Neuro-immuno-regulation of inflammation in the colonic mucosa

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Linköping University
Medical Dissertations No. 1758

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Focus on mast cells and eosinophils
in bowel disorders

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Cover: Z-stack of a human section stained by immunofluorescence procedures. Eosinophils (cyan), mast cells (red) and vasoactive intestinal polypeptide (green). Acquired by Maria Teresa Casado Bedmar with LSM800 Zeiss Inverted Confocal 100X.

“Everything is either an opportunity to grow or an obstacle to keep you from growing. You get to choose.”

- Wayne W. Dyer

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Abstract

Intestinal homeostasis is key to control uptake across the mucosa and protect from harmful substances. Disturbances in the bidirectional communication between the gut and the brain are implicated in irritable bowel syndrome (IBS) and inflammatory bowel diseases (IBD), being Crohn's disease (CD) and ulcerative colitis (UC) the two most common IBD subtypes. Although these chronic bowel-relapsing inflammatory disorders present different histopathology, they share similar pathological features. Both IBS and IBD are characterized by a disrupted intestinal barrier function, a pro-inflammatory chronic condition, and an altered gut-brain axis. Despite all the scientific effort, the sequence or exact combination of events that drive these diseases are still unknown, and so is the exact role of every single component. Growing evidence suggests altered neuro-immune interactions as a pathogenic factor.

The general aim of this thesis was to elucidate the potential involvement of mast cells and eosinophils in IBS and IBD, and the neuro-immune intercellular circuit via vasoactive intestinal polypeptide (VIP) that might exacerbate mucosal inflammation and intestinal barrier disruption.

Intestinal tissues from IBS, inactive IBD, healthy controls (HC), and murine colitis were collected. Electrophysiological and permeability studies were performed using the *ex vivo* Ussing chamber technique. Tissues were processed with immunohistological procedures to study cell numbers, activation, location, and interactions in relation to VIP.

We demonstrated for the very first time an increased transcellular passage of live commensal and pathogenic bacteria through the colonic mucosa of IBS, identifying VIP as a key regulatory molecule together with mast cells activation. *In vitro* experiments revealed the ability of VIP to activate mast cells. Image analysis identified VIP-mast cells in closer proximity in IBD patients and murine colitis compared to controls. Communication between mast cells and VIP was shown upregulated in IBD and mice colitis via VIP receptor (VPAC)1. Similarities and differences between HC, IBS, and IBD were further studied. Results indicated a pronounced increased intestinal permeability in UC, even during remission, followed by IBS, compared to healthy controls. Surprisingly, permeability results did not correlate with mast cells, but with eosinophil number and activation. A further image analysis suggested an inhibitory effect of eosinophils and VIP on mast cells and an altered interaction between them under inflammatory conditions. Lastly, intestinal VIP levels were shown to increase in IBD patients after the treatment with biological

agents and were suggested as a possible biomarker for biological treatment outcome.

This thesis presents novel insights into the regulation of intestinal permeability, as well as into the pathophysiology of IBD and IBS by demonstrating the importance of neuro-immune interactions between mast cells, VIP, and eosinophils.

Altogether, our findings have broadened the knowledge of neuro-immune interactions in IBS and IBD and might have the potential to on sight lead to new therapeutic approaches thereby improving the outcomes for patients suffering from these diseases.

Keywords: mast cells, eosinophils, vasoactive intestinal polypeptide, neuro-immune, intestinal barrier, irritable bowel syndrome, inflammatory bowel diseases, Ussing chamber, advance image analysis,

Populärvetenskaplig sammanfattning

Irritabel tarm (irritable bowel syndrome, IBS) är en kronisk tarmsjukdom som kännetecknas av buksmärtor i kombination med förändrade tarmvanor. Inflammatorisk tarmsjukdom (inflammatory bowel disease, IBD) karakteriseras som en kronisk inflammation i tarmen som vanligtvis går i skov med omväxlande perioder av låg sjukdomsaktivitet. Dessa sjukdomar är komplexa och deras exakta etiologi är okänd, men olika faktorer kan tillsammans ge upphov till sjukdomen. Dessa faktorer innefattar arvsmassan, tarmens bakterieflora, slemhinnans barriär, immunförsvaret, faktorer i vår omgivning, och störd kommunikation mellan tarmen och hjärnan, via den så kallade ”tarm-hjärnaxeln”. Däremot är sambandet mellan störd immunförsvaret, tarmbarriärfunktion, hjärna-tarmaxeln, och patientens symptom fortfarande oklart.

Denna avhandling syftar på att undersöka relationen mellan specifika nervfaktorer, som till exempel vasoactive intestinal polypeptide (VIP) och immunceller, så som mastceller och eosinofiler, hos patienter med IBS och IBD.

Vävnadsbitar av tjocktarm från människor och möss samlades in för att studera hur neuro-immuna interaktioner påverkar tarmbarriären. Inledningsvis demonstrerade vi en ökad passage av levande bakterier genom slemhinnan hos patienter och att denna regleras av mastceller och VIP. Deras interaktioner analyserades ytterligare för att upptäcka mastceller och VIP kommunikation ökade hos patienter jämfört med kontroller. Sedan studerade vi likheterna och skillnaderna av immuncellerna och tarmslemhinnan mellan IBS, IBD och friska kontroller. Resultaten indikerade en mer inflammerad och läckande tarm hos patienter, jämfört med friska kontroller. Slutligen undersökte vi förändringarna av immuncellerna och VIP i förhållande till inflammation och svar på behandling. Resultaten antydde att interaktioner i tarm-hjärnaxeln av patienter under inflammation är förändrade, att eosinofiler reglerar mastcellerna och att VIP kan vara en användbar biomarkör för IBD-sjukdom.

Vi hoppas att våra fynd kan hjälpa oss för att få ett djupare perspektiv och förståelse för sjukdomarna samt att det på sikt kan leda till bättre behandling för patienterna som lider av IBS och IBD.

Popular scientific summary

Irritable bowel syndrome (IBS) is a chronic bowel disorder characterized by abdominal pain in combination with altered bowel habits. Inflammatory bowel disease (IBD) is characterized by chronic inflammation of the gut that usually exhibits alternating periods of disease activity. These disorders are complex and their exact cause is unknown. The combination of various factors could together give rise to the disease. These factors include the genetic predisposition, alterations in the bacterial flora, disruption of the intestinal barrier, altered immune responses, factors in our environment, and disturbed communication between the intestine and the brain, via the so-called "gut-brain axis". However, the relationship between the disturbed immune system, intestinal barrier function, gut-brain axis, and patient symptoms remains unclear.

This thesis aims to investigate the relationship between specific nerve factors, such as vasoactive intestinal polypeptide (VIP), and immune cells, such as mast cells and eosinophils, in patients with IBS and IBD.

Tissue from the colon of humans and mice were collected to study how neuro-immune interactions affect the intestinal barrier. Initially, we demonstrated an increased passage of living bacteria through the mucosa of patients, and that this is regulated by mast cells and VIP. Their interactions were further analyzed to detect that mast cells and VIP communication is increased in patients compared to controls. Then we studied the similarities and differences of the immune cells and the intestinal mucosa between IBS, IBD and healthy controls. The results indicated a more inflamed and leaky gut in patients, compared to healthy controls. Finally, we examined the changes in the immune cells and VIP in relation to inflammation and response to treatment. The results suggested that interactions in the gut-brain axis of patients during inflammation are altered, that eosinophils regulate mast cells, and that VIP may be a useful biomarker for IBD disease.

We hope that our findings can help to gain a deeper perspective and understanding of the diseases and that in the long run, it can lead to better treatment for patients suffering from IBS and IBD.

List of papers

This thesis is based on the following papers, referred to in the text by their Roman numerals:

- I. Vasoactive Intestinal Polypeptide and Mast Cells regulate increased passage of colonic bacteria in patients with Irritable Bowel Syndrome**
Olga Bednarska, Susanna A. Walter, Maite Casado-Bedmar, Magnus Ström, Eloísa Salvo-Romero, Maria Vicario, Emeran A. Mayer, Åsa V. Keita. *Gastroenterology* (2017)
- II. Upregulation of intestinal mucosal Mast Cells expressing VPAC1 in close proximity to Vasoactive Intestinal Polypeptide in Inflammatory Bowel Disease and murine Colitis**
Maite Casado-Bedmar*, Stéphanie D. S. Heil*, Pär Myrelid, Johan D. Söderholm, Åsa V. Keita. *Neurogastroenterology & Motility* (2018)
- III. Increased colonic epithelial permeability and mucosal Eosinophilia in Ulcerative Colitis in remission compared with Irritable Bowel Syndrome and Health**
Georgios Katinios, Maite Casado-Bedmar, Susanna A. Walter, Maria Vicario, Ana M. González-Castro, Olga Bednarska, Johan D. Söderholm, Henrik Hjortswang, Åsa V. Keita. *Inflammatory Bowel Dis.* (2020)
- IV. Eosinophils inhibitory role on Mast Cells and Vasoactive Intestinal Polypeptide as possible biomarkers for biological treatment in Inflammatory Bowel Disease**
Maite Casado-Bedmar, Maria Ling Lundström, John W. Wills, Jonathan Powell, Maria K Magnusson, Lena Öhman, Mauro D'Amato, Jonas Halfvarson, Robert Kruse, Dirk Reipsilber, Johan D Söderholm, Marie Carlson, Åsa V. Keita (Manuscript)

*Authors contributed equally

Related papers

In addition, the following list includes other papers from the author that are related to, but not included, in this thesis:

Enteric Glial Cells-Mast Cells interaction seems disturbed in the colon of Irritable Bowel Syndrome patients. Felipe Meira de-Faria, Maite Casado-Bedmar, Michael P. Jones, Susanna A. Walter, Åsa Keita (Manuscript)

Potential neuro-immune therapeutic targets in Irritable Bowel Syndrome. Maite Casado-Bedmar, Åsa Keita. Therapeutic Advances in Gastroenterology (2020)

A β -glucan-based dietary fiber reduces Mast Cell-induced hyperpermeability in ileum from patients with Crohn's Disease and control subjects. John-Peter Ganda Mall, Maite Casado-Bedmar, Martin Winberg, Robert Jan Brummer, Ida Schoultz, Åsa Keita. Inflammatory Bowel Diseases Journal (2018)

The joint power of sex and stress to modulate brain-gut-microbiota axis and intestinal barrier homeostasis: implications for Irritable Bowel Syndrome. Marc Pigrau, Bruno Rodino-Janeiro, Maite Casado-Bedmar, Beatriz Lobo, Maria Vicario, Javier Santos, Carmen Alonso-Cotoner. Neurogastroenterology and Motility (2015)

The intestinal barrier function and its involvement in digestive disease. Eloisa Salvo-Romero, Carmen Alonso-Cotoner, Maite Casado-Bedmar, Cristina Pardo, Maria Vicario. Revista Española de Enfermedades Digestivas (2015)

Abbreviations

⁵¹Cr-EDTA: 51-chromium labelled ethylenediamine tetra-acetic acid complex

AU: arbitrary units

CD: Crohn's disease

CRH: corticotropin-releasing hormone

Da: Daltons

DSS: dextran sodium sulphate

ECP: eosinophil cationic protein

EDN: eosinophil derived neurotoxin

ENS: enteric nervous system

EPO: eosinophil peroxidase

FAE: follicle-associated epithelium

FcεRI: high-affinity IgE receptor

GALT: gut-associated lymphoid tissue

GI: gastrointestinal

HC: healthy control

HMC-1.1: Human Mast Cell sub-clone 1.1

IBD: inflammatory bowel disease

IBS: irritable bowel syndrome

IFNγ: interferon-gamma

IFX: infliximab

IgAs: secretory immunoglobulin A

IL: interleukin

Isc: short circuit current

JAM: junctional adhesion molecules

kDa: kiloDaltons

LPS: lipopolysaccharide

MBP: major basic protein

NSAID: non-steroidal anti-inflammatory drugs

PAR2: protein-activated receptor 2

PBS: phosphate buffer saline

PD: potential difference

TER: transepithelial resistance

TLR: toll-like receptor

TNF: tumour necrosis factor

TRPV1: transient receptor potential channel type V1

UC: ulcerative colitis

VE: villus epithelium

VIP: vasoactive intestinal polypeptide

VPAC: VIP receptor

ZO: zonula occludens

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“All disease begins in the gut”

– Hippocrates

Physiological Background

The gut, the Sixth Sense

The gastrointestinal (GI) tract is a specialized organ in which dynamic interactions between host cells and environment occur in addition to food digestion.

The complexity of the GI tract can be simplified as a hollow continuing tube of 5-8 meters that crosses the body. It starts in the mouth, where the digestion of ingested food begins; and it goes down to the oesophagus and into the stomach, where digestion processes are carried on. The tube winds through the abdomen forming a very long but ironically called small bowel, divided into the duodenum, jejunum, and ileum, in which place further digestion and absorption occurs. This is followed by the colon or large intestine with the absorption of the majority of the water and the formation of faeces. The journey ends in the anus with the expelling of waste (Figure 1).

We could say that the lumen of the tube is essentially an extension of the external environment, loaded with potential hazards that are in constant contact with the surface of the gut, known to be the largest surface of all the organs. Traditional numbers stated a huge surface of 300-400 m². Newer calculations¹ point to a smaller, but still impressive, 30 m². Such a surface is the result of consecutive tissue folds. First, in large circular creases (Figure 1 Zoom 1); second, in smaller folds finger-like structures or so-called villi; and last, in micro-folds located on the apical side of the epithelial intestinal cells (Figure 1 Zoom 2). This feature allows gathering as many nutrients as available that will, later on, be transformed into energy and essential components, but will also expose the body to trillions of microbes that impose a huge threat of the gut integrity and thus, the balance between health and disease. The complex ecological system formed by indigenous microbial cells that reside in the digestive tract is known as microbiota. Besides the potential danger that gut microbiota can be, its presence is crucial for a normal host metabolic function, structural development and immune system maturation².

The microbiota composition varies along the GI tract. In the small intestine, the short transit time and high levels of digestive enzymes and oxygen limit bacteria growth; whereas in the colon, anaerobic conditions and the abundance of undigested complex carbohydrates support a dense and diverse community of bacteria. In the inner body and facing the intestinal hollow, neurons and immune cells are lining and literally projecting into the lumen to interpret the gut flora signals. The

bidirectional communication between the nervous system and the microbial community is known as the gut-brain axis, which modulates and controls not only the intestinal metabolism, but also the emotional states, brain mitochondrial function, and neuron survival³.

Rather than being just a digestive processing centre, we could think of the gut like a sensory organ. A “Sixth Sense” that receives and interprets information from microbiota, which co-evolves with the host to form a mutually beneficial relationship.

Intestinal barrier function

The continuous exposure of the GI tract to antigens makes necessary the existence of a defensive function. The intestinal barrier is a semipermeable structure that restricts the passage of potential hazards while allowing the absorption of nutrients, the transport of water and electrolytes, and the selective antigen uptake across the mucosa to the underlying lymphoid tissue in order to initiate a controlled mucosal immune response⁴. The balance of these two functions, digestive and defensive, are kept in harmony thanks to the coordination of cellular and extracellular mechanisms of the intestinal mucosa (Figure 1 Zoom 2). If the barrier function is disturbed, antigen and bacterial passage can be increased which in turn may cause an uncontrolled immune reaction that damages the intestinal homeostasis and consequently leads to various diseases⁵.

Extracellular elements

The lumen itself is the first defence element. Enzymes and the low pH of gastric, pancreatic and biliary fluids un-specifically degrade microorganisms and antigens.

Covering the intestinal epithelium of the colon there is a microclimate formed by an unstirred water layer and two layers of mucus. The stirred and outer layer of mucus has hydrophobic properties and contains secretory immunoglobulin A (IgAs), phospholipids, mucins and peptides with antimicrobial properties that prevent the adhesion of microorganisms. The unstirred and inner mucus layer is known as “glycocalyx” coats the intestinal epithelium facilitating the absorption of nutrients, oral antigen tolerance and epithelial renewal, hydration and protection from digestive enzymes⁵.

Finally, the excretion of water dilutes the luminal content and the peristalsis, or intestinal motility diminishes the exposure time and translocation of toxic substances.

Figure 1. Anatomical overview of the gastrointestinal tract. (Zoom 1) Structural organization of the intestines. (Zoom 2) Schematic illustration of the components that form the intestinal barrier of the small and large bowels. VE: villus epithelium, FAE: follicle-associated epithelium, ENS: enteric nervous system, IgAs: secretory immunoglobulin A.

Cellular elements

The main cellular elements of the intestinal barrier function are the gut microbiota, the intestinal epithelium, the immune system and the enteric nervous system (ENS).

The gut microbiota, which includes bacteria, archaea and eukaryote inhabiting the GI tract, compete for nutrients and the ecological niche with pathogens.

Between the gut microbiota and the inner body lays the intestinal epithelium, considered by far the strongest determinant of the physical intestinal barrier. Remarkably, the intestinal epithelium is a single monolayer of very tight and polarized cells that continuously renews every 3 - 5 days. The foundation of this monolayer is built with enterocytes, around 80% of the cell population. In less amount, scattered among enterocytes and playing important secretory roles are Goblet cells, specialized in mucus; Paneth cells, in defensins; enterochromaffin cells, in hormones and neuropeptides; and membranous or microfold (M) cells. M cells are specialized intestinal epithelial cells that sample luminal antigens for immune surveillance by capturing the particles and transporting them across the mucosal barrier into the lamina propria⁶. M cells are only located in the small intestine, in a particular epithelium called follicle-associated epithelium (FAE). The FAE presents a biochemical face to the outer milieu distinct from the surrounding villus epithelium (VE) (Figure 1 Zoom 2).

Underneath the intestinal epithelium is the lamina propria, a loose connective tissue that accommodates the immune system, also known as gut-associated lymphoid tissue (GALT). GALT comprises the Peyer's patches in the ileum, lymphoid follicles in the colon, and diffuse GALT all along the gut. Peyer's patches are big aggregates of lymphoid cells that form a sub-epithelial dome lining by FAE, which transfers luminal antigens, inducing immune tolerance and defence against pathogens. In contrast, diffuse GALT contains lymphocytes, in between or above enterocytes, that monitor and respond to luminal antigens. Other immune cells such as macrophages, dendritic cells, eosinophils, and mast cells share their space in the lamina propria⁵.

A controlled passage of antigens creates a controlled immune response that leads to tolerance. However, when barrier integrity is compromised, the free passage of microorganisms causes disproportionate inflammation to eliminate the insult. The uncontrolled inflammatory response can be modulated by the ENS. The ENS is an intertwined network of neurons and glial cells located in two major plexuses in the muscle layer and the submucosa. Although the ENS is not always considered as part of the intestinal barrier function, its role controlling motor, secretory and

immune activities is key for the intestinal homeostasis via the secretion of neurotransmitters⁷. The ENS is further discussed in the “Neuro-immune Background” section.

Intestinal permeability

The movement of molecules across the gut wall is known as intestinal permeability. There are several pathways for luminal substances to pass through the intestinal epithelium. The route that products take is determined by their size and hydrophobicity. The two most relevant routes are either between the cells, paracellular path; or through the cells, transcellular path (Figure 2).

Paracellular permeability

Paracellular transport refers to the passive passage of ions, water and medium-large hydrophilic molecules (400 Da – 20 kDa) between adjacent enterocytes. The paracellular route is determined mainly by the tight junction's pore size. Tight junctions are multi-protein junctional complexes found in the epithelia that form cell to cell adhesions. Tight junctions hold epithelial cells very close to each other in the apical side, polarizing the intestinal epithelium and regulating the transport of hydrophilic molecules.

Two different mechanisms have been proposed to regulate the paracellular permeability: a “pore” pathway formed by tight junction strands by which charged ions and uncharged solutes pass through, and the “leak” pathway by which small and large solutes pass through the tight junction strand breaks⁸. The paracellular pathway allows 85% of the total passive transport and prevents the intramembrane transport of proteins, lipids, and microbial-derived peptides. Dysregulation of tight junctions have been extensively proven to alter the intestinal barrier integrity^{9,10}

Several transmembrane and cytosolic proteins compose the junctional complexes. These are highly dynamic and can rapidly open and seal the barrier when required. Occludin, tricellulin, claudins, and junctional adhesion molecules (JAM) are part of the transmembrane protein complex. They connect to the actomyosin cytoskeleton via zonula occludens (ZO) scaffolding proteins ZO-1, ZO-2 and ZO-3. Below the tight junctions; adherens junctions link the cytoskeleton of adjacent cells via the prejunctional F-actin ring and cadherin proteins. Intercellular channels called gap junctions connect the interior of adjacent cells and allow the pass of ions and small molecules. Finally, in the basolateral membrane, desmosomes and hemidesmosomes are linked to intermediate filaments and anchor the epithelial cells^{5,11} (Figure 2).

It is worth to mention a few details about occludin and ZO-1 since both have been studied in this thesis.

Occludin was the first identified tight junction protein, in 1993¹². It provides structural and functional stability to the tight junctions complex and the intestinal barrier. Occludin knockout mice present intact tight junctions but a defective epithelial barrier, altered GI histology¹³ and chronic inflammation¹⁴. Also, it has been observed that occludin's expression is severely jeopardized in experimental models¹⁵ and patients^{15,16} with GI inflammatory diseases. Besides protein expression, occludin molecular dynamics can be regulated at the post-translational level. Occludin is highly phosphorylated at serine and threonine residues. Phosphorylated occludin interacts, among others, with ZO-1.

ZO-1 is an isoform of the peripheral membrane-associated proteins ZO. ZO are essential for scaffold formation and the connection of tight junctions to the cytoskeleton¹⁷. It has been shown that during oxidative processes, phosphorylation patterns are altered and tyrosine residues are phosphorylation instead, which results in the disruption of occludin-ZO-1 interactions and occludin's redistribution¹⁸.

Figure 2. Schematic illustration of the tight junction complex and the passage routes across the epithelium. ZO: zonula occludens. JAM: junctional adhesion molecules.

Transcellular permeability

Transcellular pathways allow transportation across the enterocytes in different fashions.

Small hydrophilic and lipophilic molecules can diffuse passively through the enterocyte membrane's lipid bilayer or using aqueous pores.

Active mechanisms transport nutrients and other antigens from the lumen to the inner body. Since large particles, such as proteins and bacterial products, cannot cross through the cell membrane, they are taken up by different endocytosis processes. Clathrin-coated vesicles regulate highly specific receptor-mediated endocytosis that internalizes mainly immunoglobulins and viruses. Phagocytosis is relevant for the uptake of diet and derived bacteria antigens after their bound with receptors. Micropinocytosis is a non-specific endocytosis process by which extracellular fluids can be internalized in small vesicles. Larger vesicles are resulting from macropinocytosis, an endocytosis process initiated by actin-driven extensions of plasma membrane ruffles. Lastly, lipid rafts involve the invagination of cholesterol-enriched micro-domains within the cell membrane and seem involved in the uptake of viruses and enterotoxins. Thereupon endocytosis, antigens are carried across the cytoplasm via transcytosis and driven out by exocytosis to the underlying immune system¹⁹.

Neuro-immune Background

With more than 200-600 million neurons, the ENS is the largest component of the peripheral nervous system. The communication between the gut and the brain is constant and, although the ENS is not autonomous, it is uniquely able to orchestrate local functions independently of the central nervous system. Interestingly, 90% of vagal fibres of the so-called “gut-brain axis” are afferent, suggesting that the brain is actually receiving more information than sending²⁰.

Enteric neurons are organized in microcircuits, embedded in the GI walls, forming two main plexus that lie in the submucosa and the myenteric plexus. The ENS sense, reacts and adapts to changing conditions to keep the homeostasis and modulate intestinal inflammation. Sensorial neurons, interneurons and motor neurons, supported by a glial cell network, control the intestinal motility, local blood flow changes and fluid and electrolyte secretion²¹.

Enteric neurons share anatomical niches with gut immune cells. They are continuously exposed to the external milieu, challenged with the complex task of discriminating between beneficial commensal bacteria and invading pathogens. The expression of neurotransmitter receptors by distinct immune cell subsets and cytokine receptors by neurons suggest a reciprocal communication that is likely to contribute to organ homeostasis and disease (Figure 3).

Figure 3. Summary of the main neuro-immune interactions between mast cells, eosinophils and vasoactive intestinal polypeptide (VIP) during physiological conditions. ENS: enteric nervous system, ZO1: zonula occludens. VPAC: VIP receptors.

Vasoactive Intestinal Polypeptide

Vasoactive intestinal polypeptide (VIP) is a 28 amino acids neurotransmitter, discovered in 1960 and characterized later in 1970 by Said and Mutt as a strong vasodilator and hypotensive²². Endogenous VIP was firstly identified in neurons of the central and peripheral nervous system that could be released by numerous stimuli. Further research demonstrated its presence also in immune cells including T cells²³, B cells, mast cells²⁴, and eosinophils²⁵.

General functions

The wide distribution of VIP contributes to an extensive range of physiological and pathological processes. In the gut, it exerts secretory functions²⁶, regulates smooth muscle activity, epithelial absorption and blood flow after interacting with its main receptors VPAC1 and VPAC2. Moreover, VIP presents immunomodulatory properties observed in different inflammatory and autoimmune diseases.

This neuropeptide is an extremely pleiotropic molecule, able to participate in biological processes very differently depending on the targeted cell, the anatomical location and the surrounding milieu. An example of its complexity can be seen in VIP knockout mice. VIP knockout mice present an endogenous VIP reduction that leads to reduced mucus production, cell proliferation and crypt height, with increased paracellular permeability and apoptosis that can be corrected with exogenous VIP²⁷. Differently to the colon, the small intestine presented intestinal mucosa abnormalities and motility disorders²⁸ with increased crypt depth and cell proliferation. Interestingly, exogenous VIP could not restore the ileum mucosal changes²⁷.

Inflammatory, or not inflammatory, that is the question

Regarding its inflammatory contribution, it is still uncertain if VIP is playing a pro- or anti-inflammatory role in the gut. Research in animal models is controversial and unclear.

Supporting an anti-inflammatory position, some studies with VIP knockout mice have shown to develop severe inflammation and intestinal mucosal changes due to induced colitis in a more aggravated way than normal mice. VIP exogenous treatment rescued the phenotype and protected the VIP knockout mice²⁹. Moreover, VIP can downregulate inflammation by shifting the Th1/Th2 balance³⁰ and Th17/Th2 towards a “Th2 phenotype” response³¹ by promoting Th2 proliferation and migration, and preventing or reducing Th1 infiltration³².

Supporting a pro-inflammatory role, other studies performed as well with VIP knockout mice have shown altered responsiveness of inflammatory cells to endotoxins. These mice were more resistant to lipopolysaccharide (LPS) endotoxemia due to a decreased production of pro-inflammatory cytokines interleukin (IL)-6, IL-12 and tumour necrosis factor (TNF), which in turn decreased mortality and tissue damage³³. At the same time, VIP was shown able to promote the production and release of pro-inflammatory cytokines on mast cell³⁴.

Different shreds of evidence indicate that the pro-inflammatory effects of VIP in the GI tract may be mediated mainly by VPAC1,³⁵ while anti-inflammatory effects may be mediated mainly by VPAC2 activation^{35,36} (Figure 3).

VIP effects on intestinal permeability

While a direct VIP effect on tight junctions seems to increase ZO-1 expression³⁷ and to prevent the translocation of ZO1, occludin and claudin-3³⁸, animal experiments have shown that the endogenous absence of VIP increases paracellular permeability, which can be restored with exogenous administration²⁹. Indirect VIP signalling may also affect changes in the paracellular permeability.

In line with its anti-inflammatory effects, VIP could reduce TNF release from activated macrophages via VPAC2 stimulation³⁶. In line with the pro-inflammatory theory, VIP can enhance TNF production³⁹ and activate mast cells to release products that can increase the intestinal permeability⁴⁰. See “Disruption of intestinal permeability by mast cells” section for more details.

Mast cells

Mast cells are bone-marrow-derived leukocytes. Mast cell precursors circulate in the bloodstream and migrate into peripheral tissues where their subsequent differentiation takes place.

Differentiated mast cells present a cytoplasm packed with granules that store preformed mediators. These preformed mediators include histamine, serotonin, heparin and proteases; and can be released almost instantly upon activation during degranulation process. The majority of mast cell mediators are synthesized *de novo* and released in the hours following activation. These include leukotrienes, prostaglandins and a large number of cytokines^{41*} (Figure 4).

Based on their specific proteases content, human mast cells are conventionally divided into three subtypes: mast cells containing the only tryptase; only chymase; or

containing chymase, tryptase, carboxypeptidase and cathepsin G. Mature mast cell subtypes display a high degree of plasticity that is dependent on the tissue milieu⁴². In the intestine, the majority of mast cells are tryptase positive and only a very limited number show chymase expression^{43,44}.

Figure 4. Mast cell illustration with main functions and mediators. TNF: Tumour necrosis factor

General functions

Mast cells can be found in almost all tissues, but their presence is particularly high in those tissues that interface the external environment. Mast cells critically orchestrate efficient antibacterial responses that contribute or even initiates immune responses against invading microorganisms. Thus, mast cells are considered sentinels of innate immunity. Upon activation, mast cells exert a vast range of homeostatic and pathogenic functions that include tissue remodelling, toxins degradation, allergic reactions and inflammatory processes, among others⁴⁵ (Figure 3).

Nerve-Mast cell interactions

One of the earliest neuro-immune associations ever described was in 1959 by Santiago Ramón y Cajal, and included a brief description of mast cells in injured nerve trunks⁴⁶. Their anatomical close proximity suggested the existence of a direct communication^{47,48}. There are now extensive pieces of evidence that mast cells and nerves communicate utilizing bidirectional paracrine interactions, or even forming synapses⁴⁹. Although bidirectional communication between mast cells and enteric neurons exists, Buhner *et al* revealed that a nerve to mast cell signalling is more prominent than a mast cell to nerve signalling⁵⁰. The neuronal secretions of neuropeptides, including VIP and substance P, seems to be a key component of neuro-genic inflammation.

Several studies have proven the existence of VIP-mast cell crosstalk; however, their interplay is still not fully elucidated (Figure 3). VIP stimulation of GI mast cells can lead to cell activation⁵¹, pro-inflammatory cytokine production and degranulation of mast cell mediators³⁴ that create a positive-feedback loop with the ENS via activation of nociceptors. Among the mast cell-derived mediators that act on enteric neurons, receptors are histamine, shown to generate and maintain visceral hypersensitivity⁵²; and tryptase. Tryptase can cleave protein-activated receptor 2 (PAR2) on enteric neurons and cause long-term neuronal hyper-excitability⁵³, the release of neuromodulators like substance P⁵⁴ and neuronal cell-death⁵⁵. In addition, tryptase may also limit neuronal activation by cleaving and thus inactivating VIP⁵⁶.

Disruption of intestinal permeability by mast cells

One mechanism by which mast cells seem to initiate or exacerbate inflammation is via the perturbation of the epithelial barrier integrity, which can be modulated by psychological stress. Very elegant studies have implicated stress in the increase of intestinal permeability via corticotropin-releasing hormone (CRH)-mediated mast cell recruitment and activation in humans⁵⁷ and animals^{40,58}. Mast cell stabilizers and VPAC blockers can abolish increased transcellular and paracellular permeability caused by stress. Similar approaches have been used in chemical induced-colitis in rats, where much harsher methods are employed to physically damage the intestinal mucosa. Results concluded that mast cells contribute to the intestinal barrier and transport function changes⁵⁹.

The main mast cell mediator candidate for the regulation of intestinal permeability changes is tryptase. Mast cell tryptase cleaves and activates PAR2 on enterocytes, which interacts with the adaptor protein β -arrestin-2, reorganizes the perijunctional F-actin, causes cell damage⁶⁰ and redistribution or downregulation of tight junctions. These structural changes end in the increase of paracellular permeability to macromolecules. Another mast cell-derived mediator that can regulate tight junctions is TNF. Although TNF is primarily produced by macrophages, it has been shown that many of the TNF expressing cells in the intestinal mucosa are mast cells⁶¹, proven to be as well an important source of this cytokine⁶¹. TNF increases intestinal permeability largely by inducing the myosin light chain kinase, which in turn destabilizes tight junctions⁶².

Eosinophils

Eosinophils are derived-bone marrow leukocytes which differentiation and proliferation take place by the influence of several transcriptional and growth factors⁶³. Under healthy conditions, differentiated eosinophils enter the bloodstream and migrate in response to chemokines until reaching their final destination where they fully mature and exert their functions. Eosinophils are strongly driven by eotaxin gradient to the gut, shown to be the primary tissue that gathers them. Although intestinal eosinophils are extensively present in the GI tract, their role in the gut is still not fully understood⁶⁴.

General functions

Mature eosinophils possess a very characteristic bi-lobular nucleus and a heavy granulated cytoplasm that contains a vast diversity of proteins⁶⁵. They express a wide repertoire of granule-derived basic proteins, lipid mediators, cytokines, and chemokines. The four most relevant granule-derived proteins are major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN or eosinophil protein X, EPX) and eosinophil peroxidase (EPO). Via the release of their potent inflammatory arsenal, eosinophils contribute to a vast array of functions that includes immune-regulation, tissue remodelling, antigen presentation, allergic reactions and protection against pathogens⁶⁶. The versatility of eosinophil action in health and disease is a relatively new view incorporated in 2010 by Lee *et al* with the “LIAR hypothesis” (Local Immunity And/or Remodeling/Repair). They presented and reinterpreted previous data to cease the strong traditional dogma that claimed eosinophils as an end-stage effector cell with destructive capabilities⁶⁷ (Figure 5).

Figure 5. Eosinophil illustration with main functions and mediators

Neuro-immune cross-talk with eosinophils

Resident eosinophils communicate with almost all the cells harboured in the intestinal mucosa. Even though their interactions with mast cells, nerves and epithelial cells seem relevant for the homeostasis of the tissue, not much is known about their mutual involvement in GI disorders.

Mast cells and eosinophils are the key components of allergic hypersensitivity. Their cross-talk is mediated via their released mediators and physical interactions, modulating each other's function and driving to the allergic inflammatory reaction^{68,69}. Nevertheless, eosinophil-mast cell crosstalk also occurs during inflammation in non-allergic scenarios. Mast cell-tryptase have been proven to recruit eosinophils⁷⁰ and to be up-taken by eosinophils during physical contact⁷¹. Eosinophils on the other hand are able to promote mast cells growth, trafficking and survival via the release of cytokines and growth factors, like stem cell factor and MBP^{72,73} (Figure 3). They can also induce mast cell activation and degranulation via ECP and MBP release⁷⁴. As consequent of their communication, eosinophils' survival and chemotaxis are enhanced as well as their reciprocal activation⁷⁵, which contributes to the perpetuation of inflammation.

The third-party that may be involved in the inflammation is the ENS. Human eosinophils highly express the VIP associated receptor CRTH2, instead of the conventional VPAC1 and VPAC2⁷⁶. Neuronal VIP interacts with eosinophils via CRTH2⁷⁷, inducing their recruitment and accumulation around VIP-producing terminal nerves. Interestingly, VIP's power to chemoattract eosinophils is similar to eotaxins⁷⁶, the main chemokine that directs eosinophil accumulation to the gut.

The interaction eosinophil-nerves is in fact bidirectional. Eosinophils can produce, store and release neuropeptides such as substance P and VIP that directly regulate neuronal activity⁷⁸, increasing the release and content of neurotransmitters, modifying the nervous system growth and enhancing neuronal survival⁷⁹. Neuronal plasticity can also be modified by eosinophils, causing an increase in neural activity associated with pain and motility changes⁸⁰. Although several studies suggest a link between eosinophils and neuronal homeostasis, most of the current literature available is based on airway diseases and specific research on the GI is lacking.

Disruption of intestinal permeability by eosinophils

Via the secretion of cytokines and chemokines such as eotaxin, IL-5⁸¹ and IL-18⁸², enterocytes can activate eosinophils and induce the release of ECP⁸³.

Remarkably, the effect that eosinophils have on the intestinal barrier is directly related to their amount and can result in either physiological or pathological states. Under normal conditions, enterocytes-eosinophils ratio is low. They spur the homeostasis of the epithelial barrier function by promoting the tissue healing and the microbial host defence, thanks to the stimulation of fibroblast and production of mitochondrial DNA traps, respectively. On the other hand, depletion or high ratios of eosinophils are associated with an impaired barrier⁸⁴, partially due to MBP release. Eosinophil-MBP has been identified to upregulate transcellular permeability and downregulate the tight junction protein occludin⁸⁵.

Pathological Background

Irritable bowel syndrome

Irritable bowel syndrome (IBS) is considered the most common chronic functional disorder in today's society. Global prevalence goes up to 9.2%, being more predominant among women (12% women vs 8.6% men). IBS is considered a very problematic disorder with negative impact in patient's quality of life that generates high social-economic costs⁸⁶.

Despite all the scientific effort to understand this syndrome, its etiopathology and mechanisms remain mostly unclear. To date, IBS is considered a multifactorial disorder where physiological and psychological variables, such as age, gender, stress, diet, genetic predisposition, GI infections and consumption of antibiotics, are contributors to the aetiology and perpetuation of IBS⁸⁷. Latest evidence also points to a biological dysregulation of the brain-gut axis since patients with IBS present differences in connectivity, brain structures, and functional responsiveness compared to healthy controls⁸⁸.

IBS' hallmarks are abdominal pain and bloating feeling in association with alterations in bowel habits. Many of the patients, 40-60%, also experience comorbid psychological disorders, such as depression or anxiety. Surprisingly, no macroscopic nor microscopic obvious changes are detected during endoscopy or with conventional histopathology methods, respectively⁸⁷.

The lack of biomarkers leads to a diagnosis based on the clinical symptoms, which are very heterogeneous and overlap with other GI disorders. The Rome Criteria is a symptom-based guideline created in 1989 to help diagnose IBS⁸⁹. Its latest version, Roma IV⁹⁰, states that the abdominal pain has to be for at least one day per week and be associated with stool consistency and frequency changes over the past three months, starting six months before the diagnosis. According to this, IBS can be sub-grouped depending on the prevalent stool consistency pattern: predominant diarrhoea (IBS-D), predominant constipation (IBS-C), mix type of stools (IBS-M) and (IBS-U) for inconsistent or un-subtyped IBS.

Neuro-immune contribution to IBS

As previously mentioned, no obvious macro- nor micro-inflammation are detected in the intestinal mucosa of IBS. Nevertheless, a growing number of signs point to a local micro-inflammation with an increased number of T- and mast cells⁹¹⁻⁹³ that

results in intestinal barrier dysfunction. IBS patients present a significantly lower expression or downregulation of ZO-1, occludin⁹⁴, JAM-A and claudin-1^{95,96} tight junctions proteins and, subsequently, increased paracellular permeability that has been proven to be in association with the presence of mast cells and eosinophils⁹⁵.

Some studies have suggested that neuro-immune interactions regulate IBS enteric pathophysiology (Figure 6), correlating with fatigue and depression⁹³. Increased amounts of VIP, eosinophils⁹⁷, mast cells⁹⁸ and their respective mediators⁹⁹ have been detected in IBS patients, mostly IBS-D^{100,101}, and correlated with increased intestinal permeability¹⁰², abdominal pain and depression¹⁰³. Barbara *et al* demonstrated that only mast cells near nerves are significantly correlated with severity and frequency of abdominal pain⁹⁸. Additional studies have correlated tryptase levels with visceral hypersensitivity¹⁰⁴ and with the spike discharge, or neuronal activity, induced by biopsies supernatant of IBS patients⁹⁹. Interestingly, IBS patients seem to present a hypersensitivity to tryptase that causes a remarkably high increase of the intestinal permeability¹⁰⁵. Another well-known mast cell mediator, histamine, is increased in IBS and correlate with mast cell count and abdominal pain¹⁰⁶. Interestingly, the supernatant of colonic biopsies from IBS patients, but not controls, increases nociceptive neuronal activity that can be inhibited by histamine receptor antagonists and protease inhibitors¹⁰⁷.

Figure 6. Summary of the main neuro-immune interactions between mast cells, eosinophils and vasoactive intestinal peptide (VIP) in inflammatory bowel syndrome (IBS). ENS: enteric nervous system, CRH: corticotropin releasing hormone,

It is well-established that elevated VIP levels can lead to severe secretory diarrhoea¹⁰⁸, one of the main symptom of IBS. The mechanisms by which IBS patients present an upregulated expression of VIP¹⁰⁹ is still unknown. Although it may be

directly or indirectly related to sex hormones since only IBS-D women present increased VIP levels¹⁰⁰, most evidence point to a connection with stress. Recent studies demonstrated that VIP is involved in colonic functions and diarrhoea as a downstream effector of CRH¹¹⁰ induced by stress, anxiety or depression¹¹¹.

Another important neuronal stimulus involved in IBS is stress. Psychological stress evokes the release of neurotransmitters, such as substance P or acetylcholine by nervous terminals, and activates eosinophils, inducing the expression and release of CRH. In turn, CRH can activate mast cells, whose released mediators, as discussed previously^{112,113}, cause barrier dysfunction, a common feature observed under stress conditions.

Even though increasing results point to eosinophils, and its mediators, to be of relevance in GI disorders, their connection to the pathophysiology of IBS is still to be solved.

Inflammatory bowel diseases

Inflammatory bowel disease (IBD) is an umbrella term to describe the relapsing inflammatory condition of the GI tract and consists mainly of Crohn's disease (CD), ulcerative colitis (UC) and microscopic colitis. This last one is not always as well-recognized as the other two and it is not included in this thesis either. The current frequency of IBD varies among countries. Overall, its prevalence is estimated in around 200 per 100.000 population, being higher in Western countries in the North of Europe and America, and the highest in Scandinavia. The incidence seems to be rising worldwide, causing considerable mortality and economic loss¹¹⁴. CD has an incidence of around 3 to 9 per 100.000 persons per year, with an incidence peak between the age ranges of 20 – 35 years¹¹⁵. Whereas, UC has an incidence of around 9 to 20 per 100.000 persons per year, presenting a bimodal pattern of incidence. The main onset peaks between 15 – 30 years, and a second peak occurs between 50 – 70 years¹¹⁶.

Although IBD's symptomatology is important for its diagnosis, a conclusive verdict still relies on endoscopy and histological assessment of biopsy specimens. The use of reliable biomarkers could help identify patients in early-stage, predict treatment outcomes and differentiate between UC and CD without the use of invasive methods.

IBD symptoms vary depending on the severity and localization of the inflammation. CD is characterized by a deep and chronic inflammation that can be located throughout the GI tract, whereas UC is a chronic non-transmural inflammation limited to the colon (Figure 7). The most common symptoms are abdominal pain

and cramps, bloody diarrhoea, fatigue and unintended weight loss. Many patients with IBD have IBS-like symptoms, including abdominal pain and diarrhoea. These phenomena are present mainly during remission¹¹⁷.

Figure 7. Main types of IBD included in this thesis and their corresponding inflammation pattern.

The exact causes of the initial development of IBD remain unclear. It is believed that several factors are implicated, among those are alterations of the microbiota, the malfunction of the immune system which elicits an abnormal response against the intestinal microbiota, changes in the intestinal barrier function like modified tight junction expression or alterations in the mucus layer¹¹⁸, environmental factors, stress and genetic susceptibility. It is still under discussion if intestinal barrier dysfunction in IBD patients is an early event in the disease independent of inflammation^{119,120}, or if it is a consequence of the raging inflammation¹²¹. Despite this, a large number of studies have concluded that an inherited disrupted intestinal permeability due to functional abnormalities in tight junction could result in IBD, even in the presence of a normal underlying immunity^{122,123}.

The development of the disease at a young age in most cases compel the start of long-term anti-inflammatory treatments to prevent disease flares and disease progression with intestinal complications that would end with the need for hospitalization and surgery. In recent years, the management of IBD has improved considerably due to a new approach that uses biological agents to neutralize inflammatory molecules.

To date, the most successful biological agents are those targeting TNF, both soluble and membrane-bound. TNF is one of the most important cytokines involved in inflammatory processes that together with other pro-inflammatory cytokines, like IL-6 and IL-1, results in tissue damage. More specifically, immune cells can release TNF and induce cell death, recruitment and activation of immune cells. Increased levels of TNF have been implicated in several human diseases, IBD in-

cluded¹²⁴, and tight junction disruption⁶² which in turn may result in the perpetuation of the chronic inflammation. Examples of anti-TNF therapies used in the clinical setting of IBD are infliximab (IFX), adalimumab and golimumab. Biological therapies use antibodies to neutralize TNF. These drugs have distinctive pharmacodynamics profiles that result in different therapeutic efficacies. The most beneficial outcomes are inhibition of immune cells activation, mucosal healing, restoration of the gut epithelial barrier function with reduction of histological and endoscopic disease activity, and sustained anti-inflammatory response¹²⁵.

VIP contribution to IBD

Studies of the contribution of VIP in animal colitis have shown all the possible outcomes. A protective effect with a reduction of the clinical and histopathologic severity^{29,126}, a neutral effect with no changes due to VIP treatment¹²⁷, and a harmful effect since VIP-knockout mice can be protected from induced colitis¹²⁸.

The complexity of VIP functions escalates even more in studies of IBD patients. Serum and local levels of VIP are frequently increased in IBD patients compared to controls^{129,130}, and it has been proposed as a biomarker for inflammation during the active disease phase¹³¹. Some results suggest that it may play different roles in CD and UC pathogenesis since VIP mRNA expression is higher in biopsies from UC, compared with CD¹³². Differences can also be found at various stages of active disease. Both plasma and tissue concentrations of VIP are higher in moderate and severe disease compared to mildly active UC¹²⁹. Interestingly, the stimulation of primary cultures of ENS with supernatant from non-inflamed tissues of CD patients induces more production of VIP than the stimulation with supernatant from inflamed biopsies¹³².

Not only the neuropeptide but also the phenotype of its receptors have been shown altered among patients. VPAC1 positive cells are increased in the inflammatory mucosa of IBD patients and its expression is different between UC and CD. In UC patients, cells that express VPAC1 are mostly CD-3 positive, while in CD they are CD-68 positive¹³³.

VIP therapeutic aspects have been considered and both agonists and antagonists have been suggested as promising anti-inflammatory agents. Unfortunately, therapies have not yet succeeded, probably because of its pleiotropic and wide function, and due to drug delivery issues. VIP-based drugs are rapidly degraded, limiting their bioavailability and delivery. It is known that VIP degrades quickly through enzymatic and spontaneous hydrolysis, having a half-life of 1-2 minutes¹³⁴.

Mast cells contribution to IBD

It seems to be a consensus in the literature that mast cell number and activation, hence tryptase levels, are increased in IBD¹³⁵. One of the big hints documenting the importance of mast cells tryptase in IBD was demonstrated in transgenic mice that lacked tryptase expression and whose induced colitis was markedly diminished¹³⁶. Another study discovered that tryptase inhibitors reduce mucosal injury and colon inflammation¹³⁷.

The exact molecular mechanisms by which mast cells contribute to exacerbated inflammation in IBD is still unclear (Figure 8). Comprehensive studies characterizing the distribution and phenotype of the mast cells within inflamed and non-inflamed intestinal segments of controls and IBD patients could help to clarify this matter. Inflamed tissues commonly present an increased number of mast cell with more pronounced controlled exocytosis of granules (piecemeal degranulation)¹³⁸. Sometimes, mast cell degranulation in actively inflamed tissue is such that they look “exploded” or even “disintegrated” (anaphylactic degranulation)^{139,140}, and the only remaining left behind are tryptase and chymase spread in the tissue. This could lead to a paradoxically reduced count of mast cells¹⁴¹. Mast cells from inflamed/active areas of IBD intestinal mucosa are involved in significantly greater release amounts of mediators, such as histamine and prostaglandin D2, compared with non-inflamed areas. This difference seems to be the result of an enhanced production, not greater storage. This indicates that an over-activation of mast cells may contribute to the IBD inflammatory process¹⁴².

Figure 8. Summary of the main neuro-immune interactions between mast cells, eosinophils and vasoactive intestinal peptide (VIP) in inflammatory bowel disease (IBD). ENS: enteric nervous system, CRH: corticotropin releasing hormone,

Concerning permeability, both paracellular and transcellular permeability are increased in IBD patients (Figure 8). Non-inflamed areas of CD present increased transcellular permeability, which is considered as a possible initiating pathogenic event¹⁴³. Mast cell mediators, and more specifically TNF and tryptase, may be closely involved in this phenomenon. TNF seems to be partially responsible for the decrease of ZO-1 and occludin expressions¹⁴⁴, characteristics in IBD¹⁴⁵. PAR2 signalling on the other hand is upregulated and correlates with the number of mast cell tryptase positive cells¹⁴⁶. Another interesting correlation found is between these two mediators and disease activity.

Although the term “IBD” includes both UC and CD, we must not forget that they are two different entities with different characteristics. A few studies have shown that mast cells might play a more relevant/active role in UC patients. UC samples can be differentiated from CD based on their immunohistochemical features, which, among others, include a more severe infiltration of mast cell tryptase positive¹⁴⁷ and a higher mast cell activation with enhanced and prolonged tryptase secretion¹⁴⁸.

Eosinophils contribution to IBD

The interest in the role of eosinophils in IBD has been increasing during the past years. An enhanced number of eosinophils, their activation and related cytokines are largely demonstrated in active IBD^{141,149}.

According to the pro-inflammatory and tissue-damaging paradigm, eosinophils are involved in the promotion of inflammation and intestinal motility changes as a consequent of eosinophil mediators release, which can be measured in blood, tissue and faeces. Faecal biomarkers are important non-invasive markers used in the clinics to monitor IBD disease activity^{150,151}. Specifically, faecal ECP (fECP) and EDN (fEDN) correlate with endoscopic score and are considered not only as diagnostic but also as prognostic markers^{150,152,153}. In general, released eosinophil mediators are related to increased tissue damage and activation of immune cells. Some studies have suggested that the recruitment and activation of GI eosinophils could participate in early mucosal damage in IBD¹⁵⁴. In IBD animal models, CRTH2 antagonist has shown to reduce eosinophils recruitment and with it the severity of the inflammatory condition¹⁵⁵.

Nonetheless, during inactive UC, the number of activated eosinophils is even larger than the inactive phase, which suggests a protective role of eosinophils in the repair of injured epithelium¹⁵⁶. Supporting these results, a recent study showed that IBD patients with eosinophil-predominant inflammation may have reduced risk of disease flares and hospitalization¹⁵⁷. Surprisingly, CD patients do not share this interesting activation pattern. Number and activation levels of CD intestinal eosinophils

reflects the disease activity¹⁵², being diminished during quiescent and increased during flares. *In vitro* experiments suggest that the mechanisms of eosinophils activation are closely defined by the cytokine milieu. IL-5 and other Th2 cytokines, found in higher concentrations in UC compared with CD, seem to be the main candidates responsible for the eosinophils phenotype^{149,158}.

Despite their differences, neuro-immune alterations in both diseases have been described and related to the disrupted epithelium. Already in 1980, a close physical association between eosinophils and mast cells was documented in the tissue of CD¹⁴⁰. More recently, activated eosinophils have been described expressing more neuropeptide receptors¹¹³ and in closer proximity to nerves in the mucosa of IBD¹⁵⁹, compared with controls. At least in UC patients, the neuro-immune cross-talk increases intestinal permeability, via the activation of eosinophil-muscarinic receptors and subsequent activation of mast cells¹¹³.

Aims of the thesis

The general aim of this thesis was to elucidate the potential involvement of mast cells and eosinophils in gastrointestinal disorders such as IBS and IBD, and the neuro-immune intercellular circuit via VIP that might exacerbate mucosal inflammation and intestinal barrier disruption.

The specific aims for the separate studies were to:

- I. Investigate the passage of live commensal and pathogenic microbes through the colonic mucosa of women with IBS; the role of mast cells and VIP in the regulation of bacterial passage; and the distribution of mast cells, VIP/VPACs in the colon of IBS subjects.
- II. Map the mast cells and their expressions of VPACs and proximity to VIP in villus epithelium, follicle-associated epithelium, and adjacent villus epithelium of CD patients, in the colon of patients with ulcerative colitis UC and controls. Moreover, we aimed to study this in dextran sodium sulphate (DSS) mice to further evaluate the involvement during inflammation and to study possible diverse effects of VIP in different species.
- III. Search for similarities and differences in epithelial barrier function and related immune cells between UC in remission and IBS for a deeper understanding of the disease-specific barrier function disturbances.
- IV. Investigate the alterations of eosinophils, mast cells and VIP in relation to inflammation and the response to biological treatment that could help elucidate the disease and find biomarkers.

*“Det viktiga är inte vad man gör
utan att man gör det med stil”*

- Lars Demian

Material and Methods

1. Subjects and human samples

A mix of IBS (sub-grouped in IBS-D, IBS-C and IBS-M), IBD, non-IBD and healthy control (HC) samples were studied in this thesis.

For Paper I, colonic biopsies and blood from 37 IBS with a mean age 32.2 years (range 19–55) and 20 HC with a mean age 29.9 years (range 20–48) were collected from the descending colon-sigmoid by sigmoidoscopy at the University Hospital, Linköping. All subjects were women. IBS patients fulfilled the Rome III questionnaires and had a moderate-severe IBS. Exclusion criteria for both groups were self-reported allergy, organic gastrointestinal disease, metabolic or neurologic disorders, and nicotinic or non-steroidal anti-inflammatory drugs (NSAID) intake. The Committee of Human Ethics, Linköping, approved the study (Dnr 2013/506-32) and all subjects had given their informed consent.

For Paper II, blood, 16 terminal ileal samples from CD with a median age of 53 years (range 17-74), and 12 proximal colonic specimens UC with a median age of 41 years (range 26-68), were collected during surgery at the University Hospital, Linköping. Inclusion criteria for the IBD patients were clinical and endoscopic remission and treatment for relapse within the last year. As controls, 15 ileal samples were obtained from colon cancer patients undergoing surgical removal of the colon and overlapping ileum. None of the patients had received preoperative chemotherapy or radiotherapy. Clinical pathologists confirmed the lack of tumour formation in the tissue collected, which was considered as non-IBD. Colonic samples from 10 HC, who did not have a medical history of chronic GI symptoms or disorders and no medication intake, were obtained during colonoscopy. The subjects included in this paper were both men and women. The median age was similar between IBD patients (CD: 53, UC: 41), but non-IBD were notoriously older, and HC younger (non-IBD: 71, HC: 31). Exclusion criteria for all groups were the same as for the Paper I. The Committee of Human Ethics, Linköping, approved the study (Dnr 02-154, Dnr 2011/200-31, Dnr 76-14) and all subjects had given their informed consent.

Paper III included the IBS, UC and HC samples from the two previous studies.

Paper IV included samples that belong to a larger cohort known as BIO IBD. BIO IBD is part of a prospective multicentre study conducted at national Swedish level

which aim is to identify genetics, metabolomics, miRNA, and also proteomics biomarkers for diagnosis, therapy response and disease progression in IBD. The gastroenterology departments of Sahlgrenska University Hospital (Gothenburg), Södra Älvsborg Hospital (Borås), Örebro University Hospital (Örebro), Uppsala University hospital (Uppsala) and Karolinska University Hospital (Solna) recruited 10 CD and 19 UC patients in clinical remission and collected blood and colonic biopsies at baseline and after three-months with biological treatment. The Regional Ethical Review Board, Uppsala, approved the study (Dnr 2010/313, Dnr 2010/313/2) and all patients gave their written consent.

For more details about the subjects, see each paper.

2. Faecal marker

For Paper IV, stool samples were collected and stored at -70°C . Before analysis, the samples were thawed and faecal extracts were prepared as described previously¹⁵¹. Faecal EDN was measured by our collaborators in Uppsala at Marie Carlsson lab group with an in-house ELISA assay. The levels of markers in faeces were adjusted for water content and EDN levels were expressed as micrograms per gram of semidry faeces. Normal levels were set to $\text{EDN} \leq 2.15\ \mu\text{g/g}$. The intra- and inter-assay variations were less than 10% for all assays.

3. Ussing chamber experiments

The Ussing chamber refers to an *ex vivo* technique used for the measurement of permeability and electrophysiology. It was originally created by Hans Henriksen Ussing in 1951¹⁶⁰. Dr. Ussing was a marine biologist who pioneered the development of epithelial physiology and provided the first demonstration of active transport of sodium ions based on an experimental model system with frog skin. In 1988, George M. Grass simplified and adapted the Ussing chamber technique to GI tissue¹⁶¹.

In our hands, Ussing chamber experiments start with the collection of the biopsies and their placement in cold oxygenated Krebs Buffer. Once in the lab, biopsies are mounted directly without previous manipulation, but with the awareness of the mucosal/serosal side. Mounting the tissue implies its flat placement between two films, on a $1.8\ \text{mm}^2$ opening, that is placed between two half chambers. The apical or epithelial side of the biopsies are then exposed to the “mucosal compartment”, filled with cold $10\ \text{nM}$ mannitol Krebs Buffer and isolated from the basal or lamina propria side of the biopsies, which are exposed to the “serosal compartment”, filled with cold $10\ \text{nM}$ glucose Krebs Buffer (Figure 9). The chambers are kept at 37°C , continually oxygenated with a 95% O_2 5% CO_2 gas flow. After 20-40 minutes of

equilibration, time is set to 0 and paracellular permeability marker +/- bacteria are added to the mucosal compartment. Serosal samples are collected at different time points and stored for further analysis. For Paper I, samples were pre-incubated with different blockers in the serosal compartment for 20 minutes.

2.1. Electrophysiology

A set of two platinum electrodes and agar-salt (3M NaCl 2% agar) bridges connected to reference electrodes, all in open circuit conditions, enable electrophysiological measurements: potential difference (PD), short circuit current (Isc) and transepithelial resistance (TER).

PD is the difference in the electrical charge across the epithelium as a result of the solute transport by Na⁺/K⁺ ATPase pumps. The addition of glucose to the mucosal solutions resulted in a rapid increase in the PD¹⁶². The greater the unidirectional transport of charged particles across the epithelial barrier, the higher the PD. The Isc represents the current needed to nullify the PD and is a function of the pump's activity. Since PD and Isc require active ion transport, their values can be used to monitor tissue viability. Lastly, the TER reflects the electrical resistance of the paracellular routes, the free movement of ions and solutes across the epithelium, and depends on the tightness of the junctional complexes between cells, giving an overall of gut integrity. Low TER value is indicative of increased permeability. It is noteworthy that sometimes the TER measured during Ussing chamber experiments is unchanged while paracellular permeability is increased, or vice versa. Therefore, TER cannot be equated to paracellular permeability of paracellular markers.

Figure 9. Schematic drawing and a photograph of one Ussing chamber.

Electrophysiology measurements were monitored with the software developed by LabVIEW (LabVIEW, National Instruments, Solna Sweden) every minute for 120 minutes. After 20-40 minutes of equilibration, the PD values achieved a steady state.

2.2. Paracellular permeability marker

The radioactive ^{51}Cr -chromium labelled ethylenediamine tetra-acetic acid complex (^{51}Cr -EDTA) is an inert probe with a very low molecular weight (292 Da or 11 Å), widely used as intestinal permeability marker since 1964 when A. M. Downes and W. McDonald used for the first time ^{51}Cr -EDTA to study the intestinal transit (of cows)¹⁶³. EDTA's binding to the ^{51}Cr is very strong. This fact assures that Cr passage is equal to the passage of EDTA and that no calcium can bind to the EDTA. Measurements of ^{51}Cr -EDTA are very precise and easy.

Paracellular permeability in Papers I and III were determined by adding ^{51}Cr -EDTA to a final concentration of 34 $\mu\text{Ci}/\text{ml}$ in the mucosal compartment and measuring the appearance of radioactivity in the serosal compartment at 0, 30, 60, 90 and 120 minutes after the equilibration of the chambers. Radioactivity was measured with the 1282 Compugamma gamma-counter; LKB, Bromma, Sweden.

2.3. Bacterial transcellular permeability

The bacterium *Escherichia coli* (*E. coli*) is a ubiquitous Gram-negative bacteria that have a place among the persistent intestinal bacteria population. Each *E. coli* strain demonstrates a unique nutritional program in the intestine, as well as a protective or invasive behaviour under normal circumstances that would define them as commensal or pathogenic, respectively^{164*}. *E. coli* O9:H4 (HS) has been considered a commensal strain since the 70s¹⁶⁵. It has the ability to eliminate invasive species of *E.coli*¹⁶⁶ by competing for nutrients with invading *E.coli*.

On the other hand, *Salmonella enterica* is a Gram-negative bacteria, predominately found in the intestinal lumen, that causes the most encountered cases of foodborne disease and the third of death among diarrheal disease¹⁶⁷. *Salmonella enterica* serovar Typhimurium uses multiple virulent factors to tightly adhere to the intestinal epithelium, colonise and induce intestinal inflammation¹⁶⁸.

In Paper I, we aimed to study the mucosal permeability to commensal and pathogenic live bacteria. Previous experiment, GFP expressing ampicillin resistant *E. coli* HS and *Salmonella* Typhimurium bacteria were grown in Luria-Bertani broth (or LB media) supplemented with ampicillin for 12 hours at 37°C and re-seeded in new

LB media with ampicillin until bacteria growth curve reached mid-log phase (1 – 1.30 hours). A final concentration of 10^8 CFU/ml, i.e. the normal concentration of bacteria in the ileal lumen, was added to the mucosal compartment of separate chambers. Serosal samples were collected at 0, 30, 60 and 90 minutes and measured in a fluorimeter at 488.

3. Animal model

Since the early 1970s, numerous animal models have been developed to characterize the complexity of IBD pathogenesis, describing the underlying molecular mechanisms and evaluating potential human therapies. One of the best well-established models of colitis employs DSS. DSS is a sulphated polysaccharide, a pro-inflammatory agent that induces colitis in mice by forming nano-lipocomplexes with medium-chain-length fatty acids in the colon, which interferes the intestinal barrier function and stimulates local inflammation¹⁶⁹. This model is simple and affords uniformed and reproducible lesions of the distal colon that mimics the clinical and histological characteristics of UC¹⁷⁰. Acute, chronic and relapsing models can be achieved by modifying the concentration and administration times of DSS.

For Paper II, 14 random mice received 5% DSS in their drinking water ad libitum and, as control, another 14 mice received plain water. On the 8th day after DSS-start, mice were anaesthetized with isoflurane inhalation and terminated by cervical dislocation. Animals were excluded in case of excessive weight loss (>20%), excessive rectal bleeding (>2 days), or if the score attributed to the animal was too high. Blood, ileal and colonic tissue samples were collected for further analysis.

4. Cell culture

The cell line of Human Mast Cell sub-clone 1.1 (HMC-1.1) was obtained from a patient with mast cell leukaemia in 1987 by Butterfield *et al*⁷¹. HMC-1 exhibit many characteristics of tissue mast cells such as expression of histamine, tryptase¹⁷² and heparin. Their receptor tyrosine kinase KIT, which plays an important role in the proliferation, function and survival, is mutated converting the receptor to a constitutively tyrosine phosphorylated and active state. Like immature mast cells, HMC-1 lack expression of the main receptor that mediates allergic inflammatory signaling, the high-affinity IgE receptor (FcεRI).

In vitro experiments with HMC-1.1 were performed only in Paper II to prove the possible direct effect of VIP on human mast cells. Cells were cultured in optimal conditions for 3 – 4 weeks. HMC-1.1 were then seeded at 10^6 cells/ml, acclimatized

(60 minutes) and then stimulated with different concentrations of VIP during 30, 60 and 120 minutes.

Mast cells degranulation in consequence of VIP exposure was quantified by β -hexosaminidase assay. B-hexosaminidase is a lysosomal enzyme, an acid hydrolase found in the granules of mast cells which release reflects mast cell degranulation. Based on the fact that β -hexosaminidase can hydrolyse terminal N-acetyl- β -d-glucosaminidase residues from 4-nitrophenyl N-acetyl- β -d-glucosaminidase, its activity can be quantified by a simple colourimetric assay (Figure 10). Briefly, the supernatant of stimulated mast cells was incubated with the substrate 4-nitrophenyl N-acetyl- β -d-glucosaminidase in Citrate Buffer (pH 4.5) at equal amounts. They were incubated in transparent 96-well plates at 37 °C (1 hour). The reaction was quenched with the addition of Glycine (pH 10.7). The reaction revealed a yellow colour which optical density was read at 405 nm in VERSAmax Tunable Microplate Reader, Softmax Pro5.

Results were normalized and expressed as a percentage of degranulation in relation to control samples.

Figure 10. Chemical reaction responsible for the β -hexosaminidase assay

5. Microscopy

Microscopy is the science of investigating microorganisms or other structures that cannot be seen with the naked eye. Different tissue structures, cells or molecules can be revealed depending on the specific microscopy technique employed.

5.1. Hematoxylin & Eosin staining

Hematoxylin & Eosin staining is an easy histological technique widely used to stain cell structures and components that provide a general overview of tissue sample's structures. Hematoxylin, a basic dye positively charged, stains nucleic acids with intense blue-purple colour while eosin, an acid dye negatively charged, stains pink

the cytoplasm of cells and extracellular matrix. Although these stainings are unspecific, together become a powerful tool. Eosinophils cytoplasm contains large acidophilic cytoplasmatic granules that have a high affinity for acid dyes such as eosin, consequently, eosinophils are easily identified as dark-purple two lobes nuclei cells with very pink-slightly orange cytoplasm filled with small red granules.

This technique was used in Paper III to count eosinophils in colonic tissue of IBD, IBS and HC. Biopsies were fixed in 4% paraformaldehyde for 24 hours at 4°C, embedded in paraffin blocks and sectioned at 5 µm. Slides were hydrated by submersion in decreasing concentrations of ethanol. Samples were then stained by incubating them in Harris hematoxylin (4 minutes), rinsed in water, immersion in 1% hydrochloric alcohol (10 seconds), rinsed in water, immersion in tap water with 2 to 3 ammonia drops (1 minute) and, finally, slides were incubated in eosin (1 minute), dehydrated with an increasing battery of alcohols and mounted with Pertex mounting media.

5.3. Immunofluorescence microscopy

The essence of this technique is based on the use of specific antibodies that will specifically attach to the epitopes of interest and amplify the signal with the use of photons (light). Primary antibodies detect the targeted antigens in the tissue with their variable region and leave exposed the constant region, which is host-specific. Secondary antibodies recognize and bind strongly to the constant region of the heavy chain. Commonly, secondary antibodies are conjugated with fluorophores that emit fluorescence upon photoexcitation. Table 1 contains the characteristics and details of all the antibodies used in this thesis.

A general protocol was used for Papers I – III. Biopsies were fixed, cut and hydrated by standard procedure. Slides were then incubated with background sniper (5 minutes), washed in PBS, incubated at 4°C (overnight) with the corresponding primary antibodies, washed in PBS, incubated with each respective secondary antibodies (1 hour) at room temperature, rinsed and mounted with Prolong Gold-DAPI (Figure 11).

Figure 11. General immunofluorescence protocol used to identify mast cells or eosinophils, VIP, VPACs and tight junctions.

Target	Paper	Primary antibody		Secondary antibody		
		Concentration & Host specie	Company	Concentration, Host and Target Specie	Fluoro-phore	Company
Mast cell Tryptase	I and III	1:200 Mouse monoclonal-anti-Human	Santa Cruz	1:400 Rabbit anti-Mouse	Alexa Fluor 546	Life Technologies
	II and IV			1:200 Donkey anti-Mouse	Alexa Fluor 594	Invitrogen
Eosinophil ECP	III	1:200 Mouse anti-Human	Diagnostic Development	1:200 Donkey anti-Mouse	Alexa Fluor 594	Invitrogen
	IV			1:100 Donkey anti-Mouse	Alexa Fluor	Invitrogen
VIP	I	1:25 Goat polyclonal anti-Human	Santa Cruz	1:200 Donkey anti-Goat	Alexa Fluor 488	Life Technologies
	II and IV	1:200 Rabbit polyclonal anti-Human		1:200 Donkey anti-Rabbit	Alexa Fluor 488	Life Technologies
VPAC1 / 2	I and II	1:400 Goat polyclonal anti-Human	Santa Cruz	1:200 Donkey anti-Goat	Alexa Fluor 488	Life Technologies
ZO-1	I	1:100 Rabbit polyclonal anti-Human	Life Technologies	1:200 Goat-anti-Rabbit	Alexa Fluor 546	Invitrogen
Occludin	I	1:50 Rabbit polyclonal anti-Human	Invitrogen	1:200 Goat-anti-Rabbit	Alexa Fluor 546	Invitrogen

Table 1. Characteristics and details about all the antibodies used within this thesis

Extra blocking steps with normal mouse serum and anti-mouse FAB unconjugated were needed for Paper IV in order to avoid mouse-mouse cross-activity (Figure 12).

Figure 12. Modified immunofluorescence protocol to avoid mouse-mouse cross-activity

Conventional immunofluorescence was used to detect and manually count mast cells, VIP/VPAC1/VPAC2-positive mast cells (Paper I, II and III) and the distance between mast cells and VIP (Paper II) using a widefield microscope at 400X magnification. Moreover, the thigh junctions ZO-1 and occludin were stained, evaluated with a LSM700 Zeiss Inverted Confocal at 1000X and quantified with ImageJ Fiji software.

In a bit more sophisticated fashion, a pipeline was created in the software Cell Profiler 2.2.0 to measure the activation and degranulation levels of mast cells-tryptase and eosinophils-ECP for Paper III. For that, 400X single field confocal images were taken with a LSM800 Zeiss Inverted Confocal. A total of 8-14 images of single mast cells or eosinophils per sample were collected and analysed. Briefly, the nuclei signal was used, together with tryptase or ECP signal, to delimit and segment virtual cell limits. After threshold, all tryptase or ECP signal detected inside those limits was defined as intracellular; while the signal detected outside the cellular limits were defined as extracellular and interpreted as an indication of degranulation levels. The average of integrated intensity per cell and the extracellular content surrounding the cell were measured. Finally, median values were calculated from all the averages to create a median of intensity expressed with arbitrary units (AUs).

Furthermore, a more advance image analysis called “*In situ* Cytometry” was used in Paper IV. *In situ* Cytometry is a computerized way to analyse tissue sections that enables a “flow-cytometry type” analyses. Single cells can be identified and, as in flow-cytometry, information about cell number, size and protein expression can be obtained. In advantage, since the tissue structure is intact, the interaction and cell

distribution can also be studied. The analysis used was an adaptation and simplification from John W.Wills work¹⁷³. First, 25-30 high-resolution tile scans images at 400X magnification, with 10% edge overlap, were collected on an LSM800 Zeiss Inverted Confocal (Figure 13.1). Stitched tile scans were cut up into 1.000 x 1.000 pixels individual tiles (Figure 13.2) with MATLAB, and further processed with Cell-Profiler 2.2.0. The pipeline included threshold of the images, segmentation of the tissue based on single-cell nuclei signal (Figure 13.3), identification of eosinophils (positive ECP cells) and mast cells (positive tryptase cells), quantification of intra- and extracellular ECP, tryptase and VIP and, finally, their general localization in the tissue. All the cell features were written in MATLAB objects for subsequent analysis. Here, images were reassembled and spatial localization for each detected cell in the tissue was calculated (Figure 13.4). Cells identified as mast cells or eosinophils could be displayed in scatter plots and gated manually, discarding false possible or double-positive cells (Figure 13.5). Finally, the closest distance between mast cells, eosinophils and VIP was calculated with the original MATLAB code and visualised with the module nearestneighbour.m ©2006, Richard Brown (Figure 13.6).

Figure 13. Graphic summary of the pipeline followed for the advance image analysis “*In situ* Cytometry” of mast cells, eosinophils and VIP in IBD samples.

In summary, the cell features measured and included in the following analysis were:

- Percentage of mast cell and eosinophils
- Extracellular or degranulated tryptase (tryptase^{deg})
- Intracellular tryptase per mast cell (tryptaseⁱⁿ)
- Extracellular or degranulated ECP (ECP^{deg})
- Intracellular ECP per eosinophil (ECPⁱⁿ)
- VIP, measured only outside mast cells and eosinophils
- Distance between mast cell – eosinophils- VIP

5.4. Electron microscopy

Electron microscopy is a recognized image technique employed for the characterization of nanomaterial and ultrastructure. Super high-resolution images are obtained with electron microscopes. These use a beam of electrons that either penetrates the extremely thin sample, known as transmission electron microscopy (TEM); or across the surface, known as scanning electron microscopy (SEM).

In Paper I, electron microscopy was used to identifying bacterial translocation and mast cell ultrastructure after exposing colonic biopsies to bacteria in the Ussing chamber. In Paper III, TEM was used to identify and illustrate mast cell and eosinophil activation.

Biopsies from all groups were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (24-72 hours), dehydrated and stored at 4°C until further processed following standard protocols for SEM, using an S-570 (Hitachi Ltd, Japan); and TEM, using a Hitachi H-1400.

6. Statistical analysis

All studies included in the present thesis shared the following statistics. The distribution of data was assessed using Shapiro-Wilk normality tests. Comparisons between parametric groups were performed with Student t-test, for two groups; or ANOVA test, for more than two groups followed by Post-Hoc analysis. Values were given as mean $\pm$ SEM. Comparisons between non-parametric were performed with Mann-Whitney U test, for two groups; or Kruskal-Wallis test, for more than two groups followed by Post-Hoc analysis. Values were given as median (25th-75th percentile). Correlations to determine the association between variables were conducted using Pearson's (r) test for non-parametric analysis or Spearman's (p) test for parametric data. ANOVA/ANCOVA tests and linear/logistic regression were used to determine interactions on continues variables; and Chi-square tests, for interactions between categorical variables. Statistical analyses were performed with GraphPad Prism 8 and IBM SPSS Statistics 24 Software. Differences of $p < 0.05$ were considered significant.

7. Methodological considerations

Although some of the techniques employed in this thesis are powerful tools, they present important limitations to bear in mind.

The *ex vivo* model Ussing chamber requires the use of very similar biopsies to obtain good permeability and electrophysiology results. This experiment also requires fresh and viable specimens, that are very delicate to handle and which intestinal representation corresponds to a very tiny portion of the intestinal mucosa. There are other alternative methods to measure human permeability, like multi-sugar permeability test that consists of the ingestion of a mixture of five non-invasive sugar probes that are absorbed in different points in the intestine. They can subsequently be measured in urine and reflect the intestinal permeability function.

Animal models are usually a good alternative to study causality, mechanisms and disease progression in a more complex system. Although the DSS mice model used is well-characterized, the colitis obtained is derived from an external hazard, thus not mimicking the aetiology of IBD.

The cell line HMC-1.1 is a cheap, easy and useful experimental tool that provides very detailed mechanistic studies on cellular functions. However, it is an immature cell line poorly granulated with drastically reduced levels of tryptase and chymase, compared with primary mast cells. Alternative, the mast cell line LAD2 presents a more mature profile and abundant granules. Sadly, this cell line is very slow and expensive to grow. Other mast cell lines, such as LUVA and ROSA, are very promising cell lines but their access is still very limited.

Different microscopy techniques were used in this thesis to provide information about the number, localization and activation levels of the cells of interest. Nevertheless, this methodology only describes a very limited area that, hopefully, is representative of the tissue. Moreover, microscopy is unable to provide comprehensive information about causality and interactions over time.

*“When you see the results,
you forget the pain”*

- Avi Arad

Main Results

The following section presents a brief overview of the main findings of this thesis, starting with a summary of the aims and main results of each paper. Detailed descriptions are given in the respective paper.

Paper I

- **Aims:** To investigate the barrier function of IBS patients, specifically the passage of bacteria, and its regulation via mast cell - VIP.
- **Main Results:** IBS patients present increased transcellular bacterial passage, with mast cell and VIP involved as modulating factors.

Paper II

- **Aims:** To map mast cells and their expression of VPACs and proximity to VIP in different regions of the intestine in murine colitis, UC and CD patients.
- **Main Results:** IBD patients and mice with colitis had increased number of mast cells expressing VPAC1 and more mast cells co-localizing/close to VIP. In CD, areas next to Peyer's patches were more involved in the inflammation process.

Paper III

- **Aims:** To search for similarities and differences between UC in remission and IBS patients, compared to controls, regarding barrier function and immune cells.
- **Main Results:** UC and IBS patients presented more mast cells and eosinophils, and an disturbed intestinal permeability, compared with controls. UC's increased barrier function might be closely related with eosinophil activation levels.

Paper IV

- **Aims:** To investigate the alterations of eosinophils, mast cells and VIP in relation to inflammation, IBD and biological treatment.
- **Main Results:** An increased crosstalk between eosinophils-mast cells-VIP was detected during inflammation. Eosinophils and VIP seem to play a regulatory role on mast cells. Local levels of VIP may be related with disease activity and could be a potential biomarker for treatment outcome.

Paper I -

Vasoactive Intestinal Polypeptide and Mast Cells regulate increased passage of colonic bacteria in patients with Irritable Bowel Syndrome

This is the first study demonstrating an increased transepithelial passage of live bacteria in human subjects with IBS and identifies VIP as a regulatory molecule of this mechanism.

Increased trans- and paracellular permeability in IBS

Female IBS subjects showed a greater colonic paracellular permeability and an increased permeability to both live commensal and pathogenic bacteria (Figure 14A), which endocytosis was identified through the transcellular pathway (Figure 14B).

Bacterial passages were not intercorrelated, possibly implicating different uptake mechanisms of the commensal and pathogenic bacteria strains. Interestingly, the presence of pathologic bacteria induced a decrease of the TER only in IBS patients.

Figure 14. (A) Bacterial passage through colonic biopsies mounted in Ussing chambers for 120 minutes. (B) TEM illustrating interaction of *Salmonella* (black arrow) and microvilli (white arrow) and uptaken through endocytosis. Comparisons were performed with Mann-Whitney U test, *** $p < 0.0005$

Increased VIP, Mast cells, and Mast cells expressing VPAC1

VIP levels in plasma, but not tissue, of IBS patients was higher than in controls.

Microscopy analysis of colonic biopsies revealed an increased number of mast cells compared with HC. Mast cells showed signs of activation and active release, which correlated with local tryptase levels, also found higher in patients.

IBS samples presented an increased number of mast cells expressing VPAC1, but not VPAC2, compared to controls.

Regulation of barrier function by VIP and Mast Cells

Pre-incubation with both ketotifen (mast cell stabilizer) and anti-VPACs diminished the bacterial passage in all groups (Figure 15), while only the pre-incubation with ketotifen inhibited TER decrease in IBS patients.

Occludin tight junction protein was shown downregulated in IBS compared to HC, although the addition of pathogenic bacteria decreased occludin expression in both groups. In IBS, decreased occludin expression due to bacteria was significantly inhibited by ketotifen. ZO-1 tight junction was also monitored, but no differences or expression changes were detected between groups.

Figure 15. Influence of VIP and mast cells on (A) *E. coli* HS and (B) *Salmonella* passage through colonic biopsies mounted in Ussing chambers for 120 minutes. Comparisons were performed with Mann-Whitney U test, ** $p < 0.005$, *** $p < 0.0005$ vs vehicle.

Paper II -

Upregulation of intestinal mucosal Mast Cells expressing VPAC1 in close proximity to Vasoactive Intestinal Polypeptide in Inflammatory Bowel Disease and murine colitis

In this study, we did not only find an increased number of mast cells in IBD and murine colitis, but we also described their distribution in the tissue, their expression of VPACs and their interaction with VIP.

Increased number of Mast Cells and Mast Cells expressing VPAC1

The highest amount of mast cells was detected in the colon of UC patients and murine colitis, followed by the adjacent VE to FAE of CD patients. Colonic and adjacent VE mast cell amounts of IBD were significantly increased to their respective HC areas, but not VE. Almost no mast cells were detected in the follicles.

The number of mast cells expressing VPAC1 was also increased in IBD patients. Their spatial layout followed the same distribution than the described for mast cells (Figure 16).

Figure 16. Percentage of VPAC1 positive mast cells count by immunofluorescence in regular VE and adjacent VE in (A) CD and colon in (B) UC patients. Photomicrographs illustrate the expression of VPAC1 (green) and tryptase (red). Comparisons were performed with Mann-Whitney U test, * $p < 0.05$.

Increased levels of VIP in IBD and murine colitis

The VIP plasma levels of IBD was higher than controls. When comparing IBD sub-groups, UC patients presented higher VIP than the CD.

Interestingly, when measured locally in the tissue, VIP levels followed the same pattern found for mast cells and mast cells expressing VPAC1. That is, colonic tissue from UC patients displayed the highest levels, followed by colon from murine colitis and adjacent VE of CD patients, being all those significantly increased compared to controls.

Mast cells – VIP interaction

The proximity between mast cells and VIP studies revealed a higher percentage of mast cells close to or co-localizing with VIP in IBD compared to HC. The percentage of mast cells nearby or co-localizing with VIP per area followed the same fashion than the previously described for mast cells, VPAC1 and VIP.

Lastly, *in vitro* studies demonstrated the ability of VIP inducing mast cell degranulation (Figure 17).

Figure 17. Degranulation effects of VIP on human mast cells HMC-1.1. Comparisons were performed with Kruskal-Wallis test followed with Dunn's multiple comparisons test, ** $p < 0.005$, $n = 4$.

Paper III -

Increased colonic epithelial permeability and mucosal eosinophilia in Ulcerative Colitis in remission compared with Irritable Bowel Syndrome and health

In this comprehensive work, we were able to study the similarities and differences between HC, IBS and UC in remission, comparisons that are lacking in the literature.

The intestinal barrier function of UC and IBS is significantly disrupted

Biopsies from UC in remission and IBS patients showed decreased TER and increased paracellular permeability compared with controls.

Mast cells differences between UC, IBS and controls

Colonic biopsies from both UC in remission and IBS patients showed an increased number of mast cells compared to controls. Surprisingly, intracellular tryptase amounts in mast cells were found reduced only in UC remission (Figure 18), while plasma levels of TNF were increased.

Figure 18. (A) Quantification of mast cells in colonic biopsies from UC remission, IBS and HC. (B) Representative immunofluorescence images of mast cells with virtual identification of plasma cell membrane (white outline), intracellular tryptase (red) and extracellular tryptase (green). (C) TEM illustrating ultrastructural partial degranulation (white arrows). Comparisons were performed with Mann-Whitney U test, ** $p < 0.005$.

Eosinophils differences between UC, IBS and controls

Eosinophil count using Hematoxylin&Eosin staining (Figure 19) showed that, unlike mast cells, colonic biopsies from UC remission patients had an increased number of eosinophils compared to both controls and IBS, while IBS was higher than controls (Figure 20A). These being also correlated with paracellular permeability.

Interestingly, the number of mast cells and eosinophils correlated negatively.

Figure 19. Photographs illustrating cells positive for eosin (arrows) in the colonic mucosa of UC remission, IBS and HC at 600X magnification.

Eosinophils degranulation of ECP was increased ECP in UC remission compared with controls and presented a trend, without reaching significance, in IBS (Figure 20 B-C).

Figure 20. (A) Quantification of eosinophils in colonic biopsies from UC remission, IBS and HC. (B) Representative immunofluorescence images of eosinophils with virtual identification of plasma cell membrane (white outline), intracellular ECP (blue) and extracellular ECP (magenta). (C) TEM illustrating ultrastructural partial degranulation (white arrows). Comparisons were performed with Mann-Whitney U test, ** $p < 0.005$, *** $p < 0.0005$.

Paper IV -

Eosinophils inhibitory role on Mast Cells and Vasoactive Intestinal Polypeptide as possible biomarkers for biological treatment in Inflammatory Bowel Disease

In this study we showed interactions between mast cells and eosinophils, as well as with VIP, that may be altered during intestinal inflammation. Such interactions also seem to be associated with the course of IBD and the response to biological treatments.

Eosinophils may inhibit mast cells in non-inflamed tissues

Although the percentage of mast cells and eosinophil was not different in inflamed/non-inflamed tissue, cell proximity was closer in inflamed tissues (Figure 21A). Increased cross-talk with possible inhibitory effects of eosinophils on mast cells in non-inflamed tissues was hypothesised since ECP levels correlated negatively with the number of mast cells and degranulated tryptase (Figure 21B).

Figure 21. (A) Distance in pixels between mast cells and eosinophils in inflamed and non-inflamed tissues from IBD. Comparison was performed with Student T test, ** $p < 0.01$. (B) Relationship between degranulated tryptase and degranulated ECP. Figure also includes the results from the linear regression test showing the relationship of these variables under inflammatory

Mast cells –VIP crosstalk is altered during inflammation

Amounts of VIP were higher in non-inflamed tissues (Figure 22A), and correlated positively with mast cell number, suggesting a possible mast cell-recruitment via VIP. Surprisingly, activation levels of mast cells correlated negatively with VIP only in non-inflamed tissues (Figure 22B).

Figure 22. (A) VIP amounts in inflamed and non-inflamed IBD tissues. Comparison was performed with Student T test, * $p < 0.05$. (B) Relationship between intracellular tryptase and VIP. Figure also includes the results from the linear regression test showing the relationship of these variables under inflammatory conditions.

Differences between UC and CD

The main differences found between UC and CD patients were associated with eosinophils. UC presented an increased EDN degranulation. Moreover, the number of eosinophils in UC patients was correlated positively with VIP, suggesting chemotaxis. Moreover, VIP levels correlated negatively with UC disease activity.

Changes due to biological treatment

Patients that did not respond successfully to the biological treatment after three months presented a lower number and activation of mast cells (Figure 23A). VIP levels were enhanced after treatment (Figure 23B).

Figure 23. Amount of (A) mast cells and (B) VIP at baseline and after three-months treatment in biopsies from UC patients grouped in Responders, if response to biological agents was successful; or Otherwise, if not. Comparison were performed with one-way ANOVA test, * $p < 0.05$.

*“La ciencia es todo aquello
sobre lo cual siempre cabe discusión.”*

- José Ortega y Gasset

Discussion

The body is constructed with very different elements that synchronize to form a beautiful and complex piece of machinery whose goal is to maintain the general homeostasis. The GI tract is one of the largest organs with functions that cover from the digestion of nourishments and the acquisition of energy, through the defence against any harmful microbes, to the perception of gut flora signals and modulation of the emotional state. Disturbances in the bidirectional communication between the gut and the brain are implicated in a wide range of disorders, such as IBS and IBD. Both IBS and IBD are characterized by an increased intestinal barrier dysfunction, a pro-inflammatory chronic condition and an altered gut-brain axis. Despite all the scientific effort, it is still unknown the sequence or exact combination of events that drive to these diseases, and the exact role of every single component. We hypothesised that mast cells and eosinophils, two key cells of the immune system, and their cross-talk with VIP, an extremely pleiotropic neurotransmitter, are closely involved in the maintenance of inflammation and the regulation of the intestinal epithelial barrier. Thus, the main aim of this thesis was to elucidate the potential involvement of mast cells and eosinophils in IBS and IBD, as well as the neuro-immune intercellular circuit via VIP that might exacerbate mucosal inflammation and intestinal barrier disruption.

In a rough outline, the first work included in this thesis demonstrated for the very first time an increased transcellular passage of live commensal and pathogenic bacteria through the colonic mucosa of women with IBS, identifying VIP as a key regulatory molecule together with mast cells activation. VIP-mast cell interactions were further analysed in IBD to find that mast cell-VIP proximity in patients was closer compared to controls. *In vitro* experiments revealed the ability of VIP to activate mast cells. Although several studies have made evident that mast cell role in IBS and IBD is relevant, little is known about the resemblances between them since most studies compare disease with health conditions. Consequently, we combined the data from Paper I and II with some complementary experiments to study the similarities and differences of mast cells and eosinophils. Results indicated a pronounced increased intestinal permeability in UC, even in remission, followed by IBS, compared to healthy controls. Using image analysis, we identified the eosinophil number and activation correlating with permeability results. Further optimization of the image analysis was used in our last work^(Paper IV) to investigate the alterations of eosinophils, mast cells and VIP concerning inflammation and three-months response to biological treatment. This revealed an inhibitory effect of eosinophils on mast cells and an altered interaction between mast cells and VIP under inflammatory conditions. Lastly, intestinal VIP levels were shown increased in IBD

patients after the treatment with biological agents and were suggested as a possible biomarker for treatment outcome.

The main results of this thesis are summarized in Figure 24.

Figure 24. Compilation of the main results described in this thesis. The figure illustrates the hypothesised neuro-immune interactions that occur between mast cells, eosinophils and vasoactive intestinal polypeptide (VIP) in irritable bowel syndrome (IBS) and inflammatory bowel diseases (IBD). ENS: enteric nervous system, TNF: tumour necrosis factor, VPAC1: VIP receptor 1. Novel findings are highlighted with “NEW!”.

Disrupted permeability

As mention earlier in the introduction, it is still under discussion if the intestinal barrier dysfunction that IBS and IBD patients present is an early event in the disease^{119,120}, or if it is a consequence of the raging inflammation¹²¹. Our findings from Paper I and III confirmed structural and functional alterations of the intestinal barrier in IBD and IBS patients. The tight junction occludin was significantly decreased in IBS, and the paracellular permeability was observed increased in both IBD and IBS, compared to controls. This increase was more evident in UC patients in remission compared with IBS patients. Our observations are in line with a previous study that showed increased colonic permeability to orally administrated ⁵¹Cr-EDTA in UC and IBS-D compared with controls¹⁷⁴, a fact that is widely associated with tight junction alterations^{13,175,176}. All together supports the idea that increased paracellular permeability might be a pathophysiological feature typical for both disorders.

IBS and IBD patients also present an abnormal gut microbiota or dysbiosis^{177-179*} that seems determined, at least partially, by the host immune system¹⁷⁷. It is hypothesised that a combination of an inherited disrupted intestinal permeability, an abnormal immunological response to the microbiota and episodes of gastroenteritis (about the 10% of the cases)¹⁸⁰ could result in the alteration of motility¹⁸¹, permeability, and visceral pain, characteristics in IBS and IBD.

Under physiological conditions, pathogenic but not commensal bacteria invade the intact epithelium and are recognized by a range of toll-like receptors (TLRs), which are mostly expressed in the basolateral side of epithelial cells. TLRs can recognize the flagellin of translocating bacteria. It has been shown that TLR5 activation triggers inflammatory processes containing the infection and eliminating the damaging insult¹⁸². An injured intestinal epithelium gives free access to both opportunistic commensals and pathogens to the inner mucosa. In this situation, commensal bacteria can also induce a pro-inflammatory response by activating the downstream TLR5 signalling¹⁸³.

The first work included in this thesis demonstrated for the very first time an increased passage of live commensal and pathogenic bacteria through the colonic mucosa of women with IBS. We used the bacterium *Escherichia coli* O9:H4 (*E. coli* HS) and *Salmonella enterica* serovar Typhimurium as commensal and pathogenic bacteria, respectively, to study their mucosal permeability in *ex vivo* models. Bacteria passed through the colonic mucosa of IBS tissue to a significantly higher extent in comparison to controls. Altogether suggest that bacteria and its metabolites can directly interact with the immune and the nervous systems of IBS to a greater degree than in healthy conditions as a possible result of a damaged intestinal barrier.

Unlike the previous studies¹⁸⁴, our approach used live bacteria, thereby mimicking better *in vivo* conditions. Despite that fact, it is important to mention that one of the components of the intestinal barrier, the mucus that lays on the intestinal epithelium, might be removed during Ussing chamber experiments due to the gas flow. This fact could contribute to bacterial gain access through the tissue¹⁸⁵, and could explain why there was no significant correlation between symptom severity and bacterial passage.

Besides the possibility of a damaged epithelial barrier due to experimental or intrinsic conditions, our study of the bacteria passage revealed no bacteria entering the mucosa through the paracellular space, only transcellular, suggesting that paracellular dysfunction may be secondary to the immune signals triggered by the bacteria invasion, as others have described^{186*}.

We also presume that the transcellular uptake mechanisms of commensal and pathogenic bacteria may be different from each other and enhanced in IBS patients. A newly released study from our group showed a relation between the fragmentation of the epithelial mitochondrial network and the reduction in barrier function as a result of the infection with pathogenic, but not dead or commensal, bacteria¹⁸⁷. This novel aspect could be relevant for the initiation or disease relapse of GI disorders.

Although we did not evaluate the bacterial permeability in IBD, many other studies have demonstrated an increased bacterial translocation in patients compared to controls. Further, IBD subjects show a higher incidence of pathogenic bacteria translocation, such as *Clostridium*, especially during active phase¹⁸⁸. Some of these research have been based on bacterial-culture studies. But, more recently, molecular techniques have used bacterial RNA/DNA from blood and tissues as surrogate markers enabling a deeper analysis and the identification of unculturable bacteria¹⁸⁹. As a case in point, a recent study confirmed a distinctly different microbial profile between CD and UC lymph nodes that could explain their different mechanisms of development and prognosis¹⁹⁰. These examinations would require fine-tuned *in vivo* and *ex vivo* models to help explain the underlying mechanism and linearity of the facts.

In vitro studies may be the best choice to explore these mechanisms. Clark *et al* conducted an *in vitro* study with Caco-2 cells, where they demonstrated that low concentrations of interferon-gamma (IFN- γ) induces the transcellular influx of non-pathogenic and non-invasive bacteria without altering cell viability nor TER¹⁹¹. Another main cytokine that showed to induce bacterial translocation is TNF via regulation of TLR4 expression¹⁹². Interestingly, we demonstrated that the addition of *E. coli* does not modify intestinal barrier features. The addition of *Salmonella*, on the other hand, induces a substantial decrease of the TER, an increasing trend of

the paracellular permeability of ^{51}Cr -EDTA, and a decrease of the tight junction occludin. In line with our findings, previous research has shown that *Salmonella* Typhimurium, but not commensal bacteria, causes the decrease of TER and redistribution of tight junctions in *in vitro* conditions¹⁹³. Complementary *in vivo* models with enteric bacterial dysbiosis and a low-grade inflammation without tight junction disruption are needed to support these findings.

A very recent study has shed some light on the matter and showed that the presence of intraepithelial bacteria preceded tight junction damage in IBD animal models and that bacterial transcytosis was induced by low doses of the pro-inflammatory cytokines TNF and IFN- γ , whereas high-doses caused tight junction disruption¹⁹⁴.

As previously discussed in the introduction, it is well-known that the intestinal barrier is highly regulated by immune factors^{9,45}. Evidence for mucosal immune activation in GI disorders is supported by the higher accumulation of mast cells, particularly in IBD and less evident in IBS. Here, we demonstrated a significantly increased amount of mast cells in IBS and IBD in comparison to controls. When comparing UC remission and IBS or CD remission, no differences in the number of mast cells were detected (Paper III, IV). This could confirm that mast cells are contributing factors in the pathogenesis of these GI disorders.

Functional experiments from Paper I confirmed that physiological barrier function and bacterial passage are regulated by mast cell activation via VIP, which upregulation may be stress-related. Only a few reports are examining the influence of VIP in IBS. VIP plasma levels have been reported increased in IBS compared with controls by us (Paper I) and others¹⁹⁵, and in stress animal models¹⁹⁶. Recent studies demonstrated that VIP is involved in colonic functions and diarrhoea as a downstream effector of CRH¹¹⁰ induced by stress, anxiety or depression¹¹¹.

Further, intestinal eosinophils have been shown to interact with mast cells during inflammation⁷¹. Accordingly, we detected closer proximity between these two cells in inflamed tissues of IBD-remission (Paper IV). Unlike mast cells (Paper I, III), we observed a positive correlation between the number of eosinophils and paracellular permeability in UC-remission subjects (Paper III). This might be interpreted at first that the eosinophil number could have a higher impact on paracellular permeability than mast cells. However, it could also be inferred that the increased amount and/or activation of eosinophils in inflamed tissues give rise to an enhanced secretion of CRH. In turn, CRH can activate mast cells, whose released mediators such as TNF, IL-3, IL-4, IL-5, IL-16, and IFN γ , as discussed previously^{112,113}, and cause barrier dysfunction. In our study, TNF levels were higher in plasma of UC patients compared with both IBS and HC patients. Altogether, the correlation observed between paracellular permeability and eosinophil numbers appear to be an indirect involvement of mast cells as a result of stress or enhanced nervous activity.

Neuro-immune alterations

Although it is common to find an increased number of mast cells in IBS patients, this result is not always replicable, and it has been shown to be IBS-subtype dependent¹⁹⁷. A more relevant and consistent mast cell trait that is almost always increased in IBS is the mast cell activation pattern. Thanks to the ultrastructural study of these cells, we showed a more pronounced sign of activation in IBS and IBD patients^(Paper I, III). Thus, mast cell indirect action weakening the tight junctions and increases bacterial translocation might be related to the activation state rather than the number of mast cells.

When activated, mast cells undergo a degranulation process that leads to the release of mediators. Tryptase is one of the most important mast cell pro-inflammatory mediators, commonly increased in GI disorders¹⁰². In our hands, IBS biopsies expressed higher levels of tryptase, compared to controls^(Paper I); as well as IBD-inflamed tissues, compared to non-inflamed^(Paper IV). Unexpectedly, we also found similar tryptase release in IBS, UC remission and controls^(Paper III). This last result could be due to the small sample size or methodological issues. The resolution of a confocal microscope might not be enough to identify differences in the activation levels of individual mast cells, as we did with electron microscopy. Also, it has been previously reported that a very high mast cell degranulation by an anaphylactic fashion, may lead to their miscount¹⁴¹. The quantification of total tryptase in the entire tissue, likewise in Paper IV, instead of in individual cells, could have solved this issue.

Another important mast cell mediator that promotes innate immune-mediated immunopathology and damage associated with mucosal inflammation in GI disorders is histamine via histamine 4 receptor¹⁹⁸. Elevated mucosal histamine¹⁹⁹ and its metabolite excretion²⁰⁰ correlate well with IBD patient disease activity.

Besides the mast cell number and activation levels, we also studied their anatomical distribution. In Paper III we showed that mast cells accumulate uniformly in the ileum of non-IBD individuals. While in CD, mast cells gather significantly more in VE adjacent to FAE. This suggests that adjacent VE to FAE is a more active site in CD, where probably mast cells regulate the entrance of insults and originate inflammation because of a disturbed permeability and/or an enhanced neuro-immune input.

The mast cell–nerve unit classically has represented a fundamental neuro-immune axis in the development of inflammation associated with intestinal barrier disruption. For this thesis, we focused on neuro-immune communication via VIP. Mast cells expressing VIP or near VIP were more frequently observed in adjacent VE to FAE in CD and colonic UC, compared to controls^(Paper II). Our results suggest that

the intestinal dysfunction may not be uniformly affected along the gut. Experiments performed in rats by Zhang *et al* recently showed that FAE areas are more susceptible to stress than VE, being mast cell tryptase and PAR2 expression crucial in this stress-induced barrier alteration¹⁹⁶. Similarly, our group also had previously shown that VIP increased bacterial passage in human FAE via the activation of VPACs on mucosal mast cells⁴⁰. Moreover, stress-increased intestinal permeability in rats was abolished with mast cell stabilizers and anti-VPACs⁴⁰. Here, we demonstrated that mast cells expressing VPAC1 were increased in IBS, in adjacent VE to FAE in CD and colonic UC^(Paper I, II). It is worth to mention that the activation of VPAC1 has been described to increase anion and water secretion, with resultant diarrhoea³², enhanced colitis^{35,36}, and mast cell activation^(Paper II) which may affect disease activity.

In our experience, intestinal VIP in relation to mast cells exerts mostly pro-inflammatory actions²⁰¹. Nevertheless, when studying local mast cell-VIP relation in relation to inflammation, we found new results that established a more complicated impression. In Paper IV, non-inflamed IBD biopsies presented higher amounts of VIP compared to inflamed biopsies. Exist the possibility that the observed lower levels of VIP in inflamed areas is the consequence of VIP degradation, perhaps by tryptase as previously shown in human lungs²⁰²; neuronal cell death, associated with the appearance of eosinophilic and neutrophilic infiltrates²⁰³; or VIP mRNA down-regulation by the pro-inflammatory cytokine IL-6¹³².

In agreement with our results, Jönsson *et al* showed a local production of VIP in normal and slightly/moderated inflamed but not in severe inflamed colonic mucosa of UC, together with a marked downregulation in VPAC1 receptor expression²⁰⁴. These results could indicate that VIP may be playing an anti-inflammatory role and its decrease may be associated with adverse effects in the intestinal function. Moreover, this anti-inflammatory theory would be supported by the fact that non-inflamed areas held less activated mast cells, which also correlated negatively with VIP amounts^(Paper IV). VIP pleiotropic functions have loaded the literature with contradictive results supporting the promotion and the inhibition of inflammation via VPAC1^{25,29,132,195} and VPAC2^{30,31}, respectively. In view of the fact that we did not study the status of mentioned receptors in inflamed and non-inflamed tissues, the anti-inflammatory theory that involves VPAC2 remains unsolved.

Another possibility could be that VIP anti-inflammatory effect is induced indirectly through the activation of other cells, for instance, eosinophils. It has been reported that human eosinophils highly express VIP associated receptor CRTH2, instead of the conventional VPAC1 and VPAC2⁷⁶, which is responsible for their recruitment and accumulation in the tissue⁷⁷. The positive correlation between the percentage of eosinophils and local VIP levels that we found^(Paper IV) could be an indicator of

the previously reported chemotaxis effect¹⁵⁵. Moreover, in Paper III and IV, histological staining and image analysis revealed significantly more eosinophils with higher degranulation in both inactive UC and IBS compared with controls, being higher in UC compared to IBS. In line with our results, previous studies have shown an increased release of eosinophils granules, such as ECP, in non-inflamed mucosa of patients with UC compared with controls²⁰⁵.

The hypothesis that eosinophils play a regulatory role during the remission phase is supported by growing facts. We showed that eosinophils and mast cell count correlated negatively in IBS and in UC remission^(Paper III, IV) and that eosinophils activation is increased in inactive UC compared with active¹⁵⁶. Despite the fact that we did not study eosinophils status in both phases, only during remission, we did evaluate eosinophils activation and degranulation levels in inflamed and non-inflamed biopsies to show that the ECP levels and the degranulated tryptase^(Paper IV) negatively correlate only in non-inflamed conditions. A study in murine colitis proved that eosinophil-deficient mice developed significantly more severe colitis due to a compensatory inflammatory effect from part of neutrophils, suggesting eosinophils to exert a protective effect in acute colitis via the production of anti-inflammatory lipid mediators²⁰⁶. Altogether, these findings suggest a protective role of eosinophils in the repair of the injured mucosa.

Clinical relevance

In Paper IV we identified eosinophil markers differently expressed in UC and CD, with higher degranulated ECP in CD. Even though there were too few subjects to draw solid conclusions, it is not the first time that differences have been described between UC and CD. The number and activation levels of CD intestinal eosinophils seem to reflect disease activity¹⁵², thus being diminished during quiescent and increased during flare. The differences between CD-UC, and even with IBS, seem to be closely related with the cytokine milieu. IL-5 and other Th2 cytokines, found in higher concentrations in UC compared with CD, seem to be the main candidates responsible for the eosinophils phenotype^{149,158}. A recent study profiled the systemic inflammatory proteins from UC, IBS, and healthy subjects. Results revealed a clear pro-inflammatory profile in UC patients that could be used to differentiate them from non-UC, and between active and remission phase. The systemic inflammatory profile could also distinguish between UC and IBS patients, independently of the presence of IBS-like symptoms or inflammation²⁰⁷.

Many shreds of evidence have shown an involvement of mast cell in the generation of IBS symptoms, mostly hypersensitivity. A recent study demonstrated that histamine receptors 1-mediated sensitization of transient receptor potential channel type V1 (TRPV1), a well-studied nociceptor that contributes to neuro-immune in-

flammation²⁰⁸, was directly implicated in the hypersensitivity, symptoms, and abdominal pain that IBS patients suffer²⁰⁹. Although some studies have correlated mast cell count with visceral hypersensitivity in IBS¹⁰⁴, we did not find any correlation between tryptase or mast cell infiltration and IBS or IBD symptoms^(Paper I, III).

Concerning biological agents, we found that after three-months treatment the number of mast cells was increased in patients that responded satisfactorily^(Paper IV). This finding could suggest that, for the treatment to be successful, the presence of mast cells is pertinent. Exists the possibility that mast cells are still being recruited to the tissue, but that they are not as active because of the TNF inhibition.

Surprisingly, we did find a negative correlation between UC disease activity and VIP local levels, suggesting an association between VIP and the improvement of the disease^(Paper IV). Some animal studies have shown intestinal histological recovery and reduction of pro-inflammatory cytokines because of extrinsic VIP administration^{29,210}. To our knowledge, no previous reports are correlating local VIP amounts with disease activity in UC.

Besides, we also detected an increase of this neuropeptide after three-months treatment, being its presence related to treatment outcome^(Paper IV). Although more studies are needed, VIP could be a good biomarker to predict the response to biological agents.

To conclude, the intricate neuro-immune interactions are crucial in controlling the gut homeostasis and can lead to barrier dysfunction. Today, the potential link between inflammatory mechanisms are unclear and it is implausible to conclude whether VIP-eosinophils-mast cell interactions are pathogenic, or whether if they are an attempt to control inflammation. Nonetheless, the findings from this thesis suggest mast cells to be a key cell in the alteration of the intestinal barrier function that acts after their neuro-stimulation through VIP and immune-regulation by eosinophils.

Conclusions

In the present thesis, we demonstrated that the neuro-immune communication between VIP, mast cells and eosinophils is fundamental in the regulation of the mucosal barrier function and intestinal homeostasis. More specifically:

- An exacerbated or altered neuro-immune activity can lead to barrier dysfunction and potentially cause disease.
- IBS and IBD patients present an altered barrier function with simultaneous alteration of the paracellular pathway and the transcytotic uptake route, being considered a typical pathophysiological feature that engages mast cells, eosinophils and VIP as key regulatory factors.
- Mast cells contribute to the acute and chronic inflammatory response, especially in the epithelium adjacent to the Peyer's patches. Upon activation, mast cells release a range of pro-inflammatory mediators that may lead to an inappropriate neuro-immune activation that goes on to increased bacterial passage, mucosal inflammation, neural injury and hypersensitivity.
- Eosinophils role in GI disorders is still not fully elucidated. Their presence seems directly associated with VIP and indirectly related to increased paracellular permeability. At the same time, eosinophils may play a key modulatory role on activated mast cells and repairing the tissue.
- VIP is a pleiotropic molecule involved in IBS and IBD pathology, which local amounts influence the barrier function and the treatment outcome of biological agents. Neuro-immune interactions and neuroplastic changes are conditional to the surrounding milieu and participate in GI disorders physiopathology by still not fully elucidated means.
- Although IBS, UC, and CD share pathophysiological characteristics, they are different entities with different underlying altered mechanisms that define them.

This thesis includes novel findings highlight the importance of neuro-immuno-regulation in IBS and IBD and broadens the knowledge on their pathophysiology suggesting mast cell – VIP – eosinophils communication a possible interesting therapeutic target.

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