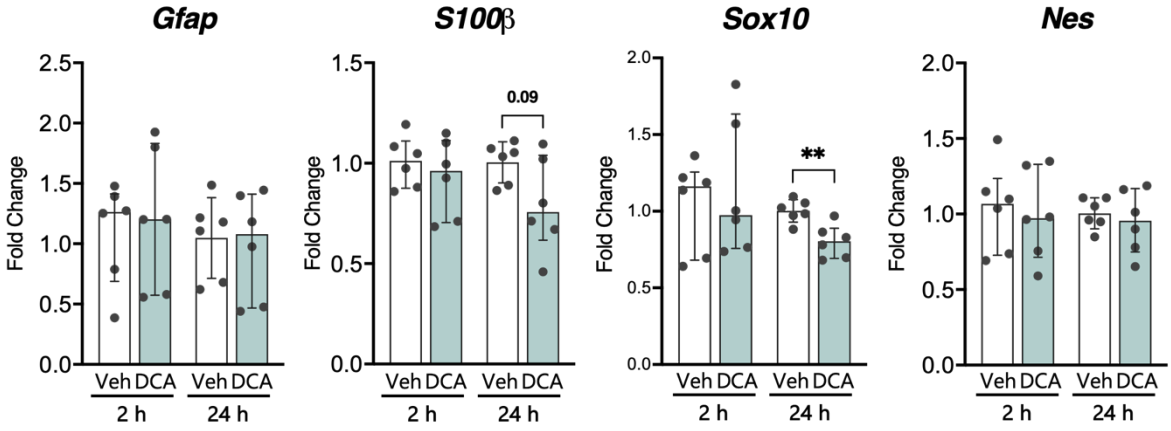

Figure 7 | EGC-specific markers expression after exposure to Caco-2-CM in the EGC cell line CRL-2690 during 6 h. CRL-2690 were stimulated with Caco-2 CM coming from chapter 2 where Caco-2 cells were exposed to DCA and its vehicle at 500 µM for 2 and 24 hours. mRNA expression of Gfap, S100β, Sox10 and Nes was assessed by qPCR. To obtain the fold-change value for each sample the ratio between target gene and the reference gene (Actb) was calculated for each sample and then normalised to the average of the vehicle group. Graphs represent data of 3 independent experiments, expressed as number of observations. Data are expressed as median (IQR). Comparison between BAs and their respective vehicles was performed using the Mann-Whitney U test. ** p≤0.01. DCA: Deoxycholic acid, Veh: Vehicle.

Table 7 | Gene expression of BARs after stimulation of EGCs with Caco-2-CM for 6 h. CRL-2690 were stimulated with Caco-2-CM (chapter 2) where Caco-2 cells were exposed to DCA and its vehicle at 500 µM for 2 and 24 h. Relative expression of BARs was analysed by qPCR.

	2h DCA/VEH - CACO			24 h DCA/VEH - CACO		
BA receptor	CM-VEH	CM-DCA	CM-DCA vs CM-VEH p value	CM-VEH	CM-DCA	CM-DCA vs CM-VEH p value
Chrm3	1.10 (0.83-1.22)	1.13 (0.71-1.28)	0.818	0.98 (0.76-1.33)	1.56 (1.03-1.84)	0.180
Gpbar1	0.80 (0.36-2.39)	0.62 (0.50-2.25)	>0.999	0.63 (0.28-5.63)	4.23 (0.64-5.28)	0.589
Vdr	1.02 (0.54-1.82)	1.22 (0.67-1.80)	0.589	0.82 (0.63-1.91)	2.54 (0.86-3.34)	*0.064*

Sample expression was normalised to the house-keeping gene *Actb* being ΔCt=Ct target gene – Ct endogenous gene. Fold change with respect to the vehicle is shown and expressed as median (IQR). Each condition CM-DCA was compared with its respective vehicle using the Mann-Whitney test. p values ≤0.05 were considered significant. CM: conditioned media; DCA: Deoxycholic acid; VEH: Vehicle.

5. DISCUSSION

In this study, we investigated the interaction between glial cells and BAs, in order to gain insight into the contribution of EGCs to the pathophysiology of IBS-D with BA malabsorption. We found for the first time, that EGCs express BARs, suggesting that the ENS responds to BAs coming from the gut lumen. Indeed, our experimental approaches allowed us to identify the response of EGCs to unconjugated BA exposure, directly, by exposing EGCs to BAs and, indirectly, by exposing EGCs to epithelial cell-derived media after incubation of IECs with BAs. Our data provide important insights into the modulation of the EGC phenotype and signalling induced by BAs. Direct exposure of EGCs to unconjugated BAs modified the expression of EGC-specific markers via BARs. Moreover, DCA promoted differential regulation of EGC-specific marker expression through its interaction with IECs.

Alterations in intestinal luminal BA profiles are associated with impaired intestinal barrier integrity (51), and increased intestinal epithelial barrier permeability is involved in the pathogenesis of IBS-D. Barrier dysregulation allows the translocation of dietary components, microbial-derived antigens and metabolites such as BAs from the intestinal lumen to the lamina *propria* (52–54). In this context, BAs may act directly to activate cell receptors and signalling pathways within those cells located in the lamina *propria* including the EGCs. Previous unpublished results from our group have described both, the differential expression of TJPs and the phenotype of EGCs in the jejunal mucosa of patients with IBS-D with or without BAD (Chapter 1). In addition, we have reported altered intestinal epithelial barrier integrity in an *in vitro* model following long-term treatment with unconjugated BAs (Chapter 2). To explore the potential involvement of the ENS in these disorders, we decided to investigate the response of EGC to unconjugated BA in an in *vitro* model using two types of myenteric glial cells, the rat cell line CRL-2690 and primary mouse cells. In addition, we investigated the effect of BA-exposed epithelium on CRL-2690 cells. First, we found that all myenteric glial cell cultures tested expressed nuclear and membrane receptors whose specific ligands are BAs at levels similar to those found in the liver and the intestine of each species studied. Notably, although *Nr1h4* was detected in CRL-2690 cells we were unable to measure its expression in CRL-2690 cells. Since BARs are present in both glial populations, we then tested the potential molecular changes in myenteric glial markers induced by BAs.

Effect of BAs on enteric glial phenotype in CRL-2690 and primary EGCs

In the literature, both preclinical and *in vitro* studies have reported that each BA exerts dual effects through the different BARs, depending on its concentration and tissue cell type (55,56), regulating different physiological processes or contributing to pathophysiologic mechanisms. Indeed, several studies in rodents have shown a dose-dependent effect of BAs on apoptosis, endoplasmic reticulum stress response and visceral hypersensitivity in hepatocytes, cardiomyocytes and mast cells (57–60). However, data on the response of EGCs to BA exposure are not yet available, so a dose-response study design was chosen. We first investigated the effect of increasing concentrations of both primary and secondary unconjugated BAs on rat myenteric glial cell signalling. We used the concentration range based on previous data from dorsal root ganglia neurons, which showed increased Ca^{2+} responsiveness to various BAs in the concentration range of 10-100 µM through Mas-related G protein-coupled receptors (61–63). To the best of our knowledge, our study is the first to demonstrate that unconjugated BAs modulate gene expression levels of myenteric glial cell markers. These results suggest that the response of EGCs to treatment with any concentration of BA can be considered specific, without any contribution to modulation of cell proliferation or cell death. Although the majority of expression changes occurred after exposure to the highest concentration of BA, 100 µM, we also detected changes at concentrations as low as 10 µM. In fact, only lower concentrations of BA induced changes in purinergic receptor gene expression as well as in EGC-specific markers, although there was no association between them, suggesting that the BA-modulated EGC phenotype may not be attributable to ATP-dependent genes.

In the field of neurogastroenterology, there is a growing interest in understanding the downstream effects and the functional consequences of the activation of the different phenotypes of EGCs under physiological and pathophysiological perturbations in the gut. The reactive phenotype of EGCs, depending on glia localisation within and throughout the GI tract, is usually associated with upregulation of the inflammatory response and changes in the expression of EGC markers with either neurotoxic or neuroprotective roles (24,64,65). Different experimental studies have attempted to address the specific characterisation of expression of molecular markers and to establish a criterion for describing all these states, which are not yet clearly defined. To gain insight into the biology of EGCs, we compared the molecular phenotype of the two cultures of myenteric glial cells, and also determined towards which phenotype the EGCs switch to when exposed to the different BAs.

Our results revealed a differential response of glial cells to BAs depending on the type of enteroglial cell culture. The different expression patterns observed between the two glial cell cultures should be interpreted with caution. Although the cell line is reported to closely resemble primary enteric glia, we found some non-significant differences in the baseline expression levels of EGC markers between the two glial cell cultures. In general, the distribution of gene expression data from EGC primary cell cultures was less scattered than that from CRL-2690, providing a better model for assessing molecular changes in EGCs in response to BAs. These discrepancies could arise because cell lines may differ in their molecular profile and activation responses compared to the cell type from which they are derived, depending on their isolation and transformation procedures. Indeed, different physiological cell responses have been described between cell lines and primary cultures of microglia, oligodendrocytes and neurons (66–69). Furthermore, the both models used in this study are derived from different species and, although they are developed from the myenteric plexus of the small intestine, the cell line was derived from the jejunum and the primary culture from the entire small intestine, which may result in different glial phenotypes, given the enormous region-specific plasticity of the EGC (70).

Regarding to the contribution of BAs in the molecular changes in primary EGC cell cultures both, primary and secondary BAs, induced a downregulation of the different EGC-specific markers, except for the primary bile BA CA and the secondary BA DCA, the former did not significantly alter any of them and the latter induced an upregulation of *Gfap* expression. It has been documented that two of the most potent and abundant unconjugated BAs in the faeces in healthy individuals are DCA and CDCA (71). Consistent with this, we observed that treatment with the primary BA CDCA induced more profound molecular changes than the other BAs, and mainly in mouse primary cells, altering the expression of the four EGC markers evaluated. Therefore, our data show that BAs induce changes in the molecular biology of EGCs, a novel finding as no previous study has shown an effect of BAs on the expression of myenteric glial cell markers.

Studies focusing on gliosis mainly rely on the assessment of changes in morphology and expression of EGC markers, in particular the stimulation of GFAP (72) and, more recently, nestin (73,74) which are considered relevant, whereas changes in S100β expression are more variable. The fact that CDCA is able to reduce *Gfap*, *Nes* and *S100β* gene expression levels suggests that this primary BA may have an effect on EGC gliosis.

Accumulating evidence points to GFAP and S100β as inflammatory reactive glial markers, which explains why most of the data generated to date have focused on their transcriptional changes within the intestinal mucosa in response to the inflammatory environment that alters the intestinal epithelial barrier, and have shown an increased expression of GFAP and S100β, as we have observed for DCA and *Gfap* expression (64,75–82). Despite all this evidence, there are also several preclinical and clinical studies that have shown a down-regulation of GFAP and S100β under different conditions, which is more consistent with the general downregulation of the different EGC-specific markers observed in this study after BA exposure. Disruption of the EGC network in animal models has been associated with inflammation, necrotizing enteritis, coding of enteric neurons and intestinal permeability (83–85). A reduction in the number of GFAP-positive and S100β-positive cells has been described in the mucosa of patients with Crohn's disease and IBS, respectively (83,86). Furthermore, downregulation of these EGC markers at the transcriptional and protein levels has been reported in animal models of inflammation and chronic psychosocial stress (70,87). Interestingly, S100β appears to play an important role in modulating inflammatory response of astrocytes, and its downregulation results in neuroprotection (88–90). However, whether the differential expression of these markers has any functional relevance or simply reflects EGC sensitivity remains unknown.

On the other hand, we found that both secondary BAs, together with primary CDCA, were able to reduce *Sox10* gene expression levels, and CDCA significantly downregulated *Nes*. Recent evidence points to EGCs as neuronal progenitors following pathological stimuli, highlighting the neurogenic potential of the expression of the transcription factor Sox10 (14,22,91) and nestin by EGCs (23,92,93). It is not clear whether these are differentiated EGCs with specific functions or premature EGCs. Our results suggest that BAs may reduce the neurogenic potential of EGCs, and that CDCA would have a greater effect on the neurogenic capacity of EGCs. However, it has been shown that nestin-positive progenitors do not express Sox10 (23), indicating that there are multiple neuronal progenitor populations with different phenotypes. Therefore, we might expect the effect of CDCA to be identified in different cell populations of neural origin.

Effect of BAs on BARs in primary EGCs

We then focused on the molecular changes in gene expression of several BARs in primary mouse glial cells. Among the BARs, only CHRM3 have been previously shown to be expressed in EGCs (48), contributing to cholinergic pathways in the ENS, and GPBAR1 and CHRM3

expression in myenteric neurons (46,47). However, the expression of other BARs in the ENS and the type of cellular signalling that is triggered remains to be investigated. Our data show, for the first time, that *Nr1h4* and *Vdr* are expressed in myenteric glial cells from two rodent species and confirm that *Gpbar1* and *Chrm3* are also present.

Thus, after treatment with different BAs at 100 µM for 24 h, our *in vitro* experiments suggest that *Nr1h4* and *Vdr* are mainly involved in BA-induced EGC reactivity. We have shown a downregulation of both BARs after exposure to CDCA, LCA and DCA in EGCs, similar to a previous study on a human cholangiocarcinoma cell line which described a decreased expression of *Nr1h4* after stimulation with glycine conjugates of CA, DCA and CDCA at 200 µM for 48 h, whereas free BAs had the opposite effect in the same study (94). The lack of significant changes induced by CA could be explained by the fact that this BA has a low affinity for Nr1h4 and Vdr (34). Interestingly, previous studies have shown that *Vdr* is only activated by any other BA that LCA in human hepatocytes (95,96), as well as the lack of changes in *Vdr* mRNA expression in the intestine of mice given oral LCA (97). In contrast, our data show that CDCA and DCA induce a similar and significant regulation of *Vdr* expression as LCA in myenteric glial cells. In addition, we also observed that treatment with both secondary BAs and the primary BA CDCA induced a similar but non-significant reduction in *Chrm3* gene expression. To date, Chrm3 has previously been reported to interact with the secondary BAs DCA and LCA or their conjugates (42), although in the present study our results also suggest a potential interaction with CDCA.

Both Nr1h4 and Vdr are nuclear receptors, and therefore also transcription factors capable of mediating the response to BAs by regulating the expression of a wide range of genes. Consistent with this, we observed a positive correlation between the expression levels of *Nr1h4* and *Vdr* and those of the main glial markers in response to the treatment with the different BAs. These results suggest a potential role for these receptors in BA-induced changes in EGC phenotype, although the pathways by which they are able to modify glial plasticity remain to be elucidated. Even though the secondary acids LCA and DCA are strong activators of Gpbar1, and here these BAs elicited significant changes in *Gfap*, *S100β* and *Sox10* gene expression suggesting a role for this G protein-coupled receptor, our data showed neither a change in *Gpbar1* gene expression levels nor an association with any of the mRNA transcripts of the major glial markers. In contrast to existence of evidence supporting its role in the regulation of various physiological functions in the gut (30), our findings seem to be apparently discordant.

Not only have we described for the first time that EGCs express multiple BARs, but we have also shown that they mediate BA-induced changes in the expression levels of EGC-specific markers. It is, therefore, important to be cautious when comparing our data with existing knowledge, as most *in vitro* studies investigating the expression of these receptors after BA stimulation have focused on other intestinal resident cell types. Our data open up a new and promising area of research where further functional studies are needed to elucidate the underlying mechanisms of changes in BARs expression and their physiological relevance in the ENS.

Effect of BAs on the inflammatory response in primary EGCs

Following our findings on the expression of BARs by EGCs, we decided to investigate whether enteric glia respond to BAs by modulating the expression of various pro-inflammatory mediators, given that EGCs are capable of exerting both pro- and anti-inflammatory effects as they express toll-like receptors and interact with intestinal resident immune cells by expressing and releasing signalling molecules such as cytokines (11). In addition, accumulating evidence suggests that innate and adaptive immune cells express BARs and that alterations in the BA pool contribute to chronic inflammatory diseases in the gut by interacting and modulating the responses of resident immune cells (31,32,98).

Notably, only CDCA and DCA increased *Il-1β* and *Il-6* gene expression by primary glia cells, respectively, thereby promoting an inflammatory environment, whereas the other BAs had no effect on the expression of derived inflammatory cytokines. Consistent with our results, CDCA induced the release of inflammatory mediators (Il-8, Il-6 and Tnf-α) by Caco-2 cells (99) and findings in mice associate DCA with exacerbation of inflammation (100–102). In this study, LCA had no effect on any of the glia-derived inflammatory cytokines, which is consistent with its novel anti-inflammatory effect and protective role in the gut (103). Notably, in the present study, only changes in key glial markers were associated with DCA-induced changes in cytokine expression, without changes in BARs expression. These results suggest that the BARs are not involved in the DCA-induced proinflammatory response in EGCs. Taken together, it is clear that the effects of BAs on gut inflammation and their mechanisms are not yet fully understood. Their effects on EGCs require further research, always bearing in mind that the effects of BAs on the inflammatory response are receptor specific and vary depending on the cell type.

BA-mediated signalling through the enterocyte in CRL-2690 cells

We also investigated the effects of epithelial-mediated exposure to the secondary BA, DCA, on EGCs. For this purpose, CRL-2690 cells were treated with Caco-2 CM to simulate the effect of epithelial downstream signalling after interaction with BAs on EGCs. In contrast to direct stimulation of CRL-2690 cells with DCA, which did not alter *Sox10* and *S100β* expression levels, only the media from DCA-treated Caco-2 cells for 24 h induced a decrease in *Sox10* and *S100β* mRNA levels as well as an increased in the expression levels of BARs. Therefore, these results suggest that the response of EGCs induced by unconjugated BAs is modulated by the epithelium. Given this huge difference, and after comparing the gene expression analysis of glial markers between CRL-2690 and primary EGCs, where primary cultures showed less variation and a greater overall trend, it would be better to perform further experiments on primary glial cultures with CM from epithelium stimulated with BAs, reproducing a more physiological model of EGC response to luminal BAs.

On the other hand, our study has several limitations: first, we cannot exclude the influence of the physiological differences in the intestinal profile of BAs, and the physicochemical properties between species (rodents and humans) (104) on the responsiveness of the two EGC cultures regarding the molecular changes induced by BAs; another limitation is the lack of experiments in our model involving the exposure of Caco-2 CM to primary glial cells. Further experiments would be necessary to confirm all the changes observed at the protein level, since our results are referred to gene expression. Furthermore, the use of agonists and antagonists of BARs would be necessary to confirm the importance of their activation in the BA-induced molecular changes in EGCs.

We must be very cautious when comparing our findings with existing data in other cell types, as it is important to remember that BAs induce different responses that are cell type specific. Nevertheless, our results are promising and open up a new area of research focusing on the role of BAs on the ENS and, in particular, on the EGC. The interaction between BAs and their receptors on the EGC and its impact on EGC plasticity needs to be elucidated not only under physiological conditions, but also under pathological stimuli, with particular attention to those conditions where there is BAs overload and an altered gut barrier is present.

In conclusion, this study demonstrates that BAs modulate the phenotype of EGC and highlights the need to develop additional studies to better describe this relationship. A deeper analysis of this cell type and its specific function after interaction with BAs will help to better define its role in GI diseases.

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GENERAL DISCUSSION

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IBS represents a common and chronic GI disorder clinically characterised by recurrent abdominal pain and abnormal bowel habits without an underlying organic origin. The current treatment is targeted to relieve the predominant symptoms, which often leads to ineffective management. As a result, IBS patients often report a significant reduction in quality of life and make use of diverse medical resources, with a progressive increase in direct and indirect healthcare and social costs (230,235,236). In spite of intensive research, the aetiology and the underlying pathogenesis of IBS are not fully understood, which explains why there are still no specific biomarkers for diagnosis, currently based on clinical criteria. For example, different patients may have the same symptoms, which can be attributed to and explained by different pathophysiology. To date, our understanding of IBS pathophysiology has increased rapidly in recent years, with several factors identified to contribute to symptoms generation, including an increased mucosal immune response and intestinal epithelial permeability, altered gut motility, disturbed gut-brain bidirectional communication, gut microbiota imbalance, and significant psychosocial stress (244). Further understanding on how these factors interact and to what extent they are involved in the clinical outcome is essential for the discovery of biomarkers and the development of novel therapies. In the context of gut dysfunction, BAs have emerged as important elements to consider in the pathophysiology of several GI conditions, including IBS (306). Indeed, it has recently been shown that patients with IBS have an altered and increased faecal BAs profile along with a distinct microbiome (282–286). In addition, up to a quarter of IBS-D patients have coexisting idiopathic BAD (301) in association with increased fluid secretion and intestinal permeability, altered colonic transit and more severe symptoms (292,296,297).

This thesis is designed to unveil the underlying molecular mechanisms associated with gut dysfunction within the jejunal mucosa of IBS-D patients, unlike the majority of the studies, which are focused on the large intestine. Within the mucosal barrier of the small intestine, a fundamental defensive activity takes place in order to avoid passage of luminal antigens while maintaining proper absorption of nutrients and tolerance to food antigens (5). Indeed, to maintain the intestinal homoeostasis, the ENS is a relevant element located along the GI tract, where the EGCs interact with the immune and the epithelial cells, giving them the ability to regulate their activity and thus their function. For this reason, there is a widespread interest in the role of the EGCs in the development of IBS pathophysiology, although to date, the limited evidence focusing on the colonic mucosa shows conflicting results (257–259).

Interestingly, our group has shown small intestinal structural and molecular alterations of the epithelial barrier and mucosal immune activation in the jejunum, suggesting the involvement of the whole intestine in the pathogenesis of IBS-D (246,250,256,307–310).

Given that a proportion of IBS-D patients have coexisting BAD, we wanted to find out which of these changes observed in the jejunal epithelial barrier were or were not due to excessive exposure to BAs and whether there was a distinct molecular signature between these two subgroups of patients. On the other hand, the study of the content of the BA in the jejunal lumen is also novel, as most research on BAs is focused on the ileum or colon. While BAs are secreted from the liver into the duodenum and pass through the jejunum, the potential deleterious effects of BAs in this segment and whether they may play a role in the pathophysiology of symptoms have not been investigated. Hence, we hypothesised that BAs in the jejunal lumen determine to different molecular regulation of epithelial barrier function and promote EGC plasticity which are actively involved in the physiopathology of IBS-D.

The protein expression analysis showed molecular changes in the phenotype of EGCs and TJPs in the jejunum of IBS-D patients, as well as a different signature depending on the BA pool composition. We present the first characterisation of the EGC molecular phenotype in jejunal mucosal tissue from IBS-D patients and our results suggest the putative role of this cell type in IBS-D, as we have described differential profile in the protein expression of the three EGC markers: an increase in GFAP and S100□ and a decrease in SOX10. In addition, we also show a differential phenotype based on the SeHCAT subset, suggesting the functional ability of this cell type to adapt to changes in the surrounding microenvironment (65,69). IBS-D SeHCAT^{+} patients showed upregulation of GFAP and CLDN2 protein expression, whereas SeHCAT^{-} patients showed downregulation of SOX10, OCLN and VILL protein expression compared to HVs. To further characterise the luminal content of BAs in the jejunum, we evaluated the concentration and composition of BAs in the jejunal fluid. Interestingly, the concentration of BAs was higher in IBS-D compared to HV, as well as between the two SeHCAT subgroups, suggesting that, in both groups, there is an alteration in BAs homeostasis. Of note, a distinct profile between the two SeHCAT subgroups and HVs was found: SeHCAT^{+} patients had a higher concentration of secondary BAs conjugated to glycine and SeHCAT^{-} patients had a greater amount of primary BAs conjugated to taurine, suggesting that, in both subgroups, there are factors that interfere with the regulation of the BA homeostasis, such as diet and changes in the intestinal microbiota (311). Our results indicate that this alteration in the BA pool profile may have an impact on the epithelial barrier at the molecular level, together with

changes in EGC biology in the jejunum. These findings deserve special attention because we found a correlation with the clinical features of the patients, also between the different SeHCAT subsets, suggesting their role in the pathophysiology of IBS-D. Therefore, in SeHCAT^{+} patients, we observed a correlation between a "reactive" phenotype of EGCs together with an upregulation of CLDN2 and loose stools and higher number of bowel movements, reflecting their potential role in altered secretion and motility. These findings are consistent with those previously reported in patients with BAD, who have a more diarrhoeal phenotype (292,296,297). Whereas the molecular dysregulation related to the epithelial barrier and EGCs may be more related to visceral hypersensitivity and alterations in the brain-gut axis in the SeHCAT^{-} group. We suggest that the effect of BAs, as a luminal-derived mechanism involved in the barrier dysfunction is not only limited to the colon. Notably, previous BAs studies in IBS-D have mostly used faecal BAs or serum 7aC4 levels to screen for BAD. Therefore, the use of the SeHCAT test to stratify our study groups gives this work an exceptional value and a completely new point of view in the study of IBS-D. However, it is important to be cautious when interpreting these molecular changes, as correlation does not imply causation and further research is required to define their functionality and the interaction between the EGC plasticity and TJPs dysregulation in relation to BAD, as well as the mechanisms by which BAs could modulate. We would also consider necessary to perform further analysis focused on other biotransfomed forms of BAs.

Different luminal BAs are capable of disrupting epithelial barrier function but the pathways and mechanisms contributing to those remain to be elucidated (312,313). Considering the differential TJPs expression under the presence or absence of BAD in the jejunal mucosa of IBS-D patients, we decided to investigate the effects of short and long-term exposure to BAs on the epithelial barrier function using the Caco-2 cell line. High concentrations of CDCA and DCA disrupted epithelial barrier integrity and increased epithelial permeability by altering the expression of TJPs and BARs. This calls for further analysis to elucidate which pathways and mechanisms are involved in BAs-mediated barrier dysfunction not only through BARs signalling. Thus, according to the different proportion of conjugation type of BAs observed in the jejunal fluid of IBS-D patients, it would be interesting to repeat this experimental design and test glycine- and taurine-conjugated BAs, since their solubility and toxicity differs (314), as well as to test the sulphated forms of BAs (145).

In order to gain insight into the contribution of EGCs to the pathophysiology of IBS-D associated with BAD, we next explored the interaction between EGCs and BAs. Our novel

findings revealed that EGCs express the BARs GPBAR1, NR1H4 and VDR and confirm the expression of CHRM3 (218). In addition, we show that EGCs respond to luminal unconjugated BAs, showing a BA-induced EGC reactivity by changes in the gene expression of myenteric EGC markers where the nuclear BARs NR1H4 and VDR are mainly involved. In addition, we have shown that EGCs also respond to the epithelial downstream signalling although with a different pattern of plasticity. We have reproduced completely different intestinal scenarios: BAs crossing the intestinal barrier facilitated by an altered epithelium and interacting directly with the EGC in the lamina *propria*, and epithelial signalling responding to BAs stimulating the EGC in the lamina *propria*. Our results open up a new and promising area of research in enteric neuroscience where the underlying mechanisms of the interaction between BAs and EGCs and its functional consequences to GI homeostasis and disease deserve to be evaluated.

We propose both, the BAs and the EGC, as important elements to be considered in the pathophysiology of IBS-D. Barrier dysregulation with increased permeability, which can be induced by BAs, allows the translocation of dietary components, microbial-derived antigens and metabolites, such as BAs, from the intestinal lumen to the lamina *propria* (29,35,245). Consequently, BAs can act directly to activate cell receptors and signalling pathways within those cells located in the lamina *propria*, including EGCs. EGCs respond to BAs via BARs by altering their plasticity and, although their functional relevance is unknown, they may signal and respond to immune cells and enteric neurons located in the lamina *propria* as well as to the epithelium and the CNS. At the same time, EGCs interact with the microbiota (87) and their derived metabolites as BAs, whose different profile may determine EGC plastic changes.

In patients with BAD, the secondary BAs conjugated to glycine have a greater impact on barrier function and EGC plasticity, resulting in a more severe diarrhoeal phenotype. From the results of this thesis, we suggest that the secondary BA DCA is an important factor to consider in the pathophysiology of IBS-D with BAD, which has been found as one of the most abundant BAs in the stools of IBS-D patients (293). Treatment with DCA induced the most significant alteration in the integrity of the epithelial barrier in Caco-2 cells, triggering an increase in the TJP CLDN2, which was also observed at the protein level in the jejunal mucosa of the IBS-D SeHCAT^{+} patients. In addition, DCA also induced an increase in the EGC marker GFAP in both *in vitro* EGC models, which was also observed at the protein level in the jejunal mucosa of IBS-D SeHCAT^{+} patients. Furthermore, its glycine-conjugated form was only found in higher concentrations in the jejunal fluid from IBS-D SeHCAT^{+} patients in contrast to

healthy controls. On the other hand, DCA-mediated epithelial signalling also had an impact on the EGC phenotype, although it did not modulate GFAP expression, it did reduce the expression of another EGC marker, SOX10.

We are aware of the limited small sample size of this study, mainly, the limited number of participants in the analysis of the jejunal mucosa as well as the small number of *in vitro* experiments. Nevertheless, our results are promising and support further research focusing on the role of BAs on ENS plasticity and, in particular, on the EGC. The interaction between BAs and their receptors on the EGC and epithelium and its impact on EGC plasticity needs to be elucidated not only under physiological conditions, but also under pathological stimuli, with special attention to those conditions where there is BAs overload and "leaky" gut is present, as described in IBS-D. These results also highlight the need to test for BAD in IBS-D for better characterisation and a more appropriate treatment, as IBS-D patients with BAD suffer from more severe symptoms and the two entities differ in terms of therapeutic management, as BAD symptoms can be relieved (133,302,305).

In conclusion, a distinct jejunal EGC phenotype has been described in IBS-D with or without BAD. Our findings contribute to the understanding of the role of EGCs and BAs in the complex IBS pathophysiology. However, to gain further insight into of the effect of BAs on intestinal epithelial barrier and glial cell physiology, additional studies would be required to provide a comprehensive understanding of this disorder, which is essential for the identification of potential biomarkers and therapeutic targets to improve the diagnosis and individualised management of IBS-D.

CONCLUSIONS

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The results of this thesis have generated the following conclusions:

1. IBS-D is characterised by a distinct molecular phenotype of enteric glial cell and expression of tight junction proteins in the jejunal mucosa associated with bile acid diarrhoea.

2. IBS-D is characterised by differential bile acid pool profile in the jejunal fluid as compared to healthy controls.

3. The identified differential molecular phenotypes of jejunal neuroplasticity and barrier integrity in the jejunal mucosa, as well as in the bile acid profile, are associated with gastrointestinal and psychological symptoms in IBS-D, suggesting their involvement in its pathophysiology.

4. Bile acids modulate epithelial barrier integrity and paracellular permeability in association with changes in tight junction proteins and bile acid receptors' expression.

5. Enteric glial cells do express bile acid receptors.

6. Enteric glial cells respond to bile acids by modifying the expression of enteric glial cell-specific markers via bile acid receptors.

7. Deoxycholic acid promotes differential regulation of enteric glial cell-specific marker expression through intestinal epithelial cells signalling.

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ANNEX 1

SUPPLEMENTARY DATA CHAPTER 1

Table S 1 | Inclusion and exclusion criteria for the study participants.

	HV	IBS-D
Inclusion criteria		
Age: 18-60 years	Yes	Yes
Rome III criteria for IBS-D	No	Yes
Naïve (newly-diagnosed)	No	Yes
Acceptance of the study protocol	Yes	Yes
Exclusion criteria		
Clinical history of food allergy	Yes	Yes
Positivity to SPT to foodstuff	Yes	Yes
Infectious gastroenteritis	Yes	Yes
Gastrointestinal comorbidities	Yes	Yes
Pregnancy	Yes	Yes
Major psychiatric disorders	Yes	Yes
Use of medication (steroids, immunosuppressive drugs, anti-histaminic and mast cell stabilizers)	Yes	Yes

HV: Healthy Volunteer; IBS-D: Diarrhoea-predominant Irritable Bowel Syndrome; SPT: Skin Prick Yest.

Table S 2 | Clinical and demographical characteristics of participants. F: Female; HV: Healthy Volunteer; IBS: Irritable Bowel Syndrome; IBSS; IBS-D: Diarrhoea-predominant Irritable Bowel Syndrome; IBS symptom severity score; M: Male; SeHCAT: 75-Selenium Homocholic Acid Taurine.

	Sex	**Age**, years	**SeHCAT**, 7-day % retention	**IBS-SSS**	**Severity of the abdominal pain**, score	**Frequency of the abdominal pain**, n° of days	**Severity of abdominal bloating**, score	**Bowel habit dissatisfaction**	**IBS interference with life**	**Bowel movements**, n° /day	**Stool form,** Bristol score	**Functional dyspepsia**, yes / no	**Becks Depression Inventory**, score	**Cohen Scale**, score	**Holmes-Rahe Scale**, score
HVs															
HV1	F	20	-	30	0	0	0	30	0	1	2.5	no	0	11	25
HV2	F	19	-	8	0	0	0	8	0	1.5	3.5	no	0	21	64
HV3	M	44	-	0	0	0	0	0	0	1.5	4	no	1	7	25
HV4	M	35	-	15	0	0	0	14	1	1	4	no	0	17	97
HV5	F	2	-	2.5	0	0	2.5	0	0	2	4	no	0	9	25
HV6	M	43	-	13	0	0	0	8	5	2	3.5	no	0	12	93
HV7	M	19	-	3	0	0	0	3	0	2	4	no	0	9	61
HV8	M	27	-	18	0	0	0	18	0	2	3	no	0	14	50
HV9	M	25	-	4	0	0	2	0	2	0.5	3.5	no	1	15	136
HV10	F	28	-	2	0	0	0	2	0	2	3	no	0	19	33
HV11	F	24	-	12	0	0	0	12	0	1	3	no	5	16	261
HV12	M	25	-	8	0	0	1	5	2	1.5	3.5	no	0	22	58
HV13	F	24	-	30	0	0	0	30	0	2	4.5	no	0	16	114
HV14	F	19	-	15	0	0	0	15	0	1	3,5	no	0	16	85
HV15	F	54	-	0	0	0	0	0	0	2	4	no	0	4	88
HV16	M	31	-	10	0	0	0	10	0	1	5	no	0	19	109
HV17	M	29	-	0	0	0	0	0	0	1	3	no	0	15	151
HV18	M	47	-	0	0	0	0	0	0	1	4	no	4	20	85
HV19	M	64	-	0	0	0	0	0	0	1	4	no	9	26	230
HV20	M	22	-	-	0	0	-	-	-	-	-	no	0	24	154
HV21	M	41	-	0	0	0	0	0	0	1	5	no	6	24	267

	Sex	Age, years	SeHCAT, 7-day % retention	IBS-SSS	Severity of the abdominal pain, score	Frequency of the abdominal pain, n° of days	Severity of abdominal bloating, score	Bowel habit dissatisfaction	IBS interference with life	Bowel movements, n° /day	Stool form, Bristol score	Functional dyspepsia, yes / no	Becks Depression Inventory, score	Cohen Scale, score	Holmes-Rahe Scale, score
HV22	M	36	-	0	0	0	0	0	0	1	4	no	1	13	64
HV23	F	29	-	0	0	0	0	0	0	-	-	no	4	25	95
HV24	M	29	-	0	0	0	0	0	0	1	4	no	-	-	142
IBS-D SeHCAT^{+}															
IBS-D1	F	38	4.98	430	73	10	70	100	87	7.5	6.5	yes	14	15	103
IBS-D2	F	24	9.70	325	54	5	52	93	76	3.5	6.5	no	15	27	179
IBS-D3	F	37	5.38	271	40	5	32	75	74	4.5	7	no	4	20	78
IBS-D4	M	29	10.01	236	75	6	4	48	49	3	6.5	no	11	22	770
IBS-D5	M	31	11.00	374	58	10	82	72	62	3.5	5.5	yes	2	10	138
IBS-D6	M	45	2.85	131	2	1	2	55	62	3.5	7	no	3	1	15
IBS-D7	M	23	6.80	128	17	1	0	54	47	1	3	no	4	13	129
IBS-D8	M	41	9.80	211	37	4	0	84	50	2	6	yes	4	19	109
IBS-D9	M	33	10.46	159	27	2	33	35	44	2.5	5.5	no	2	28	160
IBS-D10	M	55	3.28	225	28	3	28	63	76	4	5.5	no	17	10	25
IBS-D11	F	47	6.12	281	30	9	23	68	70	2	6	no	14	41	420
IBS-D SeHCAT^{-}															
IBS-D12	M	37	33.00	355	80	10	35	50	90	3.5	4.5	no	10	18	58
IBS-D13	F	52	29.17	316	13	3	100	100	73	2	6	yes	9	13	25
IBS-D14	F	30	20.09	325	65	8	76	21	83	1	4.5	yes	8	25	60
IBS-D15	M	42	30.05	264	55	8	49	9	71	4	3	no	14	20	86
IBS-D16	F	39	34.28	272	68	8	34	32	58	2	4	no	8	24	194
IBS-D17	F	21	33.83	130	15	3	36	18	31	1	4.5	yes	1	11	25

	Sex	Age, years	SeHCAT, 7-day % retention	IBS-SSS	Severity of the abdominal pain, score	Frequency of the abdominal pain, n° of days	Severity of abdominal bloating, score	Bowel habit dissatisfaction	IBS interference with life	Bowel movements, n° /day	Stool form, Bristol score	Functional dyspepsia, yes / no	Becks Depression Inventory, score	Cohen Scale, score	Holmes-Rahe Scale, score
IBS-D18	F	39	54.29	176.5	11	2	13,5	73	59	1	4.5	no	13	34	123
IBS-D19	M	37	30.43	110	17	4	17	11	25	2	5.5	yes	17	21	318
IBS-D20	F	34	33.62	140	10	2	32	55	23	2	6.5	no	4	17	172
IBS-D21	F	21	57.00	230	60	2	20	80	50	3.5	5.5	no	5	16	20
IBS-D22	F	21	68.91	372	61	6	81	82	88	0.5	3	yes	24	35	41
IBS-D23	M	31	35.40	221	23	3	18	77	73	3	4	no	7	17	159
IBS-D24	M	41	20.00	62	0	0	27	15	10	1	6	no	10	16	310
IBS-D25	F	33	30.00	294	6	10	6	96	86	6	5	no	1	24	126

Table S 3 | Experimental procedures performed with participant biological samples.
1: Mucosal immune populations cell counts in the jejunum by H&E and IHQ; 2: Gene expression analysis in the jejunal mucosa by qPCR; 3: Protein expression analysis in the jejunal mucosa by WB. 4: S100β and SOX10 stainings in the jejunal mucosa by IHQ; 5: BAs levels in the intestinal fluid by LC-MS/MS. HV: Healthy Volunteer; IBS-D: Diarrhoea-predominant Irritable Bowel Syndrome.

Participant	Experimental procedure
HV1	2, 3, 5
HV2	1, 2, 3, 4
HV3	1, 2, 3, 4, 5
HV4	1, 2, 4
HV5	1, 2, 3, 4
HV6	1, 2, 4
HV7	1, 2, 3, 4
HV8	1, 2, 4
HV9	1, 2, 3, 4
HV10	1, 2, 3, 4
HV11	1, 2, 3, 4
HV12	1, 2, 3, 4, 5
HV13	1, 2, 4
HV14	1, 5
HV15	1, 5
HV16	5
HV17	1, 5
HV18	1, 5
HV19	1, 5
HV20	1, 5
HV21	1, 5
HV22	1, 5
HV23	1, 5
HV24	1, 5

Participant	Experimental procedure
IBS-D1	1, 2, 4, 5
IBS-D2	1, 2, 3, 4
IBS-D3	1, 2, 3, 4
IBS-D4	1, 2, 3, 4, 5
IBS-D5	1, 2, 3, 4, 5
IBS-D6	1, 2, 3, 4, 5
IBS-D7	1, 2, 4
IBS-D8	1, 2, 3, 4, 5
IBS-D9	1, 2, 4
IBS-D10	1, 4, 5
IBS-D11	1, 4, 5
IBS-D12	1, 2, 3, 4, 5
IBS-D13	1, 2, 3, 4, 5
IBS-D14	1, 2, 4
IBS-D15	1, 2, 4, 5
IBS-D16	1, 2, 4
IBS-D17	1, 2
IBS-D18	1, 2, 3, 4
IBS-D19	1, 2, 4, 5
IBS-D20	1, 2, 3, 5
IBS-D21	1, 2, 3, 4
IBS-D22	1, 2, 3, 4
IBS-D23	1, 2, 4
IBS-D24	1, 5
IBS-D25	1, 5

Table S 4 | RNA Integrity Number (RIN) of samples included in the gene expression analysis to evaluate the RNA quality.

Participant	RIN
HV1	8.3
HV2	5.5
HV3	5.2
HV4	7.0
HV5	8.1
HV6	8.4
HV7	6.8
HV8	8.6
HV9	7.8
HV10	7.7
HV11	5.3
HV12	7.1
HV13	8.0

Participant	RIN
IBS-D1	7.2
IBS-D2	5.0
IBS-D3	6.6
IBS-D4	8.7
IBS-D5	7.6
IBS-D6	5.7
IBS-D7	5.3
IBS-D8	7.1
IBS-D9	7.9
IBS-D12	7.2
IBS-D13	8.1
IBS-D14	7.0
IBS-D15	8.3
IBS-D16	7.1
IBS-D17	8.2
IBS-D18	6.8
IBS-D19	7.2
IBS-D20	7.6
IBS-D21	6.2
IBS-D22	7.2
IBS-D23	5.2

Table S 5 | Gene expression assay probes used for qPCR analysis.

Gene symbol	Gene name	TaqMan assay
Enteric Nervous System		
Enteric Glial Cell		
SOX10	SRY-box 10	Hs00366918_m1
S100β	S100 Calcium Binding Protein β	Hs00902901_m1
GFAP	Glial Fibrillary Acidic protein	Hs00909233_m1
Neuron		
UCHL1	Ubiquitin C-terminal Hydrolase L1	Hs00188233_m1
Neurotrophic Factors		
GDNF	Glial Derived Neurotrophic Factor	Hs00181185_m1
BDNF	Brain Derived Neurotrophic Factor	Hs02718934_s1
Signalling pathway		
P2RX7	Purinergic Receptor P2X 7	Hs00175721_m1
GJA1	Gap Junction Protein Alpha 1	Hs00748445_s1
NGF	Nerve Growth Factor	Hs00171458_m1
NTF3	Neurotrophin 3	Hs00267375_s1
NTRK1	Neurotrophic Receptor Tyrosine Kinase 1	Hs01021011_m1
Intestinal Barrier Function		
Tight junctions		
OCLN	Occludin	Hs00170162_m1
TJP1	Tight Junction Protein 1	Hs00268480_m1
CLDN2	Claudin 2	Hs00252666_s1
Bile Acids Signalling Pathway		
NR1H4	Nuclear Receptor Subfamily 1 Group H Member 4	Hs01026590_m1
GPBAR1	G Protein-Coupled Bile Acid Receptor 1	Hs01937849_s1
VDR	Vitamin D (1,25- Dihydroxyvitamin D3) Receptor	Hs01045843_m1
FGF19	Fibroblast Growth Factor 19	Hs00192780_m1
Endogenous genes		
18S	Eukaryotic 18S rRNA	Hs99999901_s1
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	Hs02758991_g1

Table S 6 | Mucosal immune populations cell counts in the jejunum (HV/IBS-D). The number of mast cells and eosinophils is expressed as per high power field (400x) in the lamina *propria* and the number of intraepithelial T lymphocytes as per 100 epithelial cells. Data are expressed as median (interquartile range). Mann-Whitney U test was performed.

	HV	IBS-D	p value
Intraepithelial T lymphocytes (CD3 +)	20.00 (13.00-27.50)	16.00 (11.50-16.00)	0.103
Mast cells (CD117 +)	24.75 (18.05-29.83)	29.00 (17.80-38.75)	0.213
Eosinophils (eosin +)	2.00 (0.80-2.93)	1.50 (0.73-2.70)	0.410

HV: Healthy Volunteer; IBS-D: Diarrhoea-predominant Irritable Bowel Syndrome.

Table S 7 | Mucosal immune populations cell counts in the jejunum (HV/IBS-D SeHCAT$^+$/IBS-D SeHCAT$^-$). The number of mast cells and eosinophils is expressed as per high power field (400x) in the lamina *propria* and the number of intraepithelial T lymphocytes as per 100 epithelial cells. Data are expressed as median (interquartile range). Kruskal-Wallis and Dunn's multiple comparison tests were performed.

	HV	IBS-D-SeHCAT$^+$	IBS-D-SeHCAT$^+$ vs HV p value	IBS-D-SeHCAT$^-$	IBS-D-SeHCAT$^-$ vs HV p value	ANOVA p value
Intraepithelial T lymphocytes (CD3 +)	20.00 (13.00-27.50)	15.00 (11.00-19.00)	0.101	16.00 (13.75-21.00)	0.770	0.096
Mast cells (CD117+)	24.75 (18.05-29.83)	28.60 (11.50-39.00)	> 0.999	30.90 (22.98-35.95)	0.482	0.371
Eosinophils (eosin +)	2.00 (0.80-2.93)	1.30 (0.70-4.30)	> 0.999	1.50 (0.70-2.70)	> 0.999	0.703

HV: Healthy Volunteer; IBS-D: Diarrhoea-predominant Irritable Bowel Syndrome; SeHCAT: 75-Selenium Homocholic Acid Taurine.

SUPPLEMENTARY DATA CHAPTER 3

Table S 1 | Primers sequences used for qPCR analysis.

Gene symbol	Gene name	Rat primers (5'-3')		Mice primers (5'-3')	
		Forward	Reverse	Forward	Reverse
Enteric Glial Cell phenotype					
Gfap	Glial Fibrillary Acidic Protein	GGCGAAGAAAACCGCATCACC	TCCCGCATCTCCACCGTCTT	CAGCTTACGGCCAACAGTGC	AGGGTGGCTTCATCTGCCTC
S100β	S100 Calcium Binding Protein Beta	AGAGAGGACTCCGGCGGCAA	AGGGCAACCATGGCCTTCTCCA	AGAGGGTGACAAGCACAAGC	TTGTCCACCACTTCCTGCTC
Sox10	SRY-box 10	AGGACGGCGAGGCAGACGAT	CCGCTGAGCACCTGGCTGAC	CATGTCAGATGGGAACCCAGAG	CGGACTGCAGCTCTGTCTTTG
Nes	Nestin	TCTCGCTTGCAGACACCTG	AATGCAGCTTCAGCTTGGGG		
Bile Acids Receptors					
Chrm3	Cholinergic Receptor Muscarinic 3	GGACGGTCTGGGAGATTCGG	AGAGAGCCTTTGCGTTGTCT	TGCTGCGTTCTGACCAAGTG	GGACTGTGCACCCAGGAAGA
Gpbar1	G Protein-Coupled Bile Acid Receptor 1	GCAAGCCTCATCGTCATCGC	TGCCAACCCTGTGAGTAGCC	AGCCTCATCGTCATCGCCAA	CTCCATAGCCCAGGCAGCAT
Nr1h4	Nuclear Receptor Subfamily 1 Group H Member 4	ACCATTACAACGCGCTCACC	TTCCCATCTCTCTGCACTTCC	ACTACAACGCGCTCACCTGT	TTCCTTAGCCGGCACTCCTG
Vdr	Vitamin D Receptor	GCCATCTGCATTGTCTCCCC	TAGGCGGTCCTGAATGGCTT	CCACGGGCTTCCACTTCAAC	TCATGCCAATGTCCACGCAG
Neuron-Glia Signalling					
Gja1	Gap Junction Protein Alpha 1	TCATTGGGGGAAAGGCGTGA	CAGTCACCCATGTCTGGGCA		
P2rx4	Purinergic Receptor P2X 4	CCTTCCTGTTCGAGTACGACAC	TCCGTTTCCTGGTAGCCCTT		
P2rx7	Purinergic Receptor P2X 7	TCCCGCCGAAACAGAGTGAG	AGGGGCTCCTTCCGCTGATA		
Glial Inflammatory Derived Cytokines					
Il-1β	Interleukin 1 Beta			TGACCTGGGCTGTCCTGATG	TCCGACAGCACGAGGCTTTT
Il-6	Interleukin 6			TGTTCTCTGGGAAATCGTGGA	CTGCAAGTGCATCATCGTTGT
Tnf-α	Tumor Necrosis Factor-Alpha			GCCACCACGCTCTTCTGTCTA	ATGAGAGGGAGGCCATTTGGG
Endogenous genes					
Actb	Actin Beta	CTAAGGCCAACCGTGAAAAG	TACATGGCTGGGGTGTTGA	TCCTGCACCACCAACTGCTT	CACGCCACAGCTTTCCAGAG
Gapdh	Glyceraldehyde-3-Phosphate Dehydrogenase				

Table S 2 | Correlation analysis of EGC markers under exposure to different BAs. Analysis of the correlations between the gene expression of EGC markers after 100 μM BAs stimulations on primary EGCs.

		S100β			*Sox10*			*Nes*		
		rs	p	FDR	rs	p	FDR	rs	p	FDR
CDCA	*Gfap*	**0.82**	**0.002**	**0.011**	**0.66**	**0.024**	**0.029**	**0.82**	**0.002**	**0.004**
	S100β				**0.77**	**0.005**	**0.007**	**0.82**	**0.002**	**0.006**
	Sox10							*0.57*	*0.060*	*0.060*
CA	*Gfap*	**0.88**	**0.007**	**0.022**	**0.79**	**0.028**	**0.056**	**0.79**	**0.028**	**0.041**
	S100β				**0.74**	**0.046**	**0.055**	*0.67*	*0.08*	*0.083*
	Sox10							**0.95**	**0.001**	**0.007**
LCA	*Gfap*	**0.72**	**0.011**	**0.016**	**0.73**	**0.010**	**0.019**	**0.76**	**0.006**	**0.017**
	S100β				**0.67**	**0.020**	**0.024**	**0.78**	**0.004**	**0.026**
	Sox10							*0.51*	*0.094*	*0.094*
DCA	*Gfap*	**-0.80**	**0.003**	**0.015**	-0.52	*0.084*	0.168	0.38	0.218	0.327
	S100β				*0,65*	**0.026**	*0.077*	0.049	0.886	0.886
	Sox10							0.27	0.391	0.469

Comparisons were performed using the Spearman's rho correlation and the obtained p values were adjusted by the Benjamini and Hochberg method. Data are represented as: rs (spearman's rank correlation coefficient), p (p value) and FDR (false discovery rate). FDR of ≤0.05 was considered significant and it is shown in bold in the table.

Table S 3 | Correlation analysis of EGC markers and inflammatory cytokines. Analysis of the association between the gene expression of EGC markers and inflammatory-derived cytokines after 100 μM BAs stimulations on primary EGCs.

		Il1β			*Il6*			*Tnfα*		
		rs	p	FDR	rs	p	FDR	rs	p	FDR
CDCA	*Gfap*	**-0.83**	*0.015*	*0.065*	-0.43	0.218	0.305	-0.26	0.470	0.580
	S100β	-0.60	0.132	0.214	-0.66	0.044	0.131	-0.53	0.123	0.235
	Sox10	-0.60	0.132	0.232	-0.14	0.707	0.743	-0.24	0.513	0.599
	Nes	-0.64	0.096	0.224	-0.45	0.191	0.287	-0.35	0.330	0.434
CA	*Gfap*	0.029	1.000	1.00	**0.76**	*0.037*	*0.086*	**0.81**	*0.022*	*0.076*
	S100β	0.37	0.497	0.653	**0.81**	*0.022*	*0.091*	0.67	0.083	0.134
	Sox10	-0.2	0.171	0.789	0.55	0.714	0.256	**0.83**	*0.015*	*0.081*
	Nes	-0.26	0.658	0.768	0.55	0.171	0.239	**0.86**	*0.011*	*0.075*
LCA	*Gfap*	-0.07	0.865	0.908	0.00	1.000	1	-0.26	0.435	0.913
	S100β	-0.09	0.811	0.897	-0.28	0.379	0.884	-0.40	0.225	0.675
	Sox10	0.10	0.785	0.916	0.12	0.716	0.884	-0.31	0.356	0.934
	Nes	-0.22	0.537	0.939	-0.24	0.457	0.873	0.15	0.673	0.942
DCA	*Gfap*	0.45	0.230	0,345	**0.74**	*0.008*	*0.055*	0.60	0.073	0.257
	S100β	-0.63	0.076	0.110	**-0.90**	**<0.001**	**0.004**	-0.56	0.096	0.202
	Sox10	-0.63	0.076	0.196	-0.46	0.134	0.256	0.01	1.000	0.955
	Nes	-0.12	0.776	0.857	0,063	0.851	0.894	0.32	0.368	0.483

Comparisons were performed using the Spearman's rho correlation and the obtained p values were adjusted by the Benjamin and Hochberg method. Data are represented as: rs (spearman's rank correlation coefficient), p (p value) and FDR (false discovery rate). FDR of ≤0.05 was considered significant and it is shown in bold in the table.

ANNEX 2

ORIGINAL ARTICLES

Lysosomal lipid alterations caused by glucocerebrosidase deficiency promote lysosomal dysfunction, chaperone-mediated-autophagy deficiency, and alpha-synuclein pathology.

Navarro-Romero A, Fernandez-Gonzalez I, Riera J, Montpeyo M, **Albert-Bayo M**, Lopez-Royo T, Castillo-Sanchez P, Carnicer-Caceres C, Arranz-Amo JA, Castillo-Ribelles L, Pradas E, Casas J, Vila M, Martinez-Vicente M.

NPJ Parkinsons Dis. 2022 Oct 6;8(1):126. doi: 10.1038/s41531-022-00397-6.

Mucosal Plasma Cell Activation and Proximity to Nerve Fibres Are Associated with Glycocalyx Reduction in Diarrhoea-Predominant Irritable Bowel Syndrome: Jejunal Barrier Alterations Underlying Clinical Manifestations.

Pardo-Camacho C, Ganda Mall JP, Martínez C, Pigrau M, Expósito E, **Albert-Bayo M**, Melón-Ardanaz E, Nieto A, Rodiño-Janeiro B, Fortea M, Guagnozzi D, Rodriguez-Urrutia A, Torres I, Santos-Briones I, Azpiroz F, Lobo B, Alonso-Cotoner C, Santos J, González-Castro AM, Vicario M.

Cells. 2022 Jun 28;11(13):2046. doi: 10.3390/cells11132046.

REVIEWS

Eosinophils in the Gastrointestinal Tract: Key Contributors to Neuro-Immune Crosstalk and Potential Implications in Disorders of Brain-Gut Interaction.

Salvo-Romero E, Rodiño-Janeiro BK, **Albert-Bayo M**, Lobo B, Santos J, Farré R, Martinez C, Vicario M.

Cells. 2022 May 14;11(10):1644. doi: 10.3390/cells11101644.

Present and Future Therapeutic Approaches to Barrier Dysfunction.

Fortea M, **Albert-Bayo M (co-first autor)**, Abril-Gil M, Ganda Mall JP, Serra-Ruiz X, Henao-Paez A, Expósito E, González-Castro AM, Guagnozzi D, Lobo B, Alonso-Cotoner C, Santos J.

Front Nutr. 2021 Oct 28;8:718093. doi: 10.3389/fnut.2021.718093.

The Role of Purported Mucoprotectants in Dealing with Irritable Bowel Syndrome, Functional Diarrhea, and Other Chronic Diarrheal Disorders in Adults.

Alonso-Cotoner C, Abril-Gil M, **Albert-Bayo M**, Mall JG, Expósito E, González-Castro AM, Lobo B, Santos J.

Adv Ther. 2021 May;38(5):2054-2076. doi: 10.1007/s12325-021-01676-z. Epub 2021 Mar 18.

Intestinal Mucosal Mast Cells: Key Modulators of Barrier Function and Homeostasis.

Albert-Bayo M, Paracuellos I, González-Castro AM, Rodríguez-Urrutia A, Rodríguez-Lagunas MJ, Alonso-Cotoner C, Santos J, Vicario M.

Cells. 2019 Feb 8;8(2):135. doi: 10.3390/cells8020135.

CONGRESS PRESENTATIONS

Fenotipo diferencial de la célula entérica glial en el yeyuno del Síndrome del Intestino Irritable y su asociación con el fenotipo clínico y la severidad de los síntomas.

Albert-Bayo M, González-Castro AM, Expósito E, Alonso-Cotoner C, Pigrau M, Nieto A, Rodríguez N, Guagnozzi D, Serra X, Henao A, Abril M, Ganda-Mall JP, Aldea C, Azpiroz F, Santos J, Vicario M, Lobo B.

XXXI Congrés de la Societat Catalana de Digestologia 2022. June 2022. Girona. Societat Catalana de Digestologia. Poster presentation.

Fenotipo diferencial de la célula entérica glial en el yeyuno del Síndrome del Intestino Irritable y su asociación con el fenotipo clínico y la severidad de los síntomas.

Albert-Bayo M, González-Castro AM, Expósito E, Alonso-Cotoner C, Pigrau M, Nieto A, Rodríguez N, Guagnozzi D, Serra X, Henao A, Abril M, Ganda-Mall JP, Aldea C, Azpiroz F, Santos J, Vicario M, Lobo B.

XII Jornada científica del VHIR 2021. December 2021. Barcelona. Vall d'Hebron Institut de Recerca. Poster presentation.

Fenotipo diferencial de la célula entérica glial en el yeyuno del Síndrome del Intestino Irritable y su asociación con el fenotipo clínico y la severidad de los síntomas.

Albert-Bayo M, González-Castro AM, Expósito E, Alonso-Cotoner C, Pigrau M, Nieto A, Rodríguez N, Guagnozzi D, Serra X, Henao A, Abril M, Ganda-Mall JP, Aldea C, Azpiroz F, Santos J, Vicario M, Lobo B.

XV Jornadas Científicas del CIBER Enfermedades Hepáticas y Digestivas 2021. Noviembre 2021. Barcelona. CIBER Enfermedades Hepáticas y Digestivas. Poster presentation.

Differential enteric glial cell phenotype in the jejunum of diarrhoea-prone Irritable Bowel Syndrome and its association with clinical phenotype and symptom severity.

Albert-Bayo M, González-Castro AM, Expósito E, Alonso-Cotoner C, Pigrau M, Nieto A, Rodríguez N, Guagnozzi D, Serra X, Henao A, Abril M, Ganda-Mall JP, Aldea C, Azpiroz F, Santos J, Vicario M, Lobo B.

UEG week 2021. October 2021. Virtual format. United European Gastroenterology. Oral presentation.

Distinctive plasticity of enteric glial cells in the jejunal mucosa of diarrhoea-prone Irritable Bowel Syndrome patients with and without bile acid diarrhoea.

Albert-Bayo M, González-Castro AM, Expósito E, Alonso-Cotoner C, Rodríguez N, Serra X, Abril M, Santos J, Lobo B, Vicario M.

NeuroGASTRO 2021. September 2021. Virtual format. European Society of Neurogastroenterology and Motility. Oral presentation.

Mucosal IgG production and plasma cells-nerves interaction: potential mechanisms of gut-brain axis dysfunction in diarrhoea-prone irritable bowel syndrome.

Pardo C, Melón E, **Albert-Bayo M**, González A.M, Expósito E, Martínez C, Nieto A, Pigrau M, Galán C, Fortea M, Segú H, Rodríguez A, deTorres I, Azpiroz F, Alonso C, Santos J, Vicario M.

UEG week 2019. October 2019. Barcelona. United European Gastroenterology. Poster presentation.

Mucosal IgG production and plasma cells-nerves interaction: potential mechanisms of gut brain axis dysfunction in diarrhoea-prone irritable bowel syndrome.

Melón E, Pardo-C, **Albert-Bayo M**, González A.M, Pigrau M, Nieto A, Azpiroz F, Santos J, Vicario M.

NeuroGASTRO 2019. September 2019. Lisboa. European Society of Neurogastroenterology and Motility. Poster presentation.