Universitat Autònoma de Barcelona

MOLECULAR MECHANISMS AND EFFECT OF ACUTE AND PSYCHOSOCIAL STRESS ON THE INTESTINAL BARRIER FUNCTION. IMPLICATIONS ON THE IRRITABLE BOWEL SYNDROME

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MOLECULAR MECHANISMS AND EFFECT OF ACUTE AND PSYCHOSOCIAL STRESS ON THE INTESTINAL BARRIER FUNCTION. IMPLICATIONS ON THE IRRITABLE BOWEL SYNDROME.

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HACEN CONSTAR

Que la tesis titulada “Molecular mechanisms and effect of acute and psychosocial stress on the intestinal barrier function. Implications on the irritable bowel syndrome.” presentada por **Marc Pigrau Pastor** para optar al grado de Doctor, se ha realizado bajo su dirección, y al considerarla concluida, autorizan su presentación para ser juzgada por el tribunal correspondiente.

Y para que conste a los efectos firman la presente.

Barcelona, Septiembre de 2018

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“Science is not only a disciple of reason but, also, one of romance and passion”.
Stephen Hawking (1942-2018)

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A Ceci y a mis padres.

ABBREVIATIONS

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5-HT: 5-hydroxy tryptamine
ACTH: adrenocorticotropic hormone
ADHD: attention deficit and hyperactivity disorder
AJ: adherens junctions
ANOVA: analysis of the variance
AVP: arginine-vasopressin
BDI: Beck's depression Inventory
BGA: brain-gut axis
BMAL1: brain–muscle–arnt-like protein 1
BMI: body mass index
bpm: beats per minute
CB1: cannabinoid 1 receptor
CGRP: calcitonin gene-related peptide
CLOCK: circadian locomotor output cycle kaput
CLRs: C-type lectins receptors
CNS: central nervous system
CP: canonical pathway
CPS: cold pain stress
CRH: corticotrophin-releasing hormone
DAMPs: danger-associated molecular patterns
DBP: diastolic blood pressure
DHEA: dehydroepiandrosterone
ENS: enteric nervous system
F: female
FC: fold change
FD: functional dyspepsia
FE: fractional excretion
FGID: functional gastrointestinal disorders
Fx: functions
GALT: gut-associated lymphoid tissue
GI: gastrointestinal
GR: glucocorticoid receptor
H-R: modified social readjustment scale of Holmes-Rahe

ABBREVIATIONS

HPA: hypophysis-pituitary-adrenal
HPF: high power field
HR: heart rate
HV: healthy volunteer
IBD: inflammatory bowel disease
IBS: irritable bowel syndrome
IBS-C: constipation-predominant IBS
IBS-D: diarrhea-predominant irritable bowel syndrome
IBS-SSS: irritable bowel syndrome severity score scale
IELs: intraepithelial lymphocytes
IgA: immunoglobulin A
IPA: Ingenuity pathway analysis
JAMs: junctional adhesion molecules
LC: locus ceruleus
LIMMA: comparative analysis and linear models for microarray data
LMR: lactulose-mannitol ratio
LPS: lipopolysaccharide
LS: low stress
M: male
MBP: median blood pressure
MC2-R: melanocortin type 2 receptor
MR: mineralocorticoid receptor
MS: moderate stress
NE: norepinephrine
NGF: nerve growth factor
NLRs: NOD-like receptors
NOD: nucleotide-binding and oligomerization domain
PAMPs: pathogen-associated molecular patterns
PAR2: protease-activated receptor 2
PER: periods
PI-IBS: post-infectious irritable bowel syndrome
POMC: pro-opiomelanocortin
PPIA: cyclophilin
PRRs: pattern recognition receptors
PSS: perceived stress scale
RCT: randomized controlled trial

RIG-I: retinoic acid inducible gene-I

RLRs: RIG-I-like receptors

RT-qPCR: quantitative real time polymerase chain reaction

S: stress

sd: standard deviation

SBP: systolic blood pressure

SIBO: small intestinal bacterial overgrowth

SSRIs: selective serotonin re-uptake inhibitors

SSRS: modified social readjustment rating scale.

TCAs: tricyclic antidepressants

TJs: tight junctions

TLRs: toll-like receptors

TRPV1: transient receptor potential cation channel subfamily V member 1

VIP: vasoactive intestinal peptide

WAS: water avoidance stress

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SUMMARY

SUMMARY

Psychosocial stress is becoming a huge health problem in modern societies in the last years. In physiological conditions, stress represents a threat to the internal homeostasis, and in response to stress a coordinated response involving autonomic, endocrine, and immune systems is generated to maintain stability. However, stress overexposure in susceptible individuals, impairs this adaptive response, and could lead to the development of disease. One of the most vulnerable organs to stress is the gastrointestinal tract (GI), as most common gastrointestinal disorders, functional gastrointestinal disorders (FGID), are considered stress-sensitive disorders although the underlying mechanisms still remain unknown. In the last years, increasing evidence supports the interaction between stress and the GI tract, as both acute and chronic stress have been shown to increase ion and water secretion, impair epithelial permeability, and modify intestinal microbiota leading to intestinal barrier dysfunction and mucosal immune activation that have been related to IBS development.

The aim of this thesis is to describe how stress impacts on gastrointestinal function and to describe cellular and molecular components of the stress response that can predispose to the development of FGID. In this study, we first retrospectively determine the prevalence of chronic psychosocial stress in diarrhea-predominant irritable bowel syndrome (IBS-D) in our area and analyze the effect of chronic psychosocial stress and sex in the severity of GI function. The analysis revealed that chronic psychosocial stress is more prevalent in IBS-D subjects than in healthy volunteers. Moreover, females displayed more dyspeptic symptoms than males in the IBS-D group. Subgroup analysis by comorbid factors showed a more severe IBS-D in patients that had concomitant depression.

We also performed a prospective study to investigate the molecular mechanisms involved in the intestinal mucosa's response to stress. Two biopsies were obtained before and after stress. Throughout the study, autonomic, hormonal and psychological responses to cold pain stress (CPS) were monitored. Mucosal RNA from the intestinal mucosa was isolated and submitted to microarray analysis followed by differential gene expression and biological pathways identification. The analysis revealed the influence of circadian rhythm, intestinal barrier function and inflammation on the response to stress, three factors associated to FGID development. PCR validation confirmed these results, but also demonstrated that stress was also able to modify tight junction related genes. Subgroup

analysis revealed that psychosocial stress and sex determine a differential response to stress.

Finally, we performed a clinical study to asses if these molecular changes were associated with increased gut permeability by measuring the excretion of lactulose and mannitol after stress. The study identified that CPS not only modifies intestinal barrier function at a gene level, but also impairs intestinal permeability. Interestingly, chronic psychosocial stress levels and sex differentially affected intestinal permeability suggesting that this differential response is linked to the female predominance in IBS.

In conclusion, chronic psychosocial stress plays a role in IBS-D pathophysiology, and depression and dyspepsia are two comorbid conditions that worsen IBS-D symptoms. Acute stress alters circadian rhythm, mucosal inflammatory and intestinal barrier gene expression which could lead to intestinal dysfunction and to the development of FGID. Strategies directed to identify comorbidities in IBS-D patients and to reduce chronic psychosocial stress could be useful to manage IBS-patients.

RESUMEN

RESUMEN

El estrés psicosocial se está convirtiendo en uno de los grandes problemas de salud en las sociedades modernas en los últimos años. En condiciones fisiológicas, el estrés representa una amenaza para la homeostasis interna, y en respuesta al estrés, una respuesta coordinada que implica a los sistemas autonómico, endocrino e inmunitario es generada para mantener la estabilidad del medio. Sin embargo, una sobreexposición al estrés en individuos susceptibles, altera esta respuesta adaptativa, y puede dar lugar al desarrollo de enfermedades. Uno de los órganos más vulnerables al estrés es el tracto gastrointestinal (GI), ya que las enfermedades más comunes del tracto gastrointestinal, las enfermedades funcionales digestivas (*functional gastrointestinal disorders*, FGID), son consideradas enfermedades sensibles al estrés, pero los mecanismos subyacentes aún siguen siendo desconocidos. En los últimos años, numerosas evidencias apoyan esta interacción entre el estrés y el tracto GI, ya que tanto el estrés agudo como el crónico han demostrado aumentar la secreción de agua e iones, alterar la permeabilidad intestinal y modificar la microbiota intestinal, dando lugar a la disfunción de la barrera intestinal y a la activación de la inmunidad a nivel de la mucosa intestinal, dos factores que se han relacionado con el desarrollo del síndrome del intestino irritable (SII).

El objetivo de esta tesis es describir como el estrés influye en la función GI y describir los componentes celulares y moleculares de la respuesta al estrés que pueden predisponer al desarrollo de las FGID. En este estudio, primero realizamos un estudio retrospectivo para determinar la prevalencia en nuestra comunidad de estrés crónico psicosocial en pacientes con SII con predominio de diarrea (SII-D) y para evaluar el efecto del estrés crónico psicosocial y el sexo en la severidad del SII-D. El análisis reveló que los sujetos con SII-D presentaban una mayor prevalencia de estrés crónico psicosocial que los voluntarios sanos. Asimismo, las mujeres afectas de SII-D presentaban más síntomas dispépticos que los sujetos varones del mismo grupo. El análisis por subgrupos demostró que las comorbilidades condicionaban una mayor severidad clínica del SII-D en aquellos pacientes con depresión.

Posteriormente, se realizó un estudio prospectivo para investigar los mecanismos moleculares subyacentes a la respuesta de la mucosa intestinal al estrés. Para ello, se obtuvieron una biopsia antes y otra después del estrés. Durante todo el estudio se monitorizaron variables autonómicas, hormonales y psicológicas de respuesta al estrés por frío (*cold pain stress*, CPS). Se aisló el RNA de la mucosa intestinal y se realizó un

estudio de *microarray* seguido de un análisis de la expresión génica diferencial e identificaron las vías biológicas implicadas en la respuesta al estrés. El análisis reveló la influencia del ritmo circadiano, la función barrera intestinal y la inflamación en la respuesta al estrés, tres factores que han sido asociados al desarrollo de FGID. La validación por PCR confirmó estos resultados pero también demostró que el estrés era capaz de modificar la expresión de genes relacionados con las uniones estrechas. El análisis por subgrupos de estrés crónico psicosocial y sexo objetivó que estos dos factores determinan una respuesta diferencial al estrés.

Finalmente, realizamos un estudio para investigar si los cambios observados a nivel molecular se asociaban a un aumento de la permeabilidad intestinal medida mediante el test de lactulosa-manitol después del estrés. El estudio identificó que el CPS no sólo era capaz de modificar la función de barrera intestinal a nivel génico sino que también era capaz de aumentar la permeabilidad intestinal. Además, el análisis por subgrupos por estrés crónico psicosocial y por sexo identificó que estos dos factores afectan diferencialmente la respuesta al estrés por frío, sugiriendo que esta respuesta diferencial podría estar relacionada con la mayor prevalencia de SII en mujeres.

En conclusión, el estrés crónico psicosocial tiene un papel en la fisiopatología del SII-D y la depresión y la dispepsia son dos comorbilidades que empeoran la sintomatología en los pacientes afectos de SII-D. El estrés agudo altera la expresión de genes relacionados con el ritmo circadiano, la respuesta inflamatoria y la regulación de la barrera intestinal, lo que podría dar lugar a la disfunción de la barrera intestinal y al desarrollo de las enfermedades funcionales digestivas. Estrategias dirigidas a identificar y tratar las comorbilidades en los pacientes con SII-D y a reducir el estrés crónico psicosocial podrían ser útiles en el manejo clínico de los pacientes con SII.

INTRODUCTION

INTRODUCTION

1. The intestine

The intestine is a complex organ, composed by different tissues, which main functions are the nutritional and energetic support of the body and the immune surveillance and defense. The digestive function can be divided into nutrient digestion and absorption, water and electrolyte transportation, protein secretion into the intestinal lumen, and intestinal motility. The intestine also acts as a physical barrier to avoid the passage of toxic or potentially harmful substances from the lumen into the internal *milieu.* These two roles are performed in a synchronized and safety manner thanks to the particular anatomy and function of the intestinal mucosa.

1.1. ANATOMY OF THE INTESTINE

Four different layers can be differentiated in the intestinal wall:

- The mucosa is the part in direct contact with the intestinal lumen and it is continuously exposed to a broad variety of antigens derived from the environment and the commensal microbiota. It can be divided into three different compartments: the glandular epithelium, the lamina propria, which is a basal membrane constituted by connective tissue, blood vessels and immune cells and the muscularis mucosae, a thin muscle layer that separates the mucosa from the submucosa.
- The submucosa is a connective tissue layer that contains blood and lymphatic vessels, nerve fibres and ganglia, which form the submucosal plexus (Meissner).
- The muscularis propria layer is made of two layers of smooth muscle cells responsible for contractility and peristaltic movement of luminal contents through the gastrointestinal (GI) tract. The inner layer is a circular coat and the outer longitudinal coat is arranged in a helicoidally pattern. Between both layers it can be found the myenteric plexus (Auerbach).
- The serosa is constituted of a single layer of mesothelial cells supported by connective tissue.

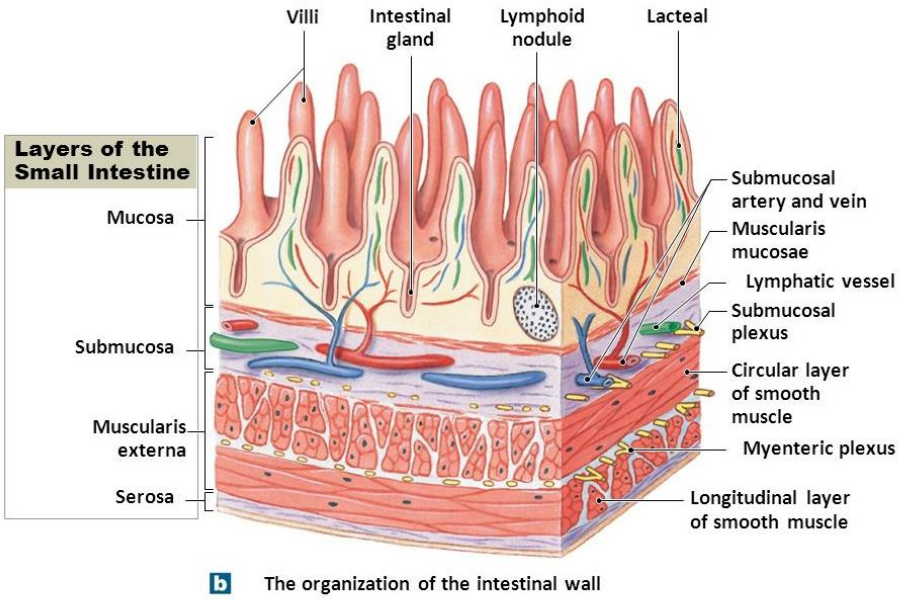


Figure 1: Representation of small intestine structure. From: (Pearson Education Inc, 2015).

1.2. THE ENTERIC NERVOUS SYSTEM

The innervation of the GI tract is different from other peripheral organs, as it has an intrinsic nervous system, the enteric nervous system (ENS), which can function independently from the central nervous system (CNS), although bidirectional brain-gut communication is the most common way of operating. Notably, 90% of vagal fibres between the gut and the brain are afferent, which suggests that the brain in this system acts more as a receiver than a transmitter (M. Rao & Gershon, 2016). Signals arriving at the brain can initiate vasovagal reflexes at the CNS, which, in response to enteric stimuli, can regulate motility patterns. Moreover, gut-to-brain signalling transmits sensations of nausea, bloating or satiety. But the vast majority of information sent from the intestine to the CNS is homeostatic and still could alter behaviour. There is evidence that luminal contents such as commensal bacteria can affect behaviour and brain development through the modulation of the enteric nervous system, the potential pathway of signalling for this modulation is the vagus nerve (Forsythe, Bienenstock & Kunze, 2014). Vagus nerve stimulation has been used to treat depression and to improve learning and memory in animals and humans (George et al., 2000; Rush et al., 2000).

The ENS controls motility, secretion of water, peptides and hormones, changes in local blood flow in the GI tract, and interacts with the gut immune system. Neurotransmission in the ENS is complex as there are more than 30 different known neurotransmitters involved in the regulation of the GI tract (Furness, 2012). The most important among them are acetylcholine, nitric oxide, serotonin, noradrenalin, somatostatin, substance P, and cholecystokinin.

1.3. THE INTESTINAL BARRIER FUNCTION

The intestinal barrier is the largest interface between the internal *milieu* and the external environment. It is composed by several levels of protection aiming to guarantee homeostasis by limiting the host contact with pathogens. It acts as a physical barrier, exerts basic weeping off functions, such as intestinal peristalsis and water secretion, and develops immunological surveillance. The intestinal barrier's selective permeability is its most important characteristic for maintaining homeostasis, as it limits the host contact with antigens and pathogens while on the other hand, allows the absorption of nutrients and water. The control of the homeostasis relies on multidirectional communication between the different components of the intestinal barrier. This complex network is regulated by the central and the enteric nervous systems, which interact with the immune system, the smooth muscle and the epithelium, to regulate immune responses, absorption and secretion, motility, and also visceral sensitivity.

From a morphological point of view the intestinal barrier is made up of several structural.

1.3.1. Mucus

The entire intestinal mucosal surface is covered by a layer of mucus gel produced by goblet cells. Mucus thickness increases along the length of the intestine according to the concentration of luminal bacteria (Atuma, Strugala, Allen & Holm, 2001). Mucus is composed of the negatively charged mucins (being MUC2 the most abundant mucin), phospholipids, water, a variety of trefoil factors, digestive enzymes, and antimicrobial agents such as antibacterial peptides including cathelicidins, defensins, and the secretory immunoglobulin A (Johansson, Sjövall & Hansson, 2013). Mucus has a protective role by preserving the epithelial lining from mechanical friction and autodigestion. Moreover it guards the epithelium against the invasion of microorganisms and against the impact of dietary and environmental antigens and toxins present in the lumen by binding itself to glycocalix, and forming a viscoelastic gel with hydrophobic and surfactant properties.

Likewise, mucus also contributes to epithelial renewal, differentiation, and integrity and also enhances oral tolerance by imprinting dendritic cells with anti-inflammatory properties (Shan et al., 2013). Also the mucus helps to regulate gut permeability.

1.3.2. Epithelial lining

The epithelium from the small intestine is composed by a single polarized continuous layer of columnar cells of at most 20 μm thick that coats the intestinal surface and stands between the intestinal lumen from the internal *milieu*.

Its primary function is to act as a physical barrier, but also regulates the absorption of water, electrolytes and dietary nutrients. There are two different routes by which passage of molecules from the intestinal lumen to the lamina propria takes place. The first one is the paracellular pathway, that allows to diffuse small molecules (<600 Da) through tight junctions (TJs), which are located between adjacent intestinal epithelial cells. The second one is the transcellular pathway, which, via endocytosis or exocytosis processes, allows the passage of larger particles through the epithelial cells.

The intestinal epithelium is not only composed by the absorptive enterocytes, it also contains other stem cell-derived cellular types: goblet cells, Paneth cells, enteroendocrine cells, and M cells (covering the lymphoid follicles and their mission is to transport luminal antigens in order to achieve antigen presentation). To ensure intestinal barrier integrity, pluripotential stem cells, located in the intestinal crypt, lead to epithelial cells, which migrate to the tip of the villus where final differentiation takes place, renewing the epithelial population every 3–5 days. This proliferation, differentiation and migration process is highly regulated by different signalling cascades such as the wnt and the Notch pathway, which are essential in the maintenance of the intestinal epithelium.

1.3.3. Tight junctions

In order to maintain the paracellular pathway sealed, enterocytes are tightly bonded to each other through the apical junctional complex, composed of TJs and subjacent adherens junctions (AJ), and desmosomes (Groschwitz & Hogan, 2009; Turner, 2009). The TJs and AJ complexes are important in the regulation of cellular proliferation, polarization and differentiation. But while the AJs and desmosomes are more important in the mechanical linkage of adjacent cells, the TJs selectively regulate paracellular ionic solute transport. AJ are composed by the transmembrane protein cadherins (e-cadherin) that interacts with catenins, which binds to the cytoskeleton. The TJs complex is more

intricate as they are multi-protein complexes composed by transmembrane proteins (claudins, occludins and junctional adhesion molecules (JAMs)), which interact with peripheral membrane (scaffolding) proteins (zonula occludens), which bind to the cytoskeleton. Actin contraction leads to increased permeability to electrolytes and small molecules. This process is a key step in the immune tolerance induction as it allows a controlled amount of small particles (less than 400 daltons) to penetrate across the epithelium to reach the lamina propria. The role of occludin is still no yet well defined, but it seems to regulate the integrity of the TJs, as occludin-deficient mice do not show alterations in TJs assembly and permeability (Balda et al., 1996; McCarthy et al., 1996). The claudin family of transmembrane proteins consists of 24 members with a molecular weight ranging from 20 to 27 kDa. Each member shows a specific organ and tissue distribution. They determine the strength, size, and ion selectivity of TJs along the crypt–villus axis and throughout the length of the intestine (Turner, 2009). The family of JAMs has been implicated in the construction and assembly of TJs, in the regulation of intestinal inflammation, by regulating the process of trans-endothelial migration of leukocytes, and permeability. Disruption of the junctional complex can lead to increased intestinal permeability, which may facilitate immune activation and, in consequence, contribute to the development of chronic inflammation in the gut (Laukoetter et al., 2007; Van Itallie et al., 2006).

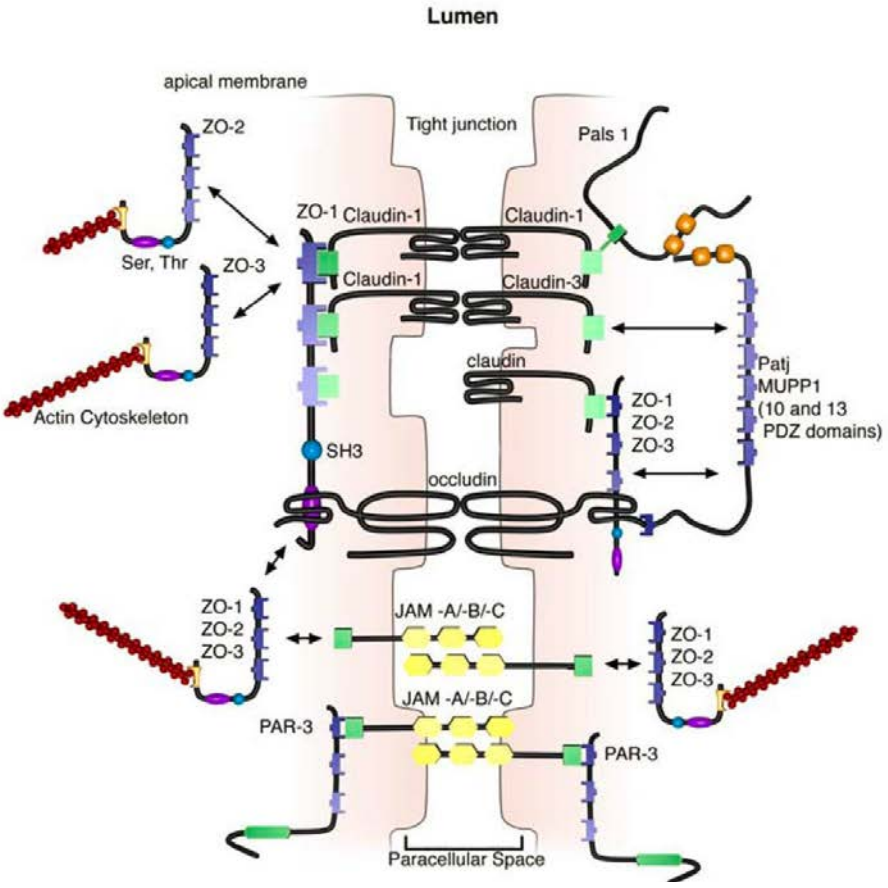


Figure 2: Tight junctions and tight junction proteins. From: (Groschwitz & Hogan, 2009).

1.3.4. The intestinal immune system

The immune system is designed to protect the organism against external and internal threats such as pathogen microorganisms, toxic substances or tumoral cells. This safeguard is achieved with two different responses, the innate immune response and the adaptive immune response.

As previously stated, the intestinal mucosa is, as the rest of mucosal sites of the body, in close contact with the external environment. It harbours billions to trillions of microorganisms. It is also in contact with food antigens, both factors representing an enormous antigenic load that, in order to maintain homeostasis, the intestinal cells have to differentiate between potentially harmful elements from those that may exert a beneficial effect and must be tolerated. This feature of mucosal surfaces favours the development of specialized lymphoid and other cell populations in order to provide generalized immunization at all mucosal surfaces, and is known as the mucosal-associated lymphoid

tissue. The intestine harbours the gut-associated lymphoid tissue (GALT) where about 70% of whole body's immune cells reside.

1.3.4.1. Innate immunity

The innate immune response is the first host defense to face a wide variety of pathogens and it is present in both, animals and plants. The innate immune system protects the host by acting immediately to threats in a non-specific manner. The main components of this immune response are the pattern recognition receptors (PRRs) and the antimicrobial peptides.

PRRs recognize evolutionary conserved molecular patterns present in microorganisms (pathogen-associated molecular patterns (PAMPs)) or cell-derived molecules released as a result of tissue damage (danger-associated molecular patterns (DAMPs)) participating in early microbial-sensing when the pathogen escapes the first line of intestinal defense, constituted by the mucus and the antimicrobial peptides. PRRs include 5 different types of receptors; Toll-like receptors (TLRs), nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), C-type lectins receptors (CLRs), and cytosolic DNA sensors (Fukata & Arditi, 2013; Han & Ulevitch, 2005; Y. K. Kim, Shin & Nahm, 2016).

The TLR family consists of at least 13 transmembrane receptors that get activated upon PAMP recognition. This interaction initiates a downstream cascade that signals specific molecules responsible for activating innate immune responses (macrophage activation and induction of antimicrobial peptides for various cell types) and adaptive immune responses (induction of T cell responses and maturation of dendritic cells). In many tissues, mast cells, dendritic cells, monocytes/macrophages and B cells express TLRs. All members of the TLR family have been identified to recognize a great variety of microbial components and in terms of functionality they share a common signalling pathway which includes effector proteins and kinases such as MyD88, IRAK1, TAK1, IKK, IκB and NFκB. TLR2 and TLR4 are responsible to generate immune responses against bacterial cellular membrane from gram negative bacteria (lipopolysaccharide, LPS) and also from gram positive bacteria.

NOD1, NOD2 and NALP3 are the most studied PRRs from the NLR family. All NLR are intracellular but while NOD1 and NOD2 recognize intracellular bacterial products, NALP3 responds to different stimuli to form a multi-protein complex named inflammasome, which promotes the secretion of cytokines from IL-1 family. NOD1 is expressed by intestinal

epithelial cells and recognizes part of the peptidoglycan from gram-negative bacteria as well as some gram-positive bacteria; whereas NOD2 expression is predominantly found in monocytes and Paneth cells and identifies muramyl dipeptide, which is found in all bacteria. NOD1 and NOD2 induce activation of both innate and adaptive immune responses through activation of NFκ-B and MAPKs pathways. The important role of NOD2 in maintaining homeostasis and barrier function has also been demonstrated by studies performed in NOD2-deficient mice which display a diminished ability to prevent intestinal colonization by pathogenic bacteria and also an increased load of commensal resident bacteria (Petnicki-Ocwieja et al., 2009).

RLRs are cytoplasmic RNA helicases critical in viral RNA recognition. Together with DNA sensors, they modulate the production of interferons and cytokines in the host antiviral response (M. R. Thompson, Kaminski, Kurt-Jones & Fitzgerald, 2011).

CLRs are transmembrane proteins that recognize carbohydrates or present a similar structure to C-type lectin-like domains (Hardison & Brown, 2012). CLRs are divided into type-I and type-II transmembrane receptors and soluble receptors. Upon bacterial or fungal infection, CLRs promote the production of pro-inflammatory mediators, fungal binding and phagocytosis, neutrophil influx, macrophage maturation and T-cell differentiation (Deng et al., 2015; Hardison & Brown, 2012). CLRs and TLRs act synergistically to ensure optimal proinflammatory responses against fungus, bacteria, viruses, helminths and protozoa.

Cytosolic DNA sensors recognize intracellular DNA from both bacteria and viruses in different cellular compartments (M. R. Thompson et al., 2011). Although cytosolic DNA sensors are expressed in a variety of cell types, few cytosolic sensors have been identified in the epithelium or the intestinal mucosa. Moreover, and due to the variety of receptors capable of DNA sensing, their classification is still unclear.

Defensins and cathelcidins are the two main types of antimicrobial peptides constitutively expressed in intestinal epithelial cells and may be also inducible in immune cells such as phagocytes and Paneth cells, which release them into the intestinal lumen (Ho, Pothoulakis & Koon, 2013). Defensins are a family of small cationic peptides (29–45 amino acids) present in prokaryotes and eukaryotes exerting a potent and wide activity spectrum against bacteria (gram-positive and gram-negative), yeast, fungi, virus, parasites and even tumoral cells. Their mechanism of action is through permeabilization of the bacterial cell membrane. Furthermore, defensins also act as regulatory and effector

cells of the immune response by inducing the release of inflammatory mediators on mast cells and phagocytic cells. Defensins also communicate with T cells and dendritic cells to increase antigen-specific cell response. Of a total of 35 types of cathelcidins identified, only one is present in humans. The human cathelcidin hCAP18/LL37 is neutrophil specific, but it is also expressed in other cell types. Its expression can be induced by bacterial components and can be inhibited by intestinal cell infection by *Shigella spp*.

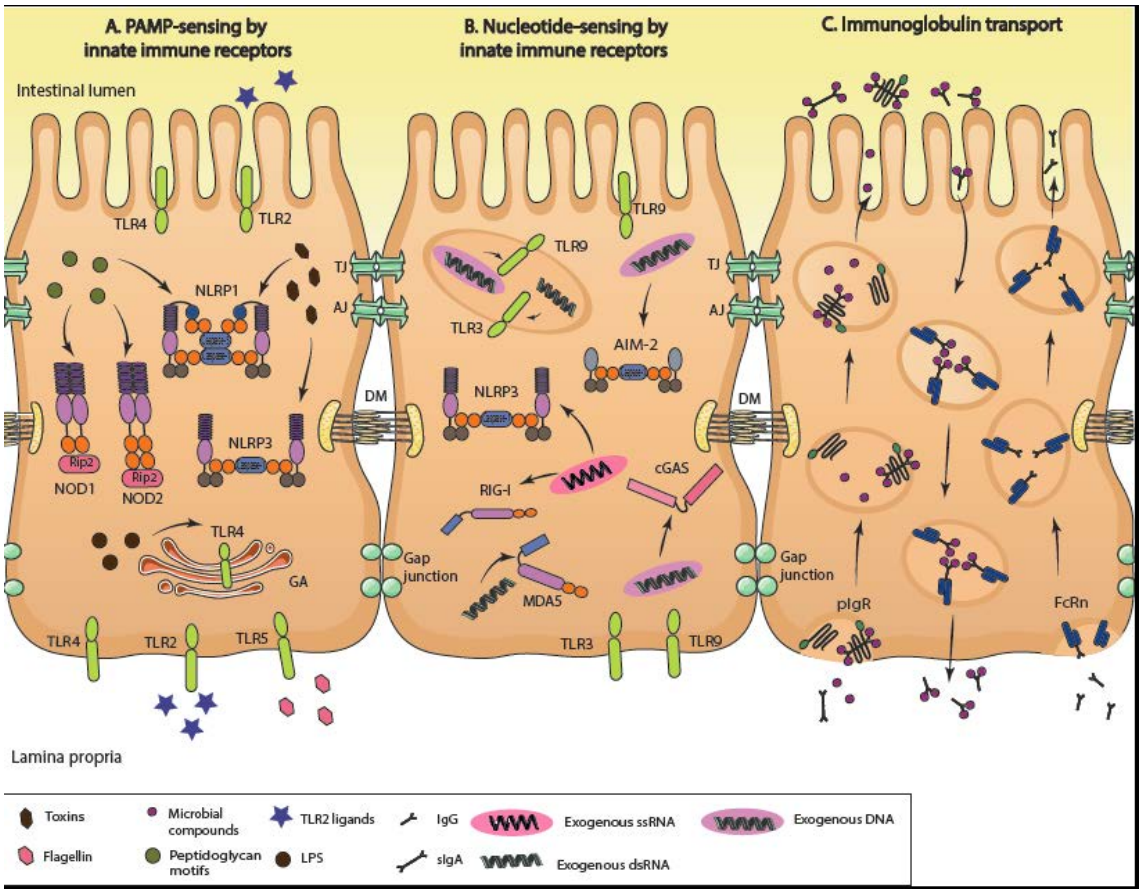


Figure 3: Figure. Representation of ligands and location of most notable receptors in human intestinal epithelial cells (IEC). A. PAMP-sensing by innate immune receptors. B. Nucleotide-sensing by innate immune receptors. C. Immunoglobulin transport. From: (Pardo-Camacho, González-Castro, Rodiño-Janeiro, Pigrau & Vicario, 2017)

1.3.4.2. Adaptive immunity

Adaptive immune response is present only in vertebrates and constitutes a second line of defence against pathogens. Antigens bind to specific receptors, which activate B and T lymphocytes, which will expand clonally to initiate a directed immune response to confer protection against exposure to the same antigen. In terms of functionality, two compartments can be distinguished: the inductive compartment, where the antigen is presented and naive T and B-lymphocytes are activated; and the effector compartment, where cells that have been sensitized to different antigens differentiate to accomplish the

destruction of pathogens. The inductive sites are organized structures located along the GI tract (Peyer's patches, isolated lymphoid follicles, and lymph nodes), while populations of cells distributed in the lamina propria and the intestinal epithelium constitute the diffuse sites.

1.3.4.2.1. Organized immune inductive sites of the GALT

Peyer's patches are macroscopic lymphoid aggregates found in the antimesenteric border of the intestine at the submucosal levels. M cells are a special type of epithelial cell on Peyer's patches, which facilitate the capture of luminal antigens and microorganisms and their transport to contact the underlying immune cells. The interfollicular areas are mainly made up of mature dendritic cell, macrophages and mostly T CD4+ lymphocytes. The follicles are composed of IgA precursor B cells, being B cells IgM+/IgD+, T CD4+ lymphocytes and dendritic cells responsible of antigen presentation and lymphocyte activation.

The mesenteric lymph nodes are the largest of the whole human body. The lymphocyte population inside the lymph nodes are organized in a well structure manner. The cortex includes primary follicles, rich in mature B cells, and secondary follicles, also known as germinal centres, where a high proliferation rate and high abundance of IgD+ B lymphocytes, but also macrophages, dendritic cells and CD4+ T cells can be found. The paracortex harbors T lymphocytes and dendritic cells. Finally, the most internal part of the lymph node, the medulla, is composed of B and T lymphocytes as well as plasma cells.

1.3.4.2.2. Diffuse GALT

Diffuse GALT is composed of two populations of leukocytes distributed at both sides of the basal membrane. The intraepithelial lymphocyte population is found between epithelial cells, above the basal lamina. The majority of intraepithelial lymphocytes are CD8+ T cells which constantly monitor and/or respond against luminal antigens or bacteria, acting as surface gatekeepers of the intestinal barrier. Below the basal membrane, in the *lamina propria* reside the *lamina propria* lymphocytes along with many other types of immune cell, such as dendritic cells, mast cells, macrophages, and eosinophils. Lamina propria immune cells constitute a much more heterogeneous population, approximately 50% of which correspond to plasma cells, 30% to T lymphocytes, and the remaining 20% to macrophages, dendritic cells, mast cells and eosinophils.

2. Stress

2.1. CONCEPT AND HISTORICAL EVOLUTION

In order to understand the concept of stress it is necessary to understand the concept of homeostasis. Homeostasis is derived from the Greek words *homeo* (means similar) and *stasis* (means position or stability) and it was first defined by Cannon in 1929 as *maintenance of nearly constant conditions in the internal environment*. This term is so general that it includes those reactions that happen at a certain time point in the organism in order to keep constant conditions. Thus, stress can be defined as the effect produced by anything that threatens homeostasis generating, therefore, the stress response. In physiological conditions, the stress response implies a group of adaptive responses that returns the body to a homeostatic state, but when this stress response are quantitatively or qualitatively inadequate, that response can lead to behavioral alterations and provoke a dysfunction of the physiological control system: the neuro-immune-endocrine axis (Chrousos & Gold, 1992; Habib, Gold & Chrousos, 2001). Therefore stress, although nowadays has negative connotations, it is a body reaction to cope with the changing environment.

The concept of stress and the stress response evolved ever since the physiologists Claude Bernard and Walter Cannon made the first contribution to the field. Claude Bernard was the first scientist to introduce the concept of *"Millieu Interieur*", which describes the existence and importance of activation and cooperation of different systems, which counteract the destabilizing factors in order to maintain an internal equilibrium for the functioning of the living organisms. Through experimental design, he demonstrated that the sympathetic-adrenal axis reacts to emergency situations through the liberation of adrenaline to the blood in order to activate mechanisms whose aim is to protect the organism and to warrant survival. This view of the stress response as a group of stimuli proposed by Cannon and Bernard was also followed by Holmes & Rahe years later.

But at that moment of the history the term stress was still not used in medicine. It was not until 1936 when Hans Selye took it from physics and used it to describe the mutual actions of forces that take place across any section of the body, conceptualizing the term stress as a response. Selye's work started when he observed that patients with severe diseases frequently had also a group of symptoms common in all of them, such as weight loss or GI alterations. Although Selye was the first one in using the word stress in medical

terminology, in his first work published in Nature in 1936, the term stress did not appear but he described a group of general adaptation and maladaptation traits that consisted in four different stages, starting with a first alarm state, a resistance, an adaptation and finally (if the stimuli continued) a stage of maladaptation leading to the death of the animal. To do so, he used rats which were exposed to cold environment, surgery, intense exercise or infection. Those rats showed an increase in suprarenal gland cortex, a reduction in thymus and lymphatic nodes and several GI symptoms, one of them was gastric ulcerations and he named this whole reaction as General Adaptation Syndrome.

Nevertheless, several years later, John W. Mason demonstrated that the body response to a stress is not general and unspecific. To do so, he applied gradually and progressively, to minimize the emotional stress, different stimuli (cold, heat or food deprivation) to monkeys and he proved that the neuroendocrine response was different and it was dependent on the stimulus applied, concluding that Selye's observations were only a consequence of the emotional response produced by the psychological malaise associated to those stressful situations (Mason, 1968b, 1968c, 1968a) However, he did not take into consideration that when applying a stimulus gradually, an adaptation to it is generated and that could explain the different stress responses described by Mason and Selye.

The concept of stress response evolved when Weiss described the effect of psychological factors. To do so, Weiss demonstrated that the neuroendocrine response observed in rats in which a physical stress was applied (electrical shock) was more dependent on the incapability of the animals to control the situation more than the effect of physical stress *per se* (Weiss, 1972a, 1972b). According to Weiss, how an individual perceives its capacity to control a stressful situation determines stress response and its consequences.

In the early 1980's, Richard Lazarus defined stress as a two-way process, which includes the production of stressors by the environment and the response of an individual exposed to these stressors. He introduced the concept of psychological stress that he called: the theory of cognitive appraisal. According to Lazarus' theory, stress response was produced when demands (pressure) exceeded subject's capacity to handle or cope with them (resources) (Lazarus, 1993). Consequently, a situation is perceived as stressful only when it is perceived as threatening, dangerous or challenging, and sometimes stress appears even though the subject has the situation fully controlled. Therefore, the same situation does not produce the same response in every single individual because the response depends on how the subject evaluates the situation (appraisal), which derives in different

actions to handle them (coping) (Lazarus, n.d.), and that will determine the inter-individual differences. Again, this concept confronts Selye's theory.

Regardless of the contributions in the past century made by all these authors to the stress field and the recent studies shedding more light on the knowledge of its physiology, a universal accepted definition for the concept of stress is still lacking.

2.2. HOMEOSTASIS-HPA AXIS

Maintenance of tight physical, molecular, and chemical conditions in the internal *milieu* is critical for the adequate functioning of living organisms. This equilibrium, namely homeostasis, involves an increasing number of behavioral responses, biological functions, mechanisms, and pathways (Table 1) intended at promoting the survival of the organisms by orchestrating and integrating every generated response to endogenous, environmental, physical or psychological stressors in a very dynamic interaction. When a stressor exceeds "normal" severity or exposure time, the homeostatic systems activate organism compensatory responses to deal with that threat. The optimal homeostatic system activity is called eustasis (homeostasis) but if there is a deficient or excessive activation of the homeostatic system, the equilibrium (homeostatic state) is lost and the risk of developing pathological conditions significantly increases. This phenomenon is called allostasis (different homeostasis), distress or dyshomeostasis. The response to a stressor to get back to the homeostasic state in a living organism is an innate stereotypic response, which takes place in the CNS and in various peripheral tissues. The principal structures involved in the stress response are commonly known as the Hypothalamic-pituitary-adrenal (HPA) axis and it includes three structures: the paraventricular nucleus of the hypothalamus, the anterior lobe of the pituitary gland, the adrenal, gland and also the locus ceruleus (LC)-norepinephrine system (central sympathetic system). When a stress stimulus appears, the paraventricular nucleus secretes into the hypophysial vessels two different neuropeptides: corticotrophin-releasing factor (CRF) and arginine-vasopressin (AVP), which are the main directors of the stress response. CRF accesses the anterior pituitary gland inducing the release of adrenocorticotropic hormone (ACTH) into the systemic circulation while AVP exerts a synergistic effect by contributing to the release of ACTH. Once in the bloodstream, circulating ACTH binds to the melanocortin type 2 receptor (MC2-R) in the adrenal cortex where stimulates glucocorticoid synthesis and secretion. Glucocorticoids are the final effectors of the HPA axis, and have two main actions: to regulate the HPA axis basal activity by inhibiting it; and to regulate metabolic,

immune, behavioral and cardiovascular processes during homeostasis and after the stress response.

PHYSICAL ADAPTATION	BEHAVIORAL ADAPTATION
Adaptive redirection of energy • Oxygen and nutrients directed to the central nervous system and stress body site(s) • Detoxification from toxic products • Contention of the stress response • Containment of the inflammatory/immune response • Increased respiratory rate • Increased heart rate and blood pressure • Altered cardiovascular tone • Increased lipolysis and gluconeogenesis • Inhibition of growth • Inhibition of reproductive system	Adaptive redirection of behavior Acute facilitation of adaptive and inhibition of non-adaptive neural pathways • Increased arousal and alertness • Increased cognition, vigilance and focused attention • Containment of stress response • Suppression of feeding and reproductive behavior

Table 1. Physical and behavioral adaptation during stress. Adapted from (Chrousos & Gold, 1992).

The stress system does not only function in response to a threat, it also has a basal circadian rhythm. In order to have a regular social interaction, well-being perception, and task development, it is necessary the homeostasis of the circadian rhythm as well as a proper response to stressful stimuli.

Conversely, inappropriate basal activity and/or responsiveness of the stress system might impair body composition, growth and development as well as might contribute to the pathophysiology of many allergic, immunological, behavioral, metabolic, endocrine and GI disorders. However, other factors such as genetic and environmental may play also an important role in the development of stress-related diseases. Therefore, stress can lead to acute or chronic mental and physical conditions in susceptible individuals and these diseases are different according to the type of stress (acute or chronic, see Table 2).

Acute stress-related diseases	Chronic stress-related diseases
Pain	Neuropsychiatric
• Abdominal pain	• Anxiety
• Pelvic	• Depression
• Low-back pain	• Executive dysfunction
• Headaches	• Cognitive dysfunction
Gastrointestinal symptoms	• Sleep disorders (insomnia, daytime sleepiness)
• Pain	Cardiovascular
• Indigestion	• Hypertension
• Diarrhea	Metabolic
• Constipation	• Metabolic syndrome
Psychiatric	• Type 2 diabetes
• Panic attacks	• Obesity
• Psychotic episodes	• Atherosclerosis
Allergic manifestations	• Osteoporosis
• Asthma	Neurologic
• Eczema	• Neurovascular degenerative disease
• Urticaria	
Angiokinetic phenomena	
• Migraines	
• Hypertensive/hypotensive attacks	

Table 2. Acute and Chronic Stress related diseases.

2.3. THE STRESS RESPONSE

The stress response is extremely complex and multiple structures and systems, which makes it difficult to identify a particular neuroanatomical structure responsible for a specific stress response. In response to stress there is an activation of central and peripheral nerve circuitries, central circuits and adrenal components and mediators, which are localized and exert their effects in the periphery, are activated. Moreover, interconnecting circuits include all the structures and molecules that communicate the CNS with the periphery, being the main component are the ANS and, specifically, the sympathetic-adrenomedullary system (Tsigos & Chrousos, 2002). All these functional structures are the executive arms through which, during a threatening stimuli, the brain impacts on all body organs.

2.3.1. Central and interconnecting circuits

The HPA-limbic system is regulated by neuropeptides secreted by the hypothalamic PVN. In homeostatic conditions, corticotrophin releasing hormone (CRH) and AVP are secreted 2-3 times per hour in a circadian rhythmic and pulsatile fashion. Having higher pulses in the early morning and perturbed by changes in lighting, feeding, activity, and especially disrupted by stress. They reach the anterior hypophysis to promote the synthesis of pro-opiomelanocortin (POMC) and to release ACTH and other peptides originated from its splicing. Once ACTH is released into blood flow, it transitory elevates circulatory cortisol levels, facilitating the coordination between the brain and its effects on the periphery (E Ron de Kloet, Joëls & Holsboer, 2005). Cortisol exerts negative feedback at the hypophysis, hypothalamus and hippocampus.

The noradrenergic system is originated from cells localized in the LC, medulla and pons. Norepinephrine (NE) is their principal neurotransmitter. NE exhibits a neuromodulatory effect and also potentiates synaptic transmission in targeted tissues (Woodward, Moises, Waterhouse, Yeh & Cheun, 1991).Cathecolamines activate adrenergic receptors which trigger a multisystemic stress response ultimately directed to warrant and to increase oxygen and energy transport to the organ in which is necessary. The adrenal medulla is constituted by chromaffin cells that release epinephrine (80%) and also NE (20%) (Vollmer, 1996). They are innervated by sympathetic preganglionar cells and they act postganglionary cells of sympathetic nervous system. However, epinephrine and NE do not cross the hemato-encephalic barrier in normal conditions. Their actions in the CNS are produced by the cerebral cathecolamines through connections with the HPA axis. In the brain, epinephrine acts as an alarm system by decreasing neurovegetative functions and by increasing autonomic and neuroendocrine responses to stress. NE enhances long-term memory of aversive emotional memories and maintains alert status which can lead to anxiety syndromes (Morilak et al., 2005).

During the early stage of the stress response, cathecolamines, neuropeptides, and probably cortisol, are released in order to provide an adequate response to stress stimulus. This early stage response is monitored by CRH and its aim is to produce a hypervigilance and alert state (E Ronald De Kloet & Derijk, 2004) through activation of hypothalamic pathways, which will finally unleash the release of epinephrine from the adrenal medulla. The next step in the stress response is the adaptation and recuperation phase, where multi-synaptic networks stimulate CRH producing cells (Herman, Tasker, Ziegler & Cullinan, 2002).

Corticosteroids take part in all stages of the stress response. Corticosteroid effect is mediated by two different receptors: mineralocorticoid receptors (MR), with high affinity for corticosterone; and the glucocorticoid receptors (GR), with a low affinity for corticosterone. In the periphery, cortisol exerts its effect through the GR (Gunnar & Quevedo, 2007), while in the brain, corticosteroids bind to GR and MR (van der Laan, de Kloet & Meijer, 2009). MR are implicated in the stress evaluation and at the origin of the stress response and also maintain the basal functioning and circadian rhythm of the HPA axis (Reul & de Kloet, 1985). GR ends the response and promotes the recovery phase. GRs have a progressive activation during the 24h of the circadian rhythm and they mediate acute stress responses (Sapolsky, Romero & Munck, 2000; Young, Abelson & Lightman, 2004). The last phase of the stress response is characterized by the inhibition of the inflammatory and immune response induced at the early stage and also by the mobilization of energy resources to perform that process.

Stress also induces an increment in brain neurosteroid levels (Girdler & Klatzkin, 2007). These molecules are *de novo* produced (Baulieu, 1991) from cholesterol to Dehydroepiandrosterone (DHEA) or from peripheral steroids to brain neuroactive compounds. The neuroactive steroids have a potent anxiolytic, sedative and anticonvulsant effect in animals and humans (Pisu & Serra, 2004) through modulation of gamma-amino-butyric acid A receptors (Girdler & Klatzkin, 2007) and N-metil-D-aspartate G protein coupled glutamate receptors (Rupprecht et al., 2001).

Gonadal steroids, especially testosterone, can help to develop an effective adaptation of the HPA axis to chronic stress. In basal conditions corticosterone and testosterone are able to regulate CRH and AVP expression independently, but it is necessary the combination of both to maintain homeostasis under stressful events (Williamson, Bingham & Viau, 2005).

Blood-brain barrier prevents peripheral inflammatory molecules to reach the CNS. Stress increases blood-brain barrier permeability facilitating the passage of harmful substances, and could contribute to the development of neuroinflammatory diseases (Theoharides & Konstantinidou, 2007); also could explain the passage of drugs or toxins that in steady state do not cross the barrier (Beck et al., 2003). This process is mediated by CRH which favours the release of selective mediators (tryptase, histamine, VIP, IL-6, IL-8 or tumoral necrosis factor-α) by brain mast cells.

2.3.2. Peripheral stress-inflammation circuitry

Antigen and non-immunogenic particles processed locally at mucosal sites will determine the immune response by initiating or not an inflammatory response. As described previously, protection against these toxic or immunogenic particles is maintained by strong adhered epithelial monolayers. Just below the epithelial layer, the lymphatic and blood vessels, an abundance in immune cells (plasma cells, lymphocytes, macrophages, eosinophils, mast cells and dendritic cells, among others), and a huge nervous net made of intrinsic (submucosal plexus) and extrinsic fibres reside the lamina propia. There are also other components, such as mesenchymal cells (fibroblasts, myofibroblasts, and myocytes), endothelial cells, extracellular matrix and microbiota-derived components, with effector and regulatory actions on the local immune responses. Moreover, epithelial cells can express co-stimulatory molecules, type II major histocompatibility complex, innate immune receptors, chemotactic and inflammatory cytokines and antimicrobial peptides.

Numerous evidences support the existence of a communication between the components of the stress-inflammation circuitry (Javier Santos, Bienenstock & Perdue, 2002). This communication is performed through the release of neuropeptides, neurohormones, neurotransmitters and other molecules that play a regulatory role such as chemokines, cytokines and growth factors. The functional implication of these interactions includes the regulation of mucosal immune and inflammatory processes, through the control of secretion and absorption, transepithelial or endothelial macromolecular transportation, migration and activation of immune cells, or through intestinal microbiota's metabolic capacity (Sibille, Pavlides, Benke & Toth, 2000; Wood, 2007).

2.4. EFFECT OF STRESS ON THE INTESTINAL BARRIER

Both, chronic and acute stress can affect intestinal barrier function by increasing water and ion secretion and by modulating intestinal permeability (C. Alonso et al., 2012; Carmen Alonso et al., 2008; Barclay & Turnberg, 1988; Fiocchi, 1997; J Santos, Saunders, et al., 1999; Söderholm, Yates, et al., 2002; Vanuytsel et al., 2014). There are many processes implicated in the epithelial adaptation response to stress, but it has been demonstrated that mast cells play a key role on it (Barclay & Turnberg, 1988; J Santos et al., 1998). Stress can initiate and reactivate mucosal inflammation (Fuentes et al., 2016; Söderholm, Yang, et al., 2002) and also alter epithelial function (Qiu, Vallance, Blennerhassett & Collins, 1999); these changes have been also observed in patients with diarrhea-predominant IBS (IBS-D). Stress-induced increased intestinal permeability and

gut leakiness could break the gut barrier function, allowing passage of bacteria and luminal antigens trough the epithelium, consequently leading to activation of the immune system and generation of intestinal inflammation.

Stress and sex steroids can affect intestinal barrier function through different mechanisms as shown in Figures 4 and 5.

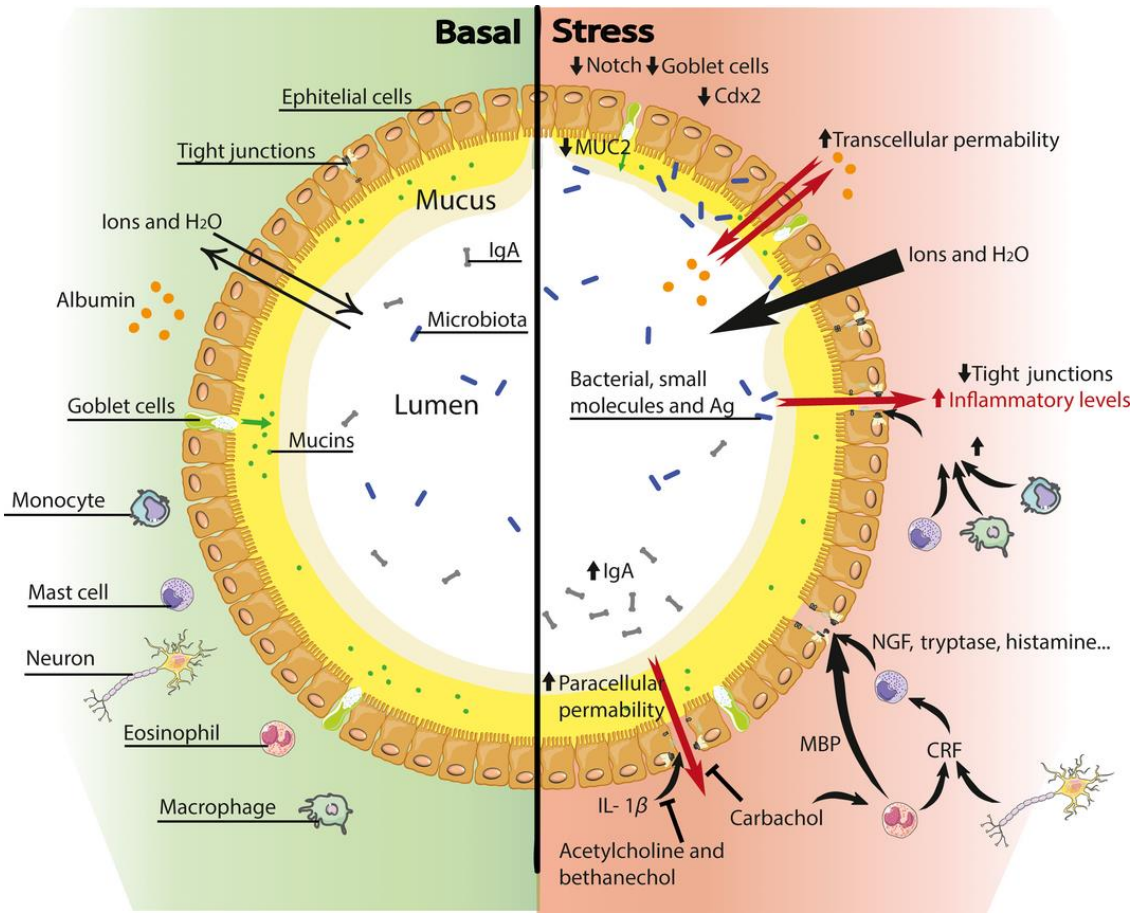


Figure 4: Stress-mediated effects on the intestinal epithelial barrier function. On the left, intestinal mucosa under physiologic conditions. On the right, stress-induced intestinal barrier dysfunction. From (Pigrau et al., 2016).

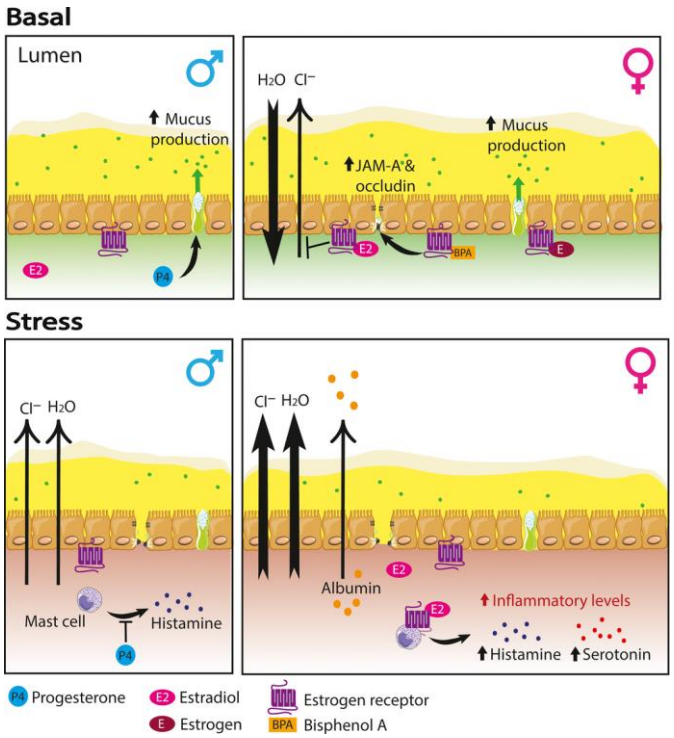


Figure 5: Stress-induced differences according to sex. From (Pigrau et al., 2016)

2.4.1. Mucus composition and function

Acute stress by immobilization increases mucin release by goblet cells in rat colonic explants (Castagliuolo et al., 1996), an effect that depends on mast cells (Castagliuolo et al., 1998). Moreover, rats with stress-induced anxiety also display increased goblet cells and increased mucus secretion (O'Malley, Julio-Pieper, Gibney, Dinan & Cryan, 2010). On the other hand, chronic stress reduces MUC2 synthesis (Shigeshiro, Tanabe & Suzuki, 2012) and predisposes to mucus depletion and reduced number of goblet cells in the distal colon of rats (Pfeiffer, Qiu & Lam, 2001; Söderholm, Yang, et al., 2002).

2.4.2. Ion and water secretion

Animal models of acute, chronic or repetitive stress (either homotypic or heterotypic), have an increase in ion and water secretion in small and large intestine (P R Saunders, Kosecka, McKay & Perdue, 1994; María Vicario et al., 2010) and mast cells play a key role in this response (J Santos, Benjamin, Yang, Prior & Perdue, 2000; Smith et al., 2010). Moreover, peripheral administration of CRF receptor agonists produced the same effects on the colon epithelium as the stress involving mast cells and adrenergic and cholinergic nerves (J Santos, Saunders, et al., 1999; P R Saunders, Hanssen & Perdue, 1997; Paul R Saunders, Maillot, Million & Taché, 2002; Teitelbaum, Gareau, Jury, Yang & Perdue,

2008). In human studies, in vivo segmental perfusion showed enhanced ion and water secretion in the human jejunum or reduced water absorption, induced by acute physical and psychological stress (Barclay & Turnberg, 1987, 1988) and this effects are mediated by mast cells and the parasympathetic nervous system (J Santos, Bayarri, et al., 1999). Moreover, in healthy women, this response was determined by background stress, as females with increased life stress exhibit less intestinal secretory responses to cold pain stress (Carmen Alonso et al., 2008).

2.4.3. Intestinal permeability

Experimental studies have demonstrated how both, acute and chronic stresses alter passage of molecules across the intestinal epithelia. Partial restraint stress, water avoidance stress (WAS), maternal separation and swimming stress increase paracellular permeability in the rat small intestine and colon (Ait-Belgnaoui et al., 2012; F Barreau, Ferrier, Fioramonti & Bueno, 2004; Xu et al., 2014). WAS and repeat restraint stress impair mucosal barrier function and induce mucosal inflammation, dysbiosis, and visceral hyperalgesia in rats (Xu et al., 2014). Studies in rodents (Keita, Söderholm & Ericson, 2010; J Santos, Saunders, et al., 1999; Javier Santos et al., 2008; Söderholm, Yang, et al., 2002; Teitelbaum et al., 2008; Zheng et al., 2009) and humans (C. Alonso et al., 2012; Carmen Alonso et al., 2008; C. Wallon et al., 2008; Conny Wallon et al., 2011) show that stress activates mast cells and eosinophils which, in the end, affect transcellular permeability across the intestine. Stress also affects TJs proteins, as chronic psychological stress rat model it increased intestinal permeability through the reduction of ZO-1, claudin-1, and occludin expression and this was mediated by corticosterone (Overman, Rivier & Moeser, 2012).

In humans, intestinal permeability can be altered by drugs like non-steroidal anti-inflammatory drugs, psychological or physical stressors such as GI infections (Dunlop et al., 2006), trauma (Spindler-Vesel, Wraber, Vovk & Kompan, 2006) and surgery (Schietroma, Carlei, Cappelli & Amicucci, 2006). The role of sex steroids in the regulation of intestinal permeability has not been completely defined, although females are often more resilient to stress than males and cycling sex hormones can affect the stress response (Brown & Grunberg, 1995; Moussa et al., 2013).

2.5. EFFECT OF STRESS ON THE IMMUNE RESPONSE

2.5.1. Regulation and inhibition of the innate and adaptive intestinal mucosa immune response.

Stress mediators can profoundly affect the immune response. Glucocorticoids supress maturation, differentiation and proliferation of all immune cells acting through signalling pathways that depend on classical intracytoplasmic receptors as well as membrane G protein coupled receptors (Tasker, Di & Malcher-Lopes, 2006). Glucocorticoids also inhibit expression of adhesion molecules and chemokines implicated in cellular trafficking. Moreover, *in vitro* experiments have demonstrated that can glucocorticoids increase the expression of TLR-2 mRNA (Sternberg, 2006). Neuroanatomical studies have shown the presence of noradrenergic nerve endings in primary and secondary lymphoid organs as well as surface adrenergic and dopaminergic receptors in immune cells (Felten et al., 1998). Norepinephrine produces local regulation on immune organs, where inhibits the production of pro-inflammatory cytokines (IL-1, IL-6, IL-12) while promotes the release of IL-10 and CXCL8, increasing NK cell, monocyte and macrophage migration and reducing dendritic cell migration (Maestroni & Mazzola, 2003). Systemic adrenalin decreases the number of monocytes, T and B lymphocytes, and circulating NK cells through β-adrenergic receptors (Oberbeck, 2006). Overall, the effects of cathecolamines and glucocorticoids are directed to down-regulate the inflammatory response.

Peripheral neuropeptides also have a regulatory function on inflammation. Growing evidences suggest that peptidergic nerve endings are juxtaposed to mast cells, lymphocytes, eosinophils, and plasma cells in the intestinal mucosa (Giovanni Barbara et al., 2004; Javier Santos et al., 2002). This close relationship has been described for fibers that contain neurokinin-A, substance P, vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), neuropeptide Y, encephalins, galanin and CRH (Mawdsley & Rampton, 2005).

CRH and urocortins are widely expressed in the GI tract, and are produced by myofibroblasts, autonomic ganglion, extrinsic nervous cells and enterochromaffin cells (Tache & Perdue, 2004). Peripheral CRH modulates the inflammatory process by acting on immune cells and on nerve endings and overall interfering in the intestinal mucosal and motor functions. This is especially evident in stress-related intestinal inflammation, but also in response to bacterial enterotoxins.

Immune cells and mucosal nerve endings can also express opioid and melacortin receptors (Bagnol, Mansour, Akil & Watson, 1997), and also immune cells can produce products derivate from the POMC gene such as endorphins and ACTH. Endogenous opioids (endorphins and encephalins) have anti-inflammatory properties. Activation of μ opioid receptors modulate immune cell proliferation, NK cell activity and inflammatory and immunoregulatory cytokine production (Heine, Maslam, Zareno, Joëls & Lucassen, 2004). Moreover, μ opioid agonists have inhibitory effects on pain, intestinal motility and secretion and they have a potent anti-inflammatory effect (Philippe et al., 2003). Endogenous cannabinoids such as anandamide y 2-araquinodilglicerol have a role in inflammation modulation. This effect it is partially related to cannabinoid 1 receptor (CB1) inhibition of cholinergic excitatory neurotransmission in the intestine decreasing intestinal motility and ion transportation (Hornby & Prouty, 2004; Sharkey & Wiley, 2016). However, their role in inflammation is likely due to an increase of signal mediated by CB1 in the enteric neurons and by an activation of transient receptor potential cation channel subfamily V member 1 (TRPV1) (McVey, Schmid, Schmid & Vigna, 2003). These receptors have recently been identified as involved in inflammation-related visceral hypersensitivity (Sanson, Bueno & Fioramonti, 2006), in the stress response (Hill et al., 2010) and in the interaction between the intestinal epithelium and the microbiota (Cani, 2012).

2.5.2. Stress-mast cell axis

The regulation of intestinal epithelial physiology implies many mediators, among which mast cell deserves especial attention. Intestinal mast cells are strategically arranged to interact with the local neuroendocrine pathways. In order to maintain homeostasis, sensitive function is performed through the detection of a huge variety of signals that come from the environment surrounding them through specific and non-specific receptors (Javier Santos, Guilarte, Alonso & Malagelada, 2005) and responding by releasing selectively or massively biologic pre-made or the *de novo* synthesized substances. The variety of mediators that mast cell can release is so large that mast cells have been implicated in almost all the inflammatory and regulatory mechanisms in the GI tract (Bischoff, 2007). In the stress-related inflammatory processes is when this capacity of bidirectional communication with the CNS, the autonomic nervous system and the enteric nervous system becomes more relevant. This has been demonstrated *in vivo* by evidencing intestinal lumen release of mast cell mediators after an acute stress, which is accompanied by an increase in epithelial secretion (J Santos et al., 1998). Furthermore, *in vitro* studies show that CRH, through the activation of CRH receptors 1 and 2, expressed

by subepithelial mast cells, increases transcellular macromolecular permeability in the human colon mucosa (C. Wallon et al., 2008). Mast cell mediators can sensitize mesenteric afferent nerve endings and nociceptor receptors (Jiang et al., 2000). Among these mast cell mediators, histamine and serotonin can induce water, mucus and electrolyte secretion into the intestinal lumen. Mast cells can also produce and release nerve growth factor (NGF), which leads to increased intestinal permeability mediated by CRH receptor 1 (Frederick Barreau et al., 2007). Moreover, NGF has a hyperalgesic effect on primary sensitive neurons. Tryptase is the most abundant serine protease in mast cell granules and it can stimulate protease-activated receptor 2 (PAR2) in nerve fibers. PAR2 can regulate enteric neurotransmission, secretion, motility, epithelial permeability and visceral sensitivity contributing to intestinal inflammation through these mechanisms (Vergnolle, 2005).

3. Functional gastrointestinal disorders as diseases related to stress and GBA dysfunction: IBS as a model.

Functional gastrointestinal disorders (FGID) are a heterogeneous group of entities mainly characterized by chronic or recurrent-intermittent symptoms not attributable, according to the classic definition, to structural or biological alterations (D A Drossman, 1995; Mitchell & Drossman, 1987).

Among FGIDs, irritable bowel syndrome (IBS) and functional dyspepsia are, probably, the most common and representative diseases. In fact, IBS represents up to 15% of primary care consultations and is one of the most common (25-30%) referrals to GI clinics (Douglas A. Drossman, Camilleri, Mayer & Whitehead, 2002; Talley, Zinsmeister, Van Dyke & Melton, 1991). IBS can affect up to 20% of adult population in developed countries (Caballero-Plasencia et al., 1999; Hungin, Whorwell, Tack & Mearin, 2003; Mearin et al., 2001). In our country, prevalence of IBS oscillates between 3.3 and 13.6% (Caballero-Plasencia et al., 1999; Hungin et al., 2003; Mearin et al., 2001). Frequently, patients suffer from more than one FGID at the same time and this fact, together with the lack of effective treatments available, implies a reduction in patients' quality of life (Gralnek, Hays, Kilbourne, Naliboff & Mayer, 2000; Whitehead, Burnett, Cook & Taub, 1996), generating a huge economic burden, up to 1% of total medical expenses in United States (Brandt et al., 2009).

Although the high prevalence and high impact of these disorders in the society, its pathophysiological mechanisms remain still to be defined. The development of new techniques to study GI physiology (barostat, motility capsule endoscopy); clinical evaluation criteria (Rome and Manning criteria); animal models (Giada De Palma et al., 2017; María Vicario et al., 2012, 2010) and also techniques to study pain and behavioural responses (functional MRI) have been crucial to start understanding these disorders. Although these advances, there are still no biomarkers or specific diagnostic tests and the diagnosis is made by positive symptomatology in the absence of other diseases based on a diagnostic criteria, the Rome Criteria, currently in their fourth version (Lacy et al., 2016), which classifies each disorder according to the anatomical region that affects.

3.1. BIOPSYCHOSOCIAL MODEL

The biopsychosocial or systems model (Engel, 1977, 1981) not only helped the scientific community to understand the bi-directional relationship between body and mind, but also set a clinical framework that integrates psychosocial and biomedical factors that explain the illness experience and also help creating a unified structure for multidisciplinary research methodology. This model can be observed in Figure 6.

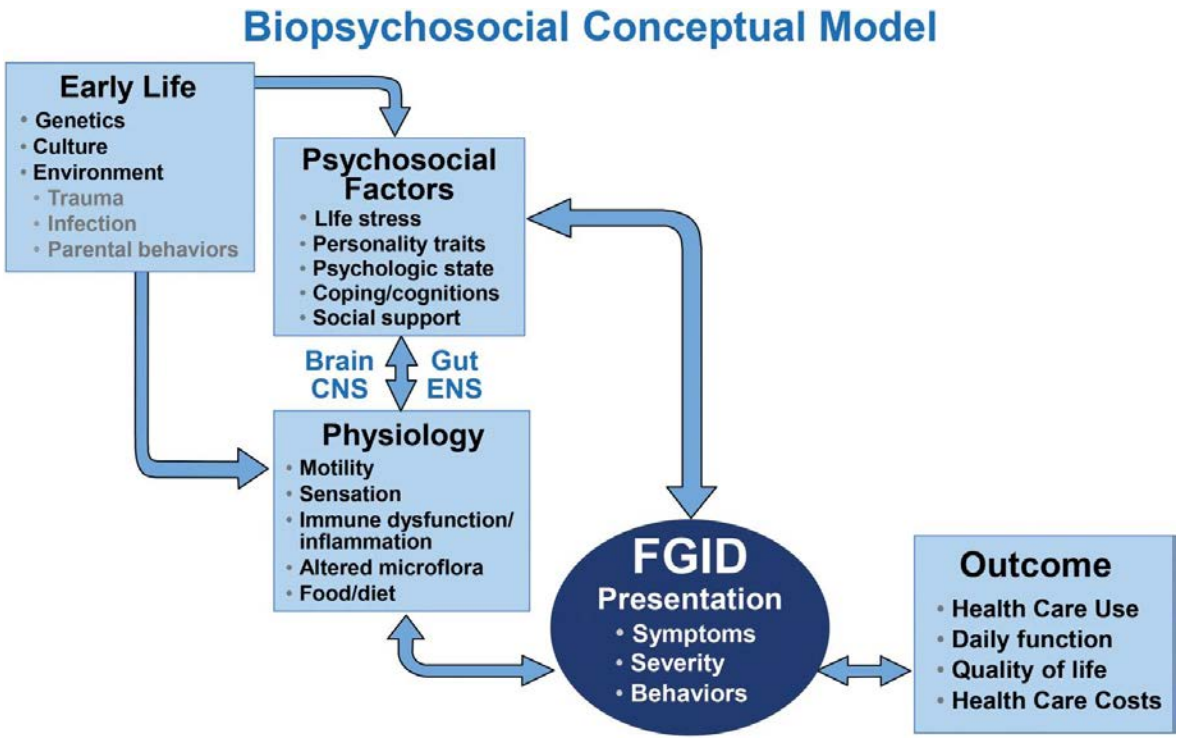


Figure 6: Biospsicosocial model. From: (Douglas A. Drossman, 2016).

3.2. IRRITABLE BOWEL SYNDROME

IBS is defined by the Rome IV criteria as the symptoms of recurrent abdominal pain on an average at least 1 day/week in the last 3 months associated with 2 or more of the following: related to defecation,; pain onset linked to a change in frequency of stool; pain onset linked to a change in form (appearance) of stool (Lacy et al., 2016). In pathophysiology research and clinical trials, a pain/discomfort frequency of at least 2 days a week during screening evaluation is recommended for subject eligibility.

According to Rome IV criteria there are four IBS subtypes (Figure 7) defined by the predominant bowel habits are based on stool form on days with at least one abnormal bowel movement, measured by the Bristol Stool Form Scale (Lewis & Heaton, 1997) (Figure 8):

- IBS with predominant constipation (IBS-C): More than 25% of bowel movements with Bristol stool form types 1 or 2 and less than one- 25% of bowel movements with Bristol stool form types 6 or 7. Alternative for epidemiology or clinical practice: Patient reports that abnormal bowel movements are usually constipation (Bristol type 1 or 2).

- IBS with predominant diarrhea (IBS-D): more than 25% Bristol stool form types 6 or 7 and less than 25% of bowel movements with Bristol stool form types 1 or 2. Alternative for epidemiology or clinical practice: Patient reports that abnormal bowel movements are usually diarrhea (Bristol type 6 or 7)

- IBS with mixed bowel habits (IBS-M): more than 25% of bowel movements with Bristol stool form types 1 or 2 and more than 25% of bowel movements with Bristol stool form types 6 or 7. Alternative for epidemiology or clinical practice: Patient reports that abnormal bowel movements are usually both constipation and diarrhea.

- IBS unclassified (IBS-U): patients who meet diagnostic criteria for IBS but whose bowel habits cannot be accurately categorized into 1 of the 3 groups above should be categorized as having IBS unclassified.

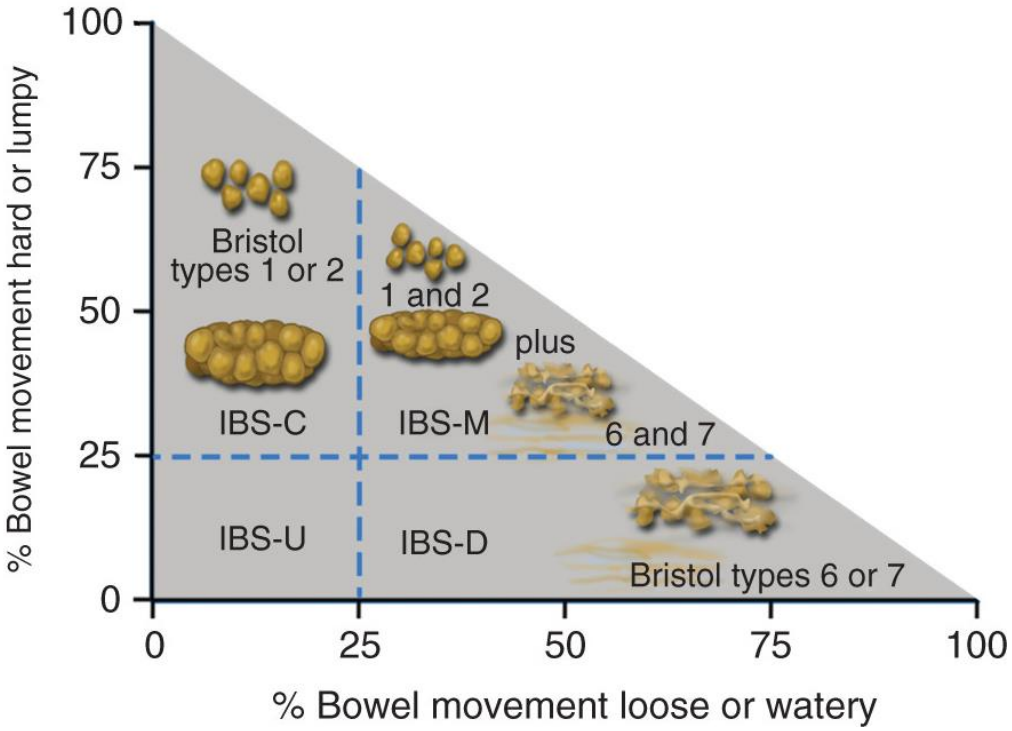


Figure 7: IBS subtypes according to Rome IV criteria. From: (Lacy et al., 2016).

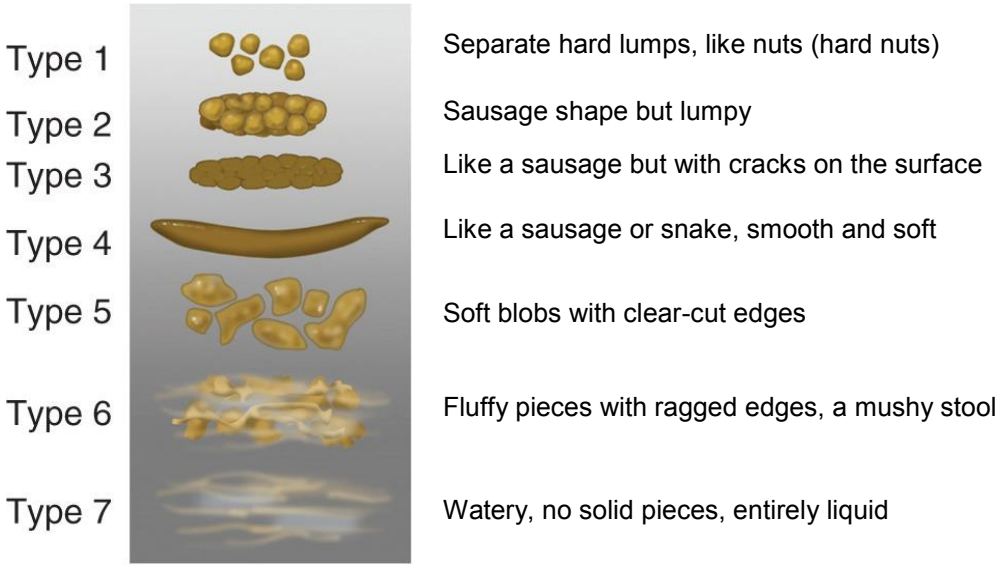


Figure 8: Bristol stool chart. From: (Lacy et al., 2016).

To classify patients with IBS-D in this thesis Rome II-III criteria were used, as there were the accepted criteria at time of subject inclusion. There are some changes between Rome III and Rome IV criteria for IBS diagnosis, these changes are summarized in Figure 9.

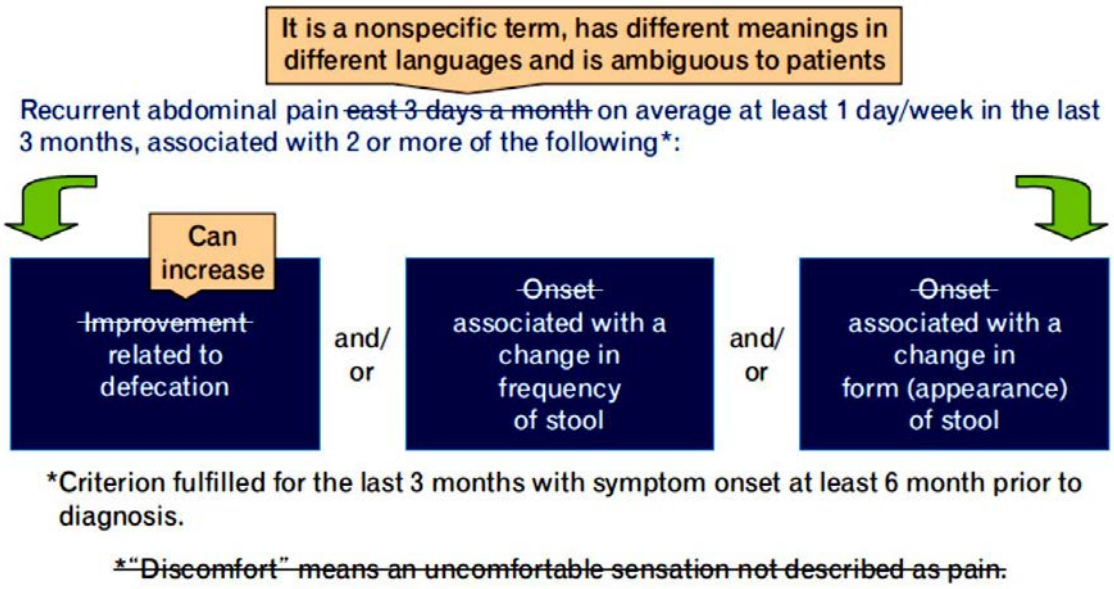


Figure 9: Changes in diagnostic criteria for IBS from Rome III to Rome IV. From: (Schmulson & Drossman, 2017).

3.3. PATHOPHYSIOLOGY

IBS is a complex disorder, whose pathophysiology is not completely described. It is produced as consequence of interaction of genetic, psychosocial, toxic, dietetic and biologic factors. Although in the past few years there a growing research interest has revealed some mechanisms of GI dysfunction, a coherent link between specific pathologies and IBS symptoms has not been established yet. There is a female gender predominance (2:1) in IBS patients (Lovell & Ford, 2012; W. G. Thompson, 1997). Although this risk of developing IBS in female subjects could be attributed to differences in psychological and neuroendocrine responses to stress, the underlying factors remain still to be found (Kelly, Tyrka, Anderson, Price & Carpenter, 2008; Kudielka & Kirschbaum, 2005).

3.3.1. Genetic factors

Genetic predisposition to develop IBS is likely, as it is not uncommon to find more than one patient with IBS in the same family (Buonavolontà et al., 2010; Yuri A Saito et al., 2010). In fact, studies performed in homozygous and heterozygous twins demonstrated a higher probability of presenting IBS on the other twin when one is affected, compared to the general population (Lembo, Zaman, Jones & Talley, 2007; R L Levy et al., 2001; Mohammed, Cherkas, Riley, Spector & Trudgill, 2005; Morris-Yates, Talley, Boyce,

Nandurkar & Andrews, 1998; Svedberg, Johansson, Wallander, Hamelin & Pedersen, 2002).

In the last few years, several genes have been associated with susceptibility to develop IBS. Most of the studies were case-control studies with a low number of subjects and the vast majority of genes described were associated with immune function and intestinal serotonin modulation (D'Amato, 2013). Despite all the efforts, only one, tumor necrosis factor superfamily member 15 has been validated as a genetic variant associated with IBS, particularly to IBS-C (Zucchelli et al., 2011). An international multicentre study using genome wide association analysis identified and validated different genes linked to IBS, being the most affected at the locus 7p22.1 (Ek et al., 2015). This study also confirmed some of the genetic associations previously observed. Although this study represents a new and powerful way to unravel the genetic susceptibility of IBS, the heterogeneity of IBS makes it difficult to generalize these results.

3.3.2. Environmental factors

Intestinal microbiota of different IBS subtypes present quantitative and qualitative differences in the stability and composition along time (Kassinen et al., 2007; Lyra et al., 2009). Although an association between IBS development and specific bacterial species has not been identified yet, an important relationship between the microorganisms and IBS development is supported by the fact that between 7 to 31 % of patients which have suffered a gastroenteritis (viral, bacterial or helminthic) develop IBS (Hanevik et al., 2014; John K. Marshall et al., 2006; Robin C Spiller, 2003; Wang, Fang & Pan, 2004; Zanini et al., 2012). Although the exact mechanisms behind symptoms generation after an infection are unclear, it has been shown that after *Campylobacter sp* infection patients presented an increase in enterochromaffin cells, T lymphocytes and intestinal permeability that will generate an increase in serotonin, which increases GI motility and mediates visceral hypersensitivity (R C Spiller et al., 2000). Another mechanism is that GI tract infection can lead to the development of bile acid malabsorption (Niaz, Sandrasegaran, Renny & Jones, n.d.; Sinha, Liston, Testa & Moriarty, 1998). There have been identified several factors associated to the development of IBS after an infection such as prolonged infection, virulence of the bacteria, smoking habit, presence of inflammatory markers, female gender, depression, or adverse life events (R. Spiller & Garsed, 2009). Moreover, patients with IBS present dysbiosis when compared with healthy volunteers (Kassinen et al., 2007), but still remains unclear if this is cause or consequence of the disease and which are the beneficial or harmful bacterial species. Intestinal dysbiosis may contribute to IBS

physiopathology by disrupting intestinal motility, perpetuating intestinal micro-inflammation, contributing to visceral hypersensitivity and overall affecting the gut-brain axis (Malinen et al., 2005). There are other factors that can modify the intestinal microbiota, contributing to IBS symptomatology such as fiber rich diet, antibiotic treatment and use of prebiotics and probiotics (Dear, Elia & Hunter, 2005; Maxwell, Rink, Kumar & Mendall, 2002; Shepherd & Gibson, 2006; Magnus Simrén et al., 2013; Törnblom, Holmvall, Svenungsson & Lindberg, 2007)

In the last few years, a debate of the role of small intestinal bacterial overgrowth (SIBO) in symptom generation in IBS patients has been generated. SIBO has been associated to meteorism, bloating and to the alteration of intestinal function through the fermentation of carbohydrates by the intestinal bacteria. Its real prevalence is unknown but could reach up to 54% of IBS patients (R. S. Choung et al., 2011). SIBO can be diagnosed by lactulose hydrogen breath test, although its specificity is low due to the large amount of false positive cases. On the other hand, there is growing evidence that non-absorbable antibiotics (Lupascu et al., 2005; Pimentel, Chow & Lin, 2000, 2003; Pimentel et al., 2011; Pimentel, Park, Mirocha, Kane & Kong, 2006) and probiotics (A. C. Ford et al., 2014) are beneficial in IBS.

3.3.3. Mechanisms of intestinal dysfunction

3.3.3.1. Visceral hypersensitivity

Visceral hypersensitivity is really common in patients with IBS. It is produced by the activation of different pathways depending if the stimulus acts on the mechanoreceptors (distension), osmoreceptors (measure changes in pH, temperature and osmolarity) or nociceptors (detect painful stimuli). Visceral hypersensitivity can be presented as allodynia (abnormal pain perception from stimuli which do not normally provoke pain, normally form a mechanical or temperature stimulus) (Moshiree, Zhou, Price & Verne, 2006), hypervigilance, hyperalgesia, and an exaggerated referred pain perception. This state of hypersensitivity is not only confined to the GI tract, but also is extended to the CNS (Ritchie, 1973). Visceral hypersensitivity can be diagnosed by the presence of a diminished pain perception threshold (Azpiroz et al., 2007); a sensitization to repeated distensions (Kwan, Diamant, Mikula & Davis, 2005) and the presence of referred to aberrant zones different than sacral area.

3.3.3.2. Intestinal motility alterations

There is a remarkable motor response against different stressing stimuli (either physical or psychological) when comparing tests performed to IBS patients with those from healthy volunteers (Douglas A. Drossman et al., 2002; Kellow & Phillips, 1987; McKee & Quigley, 1993). These findings could explain why symptoms such as diarrhea or abdominal pain are generated, but they are not enough to completely explain all the clinical manifestations of IBS. Patients suffering from IBS have an altered GI transit (accelerated in IBS-D or slowed down in IBS-C), although these observation was not observed in studies with high number of patients (Michael Camilleri et al., 2008). They also have a higher alteration of intestinal function with dietary transgression (Chey, Jin, Lee, Sun & Lee, 2001; McKee & Quigley, 1993; Narducci et al., 1986; Sullivan, Cohen & Snape, 1978) or psychosocial factors (Welgan, Meshkinpour & Beeler, 1988). Moreover there is an alteration to gas overload tolerance (Serra, Azpiroz & Malagelada, 2001) and also an excessive motor response to gastrocolic reflex, rectal distension, to stress and to the administration of cholecystokinin (Kellow, Phillips, Miller & Zinsmeister, 1988) or CRH (Fukudo, Nomura & Hongo, 1998).

3.3.3.3. Intestinal micro-inflammation

In the past few years, several studies have describe the presence of an inflammatory infiltrate (lymphocytes, mast cells, enteroendocrine cells) in the mucosa and or in the myenteric plexus from the small bowel (Guilarte et al., 2007) and the colon (G. Barbara et al., 2004; Piche et al., 2008) of patients with IBS. The presence of this inflammatory infiltrate together with immune activation and cytokine release, could contribute to the amplification or continuation of GI symptoms through processes of periphery sensitization and/or abnormal motility. This hypothesis is based on a study performed by Barbara and colleagues (Giovanni Barbara et al., 2004) where they found a close proximity between mast cells and nerve endings in patients with IBS. Moreover, other immune cell populations have been identified as increased in number and/or activation state in IBS. Eosinophilic infiltration in the lamina propria of IBS patients (K. S. Park et al., 2008) and secretion to the intestinal lumen of eosinophil cationic protein and eotaxin-1 after CRF administration in patients with IBS (Guilarte et al., 2004; Martínez, González-Castro, Vicario & Santos, 2012) have been described. A recent study from our group (Maria Vicario et al., 2014) demonstrated that not only innate immune response is implicated in IBS physiopathology, but also patients suffering from IBS have a higher number of B lymphocytes and plasma cells in the small intestine. Moreover, this cells where more

activated and plasma cells where closer to mast cells. This higher humoral activity correlated with clinical IBS symptomatology.

3.3.4. Psychological morbidity

The prevalence of psychiatric and psychological morbidity is higher in subjects suffering from IBS than in the general population (Janssens, Zijlema, Joustra & Rosmalen, 2015) and in patients with other GI diseases (Stasi, Rosselli, Bellini, Laffi & Milani, 2012). The presence of these comorbidities have been related with symptom worsening (S Elsenbruch et al., 2010; Sigrid Elsenbruch et al., 2010), a higher demand of medical care and a perpetuation of intestinal alteration. The vast majority of IBS patients associate symptoms exacerbation and outbreak with acute psychosocial events (Bennett, Tennant, Piesse, Badcock & Kellow, 1998). In the last few years there has been a growing interest to understand the function underlying mechanisms of chronic psychological stress in FGID. Epidemiological studies have associated the presence of IBS with stressful events in early life (childhood and adolescence), such as socio-economical stress in adolescence, parental marriage problems or sexual or physical abuse (Delvaux, Denis & Allemand, 1997; Irwin et al., 1996; Bruce D Naliboff et al., 2012). Moreover, there are several well described factors that favours the development of IBS in adulthood, such as being fired, divorce and social changes among others (Surdea-Blaga, B??ban & Dumitrascu, 2012). Recent observations from our group indicate that patients with IBS-D display higher levels of psychosocial stress than healthy volunteers (Guilarte et al., 2007; Martínez et al., 2013; Martínez, Vicario, et al., 2012). Moreover, healthy females with high levels of psychosocial stress display an abnormal epithelial stress in response to an acute stress (Carmen Alonso et al., 2008), which could be the initial phase to develop long-lasting alterations and could underlie a higher prevalence of IBS in females.

3.3.5. Altered intestinal barrier function

Patients with IBS present an increased *in vivo* and *ex vivo* intestinal permeability in the colon and rectum and also in the small intestine (Dunlop et al., 2006; A. S. Rao et al., 2011; Zhou, Zhang & Verne, 2009). This increase in intestinal permeability could be a key factor in IBS pathophysiology, as patients with higher intestinal permeability show higher visceral hypersensitivity (Zhou et al., 2009). Moreover, patients with IBS-D present a dysregulation of TJs expression, which is associated with distinctive mast cell activation (Martínez, Vicario, et al., 2012). There are also phosphorylation alterations in the transmembrane part of the TJs and the intestinal mucosa present ultrastructural deficiencies at the apical junctional complex. All these alterations correlate with immune

mast cell activation and also with intestinal symptoms in these patients (Martínez et al., 2013). Nowadays, it still remains unclear whether this alteration of intestinal permeability is cause or consequence of the disease, but therapeutic strategies to restore intestinal permeability are being developed.

3.3.6. Diet

Up to 70% of patients suffering from IBS refer that their symptoms or aggravation of their disease is related with the consumption of certain foods (Monsbakken, Vandvik & Farup, 2006; Nanda, James, Smith, Dudley & Jewell, 1989; M Simrén et al., 2001). This proportion is much higher than general population (G R Locke, Zinsmeister, Talley, Fett & Melton, 2000). The appearance of symptoms related with food intake could be confounded with food allergy, but several studies have evaluated food-related reactions in patients complaining from IBS and they have not found any correlation between foods that cause symptoms and allergy tests results (Dainese, Galliani, De Lazzari, Di Leo & Naccarato, 1999; G R Locke et al., 2000; Monsbakken et al., 2006; Nanda et al., 1989; M Simrén et al., 2001). In fact, several studies that evaluated the effect of dietary interventions on the IBS GI symptoms show dissimilar results (Atkinson, Sheldon, Shaath & Whorwell, 2004; Biesiekierski et al., 2013; Böhn et al., 2015; Halmos, Power, Shepherd, Gibson & Muir, 2014; Huamán et al., 2015; Jones, McLaughlan, Shorthouse, Workman & Hunter, 1982; McIntosh et al., 2016; M.-I. Park & Camilleri, 2006; Stefanini et al., 1995; Zwetchkenbaum & Burakoff, 1988).

3.3.7. Other comorbidities

Prevalence of other FGIDs is higher in patients with IBS. It is frequently associated with functional dyspepsia and it is not rare that patients with IBS present other functional diseases such as chronic fatigue, fibromyalgia, migraines, tensional headache, dyspareunia or chronic pelvic pain (Enck et al., 2016).

3.4. TREATMENT

The interaction between the patient and the treating physician is one of the key points in the treatment of IBS, especially when considering this disease from the biopsychosocial point of view (Douglas A. Drossman, 2016). To develop an adequate interaction between physician-patient allows the approach of the multiple factors that contribute and perpetuate the clinical symptomatology. This interaction is also important from an economical point of view, as it can prevent the performance of unnecessary diagnostic

tests and diminishes emergency and second opinion consultations, overall reducing health care costs.

Moreover, when deciding the treatment for a patient, is important to determine the most predominant symptom of the patient as it will condition treatment strategy. When deciding management strategy, other factors must be taking into account, such as patient preferences, previous treatments, GI symptoms pattern, severity and its impact on quality of life and also psychological comorbidities should be taken into account as it can contribute to the presence or worsening of IBS symptoms (Figure 10).
Finally, it is also important to know what IBS patients want or expect from their medical care as it will help them to manage their symptoms, improve compliance with prescribed treatments, improve patients satisfaction and overall diminish the inappropriate use of resources (Halpert, 2011).

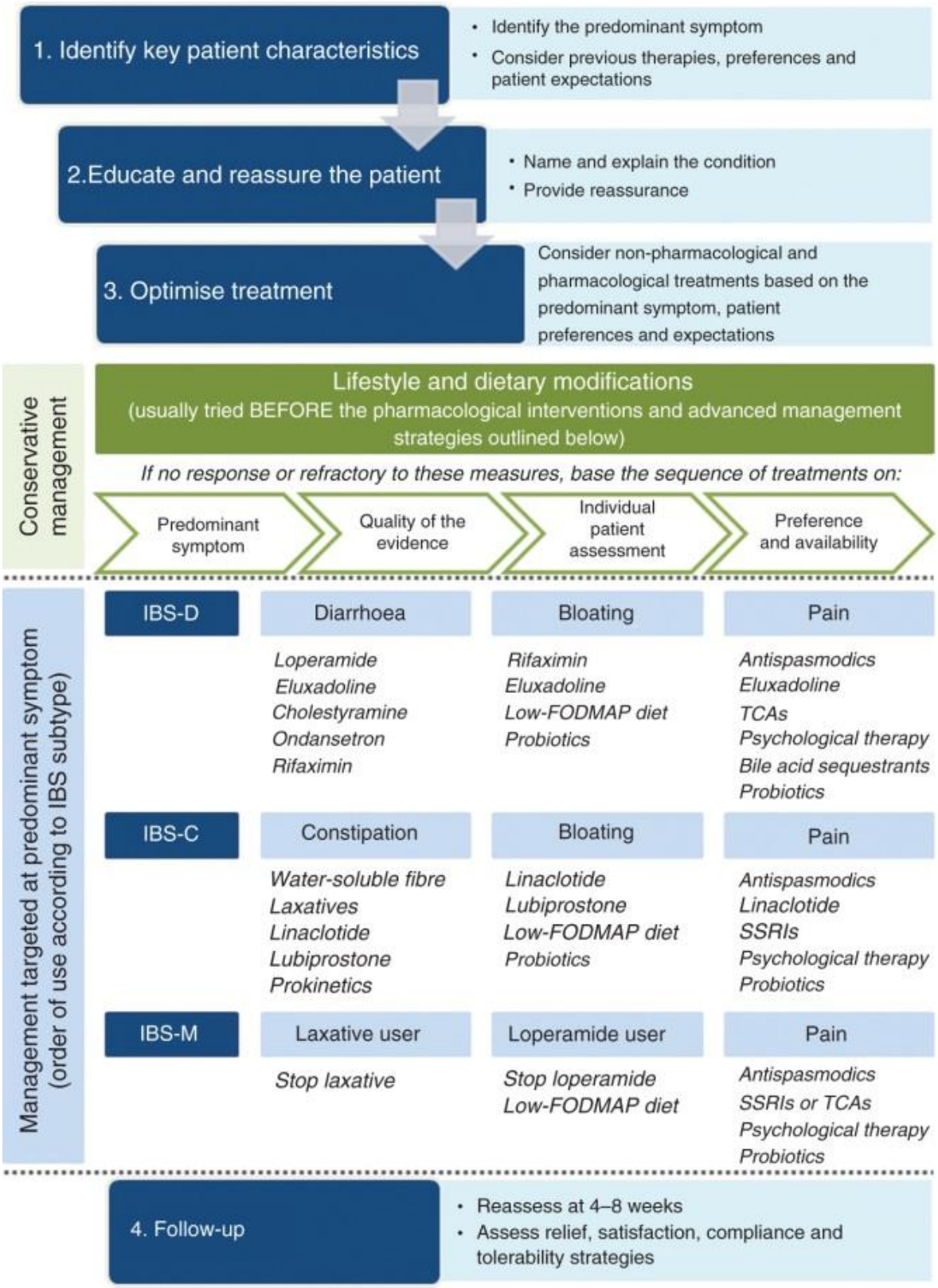


Figure 10: Management algorithm for irritable bowel syndrome. FODMAP: fermentable oligosaccharides, disaccharides, monosaccharides and polyols; SSRIs: selective serotonin re-uptake inhibitors; TCAs: tricyclic antidepressants. From: (Moayyedi et al., 2017)

INTRODUCTION

As IBS physiopathology is multifactorial implicating different systems, multiple IBS treatments have been developed to target symptoms according to underlying physiopathology. In Table 3 there is a summary of current treatments and drugs in development for IBS, along with its efficacy and the quality of data.

Therapy	Mechanism of action	Efficacy	Quality of data	Adverse events	Limitations of data
Antispasmodic drugs	Smooth muscle relaxation	May be effective	Low	More likely with antispasmodics in a meta-analysis of 22 RCTs, particularly dry mouth, dizziness, and blurred vision	No high-quality trials, heterogeneity between studies, possible publication bias, and only a small number of RCTs assessing each individual antispasmodic
Peppermint oil	Smooth muscle relaxation	Effective	Moderate	No increase in adverse events in a meta-analysis of 4 RCTs	Heterogeneity between studies
Antidepressants	Central sensory modulation	Effective	Moderate	More likely with antidepressants in a meta-analysis of 17 RCTs, particularly dry mouth and drowsiness	Few high-quality trials, heterogeneity between studies, possible publication bias, and some atypical trials included
Loperamide	μ-opioid agonist	Unknown	Low	Limited data	Few RCTs, with a small number of participants, not all of whom had IBS
Cholestyramine, colestipol, colesevelam	Bile acid sequestrants	Unknown	Low	Limited data	No published RCTs
Rifaximin	Non-absorbable antibiotic	Effective	Moderate	No increase in adverse events in a meta-analysis of 5 RCTs	Only a modest benefit over placebo in published RCTs
Eluxadoline	Mixed opioid receptor modulator	Effective	High	Serious events included acute pancreatitis and sphincter of Oddi spasm. Nausea and headache commoner with active therapy	Only a modest benefit over placebo in published RCTs; no benefit over placebo in terms of abdominal pain
Disodium chromoglycate, ketotifen Ebastine	Mast cell stabilizers	Effective	Low	Limited data	Only a modest benefit over placebo in published RCTs
Probiotics		Effective	Moderate	Limited data	Probiotics had beneficial effects on global IBS, abdominal pain, bloating, and flatulence scores. Data for prebiotics and symbiotics in IBS were sparse.
FODMAPS	Dietary intervention	Effective	Moderate	Adverse events were assessed in three RCTs only and no intervention-related adverse events were reported.	Potential for inadequate nutrient intake with stringent dietary restriction but No data on long term effects of low FODMAP diet.
Behavioural therapies (mindfulness, neurofeedback, conductive behavioural therapy, relaxation and hypnotherapy)		May be effective	Low	Limited data	Different therapies have different outcomes. More studies are needed.
Alosetron, ramosetron, ondansetron	5-HT3 receptor antagonists	Effective	High	Serious events with alosetron included ischemic colitis and severe constipation. Ramosetron and ondansetron may be	Fewer RCTs of ramosetron and ondansetron; ondansetron may have no benefit over placebo in terms of abdominal pain

				safer, although constipation commoner with active therapy	
Prucalopride	5-HT4 receptor agonist	Effective	High	Diarrhea, cramping, and cardiovascular AEs with "old generation" drugs in this class	Data available for tegaserod and mosapride, not for "new generation" drugs in this class: prucalopride, naronapride, velusetrag, YKP10811
Linaclotide	GC-C receptor agonist	Effective	High	Diarrhea commoner with active therapy, occurring in 20% of patients	None
Plecanatide,	GC-C receptor agonist	Effective	High	Diarrhea commoner with active therapy occurring in ~6% of patients	None
Ibodutant	Neurokinin NK2 antagonist	May be effective	Moderate	Promising visceral analgesic in a phase 2B trial	Awaiting phase 3 trials
TSPO inhibitor		May be effective	Low	Modest efficacy in a single proof of concept trial	Awaiting phase 2B trials
Tenapanor	NHE3 inhibitor	Effective	Moderate	Diarrhea commoner with active therapy, occurring in 12% of patients	Awaiting phase 2B/3 trials

Table 3: Summary of current treatments and drugs in development for IBS. Adapted and modified from: (Michael Camilleri & Ford, 2017). 5-HT: 5-hydroxy tryptamine; Cl-C2: chloride channel 2; FODMAP: fermentable oligosaccharides, disaccharides, monosaccharides and polyols; GC-C: guanylate cyclase C; NHE: sodium-hydrogen exchanger; RCT: randomized controlled trial; TSPO: translocator protein.

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

Background psychosocial stress and comorbidities determines barrier function in response to acute stress and clinical severity in irritable bowel syndrome.

OBJECTIVES

Main

To determine the effect of acute experimental stress on intestinal barrier function in healthy humans

Secondary

To determine the influence of mental and gastrointestinal comorbidities in clinical severity of diarrhea predominant irritable bowel syndrome and its association with chronic psychosocial stress.

To identify molecular mechanisms and pathways involved in intestinal mucosal barrier response to acute experimental stress in health.

To explore the role of chronic psychosocial stress and sex on intestinal permeability in health.

CHAPTER 1

4. Dyspepsia and depression impact on clinical severity in diarrhea-predominant irritable bowel syndrome

4.1. ABSTRACT

Background & aim: Irritable bowel syndrome (IBS) is a highly prevalent gastrointestinal disorder in western societies. In diarrhea-predominant IBS (IBS-D), Life stress, sex and mucosal immune activation have been associated with epithelial dysfunction and clinical severity. However, the interaction of susceptibility factors and clinical severity remains undefined in IBS-D. We performed an observational study in order to identify how comorbidities, sex, and mucosal immune infiltration interact with clinical outcome in IBS-D. **Material & Methods:** This is a retrospective study of IBS-D patients fulfilling Rome III criteria and healthy volunteers (HV), as a control group. Clinical assessment, including gastrointestinal symptoms, comorbidities and psychosocial stress were recorded using validated questionnaires. Mucosal leukocyte counts from the jejunum were analyzed. **Results**: Two-hundred-forty-three IBS-D patients and 164 HV were included. IBS-D patients displayed higher level of chronic psychosocial stress and depression than HV. The analysis revealed higher proportion of females with dyspepsia in the IBS-D group. Moreover, depression and stress positively correlated with abdominal pain and severity in IBS-D patients. No differences were found in the intestinal inflammatory infiltrate between IBS-D patients and HV. Subgroup analysis by comorbid factors showed a more severe IBS-D in patients that had concomitant depression. **Conclusion**: Chronic psychosocial stress features IBS-D but does not affect clinical severity, while comorbid factors such as dyspepsia or depression are directly associated with IBS-D symptom severity. Actions directed to identify factors affecting IBS should be taken into consideration in order to reduce clinical severity and, overall, enhance quality of life in these patients.

4.2. INTRODUCTION

Irritable bowel syndrome (IBS) afflicts 10-20% of the western adult population (Rok Seon Choung, Locke 3rd & Locke, 2011; Sperber et al., 2017). IBS is characterized by recurrent abdominal pain and altered bowel habits and a marked reduction in quality of life (Buono, Carson & Flores, 2017). Growing evidence indicates that factors including food, antibiotics, bile acids, infections, sex, and psychosocial events are all implicated in IBS origin (Giovanni Barbara et al., 2016; Gazouli et al., 2016), acting in genetically and epigenetically predisposed individuals to increase intestinal permeability, that via activation of local and brain immune and neuroendocrine responses, can lead to abnormal secretory and sensorimotor outputs in the gut (Enck et al., 2016; Ohman & Simrén, 2010). Epidemiological studies also yield a clear but mechanistically unexplained female predominance in IBS, particularly in post-infective and diarrhea predominant subtypes (Gwee et al., 1999; Pigrau et al., 2016; Wouters et al., 2015).

The central nervous system and the gut can impact on each other affecting clinical manifestations through the brain-gut and the gut-brain circuitry. Symptom intensity and duration have been linked to the presence of psychological alterations and chronic stress as comorbid factors (Mönnikes et al., 2001; Wouters et al., 2015). In fact, stress can alter intestinal motility (S. S. C. Rao, Hatfield, Suls & Chamberlain, 1998; Sagami et al., 2004), and disturb epithelial and secretory function (Carmen Alonso et al., 2008; J Santos et al., 1998), leading to mucosal inflammation (Qiu et al., 1999), and enhancement in visceral perception (M. J. Ford, Camilleri, Zinsmeister & Hanson, 1995; Murray et al., 2004) and pain networks (Tanaka et al., 2016). In IBS patients, a past history of traumatic stress or high levels of chronic psychosocial stress related to physical and/or sexual abuse is not uncommon (Delvaux et al., 1997; Guilarte et al., 2007). Similarly, inadequate stress coping strategies or lack of adaptability have been proposed as mechanisms that may lead to abnormal central pain processing and peripheral sensitization in this population (Knowles et al., 2017). Therefore, stress can trigger or modify clinical outcome of IBS in several ways (G Richard Locke, Weaver, Melton & Talley, 2004). Psychiatric comorbidity is also frequent in IBS and it has been related with poorer outcome and less quality of life in these patients (Kanuri et al., 2016).

Many studies also describe an increase in the number and/or activation of both mucosal and humoral immunity (Enck et al., 2016; Maria Vicario et al., 2014) in the gut wall, from the duodenum to the rectum. However, few studies have analyzed the association between microinflammation, sex and comorbidities with IBS-D severity and, unfortunately,

previous studies have included a small number of subjects, and lack a control reference group. Therefore, the main aim of this study was to determine the prevalence of psychosocial stress in a large IBS-D cohort and its relation to clinical outcome and other biological and demographic factors. The secondary aim was to evaluate the effect of sex and comorbidities.

4.3. MATERIALS AND METHODS

4.3.1. Study subjects

This is an observational and retrospective cross-sectional study of our database including IBS-D patients fulfilling Rome II or III criteria and healthy volunteers (HV) that have participated in clinical studies at the Functional Gastrointestinal Disorders Unit in Hospital Universitari Vall d'Hebron, between January 2004 and December 2017, in whom an intestinal symptom questionnaire and/or intestinal jejunal biopsy were obtained, when Patients had been recruited from the outpatient clinic and primary care-associated centers and HV by public advertisement.

Other inclusion criteria: Age between 18 and 65. Exclusion criteria: post-infective IBS-D (PI-IBS-D), pregnancy, previous major abdominal surgery, or any metabolic or structural disease or therapeutic intervention that could explain gastrointestinal symptoms. No drugs, herbs or food supplements were allowed during the week prior to the biopsy,

All participants underwent physical examination and allergy evaluation. Broad biochemical and serological profile, including anti-transglutaminase antibodies and thyroid hormones was obtained. Reasonable exclusion of gastrointestinal comorbidities, including microscopic colitis, celiac disease, and other diarrheal disorders, was accomplished by means of upper and lower endoscopy and small bowel capsule endoscopy, abdominal sonography and barium studies, when considered pertinent. HV participants disclosed no gastrointestinal symptoms (ROME negative for dyspepsia, IBS and other functional gastrointestinal disorders), did not suffer from any chronic disorder, and were not taking any treatment at the inclusion visit.

The study protocol was approved by the Ethics Committee of the Hospital Universitari Vall d'Hebron (PR (AG) 50/2017), and written informed consent was obtained from each participant.

4.3.2. Study design

We performed a retrospective analysis of all subjects (IBS-D and HV) included in our database that have participated in investigational studies at our center meeting the inclusion criteria. We first performed a descriptive analysis in each group and then compared demographic, psychosocial and clinical variables between groups. We further evaluated the effect of comorbidities on clinical severity in IBS-D. We also described the "microinflammation" in the subpopulation of IBS-D that underwent a mucosal biopsy and analyzed the potential link with stress and sex. Finally, in order to identify the factors that better characterized IBS-D we performed a random tree forest analysis by correlating biological and clinical data.

4.3.3. Clinical assessment

Gastrointestinal symptoms: IBS symptoms were evaluated using the Spanish validated questionnaire of Irritable Bowel Syndrome Severity Score Scale (IBS-SSS, Francis Score) (Almansa, García-Sánchez, Barceló, Díaz-Rubio & Rey, 2011) which measures: (a) severity of abdominal pain by a 100-point visual analogue scale; (b) Frequency of abdominal pain (number of days with pain); (c) Severity of bloating by a 100-point visual analogue scale; (d) Bowel habit dissatisfaction; and (e) interference of IBS with life in general. Moreover, stool frequency and stool consistency were assessed by the Bristol Stool Form Score (Heaton, Ghosh & Braddon, 1991). When subjects had more than one bowel movement per day, the participant was asked to mark the most frequent consistency pattern according to the Bristol Stool Form Score. Dyspepsia was diagnosed according to Rome IV criteria in the medical visit performed on the inclusion.

*Allergy: Food a*llergy was excluded by clinical history and atopy was identified by skin prick testing for 32 common foodstuffs and 24 inhalants (Leti SA, Barcelona, Spain), with histamine and saline as positive and negative controls, respectively. Subjects were categorized as atopic if they had positivity to at least one respiratory allergen or one food allergen in the absence of food allergy symptomatology.

Psychosocial stress: Background stress and depression were evaluated using 3 validated questionnaires: (1) The modified social readjustment scale of Holmes-Rahe (H-R), to assess the level of stress over the last year (Holmes & Rahe, 1967): low stress (H-R <150), moderate (H-R 150-299) or high stress levels (H-R ≥300), for this this study only were considered two groups, one with low stress (H-R <150) and the other one with moderate-high stress (H-R ≥150); (2) The perceived scale stress (PSS) of Cohen, to

assess the level of stress over the last month (Remor, 2006): no perceived stress (PSS <19), mild perceived stress (PSS 19-28), moderate perceived stress (PSS 29-38) or severe perceived stress (PSS >38); and, (3) the Beck's Depression Inventory (BDI), to evaluate the level of depression during the last week (Sanz, J., Navarro, M. E., Vázquez, 2003). According to the score, subjects can be divided into no depression (BDI <10) or depression (BDI ≥10) subgroup. This last group can be classified as mild (BDI 10-18), moderate (BDI 19-29) or severe depression (BDI 30-63).

4.3.4. Biological assessment

After an overnight fast, a single mucosal biopsy was obtained from the proximal jejunum, 5 cm distal to the angle of Treitz, using a modified Watson's capsule, as previously described (Guilarte et al., 2007). The biopsy was immediately fixed in formalin and subsequently embedded in paraffin for further microscopic examination, following standard histological procedures. Sections of 4 μm were cut and stained with hematoxylin and eosin (H&E) to assess the mucosal architecture and to identify eosinophils. Specific staining using monoclonal antibodies against CD3 or CD117 was performed as previously described (Guilarte et al., 2007) to numbers of intraepithelial T lymphocytes and mast cells, respectively. The number of eosinophils and mast cells is expressed as per high power field (HPF) in the lamina propria, and the number of T lymphocytes as per 100 epithelial cells. All tissue samples were analyzed and cells counted blindly by an expert pathologist.

4.3.5. Statistical analysis

Continuous variables, including age, body mass index (BMI), IBS-SSS, stress, and depression scores, were expressed as median (Q1-Q3). Categorical variables, such as sex, and stress level, atopy, positive skin pricks testing were expressed as percentages. For bivariate analysis between pair of categorical variables a Fisher's Exact Test, a Pearson's Chi-squared Test, a Kruskal-Wallis Rank Sum Test, or a Kendall's Rank Correlation Tau has been applied have been used when appropriate. Multivariate analysis was performed using a predictive model based on Random Forest method(Hastie, Tibshirani & Friedman, 2009). Finally, correlations were analyzed using the Spearman rank correlation test. *P*-values less than 0.05 were considered significant. Multiple testing problems have been controlled adjusting the Benjamini-Hochberg False Discovery Rate. Statistical analyses were performed using IBM-SPSS (IBM-SPSS Statistics Version 22, Chicago, IL, USA).

4.4. RESULTS

4.4.1. Participants

Of the 266 IBS-D patients and 171 HV identified in our database, 243 patients and 164 HV fulfilled the inclusion criteria and were included in the study. For the biological analysis (subjects in whom biopsies were performed), 106 IBS-D patients and 188 HV were included. Figure 1 shows the flow-chart of participant included in the study.

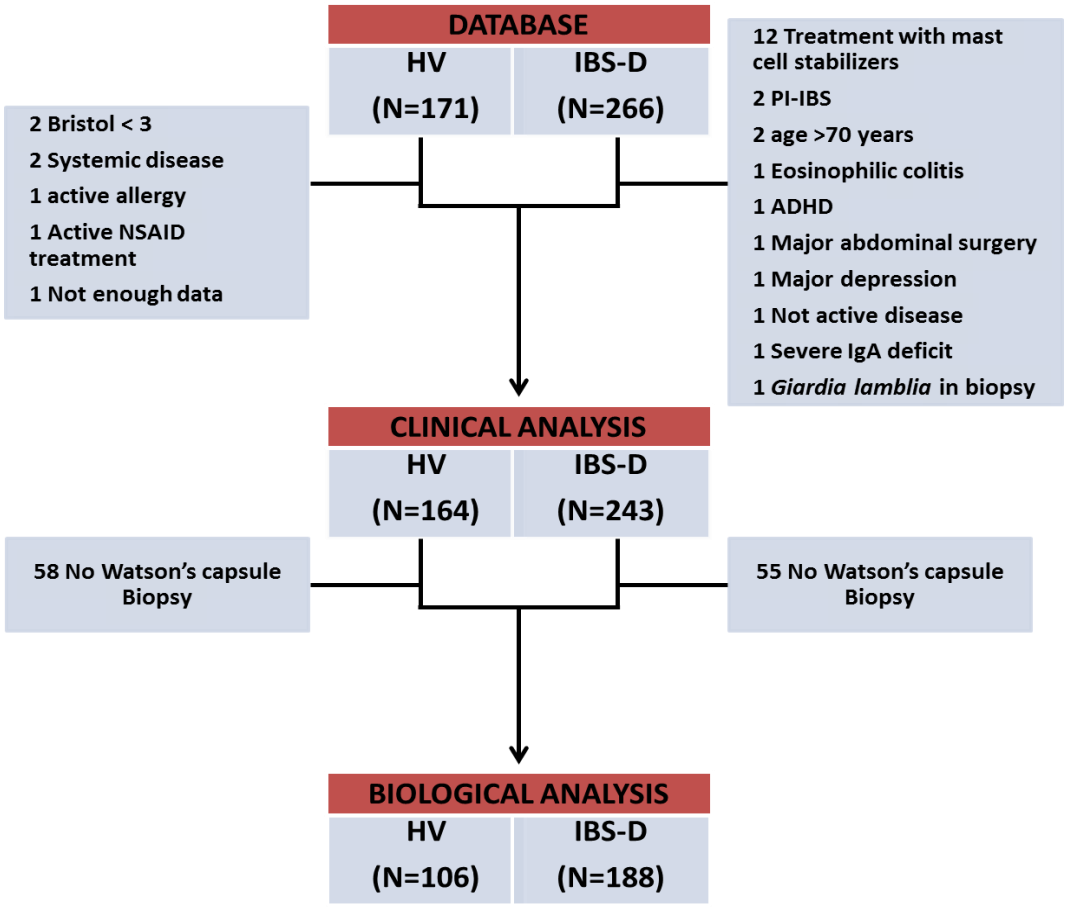


Figure 1: Flow-chart of participants included and excluded in the experimental groups and type of analysis performed in the study. ADHD: Attention deficit hyperactivity disorder; HV: healthy volunteer; IBS-D: diarrhea-predominant Irritable Bowel Syndrome; IgA: immunoglobulin A; NSAID: non-steroidal anti-inflammatory drugs; PI-IBS: post-infectious IBS.

4.4.2. Clinical analysis

Clinical and demographical characteristics of the experimental groups are summarized in table 1. No differences were observed in sex, BMI or atopy between patients and control groups. The age was 35 (28-42) and 24 (22-32) years old in the IBS-D and HV groups, respectively (P<0.001). IBS-D patients displayed higher abdominal pain (50 (27-69) vs 0

(0-0), P<0.001), increased number of daily bowel movements (3 (2-4.5) vs 1 (1-1.5), P<0.001) and looser stool consistency (5.5 (5-6) vs 3.5 (3-4), P<0.001), compared with HV. IBS-D patients showed a higher level of psychosocial stress (145 (73-227) vs. 92 (54-153), P<0.001) than HV, and it was detected a higher proportion of subjects with high stress level (48% vs 26%, P<0.001) in the IBS-D group. Moreover, patients with IBS-D scored higher depression than HV (46% vs 4%, P<0.001).

	HV (N= 164)	**IBS-D (N=243)**	***P***
Age (years)	23.94 (22.4-31.6)	34.9 (28.7-41.8)	<0.001#
Sex (F/M) In %	88 / 77 47.0 / 53.0	157 / 86 35.4 / 64.3	0.020
BMI	22.2 (20.76-25)	22.4 (20.6-25.0)	0.76
Atopy (%)	43.3	40.0	0.42
Dyspepsia (%)	-	50.4	-
Bowel movements per day	1 (1-1.5)	3 (2-4.5)	<0.001#
Stool consistency (Bristol Stool Scale)	3.5 (3.0-4.0)	5.5 (5.0-6.1)	<0.001#
IBS-SSS	90 (0.0-20.0)	264.5 (20.75-332.75)	<0.001#
Abdominal pain (intensity)*	0.0 (0.0-0.0)	50.0 (27.0-69.3)	<0.001#
Abdominal pain (days)*	0.0 (0.0-0.0)	5.0 (3.0-8.9)	<0.001#
Bloating *	0.0 (0.0-0.0)	47.0 (19.2-68.0)	<0.001#
Unsatisfaction with bowel habit *	3.0 (0.0-15.0)	71.0 (47.0-84.0)	<0.001#
Life interference *	0.0 (0.0-0.0)	72.0 (49.0-87.0)	<0.001#
H-R	92.0 (54.0-153.0)	145.0 (72.5-226.5)	<0.001#
STRESS (moderate to high in %)	26.1	48	<0.001#
PSS	16.0 (12.0-22.0)	23.0 (18.0-29.0)	<0.001#
BDI	0.0 (0.0-3.0)	9.0 (4.0-14.0)	<0.001#
Depression (% of BDI >10)	3.7	45.9	<0.001#

Table 1: Demographic data and clinical features. Data are expressed as median (Q1-Q3) except otherwise stated. BDI: Beck's depression inventory (no depression <10, depression ≥10); F, female; Holmes-Rahe stress scale (0-150, low stress; 151-300, moderate stress; >300 severe stress); IBS-D, diarrhea-predominant irritable bowel syndrome; IBS-SSS, IBS severity score system; M, male; stool consistency: 1 (hard) to 7 (entirely liquid); PSS: Perceived Stress Score. *Components of the IBS-SSS. #Differences maintained after correction for multiple comparisons.

4.4.3. Subgroup analysis: stress and sex

As the level of psychosocial stress, measured by the H-R questionnaire, was different between groups, participants were further classified into low and moderate/high level in order to evaluate the effect of background stress on the severity of IBS-D. When comparing IBS-D subjects with HV, we identified the same differences as in the first analysis, which included the entire population in each group (data not shown). As stress has an impact on IBS pathophysiology; we did a comparison in IBS-D subjects according to their psychosocial stress levels. Clinical differences between both subgroups were not found, except for levels of perceived stress and depression which were higher in the moderate-high psychosocial stress group; (20 vs 26; $P<0.001$) and (6 vs 12; $P<0.001$) respectively. Data are summarized in table 2.

To understand the role of female predominance in IBS, participants were analyzed according to sex. The prevalence of dyspepsia was higher in female than in male IBS-D patients (59% vs. 36%, $P=0.001$). Moreover, bloating was more frequent in female patients (49% vs 40%, $P=0.04$). No other differences were detected, as represented in table 2.

	IBS-D Low stress (N=119)	IBS-D Moderate-High stress (N=42)	*P*	IBS-D Male (N=86)	IBS-D Female (N=157)	*P*
Age (years)	34.5 (27.5-40.2)	35.0 (30-42)	0.16	34.4 (29.4-40.5)	35.5 (28.4-43.3)	0.41
Sex (F/M) In %	72 / 46 61.0 / 39.0	75 / 34 68.8 / 31.2	0.22	-	-	-
BMI	22.4 (20.4-24.7)	22.4 (20.2-25.7)	0.76	23.4 (21.6-25.4)	22.0 (19.5-24.8)	0.01
Atopy (%)	39.8	40.2	0.95	43.9	38.1	0.47
Dyspepsia	3.0 (2.0-4.5)	3.0 (2.0-4.5)	0.91	35.7	58.7	0.001
Bowel movements per day	5.5 (5.0-6.0)	6.0 (5.0-6.4)	0.60	3.0 (2.0-4.0)	3.0 (2.0-4.6)	0.81
Stool consistency (Bristol Stool Scale)	5.5 (5.0-6.0)	6.0 (5.0-6.4)	0.84	5.9 (5.0-6.1)	5.5 (5-6.2)	0.50
IBS-SSS	5.5 (5.0-6.0)	6.0 (5.0-6.4)	0.60	245.0 (198.0-317.5)	276.0 (204.0-378.0)	0.14
Abdominal pain (intensity)*	5.5 (5.0-6.0)	6.0 (5.0-6.4)	0.84	50.0 (25.8-70.0)	51.0 (27.3-69.0)	0.99
Abdominal pain (days)*	5.5 (5.0-6.0)	6.0 (5.0-6.4)	0.31	4.5 (2.0-9.25)	5.0 (3.0-8.0)	0.41
Bloating *	5.5 (5.0-6.0)	6.0 (5.0-6.4)	0.38	39.5 (15.0-57.5)	49.0 (19.8-72.5)	0.04
Unsatisfaction with bowel habit *	5.5 (5.0-6.0)	6.0 (5.0-6.4)	0.10	70.0 (40.3-84.0)	71.0 (48.0-84.0)	0.61
Life interference *	5.5 (5.0-6.0)	6.0 (5.0-6.4)	0.94	67.0 (48.5-84.3)	73.0 (49.5-88.0)	0.34
H-R	74.5 (47.3-113.5)	227.0 (181.0-325.0)	<0.001	141.0 (88.5-195.0)	152.5 (70.3-26.5)	0.36
STRESS (% moderate to high)	-	-	-	42.5	51	0.22
PSS	20.0 (16.0-27.0)	26.0 (20.0-31.8)	<0.001	22.0 (17.0-29.0)	23.0 (18.0-30.0)	0.51
BDI	6.0 (2.0-11.0)	12.0 (7.0-18.0)	<0.001	8.5 (3.0-13.0)	9.0 (4.0-15.5)	0.23
Depression (% BDI >10)	31.6	60.7	<0.001	42.5	47.7	0.47

Table 2: Demographic data and clinical features in IBS-D subjects, according to psychosocial stress levels and sex. Data are expressed as median (Q1-Q3) except otherwise stated. BDI: Beck's depression inventory; F, female; H-R, Holmes-Rahe; IBS-D, diarrhea-predominant irritable bowel syndrome; IBS-SSS, IBS severity score system; M, male; Stool consistency; PSS: Perceived Stress Score. *Components of the IBS-SSS.

4.4.4. Effect of comorbidities

We further analyzed the influence of depression and dyspepsia on the severity of IBS-D. Data are summarized in table 3. Overall, IBS-D patients with depression had more severe abdominal pain intensity and frequency, higher psychological stress, more dissatisfaction with bowel habit, as well as significant interference with life activities compared to non-dyspeptic IBS-D. Similarly, IBS-D patients with dyspepsia showed more severe abdominal pain intensity, frequency and bloating, more psychological stress, and a significant interference with life activities compared to non-dyspeptic IBS-D.

	IBS-D with depression (N=105)	**IBS-D without depression (N=124)**	***P***	**IBS-D with dyspepsia (N=113)**	**IBS-D without dyspepsia (N=111)**	***P***
Age (years)	36.7 (30.1-42.9)	33.6 (27.8-40.3)	0.03	35.5 (28.2-41.9)	34.1 (29.5-41.1)	0.75
Sex (F/M) In %	34/ 71 32.4/67.6	46/78 37.1/62.9	0.49	84/29 74.3/25.7	49/52 53.2/46.8	0.001
BMI	22.6 (19.8-25.4)	22.3 (20.8-24.2)	0.958	22.1.0 (19.9-24.4)	23.0 (20.8-25.5)	0.083
Atopy (%)	37.2	42.7	0.468	40.7	38.5	0.770
Dyspepsia	57.8	42.2	0.036	-	-	-
Bowel movements per day	3.0 (02.0-5.0)	3.0 (2.0-4.0)	0.149	3.0 (2.0-5.0)	3.0 (2.0-4.0)	0.054
Stool consistency (Bristol Stool Scale)	6 (5.0-6.5)	5.5 (5.0 -6.0)	0.065	6.0 (5.0-6.5)	5.5 (5.0-6.0)	0.108
IBS-SSS	300.0 (236.0-370.0)	239.5 (185.3-290.8)	0.0001	290.0 (233.5-364.5)	227.5 (179.3-271.5)	0.0001
Abdominal pain (intensity)*	60.0 (37.0-75.0)	39.5 (22.0-63.8)	0.0001	58.0 (30.0-73.0)	41.0 (22.5-62)	0.006
Abdominal pain (days)*	6.0 (4.0-10.0)	4.0 (2.0-7.0)	0.0001	6.0 (3.3-10.0)	4.0 (2.0-7.0)	0.001
Bloating *	50.0 (17.0-76.0)	40.0 (18.0-64.0)	0.147	58.0 (30.8-78.0)	32.0 (8.8-50.5)	0.001
Unsatisfaction with bowel habit *	78.0 (48.5-91.0)	63.5 (40.5-80.0)	0.006	73.0 (49.0-89.0)	65.5 (43.0-77.0)	0.139
Life interference *	79.0 (65.0-94.0)	60.0 (40.0-78.5)	0.0001	75.5 (51.5-910-100)	60.0 (43.0-77.0)	0.004
H-R	181.0 (112.3-307.5)	114.0 (59.3-177.8)	0.0001	157.0 (88.0-280)	138.0 (65.0-195.0)	0.041

STRESS (% moderate to high)	63.7	34.4	0.0001	52.3	43.5	0.224
PSS	29.0 (22.5-34.0)	19.5 (15.3-24.0)	0.0001	24.0 (18.0-29.0)	21.0 (17.0-29.0)	0.244
BDI	-	-	-	10.0 (6.0-17.0)	7.0 (3.0-13.0)	0.001
Depression (% BDI >10)	-	-	-	57.8	43.7	0.043

Table 3: Demographic data and clinical features in IBS-D patients according to the presence or absence of depression or dyspepsia. Data are expressed as median (Q1-Q3) except otherwise stated. BDI: Beck's depression inventory; F, female; H-R, Holmes-Rahe; IBS-D, diarrhea-predominant irritable bowel syndrome; IBS-SSS, IBS severity score system; M, male; Stool consistency; PSS: Perceived Stress Score. *Components of the IBS-SSS.

4.4.5. Correlation study

The correlation study identified the association between several components of the IBS-SSS: abdominal pain correlated with stool consistency (r_s: 0.2; *P*=0.011); bloating correlated with abdominal pain (r_s: 0.512; *P*<0.0001) (figure 2A); and with the number of days with abdominal pain (r_s: 0.349; *P*<0.0001) (figure 2B). No significant correlation was identified in the HV group (data not shown).

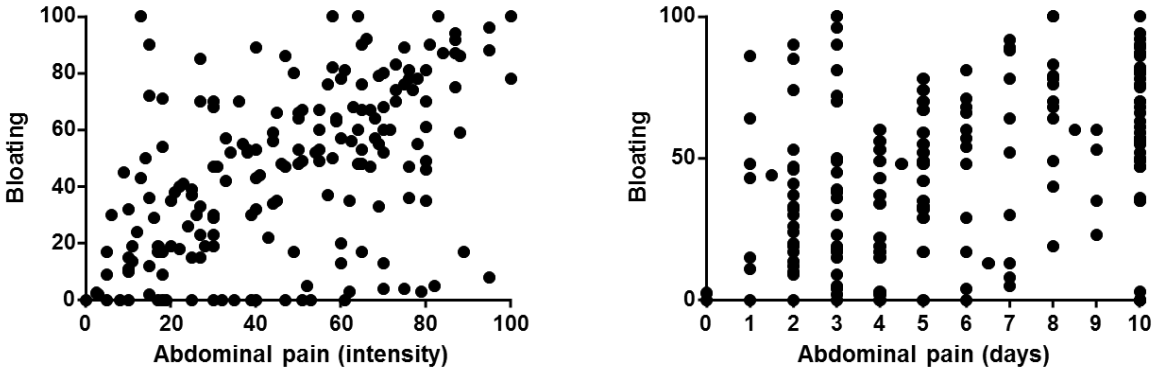


Figure 2: Correlation between clinical variables in IBS-D patients. A) Graphical representation of the correlation between bloating and abdominal pain. B) Graphical representation of the correlation between bloating and days with abdominal pain.

Clinical gastrointestinal symptoms related with IBS-D severity correlated with stress level and with depression in IBS-D, as shown in table 4, but not in controls.

		H-R	PSS	BDI
Bloating	r_s	0.198	0.119	0.234
	P	0.017	0.155	0.007
Abdominal Pain (intensity)	r_s	0.184	0.156	0.343
	P	0.017	0.043	< 0.0001
IBS-SSS	r_s	0.211	0.214	0.496
	P	0.012	0.011	< 0.0001

Table 4: Correlation between stress, depression and clinical symptoms. BDI, Beck's Depression Inventory; H-R, Holmes-Rahe; IBS-SSS, Irritable Bowel Syndrome Severity Score Scale; PSS, Perceived Stress Scale.

4.4.6. Subjective stress response to the biopsy

In those subjects submitted to the procedure of intestinal biopsy, SSRS related to the procedure was evaluated before and after the intervention. Basal SSRS was significantly higher in the IBS-D group compared to the HV. In the first five minutes after the biopsy, all participants showed equal levels of perceived stress. This intervention had no effect on the perceived stress in the HV group, while IBS-D patients perceived higher subjective stress, which diminished after the procedure by a median of 10%, as observed in figure 3.

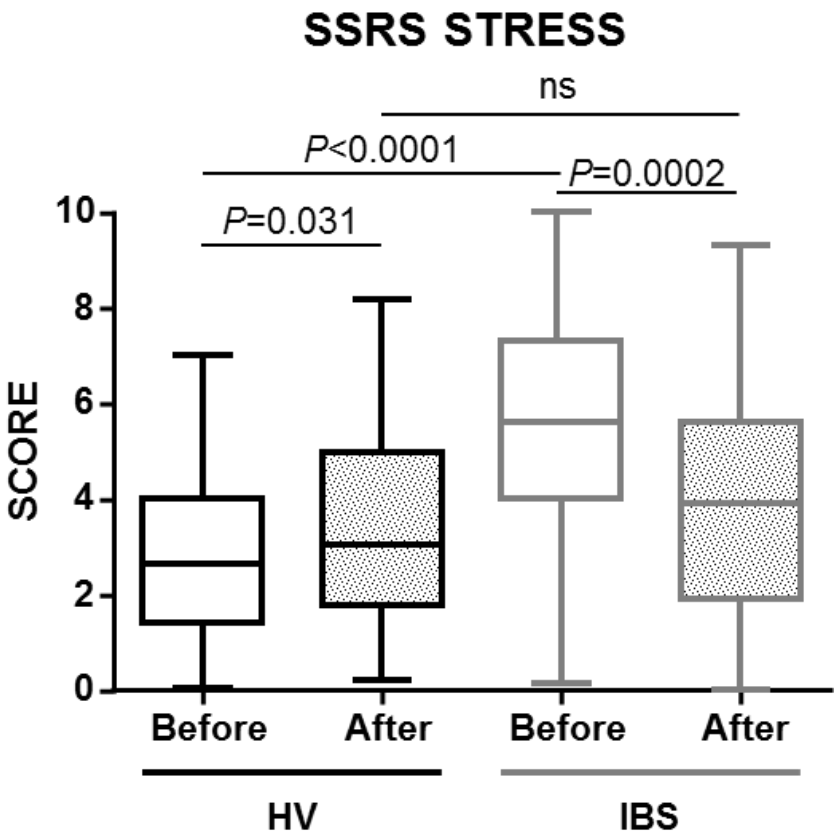


Figure 3: Perceived stress related to the biopsy procedure. The graph represents the values of the SSRS score before and after the biopsy procedure. HV, Healthy Volunteer; IBS, Irritable bowel syndrome; SSRS, Modified Social Readjustment Rating Scale.

4.4.7. Biological evaluation

As jejunal biopsies were performed only in a subgroup of subjects from our population, we performed first a comparison analysis, which determined that the demographical data of both populations was similar. Secondly, we also confirmed that differences between HV and IBS-D subjects, in the subgroup of subjects with a biopsy, were the same shown previously in the clinical analysis for whole groups.

No differences were observed in the number of eosinophils (HV: 1.75 vs IBS: 2; P=0.42), mast cells (HV: 20.6 vs IBS: 24; P=0.19) or CD3 positive lymphocytes (HV: 16 vs IBS: 18; P=0.61) in the jejunum of IBS-D patients when compared to HV subjects as observed in figure 4.

In IBS-D, the number of mast cells and eosinophils significantly correlated (r_s=0.347, P<0.001). However, the number of cells in the mucosa of patients with IBS-D or controls did not correlate with any clinical variable studied.

Life stress did not affect the number of mucosal immune cells. However, the analysis by sex identified a higher number of mucosal eosinophils in males (2.65 eosinophils/HPF) compared to IBS-D females (1.7 eosinophils/HPF, P<0.0001) and HV males (1.6 eos/HPF, P=0.025) (figure 4). We performed a subgroup analysis according to atopy levels and did not find differences in eosinophilic infiltration.

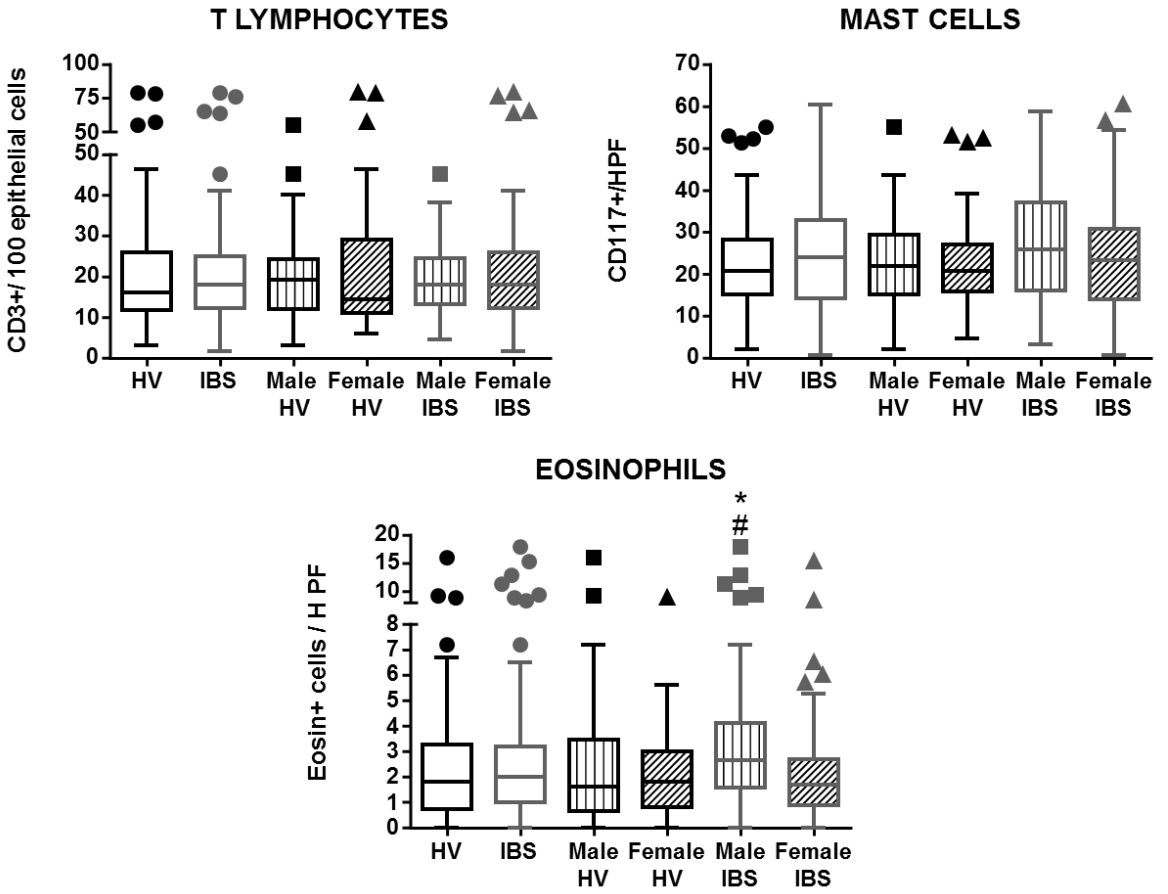


Figure 4: Comparison of mucosal immune cell counts between IBS-D and HV groups. Data are expressed as Tukey boxplot. *P=0.025 against HV males. #P<0.001 against IBS-D females.

4.4.8. Multivariate analysis

In order to identify the clinical variables that more significantly differentiate IBS-D from HV, a multivariate analysis using random forest modelling was performed. This analysis revealed that IBS-SSS, abdominal pain frequency and intensity, and stool consistency are the most significant variables. However, other variables like atopy, sex and life stress are of less importance (as observed in figure 5). Interestingly, the analysis revealed that in our population the cut-off of the IBS-SSS to properly differentiate a HV from an IBS-D subject was 100.

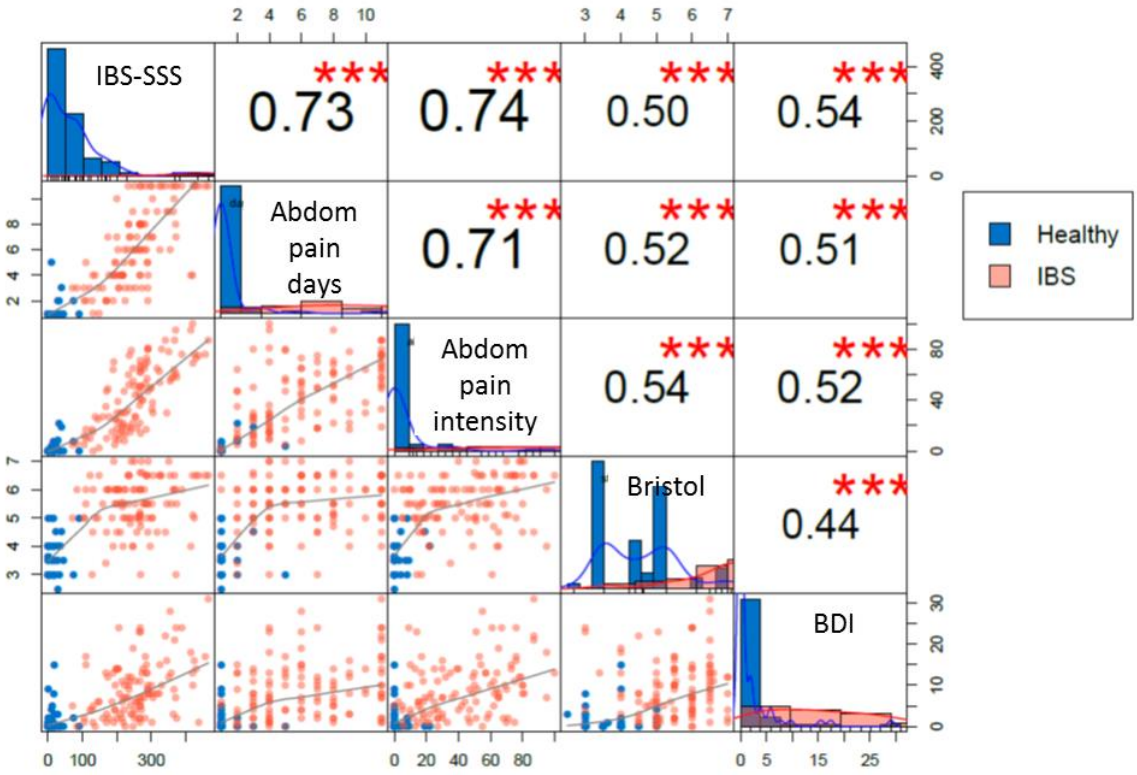


Figure 5: Visualization of the (Kendall's) correlation matrix of the top five variables (based on the Gini Index).

4.5. DISCUSSION

This is one of the largest observational studies examining the effect of gastrointestinal and psychological dysfunction on IBS-D clinical features. Our analysis unveiled that dyspepsia and psychological comorbidities contribute directly to the clinical severity of this disorder. Considering that patients with major psychiatric comorbidities were excluded it is remarkable that >40% of patients were depressed and up to 50% displayed dyspeptic symptoms and were affected by a more severe IBS-D.

Excessive or maladaptive response to stress has been proposed as a trigger for symptom generation in this disorder. The biopsychosocial model of IBS suggests that abdominal symptoms increase anxiety and depression and psychosocial factors influence intestinal manifestations (Enck et al., 2016), (Fond et al., 2014). In our study, the significant positive correlation among BDI and abdominal pain and IBS-SSS is indicative of the gut-to-brain pathway while there was only a mild correlation between stress factors and abdominal symptoms that may indicate a less active brain-to-gut pathway in our patients. Recent studies have demonstrated an association between IBS-D severity and the expression of tryptase (Martínez, Vicario, et al., 2012) and enhanced small bowel humoral immunity (Maria Vicario et al., 2014), overall supporting the theory that IBS-D patients have a differential activation of mucosal immune cells, compared to healthy controls. In the present study, no association between clinical symptoms and the number of immune cells in the mucosa was detected as we did not find any difference in the counts of mucosal immune cells between controls and patients. Therefore, it may be the state of activation rather than the number what contributes to symptoms, as already suggested by our group and others (Ohman & Simrén, 2010; Maria Vicario et al., 2014).

Sex and stress are considered two independent risk factors that influence the development of IBS. It has been shown that subjects with chronic psychosocial stress or those who had early life stress events are more prone to develop IBS, especially after an episode of acute gastroenteritis, a risk that is incremented in females. Although psychosocial stress is more prevalent in IBS-D subjects, our study suggests that chronic psychosocial stress is needed to develop IBS-D symptoms but, counter intuitively, does not seem to influence much clinical severity, as severity parameters were comparable between IBS-D with moderate-high and low stress levels. When stratifying by sex, we observed that IBS-D males had a higher eosinophil infiltrate in the jejunal mucosa when compared to IBS-D females or to healthy males. Male predominance is common in other eosinophilopathies of the gastrointestinal tract (Merves et al., 2014), what raises the

question of a differential sex-related role of eosinophilic infiltration in gastrointestinal disorders, as shown for duodenal eosinophilic infiltration of the duodenum and dyspeptic symptoms in females (Marjorie M Walker et al., 2014). Moreover, because atopy and smoking can influence the eosinophilic infiltration in the small bowel (M. M. Walker et al., 2010; Marjorie M Walker et al., 2014), we performed a specific analysis that precluded a role for both factors in this dissimilarity between male and females.

Interestingly, bloating was more severe in IBS-D patients with dyspepsia. Moreover, as described previously, there were a higher proportion of females than males with dyspepsia. This result indicates that patients suffering from IBS-D with comorbid state have more severe symptoms than patients without comorbidities. This fact is important for clinical practice as clinicians should be aware of comorbid factors and treat also them in order to improve the gastrointestinal symptoms and the quality of life of these patients.

The multivariate analysis did not identify any demographical, biological or psychological variables specifically associated with gut dysfunction that may properly differentiate IBS-D patients from HV. The random tree forest analysis identified clinical symptoms as the variables that most differentiate HV from IBS-D subjects, supporting the Rome criteria as a positive diagnostic tool. These results also emphasize the need of biomarkers research in order to diagnose IBS.

This study, as all retrospective studies, has several limitations. Some data were not available in all the subjects. Another potential selection bias of the study refers to the selection of the study population that may not reflect well the prevalence of dyspepsia and anxiety in the general IBS population. We also excluded all subjects with major comorbid psychiatric conditions, possibly underestimating the prevalence and influence of these two factors in the severity of IBS-D. Moreover, by excluding these two factors we were not able to evaluate properly the top-down pathway of the BGA.

Treating IBS is still a complicated task. General approach includes lifestyle and dietary modifications. Most available treatments are intended to alleviate abdominal pain (antispasmodics, smooth muscle relaxants, anticholinergic agents, peppermint oil) or the change in bowel habits (antidiarrheal or laxatives). Other common therapies include SSRI or tricyclic antidepressants as pain and perception modulators. In the last years there has been a growing effort to develop new treatments, some of them with already existing drugs for other diseases, more focused on the pathophysiology of this disease. Anti-inflammatory agents such as mesalazine, steroids, mast cell stabilizers (ketotifen,

disodium cromoglycate), non-absorbable antibiotics (rifaximin), pre- and -probiotics or 5-HT3 antagonists (alosetron) and dietary modifications have been used to treat this disorder with despair results, mainly with response in subgroups of patients. Recently, new drugs are emerging as first-class compounds, intended to ameliorate composite responses including mainly bowel habit and pain. In particular, in IBS-D, there is growing interest the role and effect of opioid and tachykinin and cannabinoid receptor agonism-antagonism in regulating gastrointestinal motility, secretion, and visceral sensation In this sense both FDA and EMA recently approved a poorly absorbed, peripherally active κ- and μ-opioid receptor first in class drug, eluxalodine to treat diarrhea and pain at the same time with relatively low side effects.

In conclusion, our study shows that psychosocial stress plays a role in IBS-D pathophysiology and that depression and dyspepsia are two comorbid conditions that worsen IBS-D outcome. According to these results, strategies directed to identify and early treat comorbidities in IBS-D patients may be of great benefit for IBS-D patients. Further studies designed to identify the mechanisms how psychosocial stress predisposes to the development of IBS-D are needed.

CHAPTER 2

5. Differential molecular mechanisms underlying the intestinal mucosal barrier response to acute stress. Implications for irritable bowel syndrome.

5.1. ABSTRACT

Background and aim: Vital stress is more frequent and severe in females than in males and has been postulated as one of the major predisposing factors associated with diarrhea-predominant IBS (IBS-D) and post-infectious IBS, as well as a contributor to disease severity. Moreover, recent research has identified that acute stress can severely impact intestinal barrier and that vital stress and sex also interfere in this response. Thus, the aim of this study was to identify the molecular mechanisms underlying the intestinal mucosal response to stress in the human healthy intestine, to further determine a risk to develop IBS. **Material and methods:** Twenty six (12 Females) healthy volunteers were recruited. Two jejunal mucosal biopsies were obtained in each participant: at baseline and 90 minutes after intermittent cold pain stress (CPS). Psychological stress (Subjective Stress Rating Scale), hormonal response (plasma cortisol), hand pain perception and autonomic (blood pressure and heart rate) responses were monitored throughout the experimental protocol. Mucosal RNA was isolated and analyzed by microarray technology followed by differential gene expression and biological pathways identification and qPCR validation. **Results:** CPS significantly increased heart rate, blood pressure and plasma cortisol, as well as stress perception in all participants. Stress significantly modified mucosal transcriptome, being circadian rhythm regulation the most relevant biological function *(P*<0.00001), with a significant decrease in specific clock genes expression (Fold vs. Basal: NR1D1=0.29, *P*<0.0001; NR1D2=0.56, *P*<0.0001; PER1=0.41, *P*<0.0001; PER3=0.47, *P*<0.0001; NFIL3=1.76, *P*<0.0001). Moreover, CPS altered epithelial barrier integrity gene expression (increasing CLDN2 [Fold vs. Basal: 1.35, *P*=0.0426] and decreasing SLC26A3 [Fold vs. Basal: 0.73, *P*=0.0043]), and inflammation-related genes (decreasing IL18 Fold vs. Basal: 0.80, *P*=0.0107; and increasing SOD1 Fold vs. Basal: 1.11, *P*=0.0227; and protease activity SERPINA1 Fold vs. Basal: 1.24, *P*=0.0031). Notably, significant correlation was found between gene expression of the most significant biological functions altered by stress. **Conclusion:** Acute experimental stress disrupts clock gene expression in the intestinal mucosa in association with immune and epithelial barrier alterations. These changes might help us to understand the mechanisms by which stress contributes to the development of IBS.

5.2. BACKGROUND

Functional gastrointestinal disorders (FGID) include a highly heterogenic group of diseases characterized by recurrent clinical manifestations, for which a structural and a biological base remain largely unsettled. Irritable bowel syndrome (IBS) and functional dyspepsia (FD) are the most common forms of this group of disorders, with high prevalence rates in western societies, reaching up to 15 and 20% of the population, respectively (Rok Seon Choung, Locke, Schleck, Zinsmeister & Talley, 2007; Yuri Ann Saito et al., 2000). IBS patients complain of recurrent abdominal pain that improves after defecation and a change in the number of bowel movements and/or in stool consistency. IBS is the most frequent diagnosis performed in the gastroenterology outpatient clinic (Longstreth et al., 2006) and, as many other chronic diseases, it has a clear sex predominance being more common in females with a 2:1 ratio (Chang & Heitkemper, 2002). Its pathophysiology is unclear, however alterations in gastrointestinal motility and visceral hypersensitivity have been described, and genetic, psychosocial and neurobiological factors have also been implicated. Stress is more frequent and severe in females than in males (Bourke, Harrell & Neigh, 2012) and has been postulated as one of the major predisposing factors associated to diarrhea-predominant IBS (IBS-D) and post-infectious IBS and also is a contributor to disease severity (R L Levy, Cain, Jarrett & Heitkemper, 1997; Spence & Moss-Morris, 2007). Moreover, IBS patients display mucosal microscopic inflammation when compared to controls (Chadwick et al., 2002; Guilarte et al., 2007; Maria Vicario et al., 2014) and higher levels of chronic psychosocial stress (Kennedy, Cryan, Quigley, Dinan & Clarke, 2014; Konturek, Brzozowski & Konturek, 2011) However, it is still unclear whether this association is secondary to a different neuroendocrine and psychological responsiveness to stress due to a greater sensitivity of females to vital events (Kelly et al., 2008; Kudielka & Kirschbaum, 2005).

A possible link on how stress and sex influence intestinal epithelial function has recently been described by our group, demonstrating that females with high psychosocial stress level develop a different response to acute stress than those with low stress (Carmen Alonso et al., 2008), findings also observed when comparing females with males (C. Alonso et al., 2012). This link reinforces the previously demonstrated intestinal barrier dysfunction by stress in studies in animals (J Santos, Saunders, et al., 1999; Söderholm, Yang, et al., 2002) and humans (C. Alonso et al., 2012; Carmen Alonso et al., 2008; Barclay & Turnberg, 1987). The intestinal epithelium is the largest and first barrier to luminal content and its integrity is a critical step to homeostasis maintenance. This barrier processes luminal antigens and exerts active immunological surveillance by interacting

with the underlying population of immune cells. A key cell in this surveillance is the mast cell, as it is highly involved in responses to stress by regulating, in the intestinal mucosa, the epithelial function (J Santos et al., 1998). Laboratory stress initiates and reactivates mucosal inflammation (Qiu et al., 1999), disturbs epithelial function (Carmen Alonso et al., 2008) and these changes are associated with dysbiosis (G. De Palma et al., 2015; O'Mahony et al., 2009; A. J. Park et al., 2013). The intestinal barrier dysfunction promoted by stress, might facilitate an excessive penetration of luminal antigens into the lamina propria through the epithelial layer, which could lead to chronic intestinal inflammation as a result of overstimulation of the local immune response. Despite intestinal barrier dysfunction and higher scoring of psychosocial stress in IBS-D patients than in healthy population, the specific contribution of stress to gut epithelial dysfunction, has not been determined yet. Thus, the aim of this study was to identify the molecular mechanisms underlying the intestinal mucosal response to stress in the human healthy intestine and its implications to intestinal barrier function, to further delineate a risk to develop IBS.

5.3. MATERIAL AND METHODS

5.3.1. Participants

Male and female healthy volunteers were prospectively recruited by public advertising from December 2009 through August 2012. A full medical history and a physical examination were performed to exclude participants with past history of gastrointestinal diseases or alimentary allergies. Food and respiratory allergy were ruled out using a battery of skin prick tests (Leti SA, Barcelona, Spain) for 32 common foodstuffs and 24 inhalants with histamine and saline as positive and negative controls, respectively. Subjects were eligible if they: were between 18-50 years-old, were able to understand and sign the informed consent, had negative alimentary skin prick tests, and if the pregnancy test was negative the day of the biopsy. Participants were excluded if they had amenorrhea or irregular menses, Diabetes Mellitus, received chemotherapy or radiotherapy in the 6 months previous to the study, hypo or hyperthyroidism, intestinal resection (except appendectomy), chronic and serious organic or mental illness, or any other disease initiated the month previous to the biopsy. Subjects were not allowed to take salicylates, non-steroid anti-inflammatory drugs, anticholinergic drugs or opioids 15 days prior to the study. Moreover, other drugs such as corticosteroids, antihistaminic or immunomodulatory drugs were not allowed in the last 3 months prior to the study. Written informed consent was obtained from every participant. The study protocol was approved

by the Ethics Committee at Hospital Vall d'Hebron (PR(AG)135/2008) and conducted according the revised Declaration of Helsinki.

5.3.2. Study design

Study participants were screened by medical interview and received verbal and written information about the study before giving consent. Gastrointestinal symptoms, anxiety and stress were recorded by validated questionnaires, and prick tests were performed. Figure 1 illustrates the experimental protocol carried out in eligible participants. Briefly, the day of the study, subjects collect saliva after waking up. As soon as they arrive to the hospital saliva is collected again, followed by placement of intravenous access and blood collection, together with recording of baseline autonomic variables. Then, stress questionnaires are fulfilled and, immediately after that, the biopsy tube is placed. Once past 5 to 10 cm Treitz's angle, the first biopsy is collected. Subsequently, saliva, autonomic variables, blood and stress questionnaires are collected. A second biopsy capsule is placed in the jejunum 5–10 cm distal to the Treitz's angle. Immediately, samples are collected and cold pain stress (CPS) is performed. Autonomic, psychological and biological parameters are then measured at different time points. Ninety minutes after ending the stress period, the last batch of samples is collected and a second jejunal aspirate and biopsy are obtained.

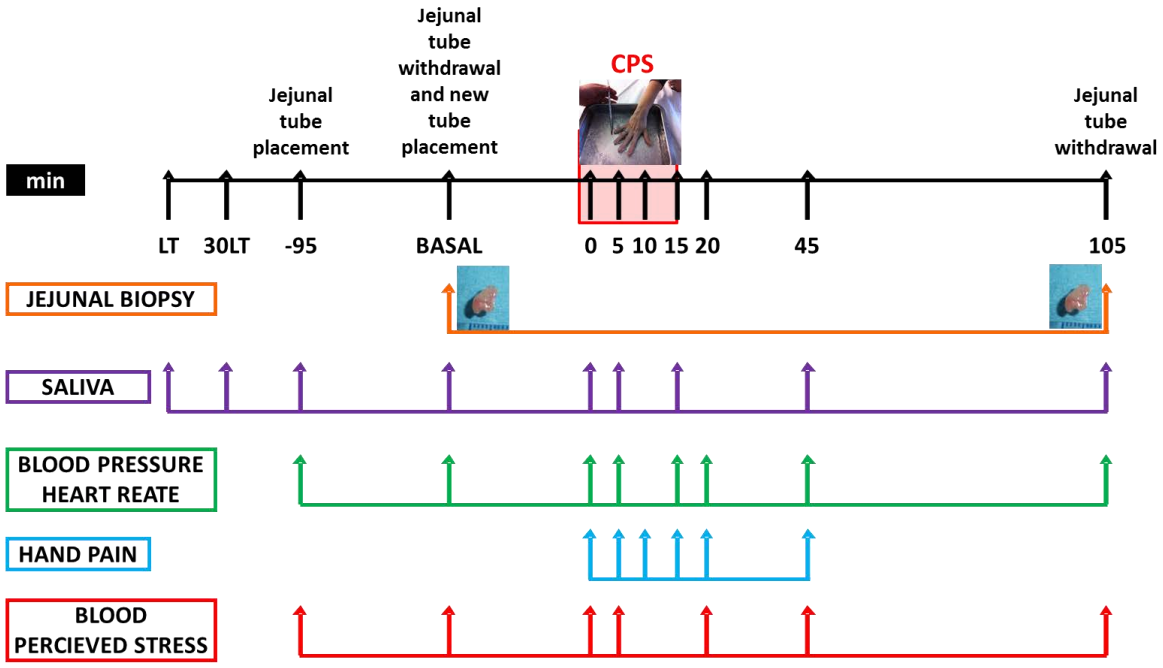


Figure 1: Experimental design. At time -95 minutes the jejunal tube is placed. At basal time jejunal biopsy is obtained and another jejunal tube is placed. At time 105 minutes a second biopsy is obtained and jejunal tube removed. 30LT: 30 minutes after waking up; BP: Blood pressure; CPS: Cold pain stress; HR: Heart rate; LT: just after waking up.

5.3.3. Psychosocial stress and depression assessment

Stress and depression background were evaluated using 3 validated questionnaires: (1) The modified social readjustment rating scale of Holmes-Rahe (H-R), to assess the level of stress over the last year (González de Rivera & Morera Fumero, 1983; Holmes & Rahe, 1967) and classify participants as: low stress (H-R <150), moderate stress (H-R 150-299) or high stress level (H-R ≥300); (2) The perceived scale stress (PSS) of Cohen, to assess the level of stress over the last month (Remor, 2006) and classify as: no perceived stress (PSS <19), mild perceived stress (PSS 19-28), moderate perceived stress (PSS 29-38) or severe perceived stress (PSS >38); and (3) the Beck's Depression Inventory (BDI), to evaluate the level of depression during the last week (Sanz, J., Navarro, M. E., Vázquez, 2003) and classify as no depression (BDI <10) or depression (BDI ≥10) subgroup.

5.3.4. Collection of biological samples

- Jejunal biopsy: Within 2 to 3 weeks after inclusion, two jejunal biopsies, one at baseline (Tbasal) and another one 105 minutes after starting the CPS (T105) were obtained in each participant using a modified Watson's capsule (Guilarte et al., 2007). Briefly, after an overnight fast, the tube was orally inserted. Proper placement, 5-10cm distal to Treitz's angle, was checked under fluoroscopic control. A tissue sample was obtained by suction with a 50mL syringe. Biopsy was then cut with a sterile blade to obtain 2 fragments for different analysis: one was embedded in 4% buffered formalin and processed for histological analysis; the other piece was placed in a tube containing 500µL of RNA later (Ambion, Madrid, Spain), kept at 4ºC for 120 minutes and stored at −80°C until processed for gene expression assays.

- Blood: Serum and plasma were isolated after blood collection at different time points from an intravenous cannula. Tubes were centrifuged following standard procedures, aliquoted, and serum and plasma were stored at -20°C.

- Saliva: Subjects were given specific instructions on how to collect saliva at home. Briefly, first collection was performed immediately after waking up. Subjects were asked to wash their mouth for 3 minutes with water, wait for 3 minutes and then collect saliva for 3 minutes. After 30 minutes they repeated the same procedure. Both samples were kept at 4ºC until arrival to the hospital. Saliva was collected by active spitting in Salivette® (Sarstedt, Nümbrecht, Germany) collection tubes. After collection, tubes were kept at 4ºC during the study period and then stored at -80ºC for at least one week. Then samples were thawed, centrifuged and aliquoted and stored at -80°C until analyzed.

5.3.5. Cold pain stress protocol and assessment of the systemic response

Physical stress was induced by the cold water pressor test (Lovallo, 1975). Briefly, participants immersed the non-dominant hand in iced water (4°C) for 45 seconds, with 15 seconds withdrawal intervals, during a 15 minutes period. The response to stress was assessed using the following parameters:

- Hand pain perception: The level of hand discomfort/pain was assessed using a visual analogue scale from 0 (no discomfort) to 10 (intolerable pain).

- Autonomic response: blood pressure and heart rate were measured with an automated sphygmomanometer (Omron M4-I; Omron Healthcare Europe B.V., Hoofddorp, The Netherlands).

- Psychologic response: the level of acute stress experienced by participants was evaluated by the Subjective Stress Rating Scale (SSRS) (B D Naliboff et al., n.d.).

- Hormonal response: Hypothalamic-pituitary-axis activation was determined by assessing plasma ACTH and cortisol concentration as well as by salivary IgA and cortisol concentration.

5.3.6. Analytical procedures

5.3.6.1. Histology and immunohistochemistry

Tissue sections were stained by hematoxylin and eosin dyes following general procedures. An expert pathologist assessed epithelial morphology and eosinophilic infiltration. In addition, the number of intraepithelial lymphocytes and mast cells per high powered field (HPF) was determined after immunohistochemical staining using anti-human CD3 and c-kit (CD117) antibodies (Dako, Barcelona, Spain), respectively, as previously described (Guilarte et al., 2007). Tissue specimens were blindly examined by the same experienced pathologist. Results are given as number of cells per HPF (mast cells and eosinophils) or per 100 epithelial cells (intraepithelial T cells).

5.3.6.2. Salivary determinations

- Immunoglobulin A (IgA) was quantified by enzyme-linked immunosorbent assay (ELISA) (Immundiagnostik K8870, Bensheim, Germany) according to manufacturer's protocol.

- Cortisol was determined by a commercial ELISA kit (Salimetrics 1-3002, Newmarket, UK) according to manufacturer's instructions.

5.3.6.3. Hormone response in serum

- Dehydroepiandrosterone (DHEA), insulin and cortisol in blood were determined in an external laboratory according to their standard operative procedure (Echevarne Laboratory, Barcelona, Spain).

- Adrenocorticotropic hormone (ACTH) was determined using an ELISA commercial kit (Cusabio CSB-E068703h, Maryland, USA) according to manufacturer's instructions.

5.3.7. Molecular biology procedures.

5.3.7.1. RNA isolation and cDNA synthesis

Biopsies were thawed in ice and lysed in TRIzol (Invitrogen, Madrid, Spain) with a bead beater (FastPrep mixer, BD medicals, Madrid, Spain), followed by RNA isolation (miRVana kit, Ambion, Madrid, Spain) and DNase treatment on-column (Qiagen, Madrid, Spain). RNA quantity and quality were confirmed by capillary electrophoresis (Agilent 2100 Bioanalyzer; Agilent Technologies, Palo Alto, Calif., USA) prior to gene array analysis. Only RNAs with RNA Integrity Number (RIN) ≥6.9 were used. Synthesis of cDNA was performed using 1µg of total RNA with the High Capacity Reverse Transcription Reagents Kit (Applied Biosystems, Madrid, Spain), following manufacturer's instructions.

5.3.7.2. Microarray

RNA from mucosal biopsies collected before and after CPS was analyzed by microarray technology in order to identify genes showing consistent differential expression [Microarray technology (Affymetrix human gene 1.1 ST, Santa Clara, CA, USA)]. The detailed protocol is described in the supplementary methods section. To identify the underlying differential profile in gene expression, we applied hierarchical clustering on the complete set of differentially expressed genes using average linkage and correlation as measures of similarity. A comparative analysis and linear models for microarray data (LIMMA) were performed for the entire probe set to further select differentially expressed genes. Probe's fold change (FC) expression was obtained as log-2 scale with an associated P-value. A false discovery rate (FDR) adjustment was not used at this point to favor discovery of a wider set of genes. A *P*-value (0.16) after LIMMA and a FC in either direction (≥1.5) was considered a probe set as differentially expressed within a

comparison group. Only genes that filled the filtering criteria as differentially expressed were further analyzed, therefore selected genes were subsequently confirmed by quantitative real time PCR (RT-qPCR).

5.3.7.3. Functional and pathway analysis.

To identify relevant biological pathways implicating those genes and proteins differentially expressed, we applied the Ingenuity Pathway Analysis methodology (IPA Software, Ingenuity® Systems, www.ingenuity.com). IPA integrates selected transcriptomic data set with mining techniques to predict functional connections and their interpretation in the context of gene networks that comprise gene interactions and related biological functions and canonical signaling pathways. Only genes with a mean fold-change of ≤0.66 and ≥1.5, compared with healthy individuals were analyzed by IPA. For network analysis, IPA provided a score according to the fit of supplied genes and the list of biological functions involved.

5.3.7.4. RT-qPCR

Gene expression was validated by RT-qPCR on an ABI PRISM® 7500 FAST Sequence Detection System (Applied Biosystems, Madrid, Spain) using validated TaqMan Gene Expression Assays and cyclophilin as endogenous control (peptidylprolyl isomerase A, PPIA) (Applied Biosystems, Madrid, Spain). Transcript quantification in each sample, including distilled water as negative control, was processed in triplicate. Gene expression was normalized to endogenous genes, and quantified using the comparative Ct method (relative quantification) and the Sequence Detector Software SDS v2.2 (Applied Biosystems). To compare gene expression differences between groups, Ct values for each experimental group was determined involving a target gene and a housekeeping gene (PPIA) that were subtracted to obtain the ΔΔCt (ΔΔCt = ΔCt T105—ΔCt T-90 = (Ct target gene T105—Ct PPIA T105)-(Ct target gene T-90—Ct PPIA T-90)). Fold change was calculated as $2^{-\Delta\Delta Ct}$. Fold change was obtained by dividing the normalized gene value by the average value in the control group. Individual data were expressed as the proportion (fold-change) with respect to baseline value of each individual (T105-Tbasal). Validated genes are indicated in the supplementary methods section (table S1).

5.3.8. Statistical analysis.

Data are expressed as mean (confidence interval) unless otherwise stated. Comparisons were made with parametric (Student's *t*-test) or non-parametric (Mann-Whitney *U*-test)

tests when appropriate. Comparison of psychologic, hormonal and autonomic variables where made using two-way repeated measures analysis of the variance (ANOVA or Kruskal-Wallis, when appropriate) where stress or sex were considered as the between-subjects factor and changes throughout the CPS protocol time were the within-subject factors. *P* values<0.05 were considered significant. All data were analyzed using commercial software (SPSS 22.0; IBM SPSS Inc., Chicago, IL, USA).

5.4. RESULTS

5.4.1. Demographical data and baseline characteristics

Twenty-six healthy volunteers were recruited initially. One participant was excluded because of impossibility to place the tube in position and one was excluded because *Giardia lamblia* was detected in the biopsies despite the absence of clinical symptoms. Twenty-four subjects were finally eligible for the study (12 female), 18 with low psychosocial stress (LS) and 6 with moderate stress (MS). Demographical, psychological, hormonal and histological data at baseline are shown in table 1.

	Median (N=24)	Q1-Q3
Age (years)	23.6	22.8-30.2
BMI (kg/m^2)	23.5	20.9-26.1
H-R	110.5	70.8-174.5
PSS	16.5	12.0-24.8
BDI	0.0	0.0-4.8
SBP (mmHg)	119.5	110.8-128.8
DBP (mmHg)	69.0	66.0-72.8
MBP (mmHg)	87	81.1-91.1
HR (bpm)	65.5	54.3-71.5
Stress *	3.4	2.3-4.2
Arousal *	3.5	2.1-4.7
Anxiety *	3.2	1.9-4.5
Anger *	3.0	1.9-4.4
Fatigue *	3.8	2.3-4.4
Attention*	2.8	1.7-4.4
Blood Cortisol(µg/dL)	8.2	6.8-10.6
Blood DHEA (ng/mL)	6.3	4.6-9.1
Blood ACTH (pg/mL)	41.8	36.6-45.3
Blood Insulin (µU/mL)	4.1	1.8-6.5
IgA saliva (µg/mL)	355.8	197.0-554.1
Cortisol saliva (µg/dL)	0.2	0.1-0.3
T Lymphocytes (CD3$^+$ cells/100 epithelial cells)	13.5	8.3-19.8
Mast Cells (CD117$^+$ cells/HPF)	23.9	18.4-29.0
Eosinophils (eosin$^+$ cells/HPF)	1.6	1.0-4.3

Table 1: Demographical, psychological, autonomic, hormonal and histological data from all participants at Tbasal. Data are expressed as median and Q1-Q3. ACTH: Adrenocorticotropic hormone; BDI: Beck's depression inventory; DBP: Diastolic blood pressure; DHEA: Dehydroepiandrosterone; HPF: high power field; H-R: Holmes-Rahe questionnaire; HR: Heart rate; IgA: Immunoglobulin A; MBP: Median blood pressure; SBP: Systolic blood pressure; *SSRS components.

5.4.2. Effect of cold pain stress

5.4.2.1. Autonomic response

CPS significantly increased systolic (SBP; F: 5.43, *P*=0.0008) and diastolic blood pressure (DBP; F: 8.1, *P*<0.0001) and did not modify heart rate (HR; F: 2.05, *P*=0.096) (figure 2). Multiple comparisons analysis, taking T0 as baseline time, showed that T5 was the only significantly different time point in SBP (T0=123 vs T5=133, *P*<0.05) and in DBP (T0=73 vs T5=85, *P*<0.01) while no changes were observed in HR (T0=64 vs T5=67, *P*>0.05).

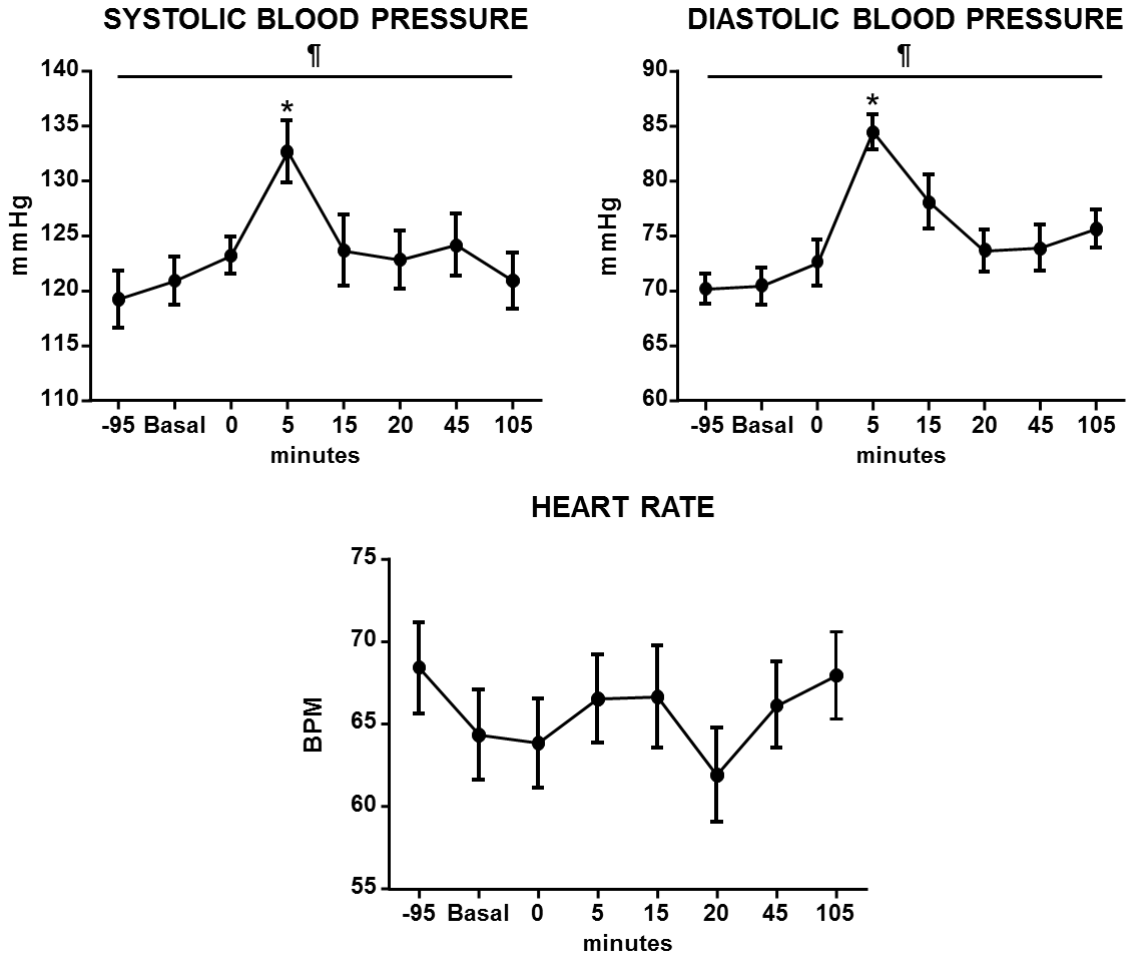


Figure 2: Autonomic response to CPS. Each dot represents the mean and the error bars are the SEM. "-95 minutes" corresponds to autonomic variables assessment upon arrival to our unit; "basal minutes" corresponds to autonomic variables assessment after first biopsy was obtained. * *P*<0.05 vs T0; ¶ ANOVA *P*<0.05; (n=21).

5.4.2.2. Effect of CPS on the psychological and pain response

CPS induced a marked raise in the perceived stress (H= 21.12; *P*=0.0017) and in hand pain perception (H= 101.07; *P*<0.0001), as observed in figure 3. Multiple comparisons analysis, taking T0 as baseline time, showed a significant increase from T5 through T20 in hand pain (T0=0 vs. T5=6.4, T10=5.99, T15=1.79, T20=1.79; *P*<0.01). Perceived stress level multiple comparison test did not show any differences when taking T0 as reference but showed that T5 and T15 were different than T105 (T105=2.56 vs T5=4.67 and T15=4.90, *P*<0.01).

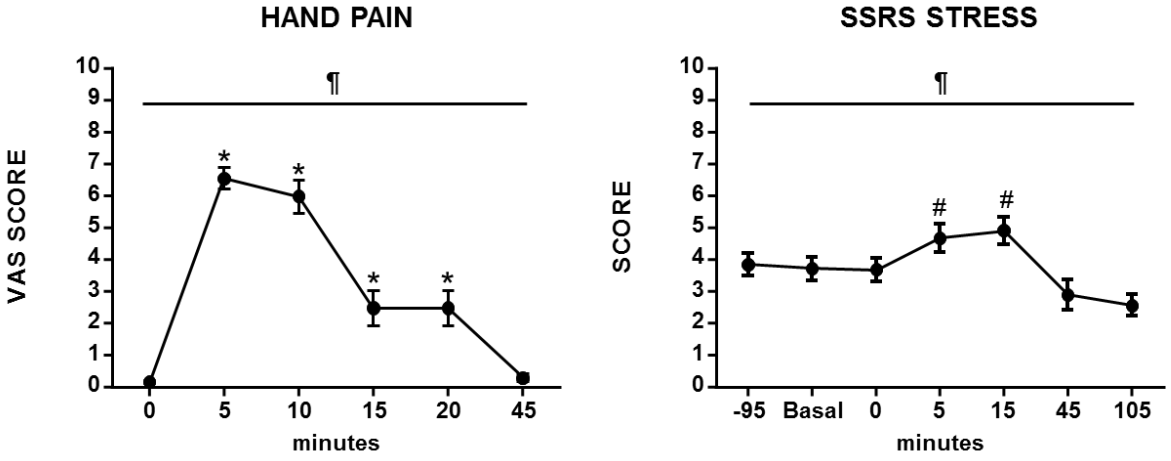


Figure 3: Psychological and hand pain perception response to CPS. Each dot represents the mean and the error bars represent the SEM. "-95 minutes" corresponds to psychological variables assessment upon arrival to our unit; "basal minutes" corresponds to psychological variables assessment after first biopsy was obtained. **P*<0.05 vs. T0; # *P*<0.05 vs T105; ¶ ANOVA *P*<0.05; (n=24).

5.4.2.3. Hormonal response

As observed in figure 4, CPS induced a blood hormonal response, as concentration of DHEA and cortisol significantly increased by CPS (F=12.28, *P*<0.0001; F=4.92, *P*=0.009; respectively); however, no differences were observed in ACTH concentration (F=0.855, *P*=0.473). Although differences were observed in blood insulin concentration, no effect of stress was observed. Stress induced a peak in salivary stress markers although no statistical significant difference was detected. Multiple comparisons analysis, taking T0 as baseline time, showed that T15 was the only different point in cortisol and DHEA (cortisol T0=8.23 vs T15=10.52 µg/dL, *P*<0.001; DHEA T0=7.85 vs T15=10.27 ng/mL, *P*<0.01). Multiple comparison tests in salivary values only showed differences between T0 and salivary collection after waking up (LT).

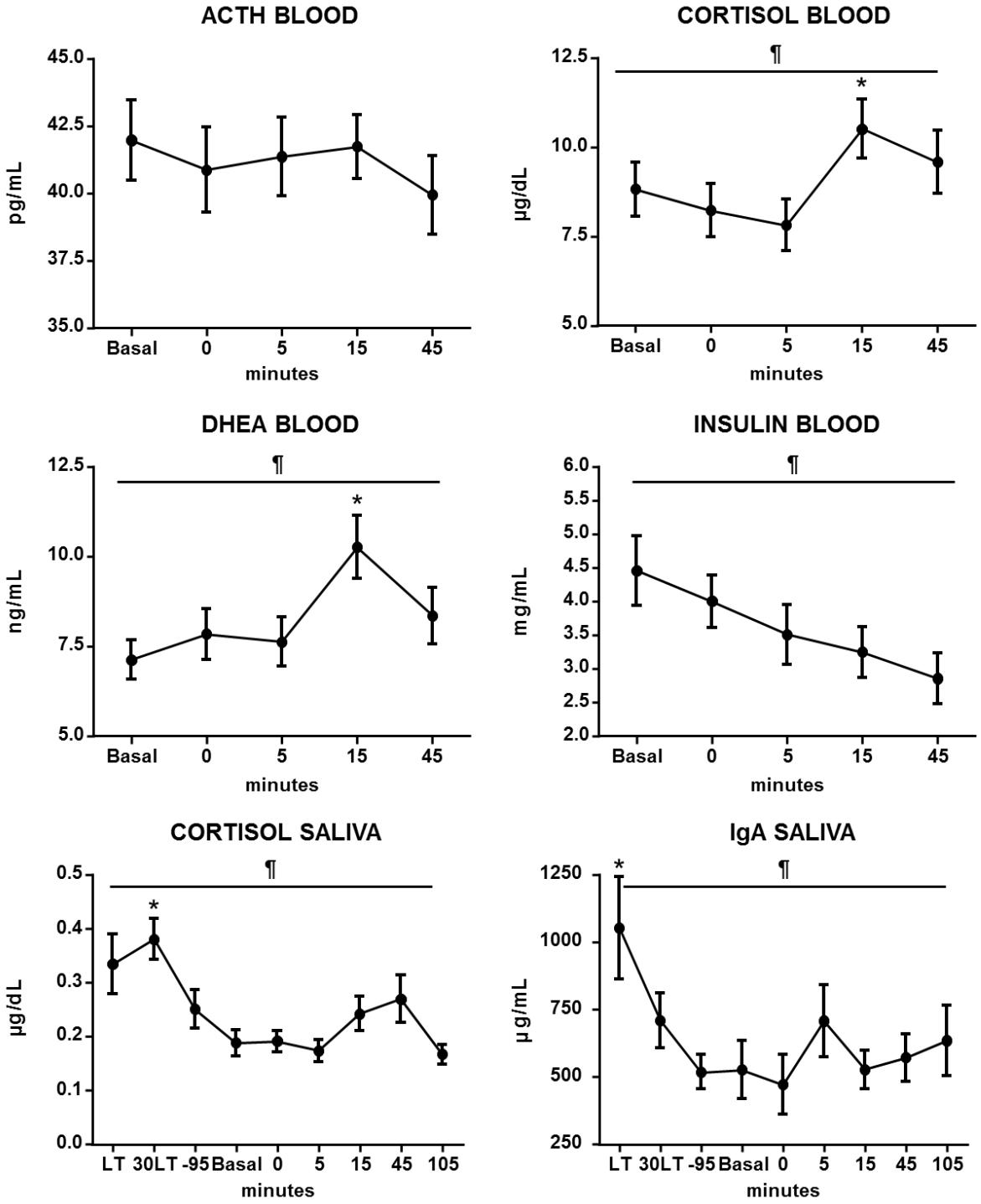


Figure 4: Hormonal response to CPS. Concentration of ACTH, DHEA, cortisol and insulin in blood and IgA and cortisol in saliva over the experimental period. Each dot represents the mean and the error bars represent the SEM. 30LT: 30 minutes after waking up; ACTH: Adrenocorticotropic hormone; DHEA: Dehydroepiandrosterone; IgA: Immunoglobulin A; LT: just after waking up. "-95 minutes" corresponds to hormonal variables assessment upon arrival to our unit; "basal minutes" corresponds to hormonal variables assessment after first biopsy was obtained. *P<0.05 vs T0; ¶ ANOVA P<0.05; (n=24).Figure 4: Hormonal response to CPS. Concentration of ACTH, DHEA, cortisol and insulin in blood and IgA and cortisol in saliva over the experimental period. Each dot represents the mean and the error bars represent the SEM. 30LT: 30 minutes after waking up; ACTH: Adrenocorticotropic hormone; DHEA: Dehydroepiandrosterone; IgA: Immunoglobulin A; LT: just after waking up. *P<0.05 vs T0; ¶ ANOVA P<0.05; (n=24).

5.4.2.4. Mucosal immune cells

Histological analysis did not show any effect of CPS on the number of mucosal T lymphocytes, eosinophils or mast cells (figure 5).

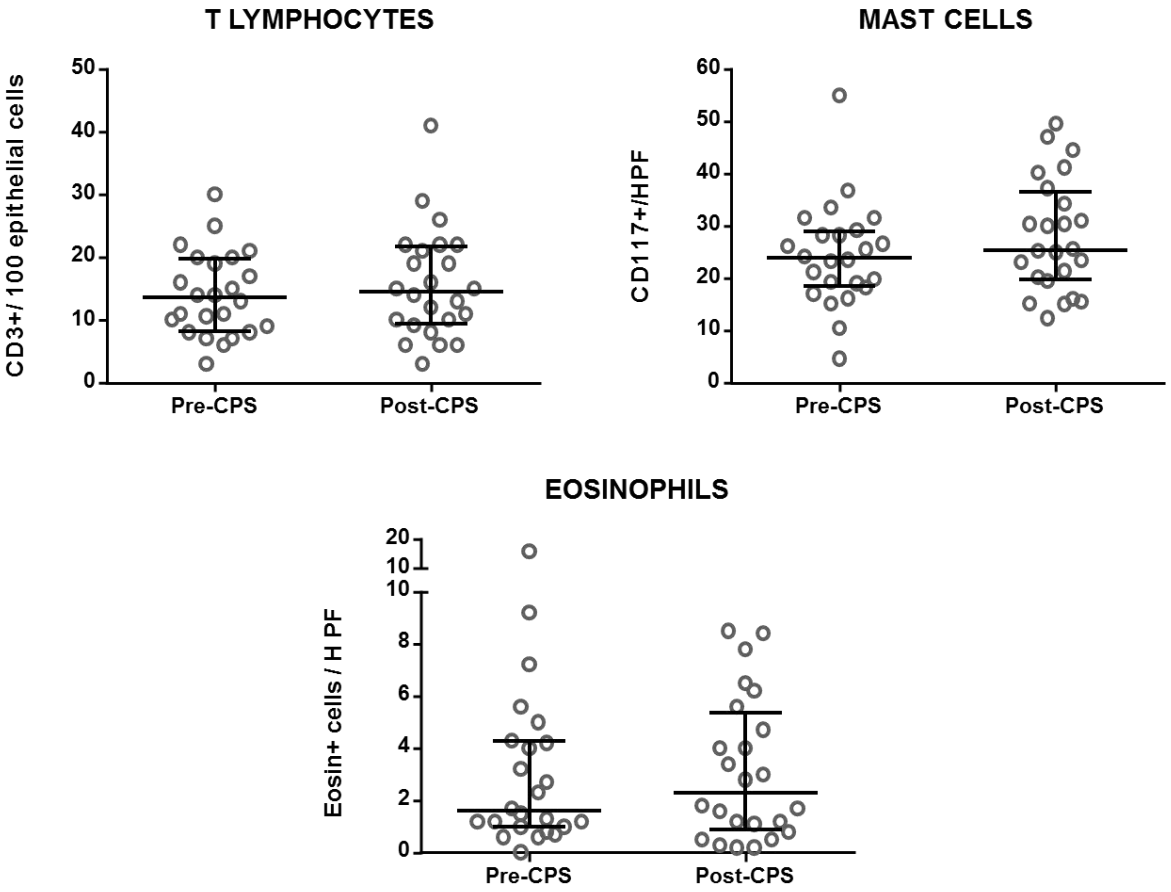


Figure 5: Leukocyte counts in the jejunal mucosa before and after CPS. The number of mast cells and eosinophils is expressed per HPF (400x) and the number of intraepithelial lymphocytes per 100 epithelial cells. Data are expressed as the median and the interquartile range; (n=24).

5.4.2.5. Jejunal mucosal transcriptome.

Eight subjects were randomly chosen for transcriptome analysis before and after the stress procedure. The groups where balanced by psychological stress level (4 with LS and 4 with MS) and by sex (2 males and 2 females in each group).

The heatmap in figure 6 represents the application of the hierarchical clustering analysis to differentiate the transcriptome in both groups. The color gradient scale ranging from red (high expression) to blue (low expression) indicates the behavior of differentially expressed genes, being the magnitude of the gene change proportional to the darkness of

the color. Each column represents a separate subject and each row represents a separate gene.

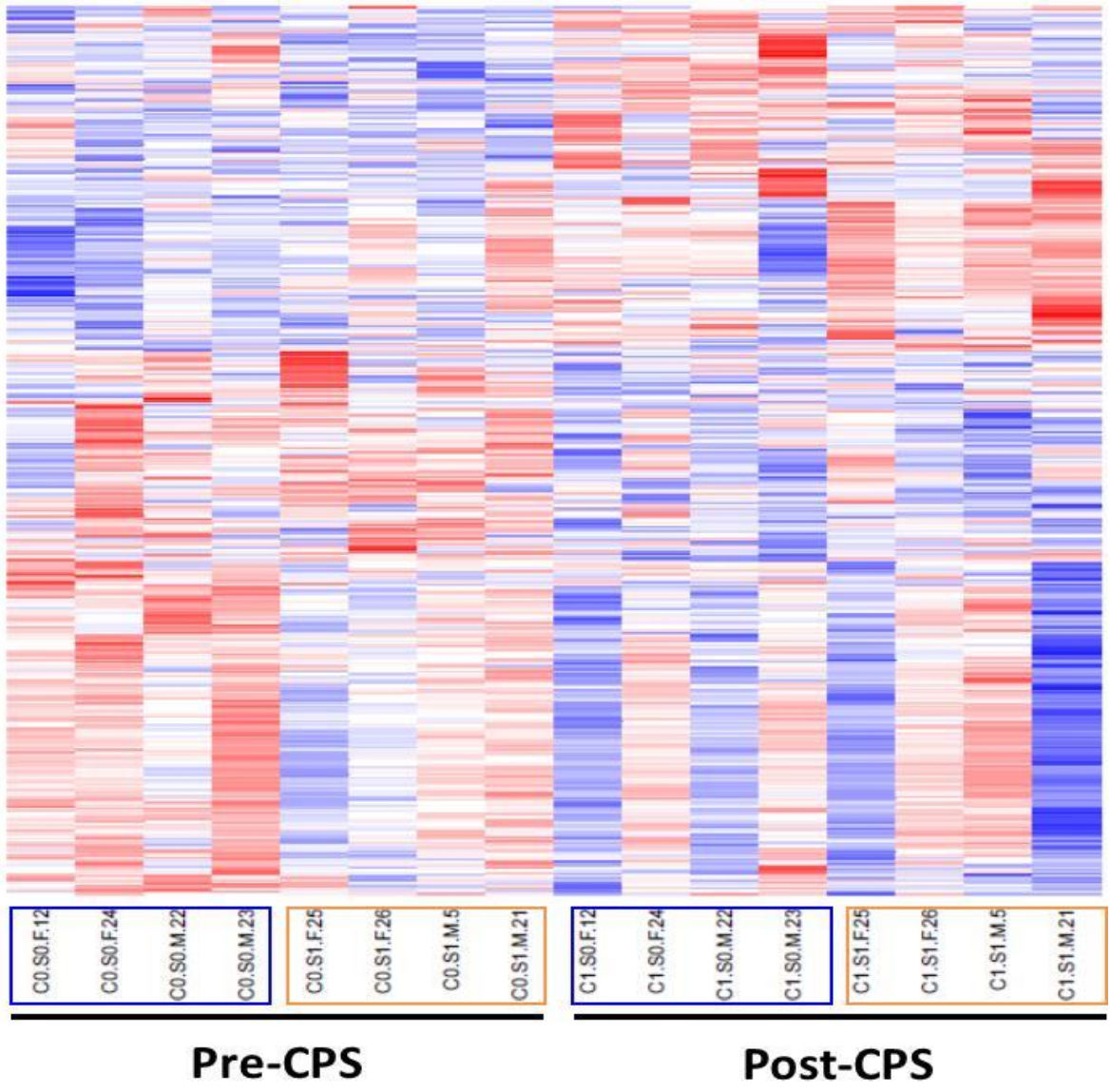


Figure 6: Heatmap of before and after CPS representing the differential gene expression profile in the jejunal mucosa of each subject. Individual samples are shown in columns and genes are represented in rows. Color grading indicates the expression level for each gene, blue and red indicate low and high level of gene expression, respectively. Square in blue indicates subjects with low life stress and square in orange subjects with moderate life stress.

A total number of 44 genes were identified as differentially expressed. Among them, 25 were defined as up-regulated and 19 were defined as down-regulated after CPS (false discovery rate-adjusted *P*=0.16). Top 20 differentially expressed genes are indicated in table 2 (A and B).

A	Entrez Gene	Gene Symbol	Gene Name	Type	Active Location	Fold Change
Up-regulated	26830	RNU5D-1	RNA, U5D small nuclear 1	other	Other	1,051
	26783	SNORA65	small nucleolar RNA, H/ACA box 65	other	Other	0,830
	6700	SPRR2A	small proline rich protein 2A	other	Cytoplasm	0,669
	1906	EDN1	endothelin 1	cytokine	Extracellular Space	0,572
	6364	CCL20	C-C motif chemokine ligand 20	cytokine	Extracellular Space	0,563
	10766	TOB2	transducer of ERBB2, 2	other	Nucleus	0,554
	5981	RFC1	replication factor C subunit 1	transcription regulator	Nucleus	0,519
	4783	NFIL3	nuclear factor, interleukin 3 regulated	transcription regulator	Nucleus	0,519
	12	SERPINA3	serpin family A member 3	other	Extracellular Space	0,497
	407034	mir-30	microRNA 30a	microRNA	Cytoplasm	0,496
	55655	NLRP2	NLR family pyrin domain containing 2	other	Nucleus	0,494
	407019	mir-27	microRNA 27a	microRNA	Cytoplasm	0,483
	406	ARNTL	aryl hydrocarbon receptor nuclear translocator like	transcription regulator	Nucleus	0,467
	142683	ITLN2	intelectin 2	other	Plasma Membrane	0,467
	2538	G6PC	glucose-6-phosphatase catalytic subunit	phosphatase	Cytoplasm	0,457

B	Entrez Gene	Gene Symbol	Gene Name	Type	Active Location	Fold Change
Down-regulated	9572	NR1D1	nuclear receptor subfamily 1 group D member 1	ligand-dependent nuclear receptor	Nucleus	-1,438
	148523	CIART	circadian associated repressor of transcription	other	Nucleus	-1,087
	5187	PER1	period circadian regulator 1	transcription regulator	Nucleus	-0,844
	8863	PER3	period circadian regulator 3	other	Nucleus	-0,722
	9975	NR1D2	nuclear receptor subfamily 1 group D member 2	ligand-dependent nuclear receptor	Nucleus	-0,717
	283232	TMEM80	transmembrane protein 80	other	Other	-0,675
	3131	HLF	HLF, PAR bZIP transcription factor	transcription regulator	Nucleus	-0,553
	6446	SGK1	serum/glucocorticoid regulated kinase 1	kinase	Cytoplasm	-0,552
	1628	DBP	D-box binding PAR bZIP transcription factor	transcription regulator	Nucleus	-0,551
	57226	LYRM2	LYR motif containing 2	other	Cytoplasm	-0,546
	6573	SLC19A1	solute carrier family 19 member 1	transporter	Plasma Membrane	-0,534
	84649	DGAT2	diacylglycerol O-acyltransferase 2	enzyme	Cytoplasm	-0,526
	3040	HBA1/HBA2	hemoglobin subunit alpha 2	transporter	Extracellular Space	-0,520
	1543	CYP1A1	cytochrome P450 family 1 subfamily A member 1	enzyme	Cytoplasm	-0,520
	6637	SNRPG	small nuclear ribonucleoprotein polypeptide G	other	Nucleus	-0,479
	2289	FKBP5	FK506 binding protein 5	enzyme	Nucleus	-0,477
	80117	ARL14	ADP ribosylation factor like GTPase 14	other	Other	-0,466
	1811	SLC26A3	solute carrier family 26 member 3	transporter	Plasma Membrane	-0,465
	6514	SLC2A2	solute carrier family 2 member 2	transporter	Plasma Membrane	-0,461

Table 2. Top up-regulated (A) and down-regulated (B) genes modulated by stress (T105-Tbasal). For each gene symbol, name, type, location and fold change, expressed as absolute log2 ratio, are indicated. *P*-Value according to multiple comparisons (LIMMA).

The biological processes and cellular functions associated to the response to CPS are shown in table 3.

Category	Functions annotation	*P*-Value	Molecules	Number of molecules
Behavior	circadian rhythm	9.05E-07	DBP, IL18, NR1D1, PER1, PER3	5
Nervous system development and function	circadian rhythm	9.05E-07	DBP, IL18, NR1D1, PER1, PER3	5
Lipid metabolism	metabolism of eicosanoid	5.79E-06	CYP1A1, DBP, EDN1, HLF, IL18, LDLR	6
Small molecule biochemistry	metabolism of eicosanoid	5.79E-06	CYP1A1, DBP, EDN1, HLF, IL18, LDLR	6
Renal and urological disease	renal failure	3.06E-05	CYP1A1, EDN1, HBA1/HBA2, IL18, LDLR	5
Cardiovascular system development and function	blood pressure	6.00E-05	DBP, EDN1, GUCY1A3, HLF, TIMP3, HBA1/HBA2	6
Digestive system development and function	mass of liver	1.13E-04	CYP1A1, G6PC, LDLR, SCL2A2	4
Hepatic system development and function	mass of liver	1.13E-04	CYP1A1, G6PC, LDLR, SCL2A2	4
Organ Morphology	mass of liver	1.13E-04	CYP1A1, G6PC, LDLR, SCL2A2	4
Cardiovascular system development and function	systolic pressure	1.21E-04	DBP, GUCY1A3, HLF, TIMP3,	4
Hematological system development and function	chemotaxis of phagocytes	3.50E-04	CCL20, EDN1, G6PC, IL18, LDLR	5
Immune cell trafficking	chemotaxis of phagocytes	3.50E-04	CCL20, EDN1, G6PC, IL18, LDLR	5
Inflammatory response	chemotaxis of phagocytes	3.50E-04	CCL20, EDN1, G6PC, IL18, LDLR	5
Cellular function and maintenance	ion homeostasis of cells	4.20E-04	CCL20, EDN1, G6PC, IL18, KCNK1, SLC26A3	6
Digestive system development and function	morphology of digestive system	5.37E-04	CYP1A1, EDN1, G6PC, LDLR, SLC2A2, SLC26A3	7
Cellular movement	cell movement of myeloid cells	8.60E-04	CCL20, EDN1, G6PC, IL18, LDLR, TIMP3	6
Hematological system development and function	cell movement of myeloid cells	8.60E-04	CCL20, EDN1, G6PC, IL18, LDLR, TIMP3	6
Immune cell trafficking	cell movement of myeloid cells	8.60E-04	CCL20, EDN1, G6PC, IL18, LDLR, TIMP3	6
Hematological system development and function	cell movement of phagocytes	8.87E-04	CCL20, EDN1, G6PC, IL18, LDLR, TIMP3	6
Inflammatory response	cell movement of phagocytes	8.87E-04	CCL20, EDN1, G6PC, IL18, LDLR, TIMP3	6
Cellular function and maintenance	cellular homeostasis	8.89E-04	CCL20, EDN1, G6PC, IL18, KCNK1, LDLR, NFIL3, SLC26A3, SLC2A2, TIMP3	10

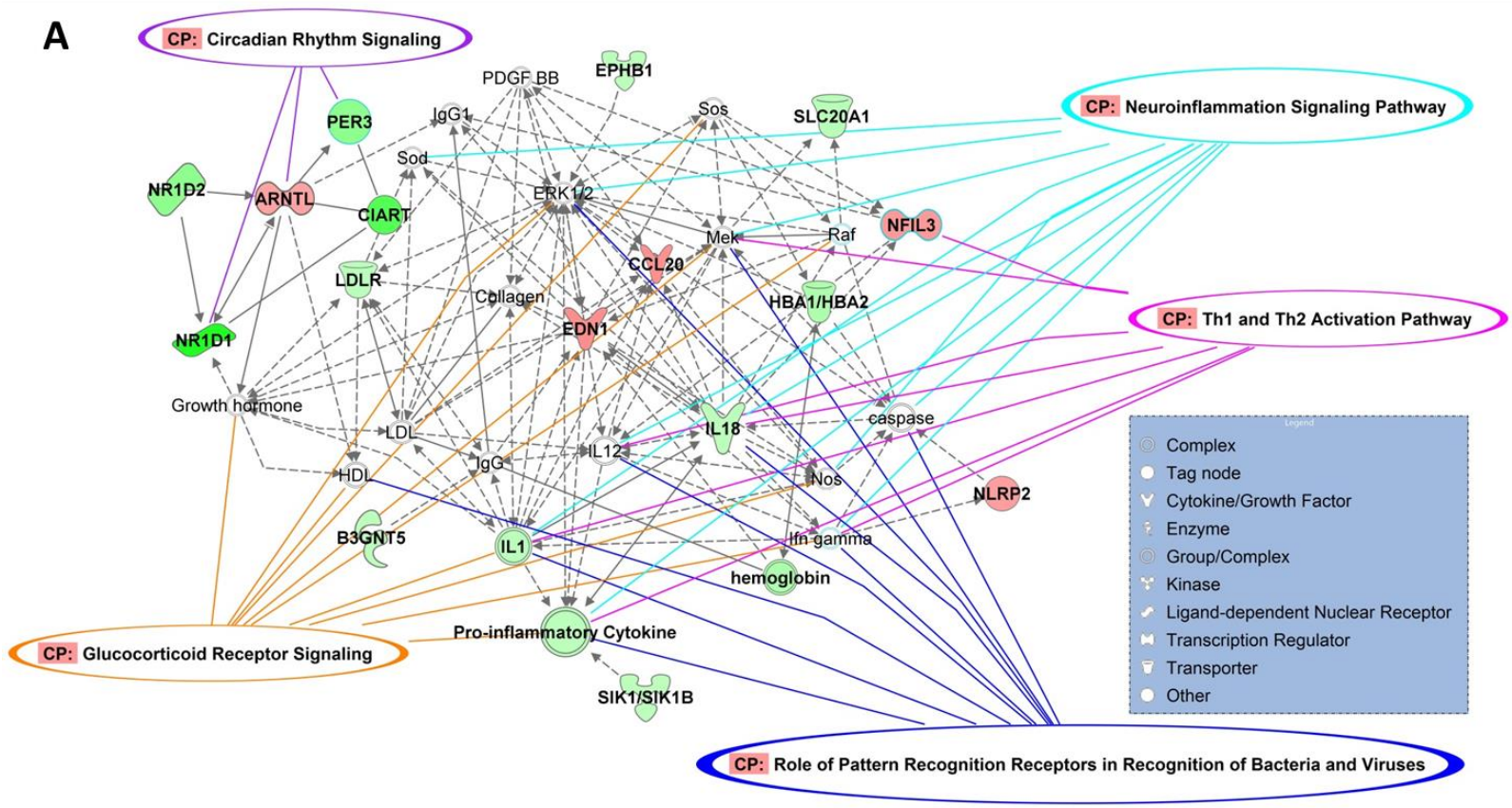
Table 3. List of the statistically relevant biological functions modulated by stress. *P*-value, the number and the symbol of genes involved within each function are reported.

Investigation of biological interaction among the 44 differentially expressed genes revealed 2 molecular networks (score>12) with a distinctive significant function in the jejunal mucosa. Table 4 represents the top-relevant and high-scoring network (score= 39) with their focus molecules and functions.

ID	Molecules in Network	Score	Focus Molecules	Top Functions
1	ARNTL, B3GNT5, caspase, CCL20, CIART, Collagen(s), EDN1, EPHB1, ERK1/2, Growth hormone, HBA1/HBA2, HDL-cholesterol, hemoglobin, Ifn gamma, IgG, IgG1, IL1, IL18, IL12 (complex), LDL, LDLR, Mek, NFIL3, NLRP2, Nos, NR1D1, NR1D2, PDGF BB, PER3, Pro-inflammatory Cytokine, Raf, SIK1/SIK1B, SLC20A1, Sod, Sos	38	16	Behavior, Nervous System Development and Function, Gastrointestinal Disease
2	AMPK, Cg, Creb, CYP1A1, DBP, ERK, estrogen receptor, FKBP5, FSH, G6PC, Histone h3, HLF, Insulin, Integrin, Lh, Mapk, mir-30, N-cor, NFkB (complex), Nr1h, P38 MAPK, PER1, PI3K (complex), Pka, Pkc(s), PLOD2, Ras, RNA polymerase II, RPL3, SGK1, SLC26A3, SLC2A2, SNORA65, TCR, Vegf	29	13	Lipid Metabolism, Small Molecule Biochemistry, Developmental Disorder
3	ABL1, Akt, beta-carotene, C9orf3, cytokine, DGAT2, EEF1B2, fructose-2, 6-diphosphate, Hmgn3, IL12 (family), IL17R, indole, Ins1, IRS, Jnk, KLB, MC4R, mir-199, Mmp, NFIL3, NRAS, octanoic acid, PDGF (family), PKC alpha/beta, PTEN, Relaxin, SERPINA3, SLC19A1, SP1, SPRR3, SPRR2A, Stat3-Stat3, Trk Receptor, tyrosine kinase, VLDL	16	8	Carbohydrate Metabolism, Nutritional Disease, Lipid Metabolism
4	ABCB4, anandamide, ARL14, ARNTL, BNIP3, DBP, DYRK2, ESPL1, glutathione peroxidase, HERC5, ITLN2, KCNK1, KRT7, ME1, mir-199, mir-210, miR-145-5p (and other miRNAs w/seed UCCAGUU), miR-4731-5p (miRNAs w/seed GCUGGGG), MPI, NDRG2, nitrite, NOP53, Pde4, progesterone, Rar, RCVRN, RPL3, RPL11, RPL7A, S100A6, TARS2, thyroid hormone receptor, TMEM80, TP53, TTK	16	8	Cancer, Cell Death and Survival, Organismal Injury and Abnormalities

Table 4. Molecular network associated with CPS. Each network contains direct and indirect interactions scored by significance. The genes differentially expressed within each one are shown in bold, up-regulated genes are indicated in red and down-regulated genes are indicated in green. ID: identification.

The analysis allowed the identification of canonical pathways (CP) and biological functions (Fx) related to the stress response in the intestinal mucosa. Figure 7 represents Network 1 and the CP (figure 7A) and Fx (figure 7B) identified by IPA.


A
CP: Circadian Rhythm Signaling
PDGF BB
EPHB1
PER3
IgG1
Sos
SLC20A1
CP: Neuroinflammation Signaling Pathway
Sod
NR1D2
ARNTL
CIART
ERK1/2
Mek
Raf
NFIL3
CCL20
LDLR
Collagen
HBA1/HBA2
CP: Th1 and Th2 Activation Pathway
NR1D1
EDN1
Growth hormone
IL18
caspase
LDL
IL12
IgG
HDL
Nos
NLRP2
B3GNT5
IL1
Ifn gamma
hemoglobin
Pro-inflammatory Cytokine
CP: Glucocorticoid Receptor Signaling
SIK1/SIK1B
CP: Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses
Legend
Complex
Tag node
Cytokine/Growth Factor
Enzyme
Group/Complex
Kinase
Ligand-dependent Nuclear Receptor
Transcription Regulator
Transporter
Other

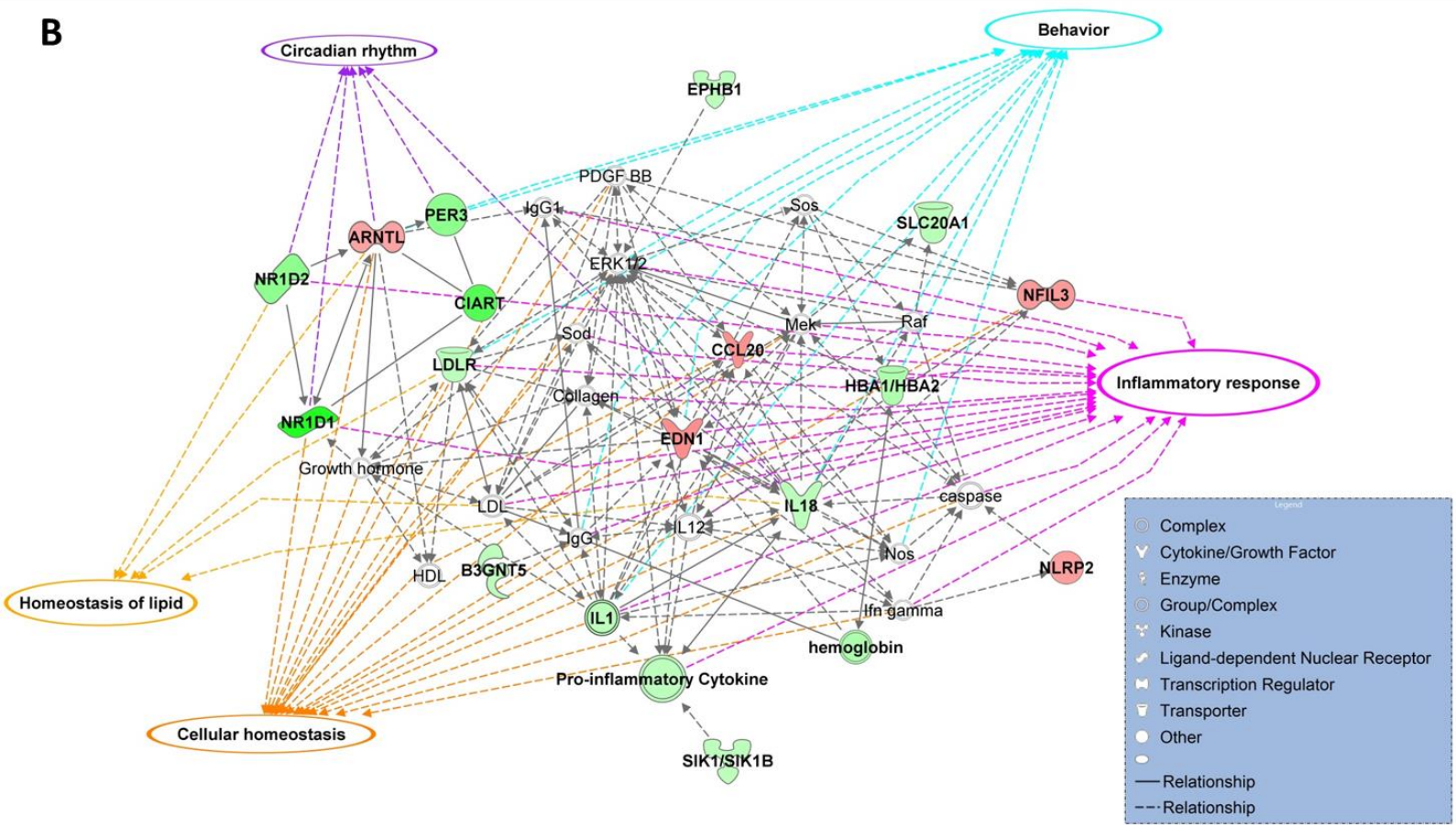


Figure 7: Relationships between differentially expressed genes in the highest scored network and related Canonical Pathways (CP) (Panel A) and Biological Functions (Fx) (Panel B). The list of differentially expressed genes in participants before and after CPS was uploaded into the IPA application. Edge (gene relationship) and node (gene) symbols are described. The intensity of the node color indicates the degree of up- (red) or down- (green) regulation. Uncolored nodes represent genes that were not identified as differentially expressed in our study and were integrated into the computationally generated networks based on the evidence stored in the IPA knowledge memory indicating relevance for this network. The network score is based on the hypergeometric distribution and is calculated with the right-tailed Fisher's exact test. The score is the negative Log of this *P* value (*P* -score = – log10 (*P* value)).

Microarray gene, as previously stated, showed that CPS induced a different gene expression profile in the intestinal mucosa. IPA analysis unraveled that the top up-regulated and down-regulated genes were clustered in different networks, being the most significant ones mainly implicated in immune and circadian clock functions. According to these findings, genes implicated in these networks and functions were used for RT-qPCR validation. Moreover, other genes implicated in immune and clock gene functions, but not differentially expressed in the array, were also analyzed. Although only one gene related to intestinal barrier function was differentially expressed (SLC26A3), and as one of our objectives was to determine the effect of stress on the intestinal barrier function, other genes related to intestinal barrier function were also assessed.

5.4.2.6. Microarray validation: Effect of CPS on clock gene and on mucosal barrier and immune function.

mRNA expression of selected genes was determined in order to validate our microarray findings. CPS down-regulated clock genes (Fold vs. Basal: NR1D1=0.29, $P<0.0001$; NR1D2=0.56, $P<0.0001$; PER1=0.41, $P<0.0001$; PER3=0.47, $P<0.0001$; NFIL3=1.76, $P<0.0001$). Moreover, CPS altered the expression of genes key to barrier function modulation by increasing CLDN2 (Fold vs. Basal: 1.35, $P=0.0426$) and decreasing SLC26A3 (Fold vs. Basal: 0.73, $P=0.0043$), and also altered the expression of inflammation-related genes by decreasing IL18 (Fold vs. Basal: 0.80, $P=0.0107$) and increasing SOD1 (Fold vs. Basal: 1.11, $P=0.0227$); as well as the protease activity gene SERPINA1 (Fold vs. Basal: 1.24, $P=0.0031$) (figure 8).

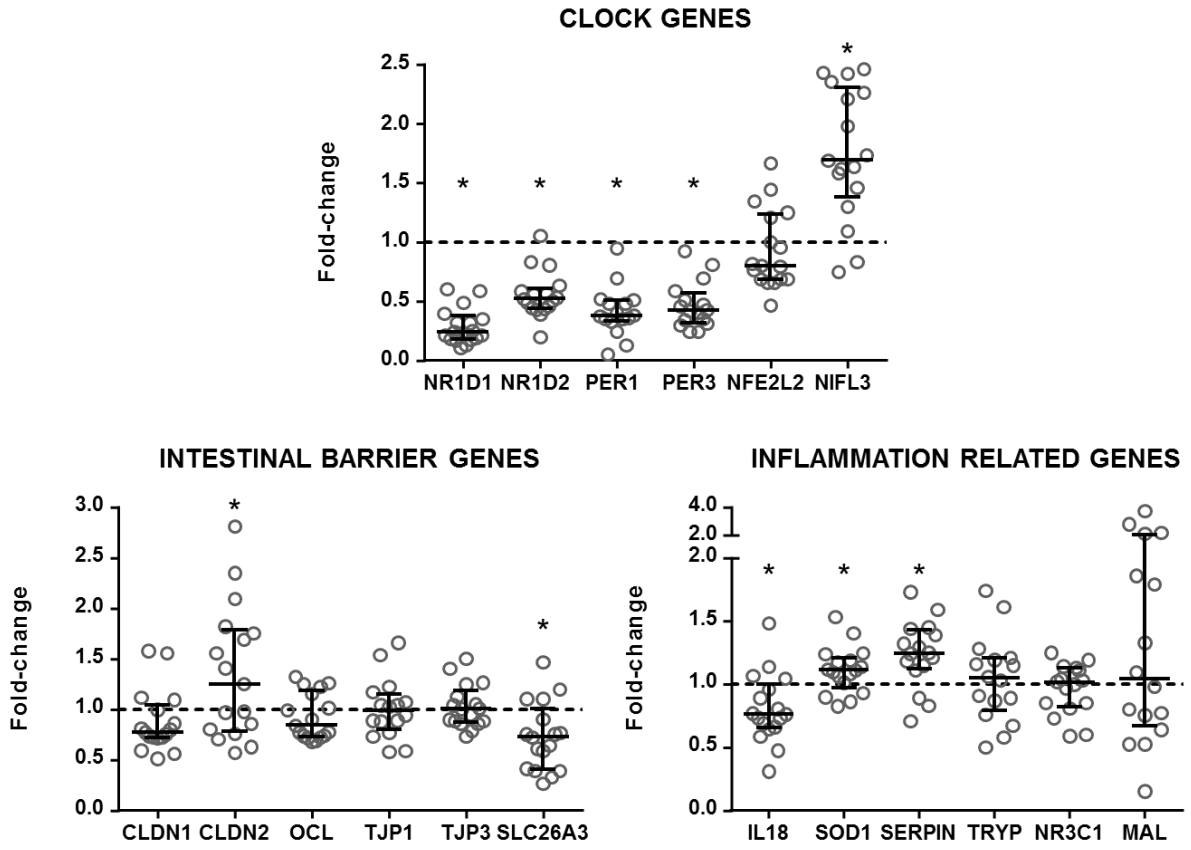


Figure 8: Fold-change expression values obtained by RT-qPCR of selected genes. Each dot represents the fold-change of each individual between pre and post-CPS and the error bars represent the SEM.

Notably, significant association was found between representative genes of the most significant biological functions altered by stress (table 5).

		CLDN2	TJP1	TJP3	SLC26A3	IL18
PER1	r	-0.20	0.57*	0.39*	0.59*	0.421*
	P	0.23	0.01	0.02	0.0001	0.009
PER3	r	-0.38*	0.35	0.31*	0.42*	0.692*
	P	0.02	0.12	0.06	0.01	0.0001
TPSAB1	r	0.60*	-0.08	-0.09	0.18	-0.68
	P	0.01	0.72	0.70	0.45	0.777

Table 5: Association between clock genes and epithelial barrier and inflammation genes. *Significant correlations (P<0.05)

5.4.3. Subgroup analysis by stress and sex

In order to assess whether chronic stress and/or sex determines a differential mucosal response to acute stress, gene expression was analyzed in groups subdivided accordingly.

5.4.3.1. Clinical and demographical data.

Subgroup analysis by stress or sex did not identify any clinical difference between participants, although males had higher BMI and different values of blood pressure than females. Interestingly, the baseline levels of several components of the SSRS where significantly different between low stress and moderate stress groups, as observed in table 6.

	LOW (N=18)	MODERATE (N=6)	*P*	MALE (N=12)	FEMALE (N=12)	*P*
Age (years)	23.3 (22.6-27.1)	24.7 (23.3-35.4)	0.257	23.9 (22.7-33.4)	23.6 (22.8-25.2)	0.729
BMI (kg/m^2)	23.5 (21.1-26.4)	24.0 (19.2-26.0)	0.815	24.9 (23.2-27.2)	21.5 (19.5-24.7)	0.02
H-R	90.5 (58.5-127.5)	232.5 (214-320.3)	<0.01	90.5 (53.3-167.3)	130.5 (84.5-204.5)	0.184
PSS	16.5 (11.8-25.3)	16.0 (12.5-22.0)	0.894	14.0 (11.0-24.8)	18.5 (13.3-24.8)	0.402
BDI	0.5 (0.0-4.3)	0.0 (0.0-8.5)	0.884	0.0 (0.0-0.8)	1.5 (0.0-6.5)	0.115
SBP (mmHg)	119.50 (110.8-128.0)	118.0 (110.3-135.5)	0.796	128.0 (119.0-135.0)	114.0 (108.0-120.0)	0.003
DBP (mmHg)	68.5 (63.75-72.0)	73.0 (69.0-83.3)	0.054	72.0 (69.0-80.0)	68.0 (63.0-69.0)	0.021
MBP (mmHg)	85.0 (80.3-89.9)	89.3 (85.3-96.2)	0.161	90.7 (8.7.-93.3)	82.0 (79.3-86.3)	0.001
HR (bpm)	65.0 (50.5-69.75)	71.0 (59.5-76.3)	0.223	66.0 (60.0-73.0)	65.0 (49.0-70.0)	0.645
Hand Pain T0	0.1 (0.0-0.3)	0.0 (0.0-0.2)	0.494	0.0 (0.0-0.3)	0.1 (0.0-0.3)	0.925
Stress *	3.1 (2.2-3.6)	4.7 (3.6-6.2)	0.028	2.9 (2.1-4.2)	3.6 (3.0-4.3)	0.26
Arousal *	3.3 (0.0-4.7)	3.7 (3.1-5.9)	0.271	2.9 (2.0-4.6)	3.5 (2.5-5.0)	0.326
Anxiety *	3.0 (1.8-3.7)	5.2 (3.3-6.1)	0.028	2.5 (1.5-4.3)	3.5 (2.9-4.9)	0.1
Anger *	2.7 (1.8-3.6)	4.1 (3.4-5.1)	0.036	3.0 (1.8-4.4)	3.1 (2.0-4.7)	0.84
Fatigue *	3.6 (2.7-4)	4.3 (3.8-6.0)	0.018	3.6 (2.0-3.8)	4.0 (2.8-5.1)	0.069
Attention *	2.7 (1.3-4.2)	4.4 (2.6-5.9)	0.062	2.8 (1.3-4.6)	2.8 (2.1-4.2)	0.644
Blood Cortisol(µg/dL)	7.7 (6.4-9.4)	10.4 (7.8-13.4)	0.205	8.8 (7.0-10.6)	8.0 (6.7-12.2)	0.525
Blood DHEA (ng/mL)	6.2 (4.6-8.4)	7.6 (4.8-10.3)	0.548	5.5 (4.6-8.9)	7.2 (5.2-9.2)	0.356
Blood ACTH (pg/mL)	41.6 (37.9-45.1)	41.9 (33.6-48.9)	0.841	41.0 (33.1-50.6)	41.9 (39.6-45.0)	0.564
Blood Insulin (µU/mL)	4.3 (2.9-7.0)	2.5 (1.0-5.2)	0.133	3.3 (1.5-6.1)	4.5 (3.2-6.7)	0.354
IgA in saliva (µg/mL)	380.1 (210.-526)	260.4 (111-926)	0.314	319.2 (161-953)	362345 (259- 537)	0.559
Cortisol in saliva (µg/dL)	0.17 (0.08-0.27)	0.19 (0.13-0.22)	0.858	0.17 (0.1-0.28)	0.20 (0.08-0.24)	0.86
CD3+ Lymphocytes	11.0 (7.8-19.3)	14.5 (12.5-22.5)	0.286	12.0 (8.0-18.5)	14.0 (9.3-20.0)	0.795
Mast Cells (cells/HPF)	23.4 (18.5-28.5)	26.9 (16.3-41.3)	0.443	24.9 (16.8-31.6)	22.7 (18.5-27.8)	0.525
Eosinophils (cells/HPF)	2.0 (0.9-4.5)	1.2 (1.0-6.0)	0.664	3.7 (1.2-6.8)	1.4 (0.9-2.6)	0.148

Table 6: Demographic and clinical characteristics of the population divided by sex and stress. Data are expressed as median and Q1-Q3. ACTH: Adrenocorticotropic hormone; BDI: Beck's depression inventory; DBP: Diastolic blood pressure; DHEA: Dehydroepiandrosterone; HPF: High power field; H-R: Holmes-Rahe questionnaire; HR: Heart rate; IgA: Immunoglobulin A; MBP: Median blood pressure; SBP: Systolic blood pressure; *SSRS components

5.4.3.2. Autonomic, psychological and hormonal response to CPS

CPS induced an autonomic, psychological and hormonal response, but no differences between groups were observed when analyzing data by stress or by sex, as observed in figures 9 and 10. Interestingly, stress perception to CPS was higher in MS than in LS subjects and only in the first ones there were differences between T15 compared to T0.

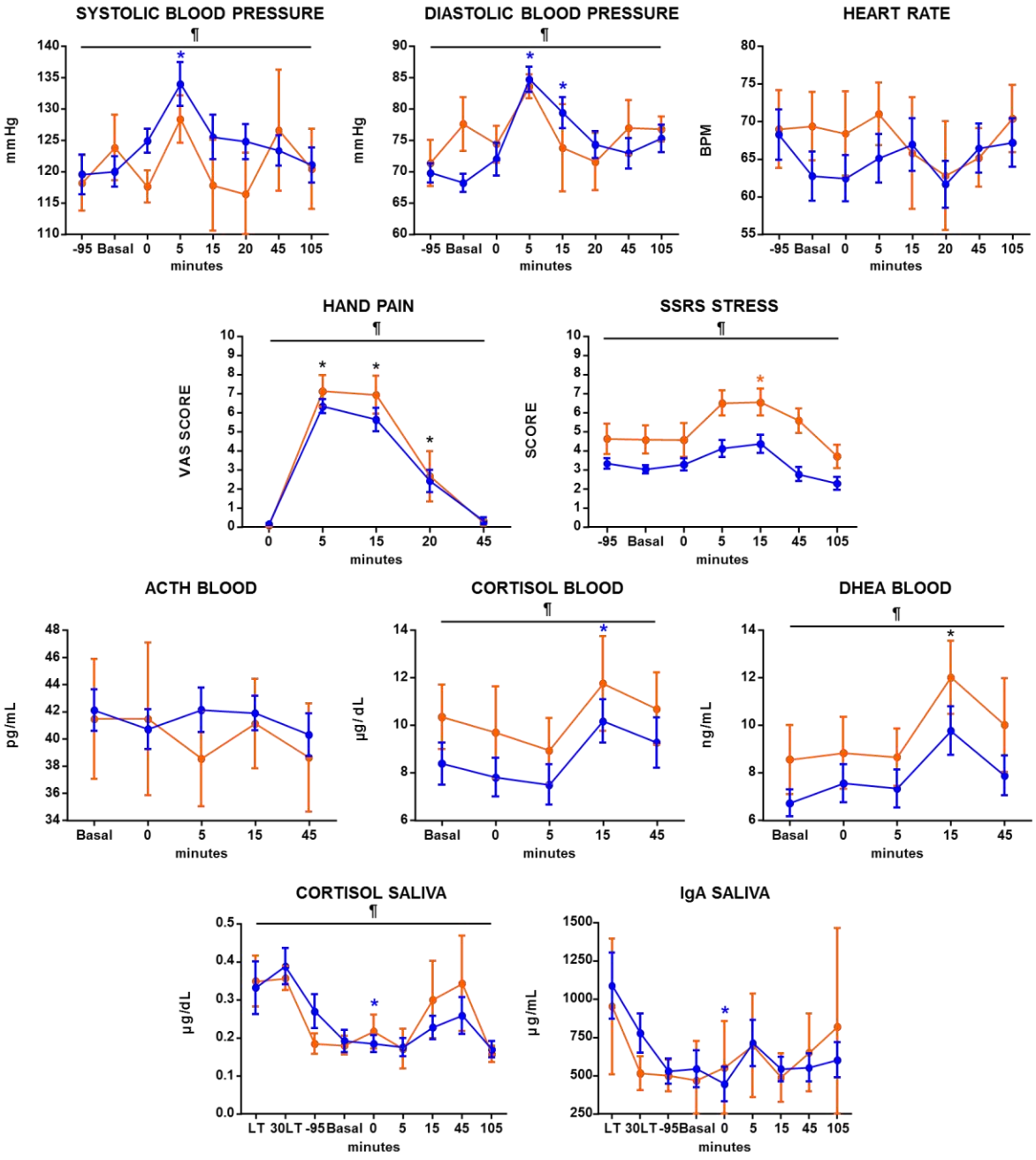


Figure 9: Systemic, psychological and hormonal response to CPS stratified by stress levels. Each dot represents the mean and the error bars represent the SEM. Blue and orange represent data from low and moderate psychosocial stress, respectively. 30LT: 30 minutes after waking up; ACTH: Adrenocorticotropic hormone; DHEA: Dehydroepiandrosterone; IgA: Immunoglobulin A; LT: just after waking up. "-95 minutes" corresponds to autonomic, psychological and hormonal variables assessment upon arrival to our unit; "basal minutes" corresponds to autonomic, psychological and hormonal variables assessment after first biopsy was obtained. *P<0.05 vs T0; in black time points different in all groups, in blue time points different only in low psychosocial stress group and in orange time points different only in high psychosocial stress group. ¶ ANOVA P value <0.05.

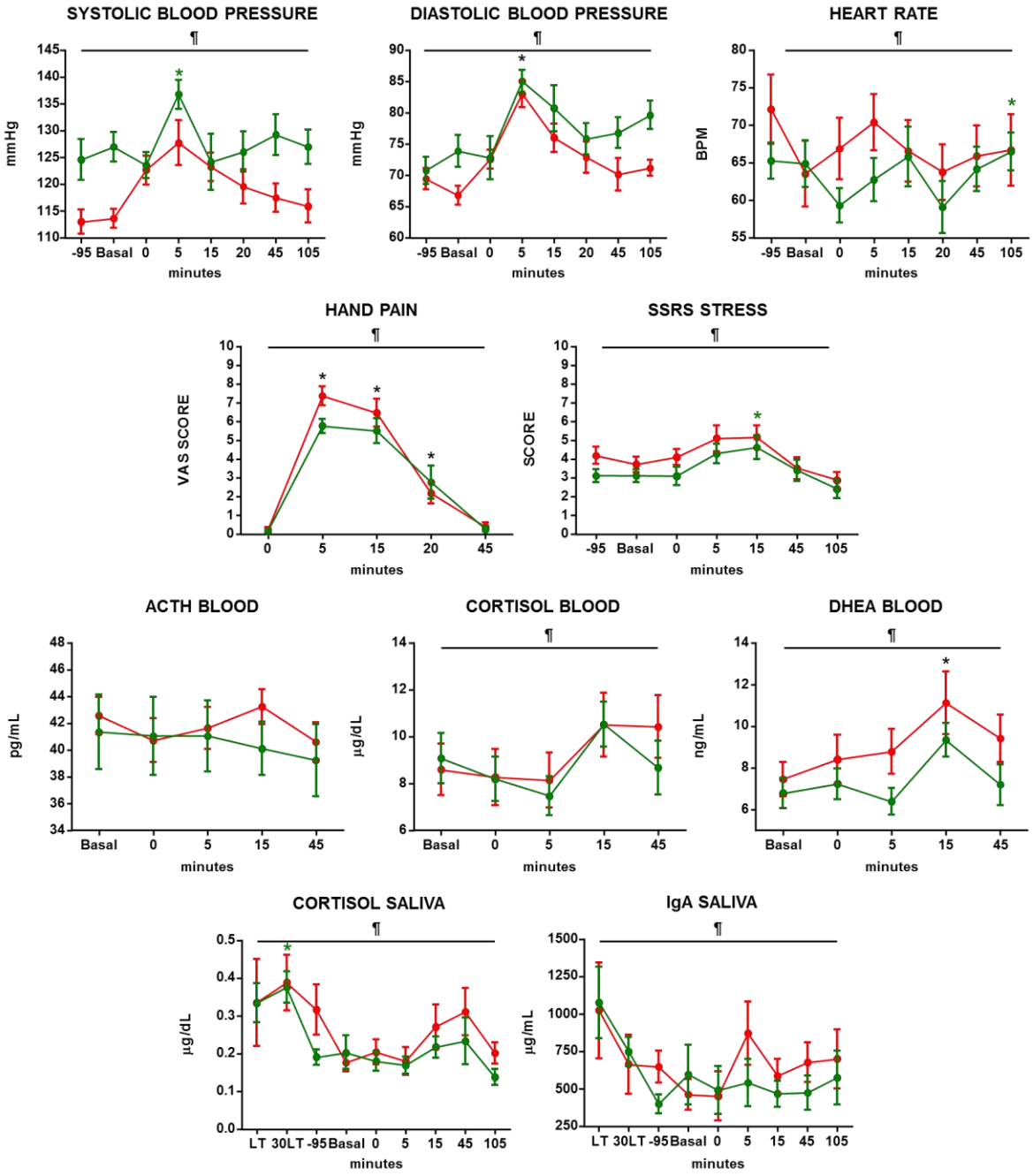
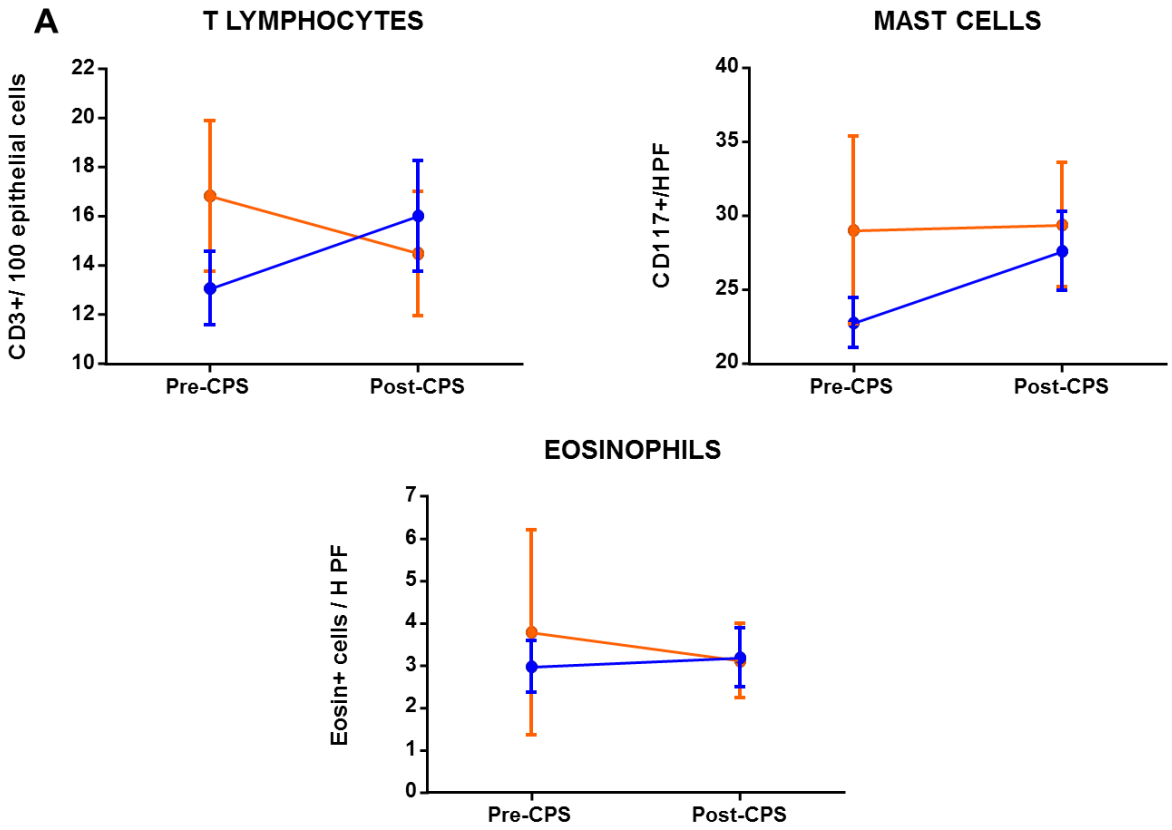


Figure 10: Systemic, psychological and hormonal response to CPS stratified by sex. Each dot represents the mean and the error bars represent the SEM. Green and red represent data from male and female subjects, respectively. 30LT: 30 minutes after waking up; ACTH: Adrenocorticotropic hormone; DHEA: Dehydroepiandrosterone; IgA: Immunoglobulin A; LT: just after waking up. "-95 minutes" corresponds to autonomic, psychological and hormonal variables assessment upon arrival to our unit; "basal minutes" corresponds to autonomic, psychological and hormonal variables assessment after first biopsy was obtained. *P<0.05 vs T0; in black time points different in all groups, in green time points different only in males and in red time points different only in females. ¶ ANOVA P value <0.05.

5.4.3.3. Mucosal immune cell infiltration

Subgroup analysis by stress and sex did not show any effect of CPS on the mucosal immune cell infiltration, as observed in figure 11.



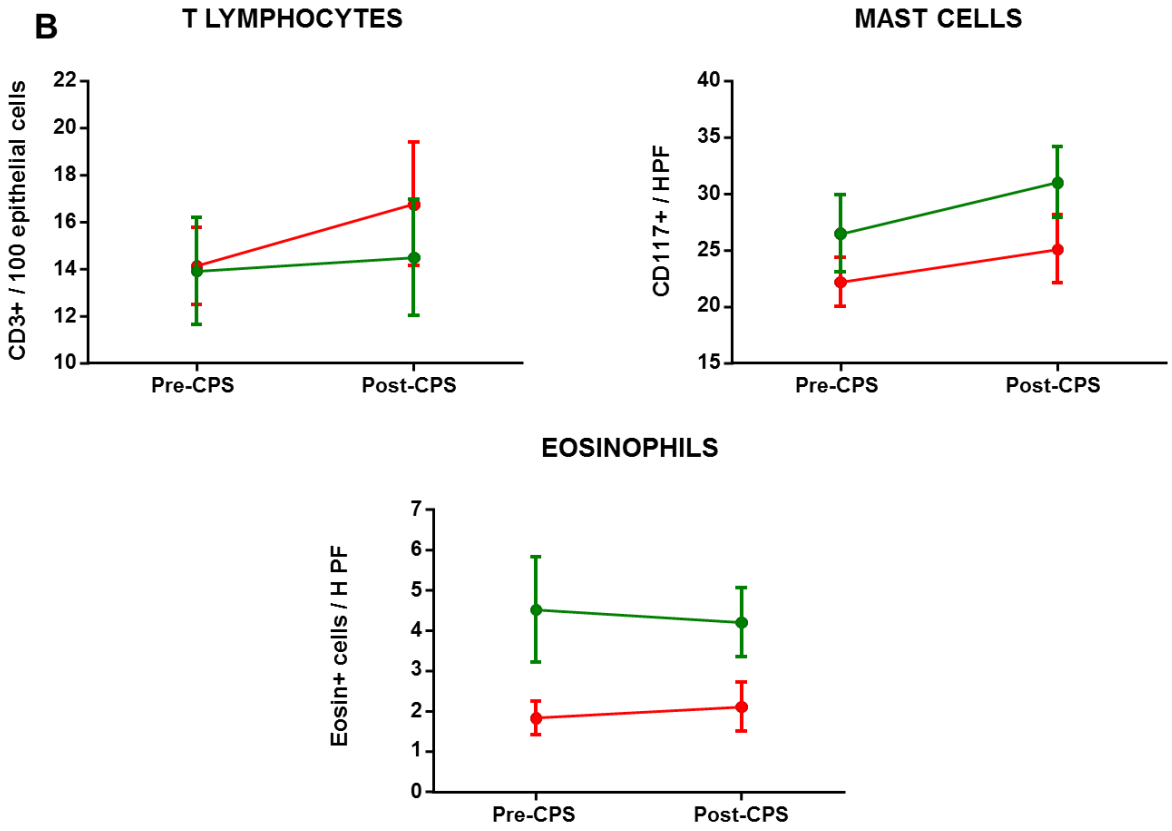


Figure 11: Representation of mucosal immune cell populations before and after CPS in subgroup analysis by stress (Panel A) and sex (Panel B). Each dot represents the mean and the error bars represent the SEM. In blue, subjects with low psychosocial stress and in orange, subjects with moderate psychosocial stress. In green, male subjects and in red, female subjects.

5.4.3.4. Mucosal transcriptome.

Chronic psychosocial stress determined a differential mucosal transcriptome induced by CPS. A total of 18 genes were identified as differentially expressed and 1 significant network was obtained in the LS group; while 45 differentially expressed genes and 3 networks were identified in the MS group (false discovery rate-adjusted *P*=0.16). Figure 12 represents the heatmap and table 7 lists the networks obtained in each analysis. Interactions among genes in each network, belonging to specific canonical pathways and biological functions are illustrated in supplementary material (fig S1 and S2).

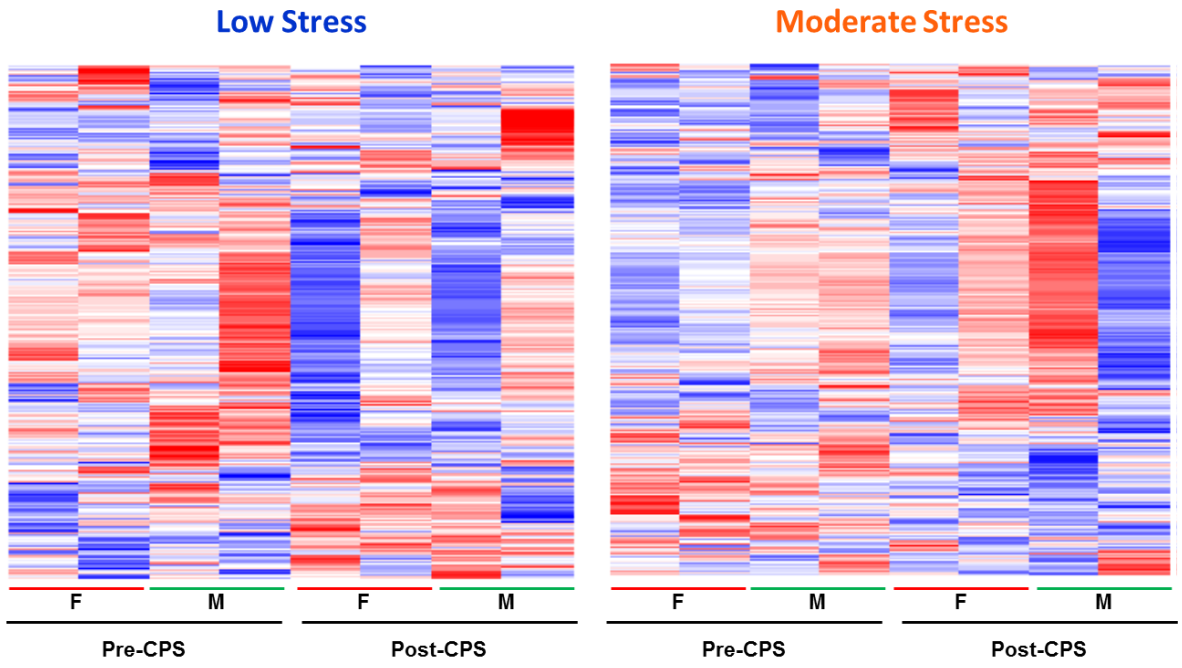


Figure 12: Heatmap of low and moderate stress groups representing the differential gene expression profile in the jejunal mucosal of each subject before and after stress. Individual samples are shown in columns and genes are represented in rows. Color grading indicates the expression level for each gene, blue indicates low level of and red indicates high level of expression. F: Female; M: Male.

	ID	Molecules in Network	Score	Focus Molecules	Top Diseases and Functions
Low stress	1	5-hydroxyeicosatetraenoic acid, Akt, APP, AVPR1A, CCL20, CIART, Cox6c, Cyb5r3, CYP1A1, DEFB103A/DEFB103B, EEF1B2, EPHA2, ERK1/2, FFAR1, HBA1/HBA2, IL17R, indole, Insulin, ion channel, KLB, MC3R, MC4R, neuroprotectin D1, NFkB (complex), nitrogen, NR1D1, NR1D2, P38 MAPK, PDGF (family), PER1, PER3, PERIOD, Pkc(s), SGK1, SLC38A3	31	11	Behavior, Digestive System Development and Function, Cellular Compromise
	1	AMPD1, AMPK, ATRX, caspase, CFH, CIART, Creb, cytokine, EDN1, ERK, ERK1/2, Histone h3, Histone h4, Ifn gamma, IL18, Insulin, Mapk, mir-30, Mmp, NFIL3, NFkB (complex), NLRP2, NR1D1, NR1D2, PDGF BB, PER1, PER3, PI3K (complex), Pkc(s), PLOD2, SERPINA3, SLC26A3, SLC2A2, Vegf, WEE1	46	18	Behavior, Nervous System Development and Function, Cell Morphology
Moderate stress	2	20-hydroxyeicosatetraenoic acid, Actomyosin, Akt, Ca2+, cyclic AMP, cyclooxygenase, CYP1B1, IGF1, Jnk, KCNJ13, L-arginine, leukotriene B4, lipid peroxide, LRPAP1, MC4R, miR-27a-3p (and other miRNAs w/seed UCACAGU), miR-4671-5p (miRNAs w/seed CCGAAGA), NFIL3, NR1D1, NUPR1, PON2, RAMP3, REG1B, Relaxin, SNORA65, SNORD53, SPRR3, SPRR2A, stearic acid, thromboxane B2, TMEM80, TMEM167A, tyrosine kinase, UTP, VLDL	25	11	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
	3	60S ribosomal subunit, ALDH1B1, CAPRIN1, CAVIN3, DRAP1, Elf5, FABP2, GEMIN5, HERC5, HIST1H2AB, HLF, miR-140-3p (and other miRNAs w/seed ACCACAG), miR-3660 (and other miRNAs w/seed CUGACAG), MYC, NFX1, OSBPL8, PDE8A, Rar, REEP3, RNF130, RPL3, RPL11, RPL18A, RPL36AL, RPL7A, TAF1C, TAF1D, TCEAL1, TDP2, thyroid hormone receptor, TIMM8B, TNFRSF8, TOP2B, TRAF3, UBC	12	6	Cancer, Hematological Disease, Immunological Disease

Table 7: Top relevant functional network in the intestinal mucosa associated with CPS according to baseline level of stress.

Sex also determined a differential response to CPS. A total of 30 genes were identified as differentially expressed in both males and females (figure 13). Only one network was identified in males and 2 significant networks were identified in females (false discovery rate-adjusted *P*=0.16), as observed in table 8. Interactions among genes in each network, belonging to specific canonical pathways and biological functions are illustrated in supplementary material (fig S3 and S4)

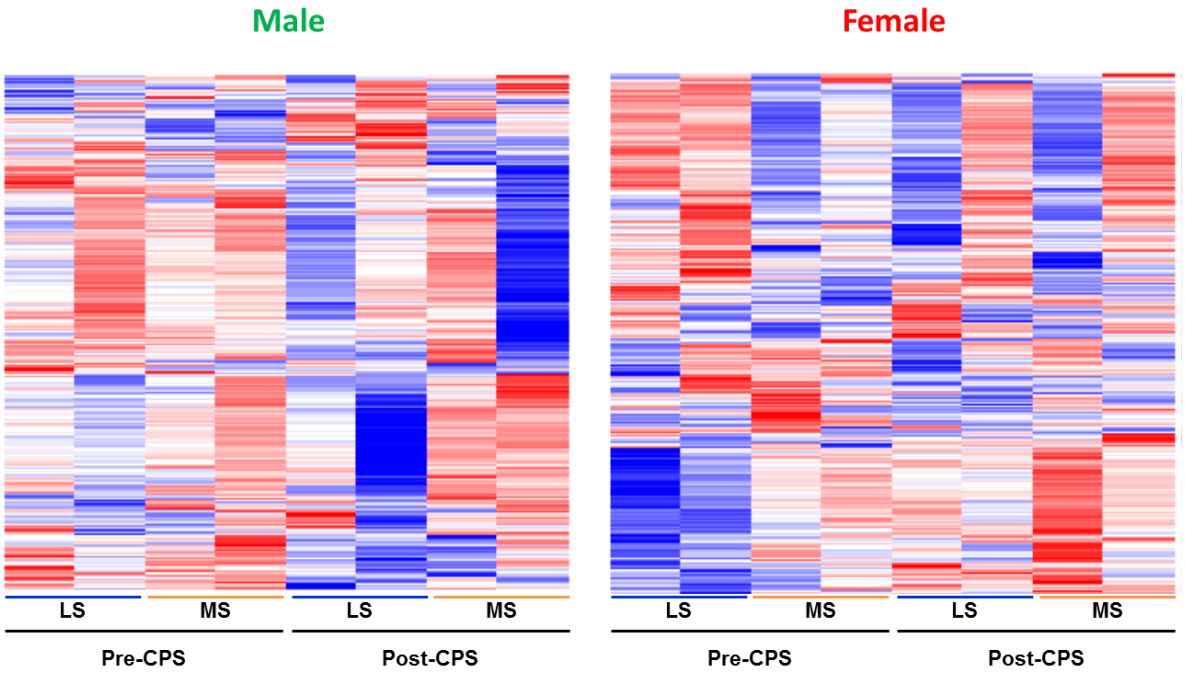


Figure 13: Heatmap of male and female groups representing the differential gene expression profile in the jejunal mucosal of each subject before and after stress. Individual samples are shown in columns and genes are represented in rows. Color grading indicates the expression level for each gene, blue indicates low level of and red indicates high level of expression. LS: Low stress; MS: Moderate stress.

	ID	Molecules in Network	Score	Focus Molecules	Top Diseases and Functions
Male	1	ADORA2A, APP, ATP5J, CDH5, corticosterone, DBI, DGAT2, DICER1, FBL, GLUL, GSR, HCAR2, HNRNPC, IL6, IL10, IL1B, KITLG, LPAR6, MAGI1, MFSD2A, mir-27, MMP8, MMP10, MMP13, NR1D1, NR3C1, PTCH2, RNASEL, SLC11A2, SMPD2, ST8SIA4, STAT3, TLR4, TNF, TRPM2	10	4	Cell Death and Survival, Nervous System Development and Function, Lipid Metabolism
Female	1	APP, ARNTL, ATG16L1, AXL, CIART, CRY2, CYSLTR2, DBP, EXOC1, EXOC2, EXOC3, EXOC5, EXOC6, EXOC7, EXOC8, FKBP5, HBA1/HBA2, IFNG, IL18, KIAA1551, L-dopa, MAL, NCAM1, NFIL3, NR1D1, PER1, PER2, PER3, PSEN1, SAMHD1, SET, TGFBR2, TNFRSF12A, TNFSF12, WDR1	28	13	Behavior, Nervous System Development and Function, Cellular Growth and Proliferation
	2	AKT1, BDNF, beta-estradiol, CASP3, CCL2, CCL5, CD40, CD69, CREB1, CRH, cyclic AMP, DLG4, dopamine, EGR1, FOS, G6PC, GRIN2A, GRIN2B, HBB, HGF, IL6, IL12B, IL1B, KLF3, LEP, NFKBIA, NOS2, PTGS2, RELA, SATB1, SGK1, SIRT1, STAT3, STAT4, TMEM80	6	4	Cellular Function and Maintenance, Cellular Development, Cellular Growth and Proliferation

Table 8: Top relevant functional network in the intestinal mucosa associated with CPS according to baseline level of stress.

5.4.3.5. Gene expression assessment according to life stress level and sex.

Finally, a comparison of the expression of inflammatory, barrier function and circadian clock genes was performed. As observed in figure 14, CPS induced a down-regulation of clock genes in both LS and MS subjects, while inflammation and epithelial barrier function genes were modified in the LS group. When comparing both groups, only differences in SERPINA1 where found between LS and MS subjects.

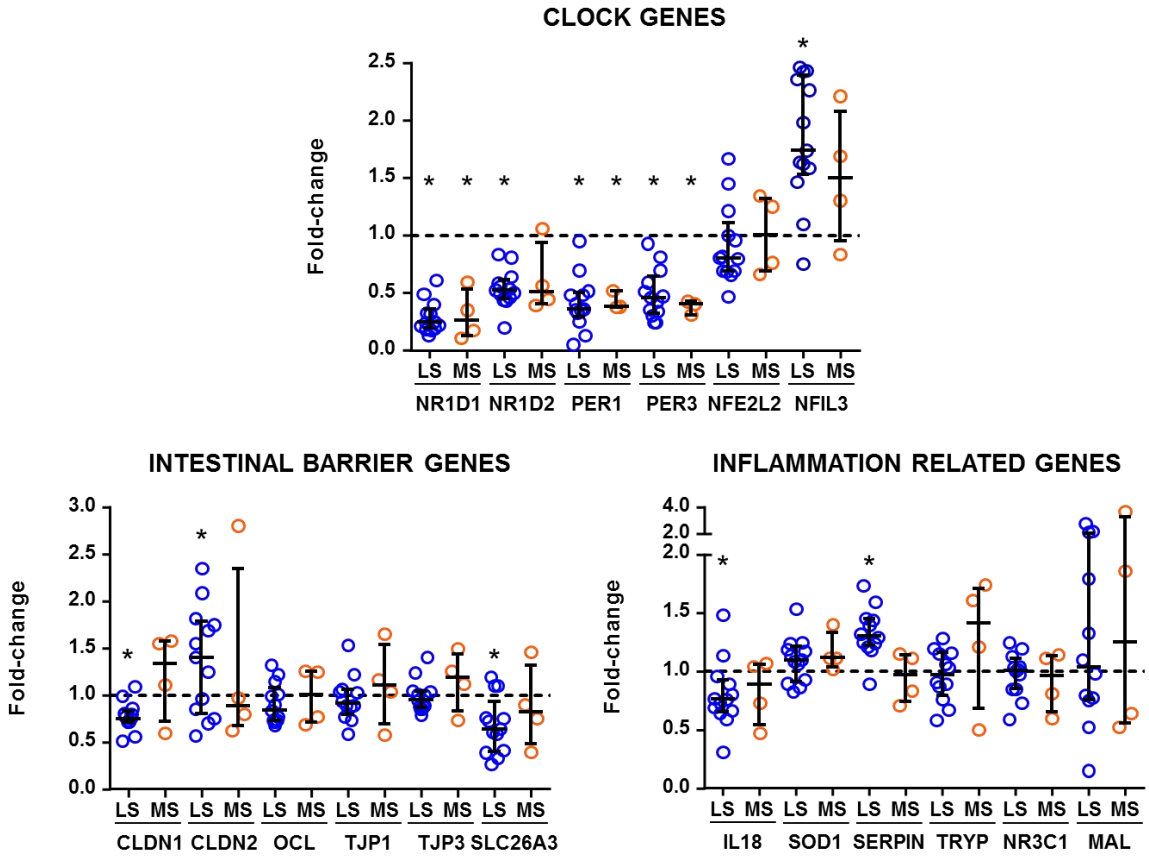


Figure 14: Fold-change expression values obtained by RT-qPCR of selected genes. Each dot represents the fold-change of each individual between pre and post-CPS and the error bars represent the SEM. In blue, subjects with low psychosocial stress and in orange, subjects with moderate psychosocial stress. * $P<0.05$.

The comparison of inflammatory, barrier function and circadian clock genes, according to sex, showed that CPS induced a down-regulation of NR1D1 and NR1D2 in both male and female subjects, but only in male PER1 and PER3 genes were significantly down-regulated (figure 15). The analysis of inflammation-related genes showed down-regulation of IL18 and up-regulation of SERPINA1 in males, and up-regulation of NIFL3 in both males and females. Regarding epithelial barrier function genes, there was a significant up regulation of CLDN2 and a down-regulation of SLC26A3 only in men. The expression of NR3C1 was also modulated by acute stress, with a borderline level of significance (*P*=0.069 in females).

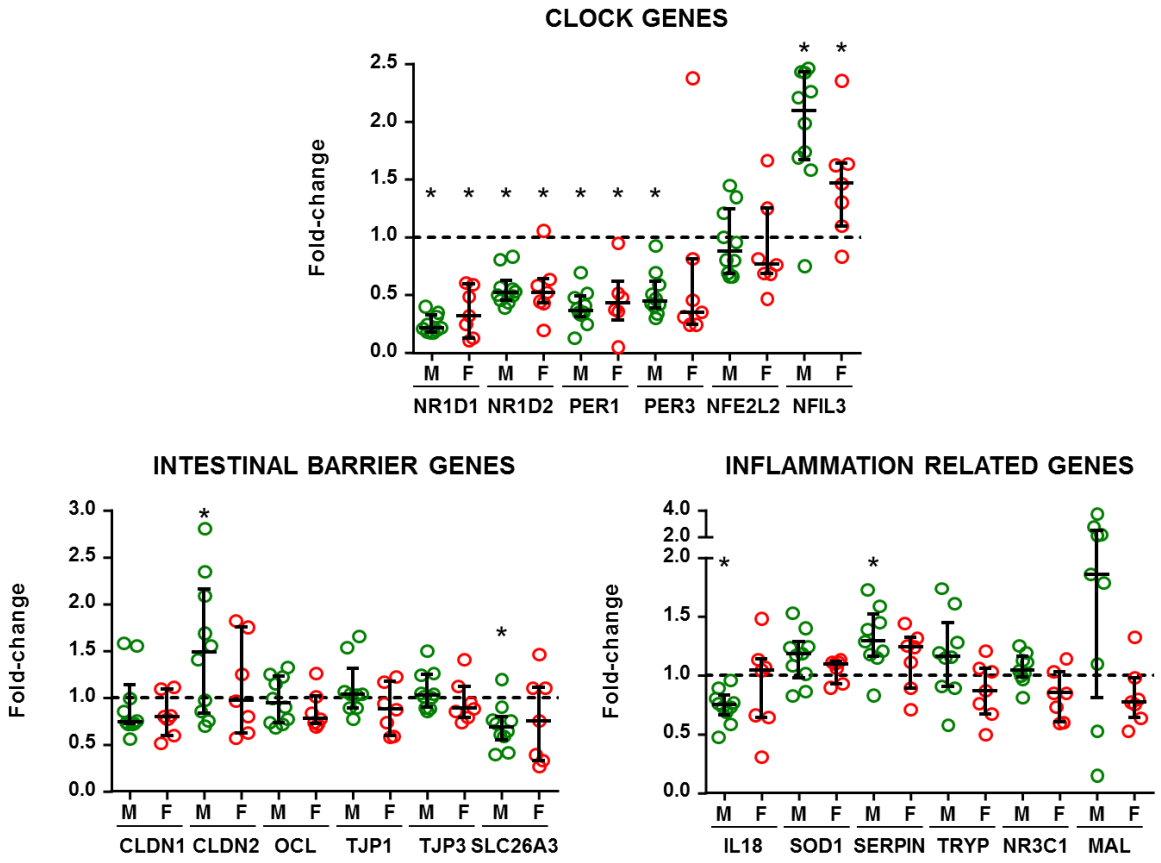


Figure 15: Fold-change expression values obtained by RT-qPCR of selected genes. Each dot represents de fold-change of each individual between pre and post-CPS and the error bars represent the SEM. Dots in green represent male subjects and dots in red represent female subjects. * *P*<0.05.

5.5. DISCUSSION

Our study demonstrated that CPS is able to induce a dysregulation of genes implicated in immune, barrier and clock gene functions. Notably, the response generated is modulated according to chronic psychosocial stress or sex.

Stress plays and important role in the pathophysiology of functional gastrointestinal disorders, especially in IBS and FD. Human and animal, ex-vivo (J Santos, Saunders, et al., 1999; Paul R Saunders, Santos, et al., 2002) and in-vivo (Vanuytsel et al., 2014), studies have shown that stress impairs intestinal permeability and gastrointestinal function, but little is known about the underlying mechanisms of this interaction. The present study demonstrates that an acute painful stimulus is able to alter the mucosal transcriptome by modifying circadian rhythm, inflammatory and epithelial barrier genes. The current work expands our previous studies on differential intestinal functional responsiveness to stress (C. Alonso et al., 2012; Carmen Alonso et al., 2008) by identifying a differential molecular mucosal response to CPS, unveiling the main pathways potentially implicated in stress-related mucosal alterations.

CPS is a well validated stress model in humans (C. Alonso et al., 2012; Carmen Alonso et al., 2008; Lovallo, 1975). In order to assess the effectiveness of the stress response in our experiment, we measured blood pressure, heart rate, and cortisol and ACTH in blood. We confirmed the stress-induced changes in theses variables, supporting the validity of CPS as a stress model. Moreover, as psychological stress is a key factor in the development of FGID (Koh et al., 2014; Rona L. Levy et al., 2006), we also confirmed that this model increases psychological stress as observed by modification of the SSRS. Overall, CPS induced a physical, autonomic, hormonal and psychological response.

At the mucosal level, CPS induced changes in gene expression related mainly with neurological disorders and immune activation. Our analysis also pinpointed circadian rhythm regulation as a new stress-regulated molecular pathway in the jejunal mucosa. In fact, our results demonstrated that stress impairs intestinal barrier function by decreasing the expression of SLC26A3, a molecule which ensures intestinal barrier function, in part through stabilizing TJs (Ding et al., 2018). Down-regulation of this molecule has been related to susceptibility to develop inflammatory bowel disease (Kumar et al., 2017). Moreover, CPS increased CLDN 2 expression which is linked to increased permeability and also has been found to be differentially expressed in IBS subjects when compared to healthy controls

(Martínez et al., 2013). These observed changes could explain one of the mechanisms by which stress can disrupt intestinal barrier homeostasis favoring the passage of luminal antigens in the lamina propria. In fact, data from animal and human studies have shown that stress can predispose to intestinal inflammation and also impairs intestinal permeability but the molecular mechanisms have not been fully studied. According to previously published studies (Miller, Rohleder & Cole, 2009; J Santos et al., 2000), our study also finds altered expression of genes related with barrier and immune functions, increasing inflammatory markers and gut leakiness. The most prominent result of our study, however, is the alteration of jejunal circadian rhythm genes by acute stress. To our knowledge, no previous work showed the effect of acute stress on jejunal clock genes expression. Clock genes are the master regulators of cellular circadian rhythm and they are regulated centrally from the suprachiasmatic nucleus of the hypothalamus (Nicolaides, Charmandari, Kino & Chrousos, 2017) and locally by peripheral clock systems (Gibbs et al., 2014). Central clock has an essential role in human physiology, controlling sleep cycle, metabolism and the activity of hypothalamic-pituitary-adrenal (HPA) axis (Golombek et al., 2013). Clock genes are a group of genes which have a strong co-regulation with feedback-loops and form the basis for the circadian rhythm regulation (Takahashi, Hong, Ko & McDearmon, 2008). Circadian Locomotor Output Cycle Kaput and Brain–Muscle–Arnt-Like protein 1 (CLOCK and BMAL1) heterodimer and other negative transcription factors, such as Periods (PER1, PER2, and PER3) and Cryptochromes (CRY1 and CRY2) are the main regulators of circadian rhythm at the molecular level. Other transcription factors can be regulated by clock genes as NFE2L2, which is a transcription factor associated with antioxidant activity (Pekovic-Vaughan et al., 2014). The mechanisms of intestinal circadian rhythm control have been much more deeply studied in animal models than in humans. Therefore, little is known about deregulation of circadian rhythm on human gastrointestinal health. A circadian condition as turn-shift work is associated with FGID (such as IBS and FD), ulcers and inflammatory bowel disease (Drake, Roehrs, Richardson, Walsh & Roth, 2004; H. I. Kim et al., 2013; Nojkov, Rubenstein, Chey & Hoogerwerf, 2010; Saberi & Moravveji, 2010; Segawa et al., 1987; Sonnenberg, 1990). Another fact that supports the role of circadian rhythm alteration in gastrointestinal disorders is the effective use of melatonin as adjuvant treatment on colonic diseases (Esteban-Zubero et al., 2017) or the beneficial effect of the probiotic VSL#3 in IBS through melatonin regulation (Wong, Yang, Song, Wong & Ho, 2015), although in this last example it cannot be excluded the effect on the microbiota or the connection between microbiota, gut and clock genes

(Voigt, Forsyth, Green, Engen & Keshavarzian, 2016). Moreover, a recent study has suggested that clock genes could be inductors of brain-gut axis dysregulation in the intestine, and they might display a pathophysiological role in IBS development and placebo effect (Lobo, Santos & Vicario, 2013).

A strong association between genes involving immune, intestinal barrier and clock functions was observed in response to acute stress. Although we found this interaction in the jejunum, it is likely that these three systems work together, as the HPA axis has an important bidirectional regulation with the circadian clock (Nader, Chrousos & Kino, 2010). Moreover, glucocorticoids can control the circadian molecular machinery, as the absence of glucocorticoids (in adrenalectomized mice) alters PER1 oscillations (Pezük, Mohawk, Wang & Menaker, 2012). In addition, treatment with glucocorticoids alters period clock genes in white adipose tissue, bronchial epithelial cells, cardiac muscle and bone (Barnea, Madar & Froy, 2013; Burioka et al., 2005; van der Veen, Shao, Xi, Li & Duffield, 2012) and can reset peripheral biological clock (Balsalobre et al., 2000). This peripheral reset on molecular circadian rhythm regulation and the presence of period genes can affect intestinal barrier homeostasis as period clock genes modify intestinal motility, tight junction expression and intestinal permeability (Golombek et al., 2013; Hoogerwerf, 2010; Kyoko et al., 2014). But, beside previous investigation, the role of clock genes in human intestinal epithelium and, specifically, on intestinal barrier function still remains unknown. Several studies have also shown that clock genes disturbance by chronic sleep disruption in animal an humans, mainly night shift workers, impairs barrier function by increasing intestinal permeability (G. R. Swanson et al., 2016) and can also favor inflammation (Poroyko et al., 2016). Several studies have demonstrated that stress can disrupt intestinal barrier making it more susceptible to bacterial infections or to inflammation (J Santos et al., 2000; J Santos & Perdue, 2000). Our study takes the effect of stress one step further, as we demonstrated that stress per se is able to modify clock genes, which play a key role in intestinal barrier homeostasis.

Sex and chronic psychosocial stress have separately been implicated in gastrointestinal diseases development and in differential response to acute stress (C. Alonso et al., 2012; Carmen Alonso et al., 2008). In our study, we analyzed the effect of these two factors on intestinal mucosal response to acute stress. Interestingly, we find that males and females showed a different molecular response to incoming stress and that chronic psychosocial stress also evoked a differential mucosal transcriptome. However, the low number of subjects

included in all the groups limited the validation of those results. Although our study was not focused on intestinal permeability measurements, the findings of the present study raise the question of what are the functional consequences of the observed molecular response. We can, however, speculate that stress increased intestinal permeability and this alteration was more evident in women and in subjects with higher chronic psychosocial stress (C. Alonso et al., 2012; Carmen Alonso et al., 2008; Vanuytsel et al., 2014). Nevertheless, further analysis at protein and ultrastructural level are needed to validate these observations.

Immune activation has a role in IBS patients as there is an increased activation of immune cells at molecular and functional levels (Giovanni Barbara et al., 2011). We found that CCL20 was differentially expressed before and after stress (table 1). CCL20 is a chemokine with antimicrobial activity (Yang et al., 2003) and also directs Treg, Th17, B-cells and immature dendritic cells to the gut mucosa. It has been observed that there is a decrease of its expression in the duodenum and an increase in the rectum of IBS-D patients (Michael Camilleri et al., 2014, 2016) as well as that it is a key gene in the pathophysiology of inflammatory bowel disease. In our study, we found that CPS up-regulated CCL20 gene expression which will induce the recruitment of immune cells to possibly act against bacteria that could reach the lamina propria due to the increased permeability (Schmuth et al., 2002). Another possible explanation is that, as CCL20 is regulated by circadian clock genes (Thu Le et al., 2017), the changes found could interfere in CCL20 gene expression and in the end predispose to IBS-D or inflammatory bowel disease development.

Despite the limited number of subjects in our study, our findings reinforce the conception that life stress is an independent factor that modifies body functionality and can predispose or modify diseases as the response was different according to chronic psychosocial stress. While low stress conditioned a response to hyperpermeability (decrease SCLA26A3 and increase in CLDN2), moderate stress had a tendency to increase in CLDN1 which has been found elevated in patients suffering from IBD (Garcia-Hernandez, Quiros & Nusrat, 2017).

There is a clear female predominance in stress-related diseases such as IBS. Genetic variations on NR3C1 have been related to maladaptive stress responses and also to major depressive disorder (Sarubin et al., 2017). Moreover, Water avoidance stress is able to down-regulate NR3C1 gene in rats after WAS and this has been related to visceral-pain (Wiley, Higgins & Athey, 2016). Moreover, Another gene that has been related to visceral pain

modification is IL-18, which its down-regulation of IL-18 diminished visceral pain in a mouse model of post-infectious IBS (Gu, Zhang & Feng, 2016). Interestingly, our subgroup analysis by sex showed a different activation of immune-related pathways between males and females, with a down-regulation of IL-18 in males and a down-regulation, although not significant, of NR3C1 in females. These findings suggest that there is a sex specific response to stress which could explain female predominance in IBS, a pain-related disorder, could be related to the different response of males and females to stress which will trigger intestinal symptomatology.

In summary, at a molecular level, acute pain stress disrupts circadian rhythm, intestinal barrier and immune function. These findings reveal a suitable mechanism by which stress is able to disrupt intestinal homeostasis favoring the development of gastrointestinal dysfunction and FGID, especially IBS. However, more studies are needed in order to determine if these transcriptomic changes induce functional changes.

5.6. SUPLEMMENTARY DATA

5.6.1. Supplementary methods: Functional and pathway analysis.

The list of genes was overlaid onto a global molecular network developed from information contained in the IPA knowledge base (IPKB). For network analysis, IPA computed a score (p-score=-log10 (p-value)) according to the fit of the set of supplied genes and a list of biological functions stored in the IPKB. The score takes into account the number of genes in the network and the size of the network to approximate how relevant this network is to the original list of genes and allows the networks to be prioritized for further studies. A score >3 ($p<0.001$) indicates a >99.9% confidence that a gene network was not generated by chance alone. The network identified is presented as a graph indicating the molecular relationships between genes/gene products. Moreover, networks are preferentially enriched for genes with the most extensive interactions, and for which interactions are specific with the other genes in the network (rather than genes that are promiscuous, those that interact with a broad selection of genes throughout IPKB). Therefore, from the list of 286 differentially expressed genes in IBS-D patients, 268 were eligible to generate networks by IPA.

The functional analysis identified the biological functions and the canonical signaling pathways that were most significant to the input data set. The significance of the association between the input data set and the functions or pathways was determined based on two parameters: (1) a ratio of the number of genes from the data set that map to the function/pathway divided by the total number of genes that map to the function/pathway and (2) a P-value calculated using Fischer's exact test determining the probability that the association between the genes in the dataset and the function/pathway is explained by chance alone.

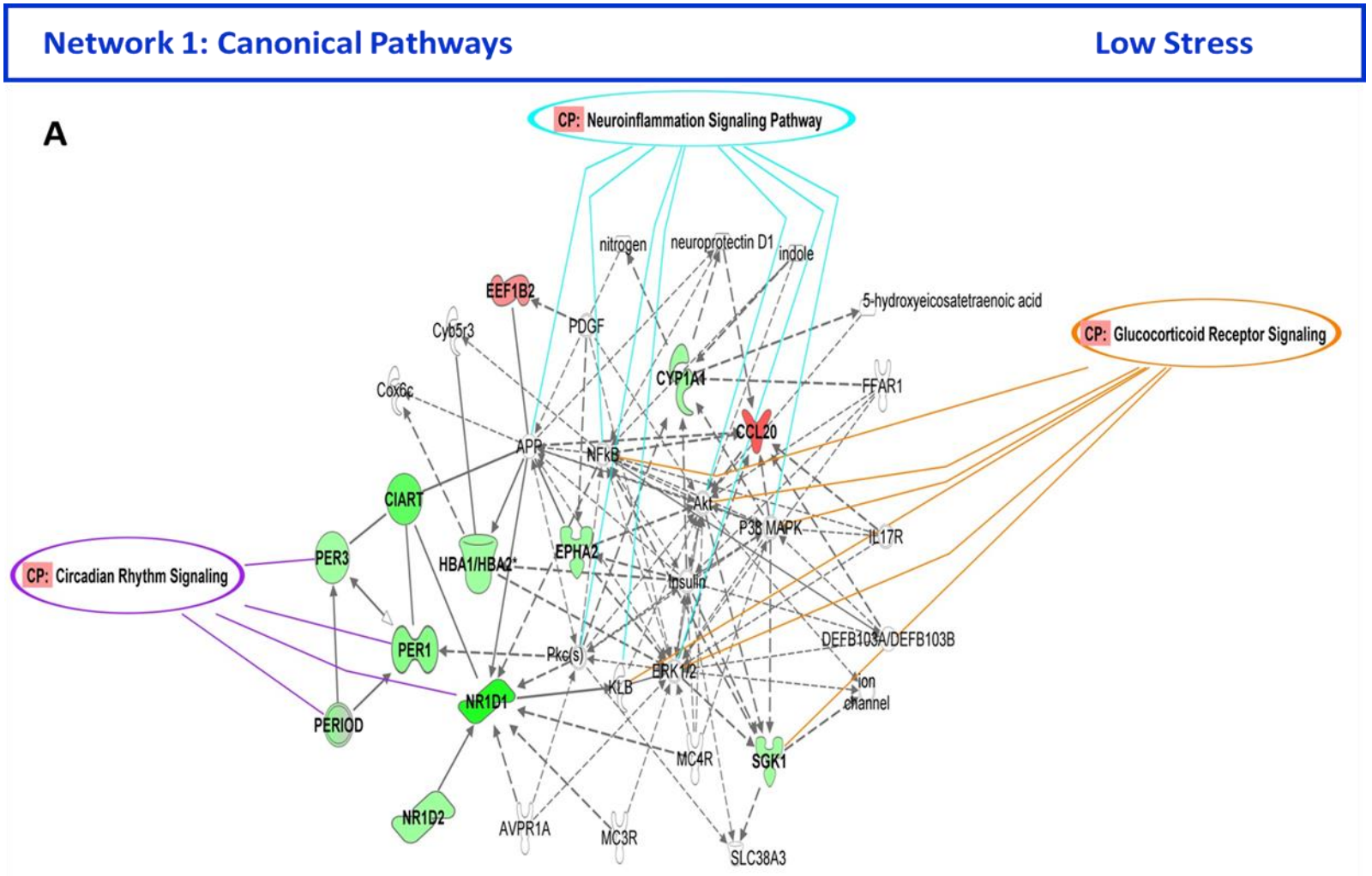
5.6.2. Supplementary table1: genes used and catalog number

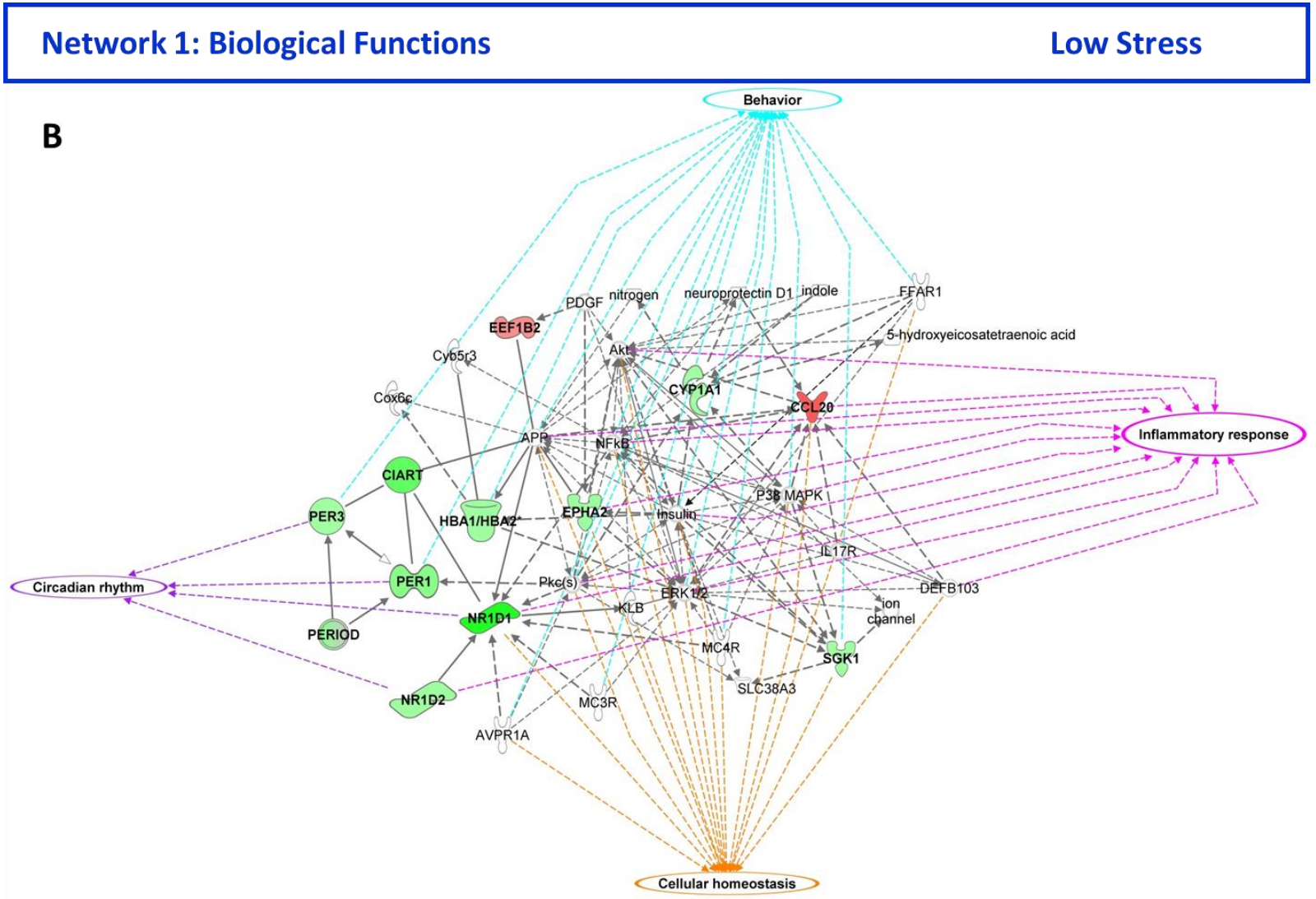
Gene	Symbol	Classification	Catalog number	Notes
Period 1	**PER1**	Clock gene	Hs01092603_m1	
Period 3	**PER3**	Clock gene	Hs00213466_m1	
Nuclear Receptor Subfamily 1 Group D Member 1	**NR1D1**	Clock gene	Hs00253876_m1	
Nuclear Receptor Subfamily 1 Group D Member 2	**NR1D2**	Clock gene	Hs00233309_m1	
Nuclear Factor, Interleukin 3 Regulated	**NFIL3**	Clock gene, Immunity-related gene	Hs00356605_g1	
Nuclear Factor, Erythroid 2 Like 2	**NFE2L2**	Clock gene, Immunity-related gene	Hs00975961_g1	
Mal, T-Cell Differentiation Protein	**MAL**	Immunity-related gene	Hs00360838_m1	
Interleukin 18	**IL18**	Immunity-related gene	Hs00155517_m1	
Superoxide dismutase 1	**SOD1**	Immunity-related gene	Hs00533490_m1	
Claudin 1	**CLDN1**	Barrier function gene	Hs00221623_m1	
Claudin 2	**CLDN2**	Barrier function gene	Hs00252666_s1	
Occludin	**OCLN**	Barrier function gene	Hs00170162_m1	
Zona Occludens 1	**TJP1**	Barrier function gene	Hs00268480_m1	
Zona Occludens 3	**TJP3**	Barrier function gene	Hs00274276_m1	
Tryptase alpha/beta 1	**TPSAB1**	Barrier function gene	Hs02576518_Gh	
Serpin A1	**SERPINA1**	Barrier function gene	Hs00165475_m1	
Solute Carrier Family 26 Member 3	**SLC26A3**	Barrier function gene	Hs00995363_m1	
Cornifelin	**CNFN**	Barrier function gene	Hs 00261196_m1	Late Cts
Glucocorticoids receptor	**NR3C1**	Stress related gene	Hs00353740_m1	
Dicer 1	**DICER1**	miRNA related gene	Hs00229023_m1	

5.6.3. Supplementary figures

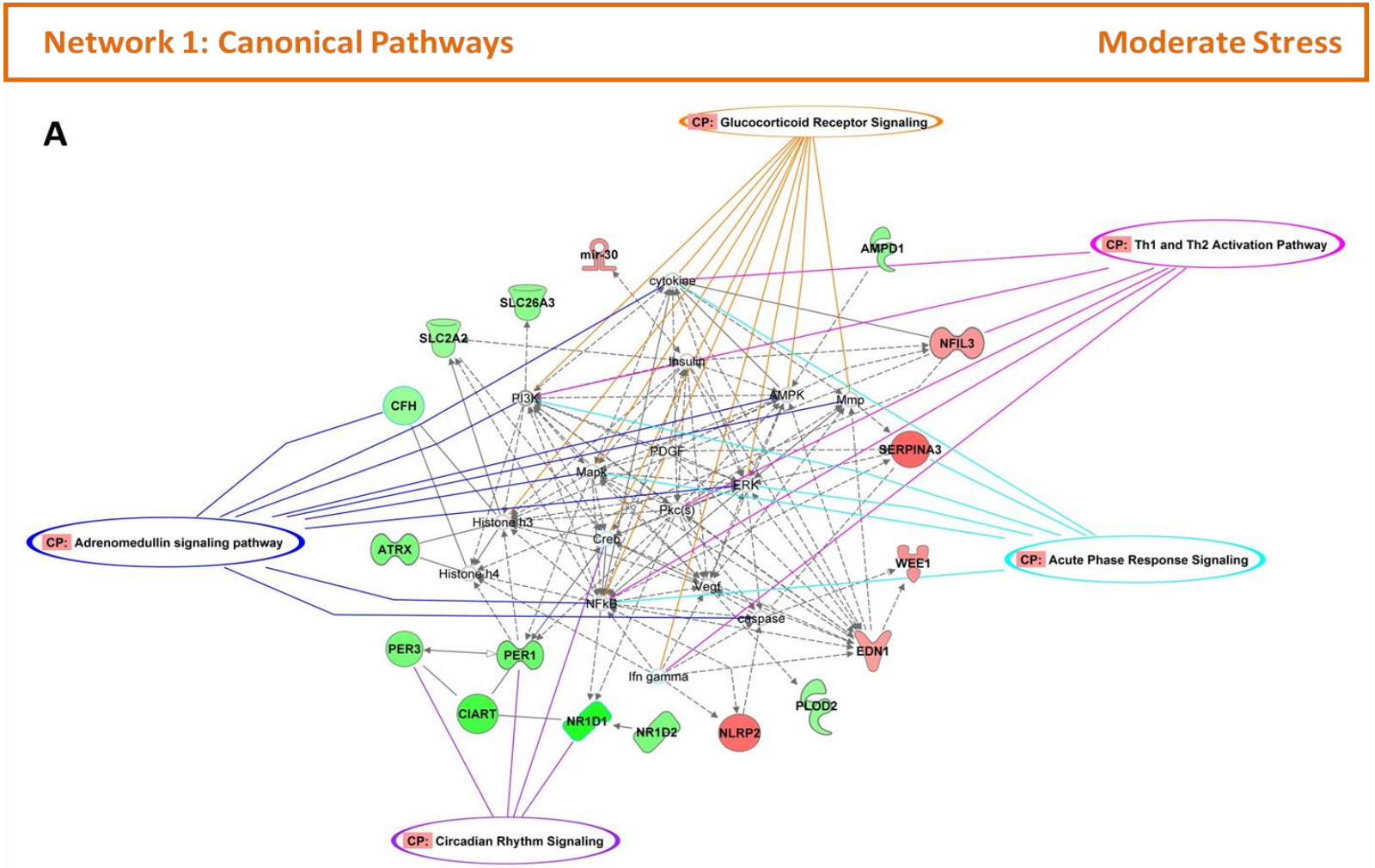
The list of differentially expressed genes in participants with low stress before and after CPS was uploaded into the IPA application. Edge (gene relationship) and node (gene) symbols are described. The intensity of the node color indicates the degree of up- (red) or down- (green) regulation. Uncolored nodes represent genes that were not identified as differentially expressed in our study and were integrated into the computationally generated networks based on the evidence stored in the IPA knowledge memory indicating relevance for this network. The network score is based on the hypergeometric distribution and is calculated with the right-tailed Fisher's exact test. The score is the negative Log of this P value (P -score = –log10 (P value)).

5.6.3.1. Supplementary figure 1: Canonical pathways and biological functions in low stress subjects

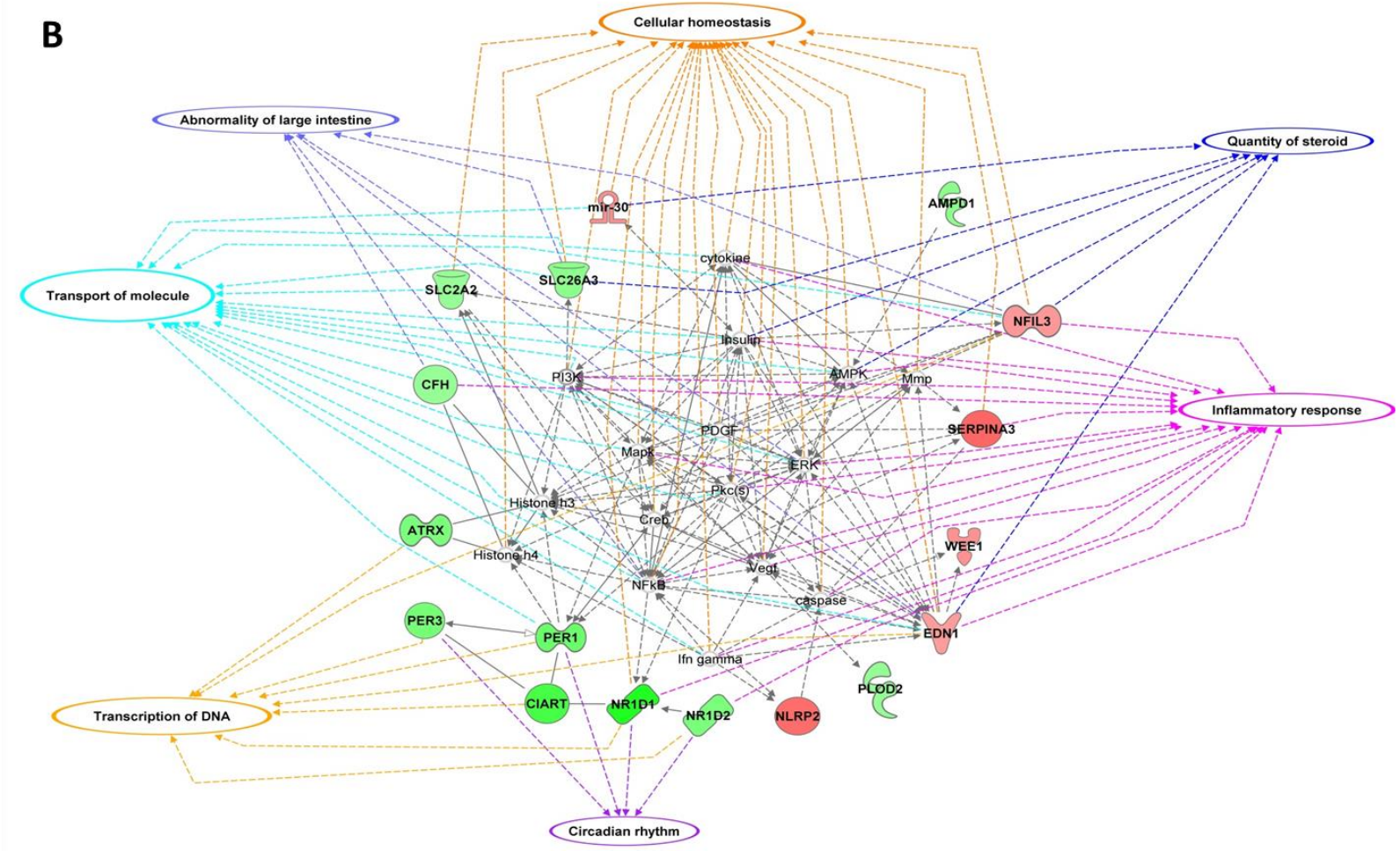



Network 1: Biological Functions
Low Stress
B
Behavior
PDGF
nitrogen
neuroprotectin D1
indole
FFAR1
EEF1B2
5-hydroxyeicosatetraenoic acid
Akt
Cyb5r3
CYP1A1
Cox6c
CCL20
APP
NFkB
Inflammatory response
CIART
P38 MAPK
PER3
HBA1/HBA2
EPHA2
insulin
IL17R
Circadian rhythm
PER1
Pkc(s)
DEFB103
ERK1/2
KLB
ion channel
NR1D1
PERIOD
MC4R
SGK1
NR1D2
SLC38A3
MC3R
AVPR1A
Cellular homeostasis

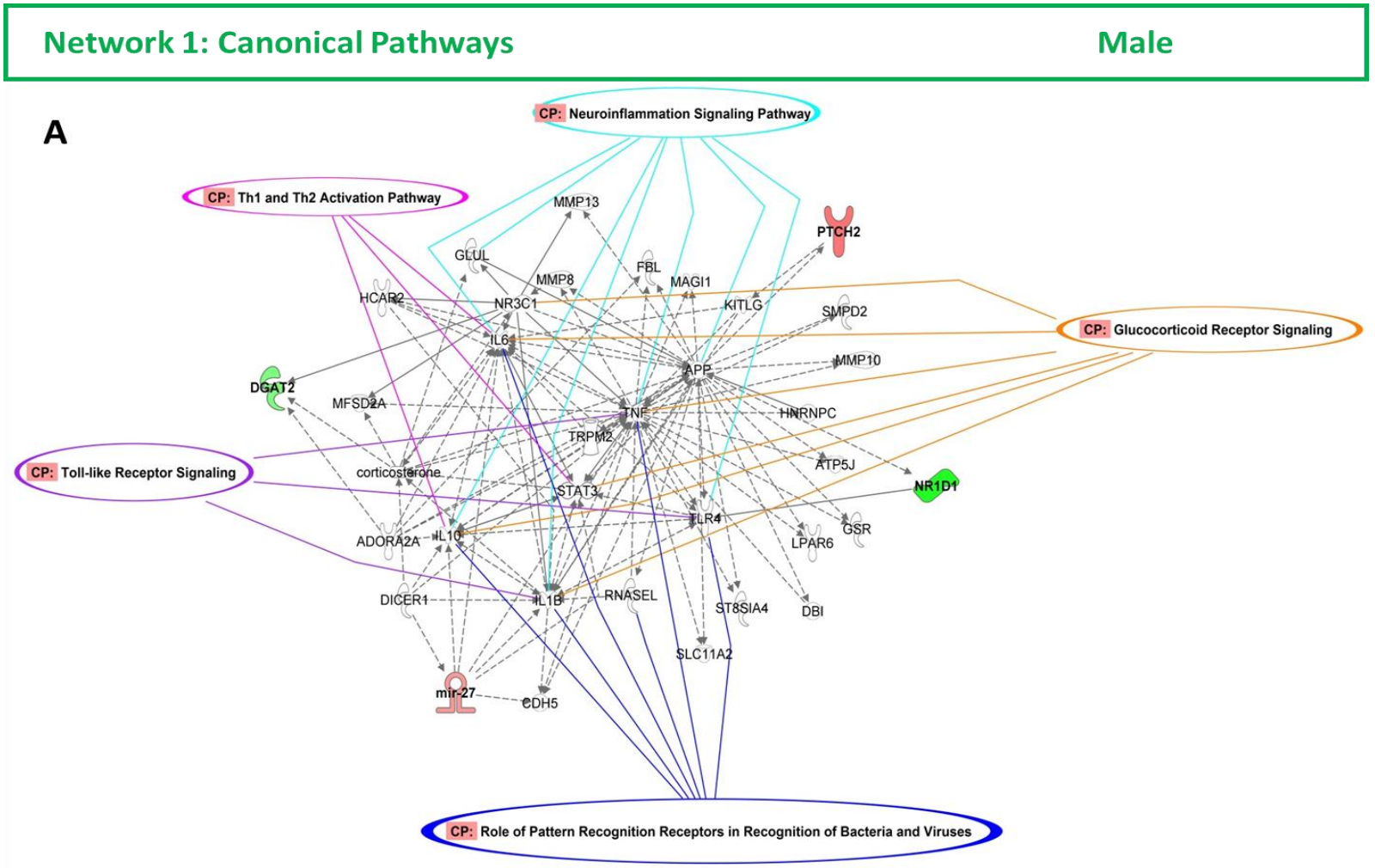
5.6.3.2. Supplementary figure 2: Canonical pathways and biological functions in moderate stress subjects

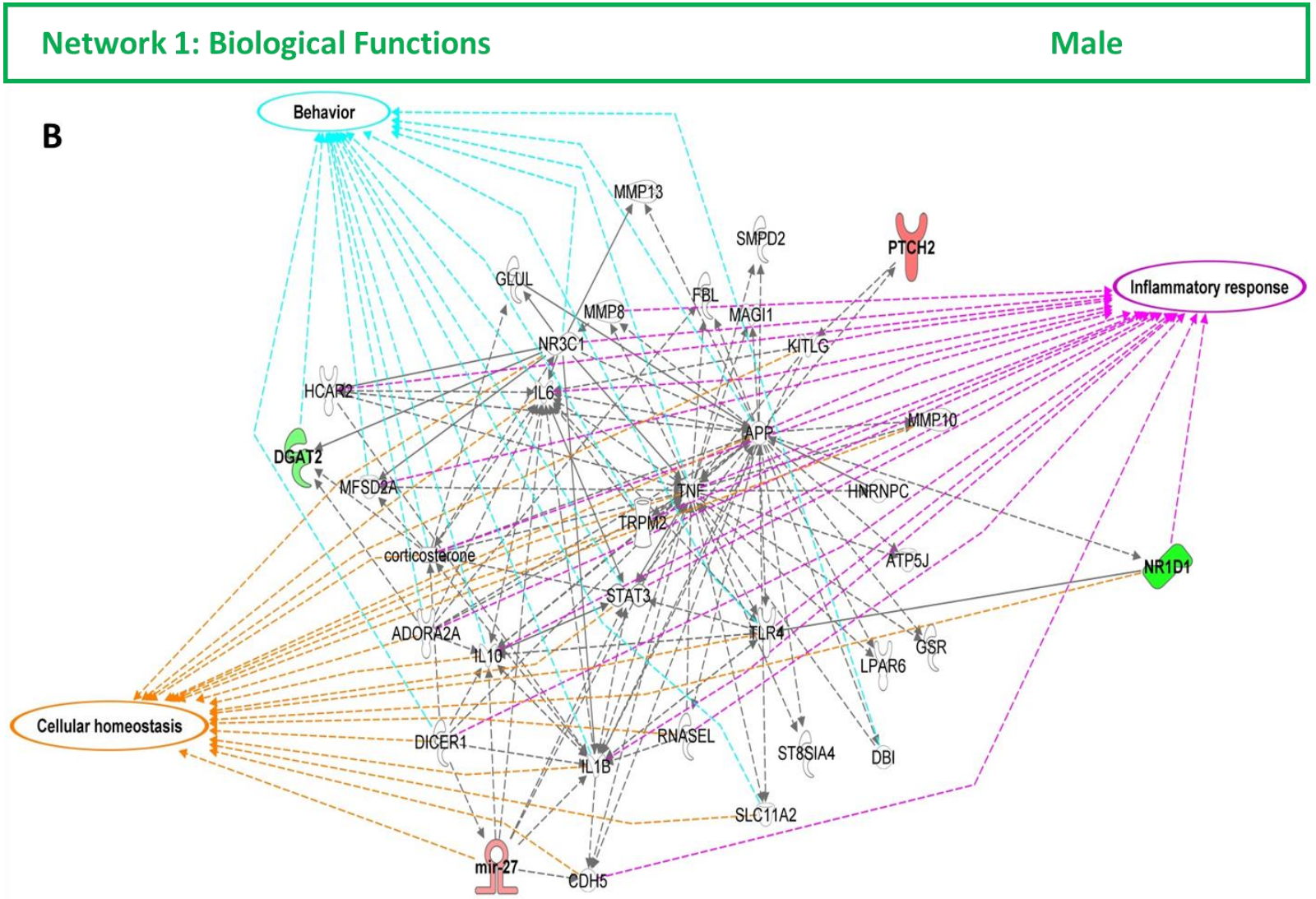


Network 1: Biological Functions **Moderate Stress**

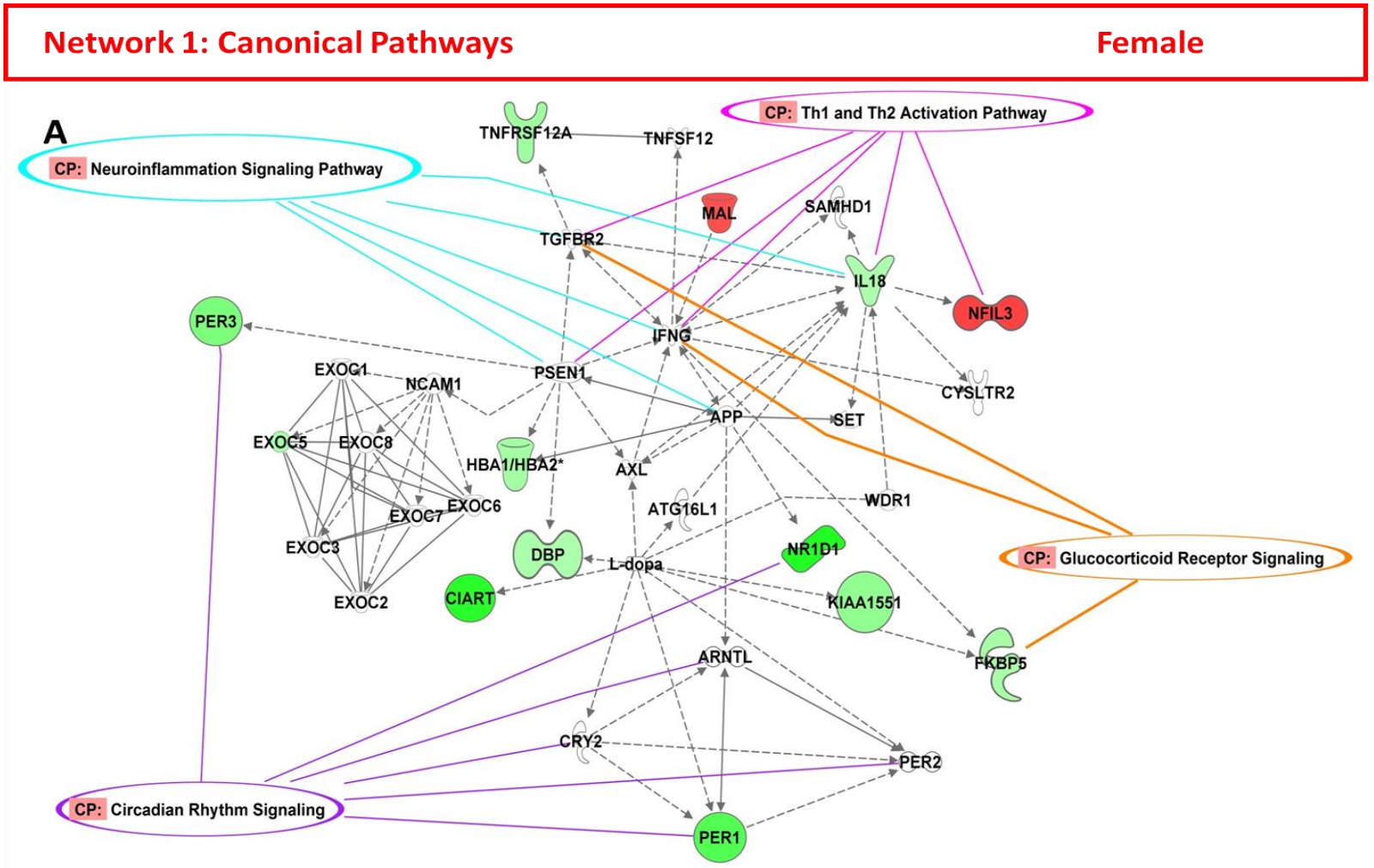


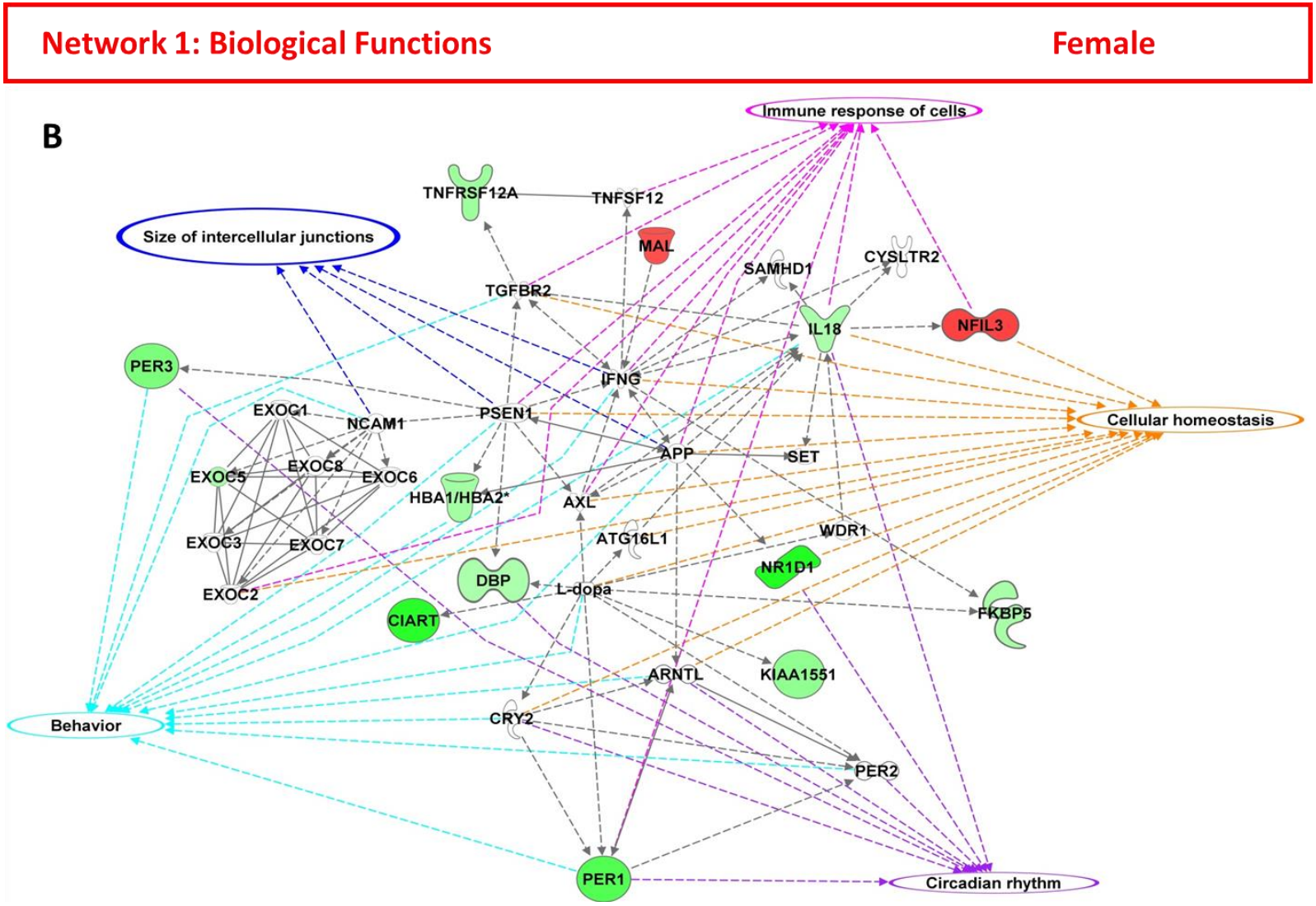
5.6.3.3. Supplementary figure 3: Canonical pathways and biological functions in males




Network 1: Biological Functions
Male
B
Behavior
MMP13
SMPD2
PTCH2
GLUL
FBL
Inflammatory response
MMP8
MAGI1
NR3C1
KITLG
HCAR2
IL6
APP
MMP10
DGAT2
MFSD2A
TNF
HNRNPC
TRPM2
corticosterone
ATP5J
NR1D1
STAT3
ADORA2A
TLR4
GSR
IL10
LPAR6
Cellular homeostasis
DICER1
RNASEL
ST8SIA4
DBI
IL1B
SLC11A2
mir-27
CDH5

5.6.3.4. Supplementary figure 4: Canonical pathways and biological functions in females




Network 1: Biological Functions
Female
B
Immune response of cells
TNFRSF12A
TNFSF12
Size of intercellular junctions
MAL
SAMHD1
CYSLTR2
TGFBR2
IL18
NFIL3
PER3
IFNG
EXOC1
NCAM1
PSEN1
Cellular homeostasis
APP
SET
EXOC5
EXOC8
EXOC6
HBA1/HBA2*
AXL
WDR1
ATG16L1
EXOC3
EXOC7
NR1D1
DBP
L-dopa
EXOC2
FKBP5
CIART
ARNTL
KIAA1551
Behavior
CRY2
PER2
PER1
Circadian rhythm

CHAPTER 3

6. Cold pain stress increases intestinal permeability in healthy volunteers: effect of chronic life stress and sex on this response.

6.1. ABSTRACT

Background and aim: Intestinal barrier dysfunction, chronic life stress and sex are key factors in the pathophysiology of functional gastrointestinal disorders (FGID). Studies in animals and humans have proved that acute stress disrupts intestinal barrier function, suggesting its contribution to the development of FGID. However, how stress exposure, especially in women, predisposes to gastrointestinal disease and the mechanisms involved are not fully described. Thus, the aim of this study was to determine the role of stress and sex on intestinal barrier function in health. **Methods:** Healthy men and women were recruited. Intestinal permeability was determined by lactulose-mannitol test from 0-2h and from 2-5h after lactulose-mannitol ingestion in two different days, at baseline and after cold pain stress (CPS) protocol. Cardiovascular, hormonal and psychological responses to CPS were measured throughout the study. **Results:** CPS induced a robust cardiovascular, hormonal and psychological response. Moreover, CPS increased intestinal permeability in both the small and the large intestine when compared to baseline. Sex determined differential blood pressure and hand pain perception. Female sex and higher levels of chronic life stress were associated with higher intestinal permeability in the small intestine in response to acute stress. **Conclusions:** CPS induces an increase in intestinal permeability in health. Increased level of psychological stress alters this response and may facilitate the passage of harmful antigens from the intestinal lumen to the internal milieu, promoting local responses that can also enhance barrier dysfunction. The lactulose-mannitol test may help to identify subjects at risk of developing FGID and to monitor intestinal barrier function.

6.2. BACKGROUND

Psychiatric comorbidity and psychosocial stress are commonly found in patients with functional gastrointestinal disorders (FGID), especially irritable bowel syndrome (IBS) and functional dyspepsia (FD). Moreover, both factors can influence the onset and the severity of these disorders (Bennett et al., 1998; Faresjö et al., 2007b; Nicholl et al., 2008). In the last few years there has been increasing interest in studying the interaction between gastrointestinal function and psychological states. The brain-gut axis (BGA) is a bidirectional communication system that comprises neural, hormonal and immunological signaling between the gut and the brain. This communication system enables stress to modulate intestinal functions such as motility and secretion and it has been considered a crucial player in the pathogenesis of FGID. Moreover, recent studies have demonstrated that this brain-gut interaction is not only exclusive of FGID as it exacerbates inflammation or intestinal symptoms in patients suffering from inflammatory bowel disease (IBD) (Bernstein, 2017; Brzozowski et al., 2016). However, specific pathways by which psychological factors modulate gut barrier function remains unclear.

The intestinal epithelial barrier plays crucial role in maintaining host homeostasis. Disruption or malfunctioning of this barrier can lead to passage of bacteria or luminal antigens into de lamina propria and promote inflammation. Intestinal barrier defects have been described in many gastrointestinal diseases such as celiac disease, IBD and IBS (Martínez et al., 2013; Martínez, Vicario, et al., 2012) and, notably, altered barrier has been associated with symptom generation and severity in these patients. Although several experimental animal models (water avoidance stress, restraint stress and crowding stress, among others) (Keita et al., 2010; Kiliaan et al., 1998; Paul R Saunders, Santos, et al., 2002; Söderholm, Yang, et al., 2002; María Vicario et al., 2012, 2010) have identified intestinal permeability as the link between psychological stress and the activation of mucosal immune responses, the mechanisms by which psychological stressors affect intestinal permeability in humans still remains unclear. Several studies have, however, focused on the effect of a stressor on intestinal physiology. Our group previously demonstrated that acute physical stress (cold pain stress, CPS) in healthy women, increased albumin release to the intestinal lumen, particularly in those subjects with moderate levels of chronic life stress (Carmen Alonso et al., 2008). Moreover, it has also been shown that females had a different response to stress when compared to males, as they had a higher macromolecular permeability than males (C. Alonso et al., 2012). A more recent study has demonstrated that acute psychological stress or peripheral administration of corticotrophin-releasing hormone increases small intestinal permeability

in humans, and that this effect can be blocked by administration of a mast cell stabilizer, indicating the involvement of local mast cells in the response to stress (Vanuytsel et al., 2014).

Thus, the aim of this study was to validate the CPS protocol as a model to assess changes in stress-induced intestinal permeability by means of the lactulose-mannitol test and to investigate whether chronic life stress and sex determine the epithelial response to stress.

6.3. MATERIAL AND METHODS

6.3.1. Participants

Female and male healthy volunteers were prospectively recruited by public advertising. A physical examination and a full medical history were performed to account for past history of any inflammatory, gastrointestinal or allergic diseases. Questionnaires to exclude gastrointestinal diseases were given to each participant. Food and respiratory allergy were ruled out using a battery of skin prick tests (Leti SA, Barcelona, Spain): 12 inhalants and 22 common foodstuffs with histamine and saline as positive and negative controls, respectively. In order to be included in this study, subjects hat to be18-60 years old; were able to understand and sign the informed consent; had no gastrointestinal disease and had negative alimentary skin prick tests. Subjects were excluded if they had any abdominal surgery (except appendectomy); or any chronic organic or mental illness. Subjects were not allowed to take salicylates, non-steroid anti-inflammatory drugs 15 days prior to the test. Alcohol and smoking was prohibited 48 hours before the test. Moreover, they were not allowed to consume lactose containing products on the day prior to the test. Written informed consent was obtained from each participant. The study protocol was in accordance with the Declaration of Helsinki and was approved by the ethics committee of the Hospital Vall d'Hebron (PR(AG)135/2008).

6.3.2. Study design

Study participants were screened by medical interview, answered gastrointestinal symptom, anxiety and stress questionnaires. Prick tests were then performed, and they received verbal and written information about the study.

Experimental design (figure 1): briefly, on the day of the study, subjects collected saliva 30 minutes after waking up and brought it in a 4°C container. Once they arrived to our facility,

they emptied their bladder and ingested an oral preparation of lactulose-mannitol. An intravenous access was placed and blood collected; baseline autonomic variables and stress questionnaires were fulfilled. Then, the CPS protocol was initiated. At each time point, autonomic variables, blood and/or subjective stress and hand pain questionnaires were collected. Urinary samples were collected at 2 hours and at 5 hours after the lactulose-mannitol ingestion.

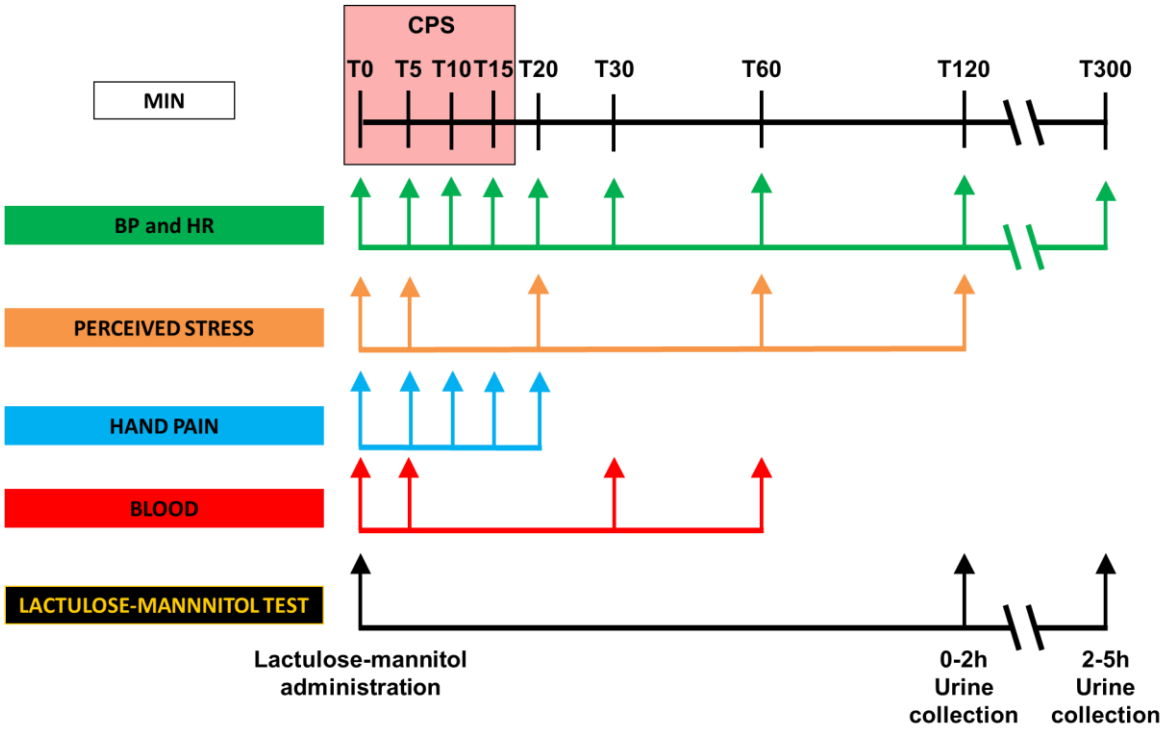


Figure 1: Experimental design. BP: Blood pressure; CPS: Cold pain stress; HR: Heart rate.

6.3.3. Psychosocial stress and depression scores

Psychosocial stress was measured using the Modified Social Readjustment Scale of Holmes-Rahe (Holmes & Rahe, 1967), which evaluates significant stressful life events in the last year of life and allows stratification of participants as suffering from low chronic stress (LS) (<150), moderate chronic stress (MS) (150-299) and high stress (≥300).

6.3.4. Intestinal permeability

Measurement of intestinal permeability was performed using the lactulose mannitol test. (Vanuytsel et al., 2014). This test consists of administrating 5g of lactulose (Duphalac®, Abbott Laboratories S.A., Madrid, Spain) and 2g of mannitol (Fagron, Spain) dissolved in 200 mL of water. Under normal conditions, lactulose and mannitol are poorly absorbed,

they are not metabolized and they are eliminated in urine after absorption. Subjects ingested the solution within 5 minutes after voiding urinary bladder. Urine was collected during the first two hours (0-2h) and thereafter, for the following three hours (2-5h) in containers kept at 4°C to prevent bacterial overgrowth. Immediately after the period finished, samples were aliquoted, filtered through a 70 μm strainer (Merck Millipore, Spain) and immediately kept at -20°C until analyzed. The urine was collected in these periods to separate sugar absorption from the two anatomical regions, as 0-2h time reflects the permeability of the small intestine, while the urine collected 2-5h time shows mostly colonic but also small intestinal permeability (Bjarnason, MacPherson & Hollander, 1995; M. Camilleri et al., 2009; A. S. Rao et al., 2011). Samples were analyzed by High-performance liquid chromatography (HPLC) in an external laboratory (Echevarne, Barcelona) according to their standard operative procedure. Fractional excretion (FE) of each individual sugar and lactulose-mannitol ratio (LMR) were calculated.

6.3.5. Blood collection

A volume of 20mL of blood was obtained at T0, and T5, T30 and T60 minutes after the beginning of CPS in specific tubes for further collection of plasma and serum. Tubes were kept on ice during the test and afterwards centrifuged an aliquoted. All samples were stored at -20°C until analyzed.

6.3.6. Cold pain stress

The CPS test was chosen to induce an acute physical stress (Lovallo, 1975). Briefly, during a 15-minute period, participants immersed the non-dominant hand in iced water (4ºC) for 45 seconds, followed by withdrawal for 15 seconds to prevent adaptation to pain.

The stress response was assessed using the following parameters:

- Autonomic response: autonomic response was evaluated by measuring systolic and diastolic blood pressure (SBP and DBP respectively) and heart rate (HR) with an automated sphygmomanometer (Omron M4-I; Omron Healthcare Europe B.V., Hoofddorp, The Netherlands).

- Hand pain perception: the level of hand discomfort/pain was assessed using a visual analogue scale from 0 (no discomfort) to 10 (intolerable pain).

- Psychological response: the level of acute stress experienced by participants was evaluated by the Subjective Stress Rating Scale (SSRS) (B D Naliboff et al., n.d.).

- Hormonal response: hypothalamic-pituitary-axis activation was assessed by plasma adrenocorticotropic hormone (ACTH) and cortisol levels by Vall d'Hebron's Biochemistry department by chemiluminescent immunometric assays (LIAISON® XL; cortisol sensitivity, 0.02g/mL, intra- and interassay CV, 7.0 and 7.7%; ACTH sensitivity, 5pg/mL; intra- and interassay CV, 6.7% and 8.2%). Baseline normal values were considered, according to the local laboratory values, as follows: ACTH 46pg/mL; cortisol 25µg/dL.

6.3.7. Statistical analysis

Data are expressed as mean (confidence interval) unless otherwise stated. Comparisons were made with parametric (Student's *t*-test) or non-parametric tests (Mann-Whitney *U* test) when appropriate. Comparison between autonomic, psychological and hormonal variables was made using a repeated measures analysis of the variance (ANOVA). To perform subgroup analysis, two-way ANOVA was used, where sex and stress were considered as the between-subjects factor and changes before and after CPS were the within-subject factors. Statistical significance's level was set at 0.05. Data are presented as median (Q1-Q3) unless otherwise stated. The statistical analysis of all data was performed using commercial software SPSS 22.0 for Windows (IBM SPSS Inc., Chicago, IL, USA).

6.4. RESULTS

6.4.1. Demographical and clinical data

A total of 30 participants (11 male; 19 females) were included. Their demographic and clinical characteristics are shown in table 1.

	Median (Q1-Q3)
Age, years	23 (22-25)
BMI	21 (20-22)
Bowel movements	1.0 (1.0-1.5)
Stool consistency	3.5 (3.0-4.0)
Holmes-Rahe, score	118.5 (60.0-161.8)
Systolic blood pressure T0, mmHg	119 (109-124)
Diastolic blood pressure T0, mmHg	72 (64-79)
Heart rate T0, bpm	63 (56-70)
Hand pain T0, VAS score	0.0 (0.0-0.2)
SSRS stress T0, VAS score	2.7 (2.0-3.8)

Table 1: Characteristics of the population. BMI: Body Mass Index; BPM: Beats per minute; LMR: Lactulose-mannitol ratio; SSRS: Subjective stress rating scale; VAS: Visual analog scale.

6.4.2. Baseline permeability

In order to evaluate the effect of CPS on intestinal permeability, a baseline permeability test was performed in all participants. Results of baseline permeability are summarized in table 2.

	Median (Q1-Q3)
Urine volume 0-2 h, L	0.08 (0.05-0.14)
Mannitol 0-2 h, mg/mL	2,474 (1,627-3,685)
Lactulose 0-2 h, mg/mL	28.8 (20.0-47.8)
LMR 0-2 h	0.030 (0.027-0.039)
Urine volume 2-5 h, L	0.60 (0.43-0.79)
Mannitol 2-5 h, mg/mL	380 (293-588)
Lactulose 2-5 h, mg/mL	14.0 (8.3-18.6)
LMR 2-5 h	0.074 (0.048-0.134)

Table 2: Intestinal permeability at baseline. Measurements performed at different time points are shown. LMR: Lactulose-mannitol ratio.

6.4.3. Effect of cold pain stress

6.4.3.1. Autonomic response to CPS

Acute stress increased systolic (F: 3.10; *P*=0.0038) and diastolic (F: 7.37; *P*<0.001) blood pressure as well as heart rate (F: 3.68; *P*<0.001), recovering baseline values 15 minutes after the CPS (T30). Multiple comparisons analysis, taking T0 as baseline time, showed that, T5 was the only different time point in systolic blood pressure (SBP) (T0=119 *vs*. T5=130; *P*<0.05) while T5 and T10 were different in diastolic blood pressure (DBP) (T0=72 *vs.* T5=86 and T10=81; *P*<0.05) and T20 and T30 in heart rate (T0=63 *vs*. T20=54 and T30=55; *P*<0.05) (figure 2).

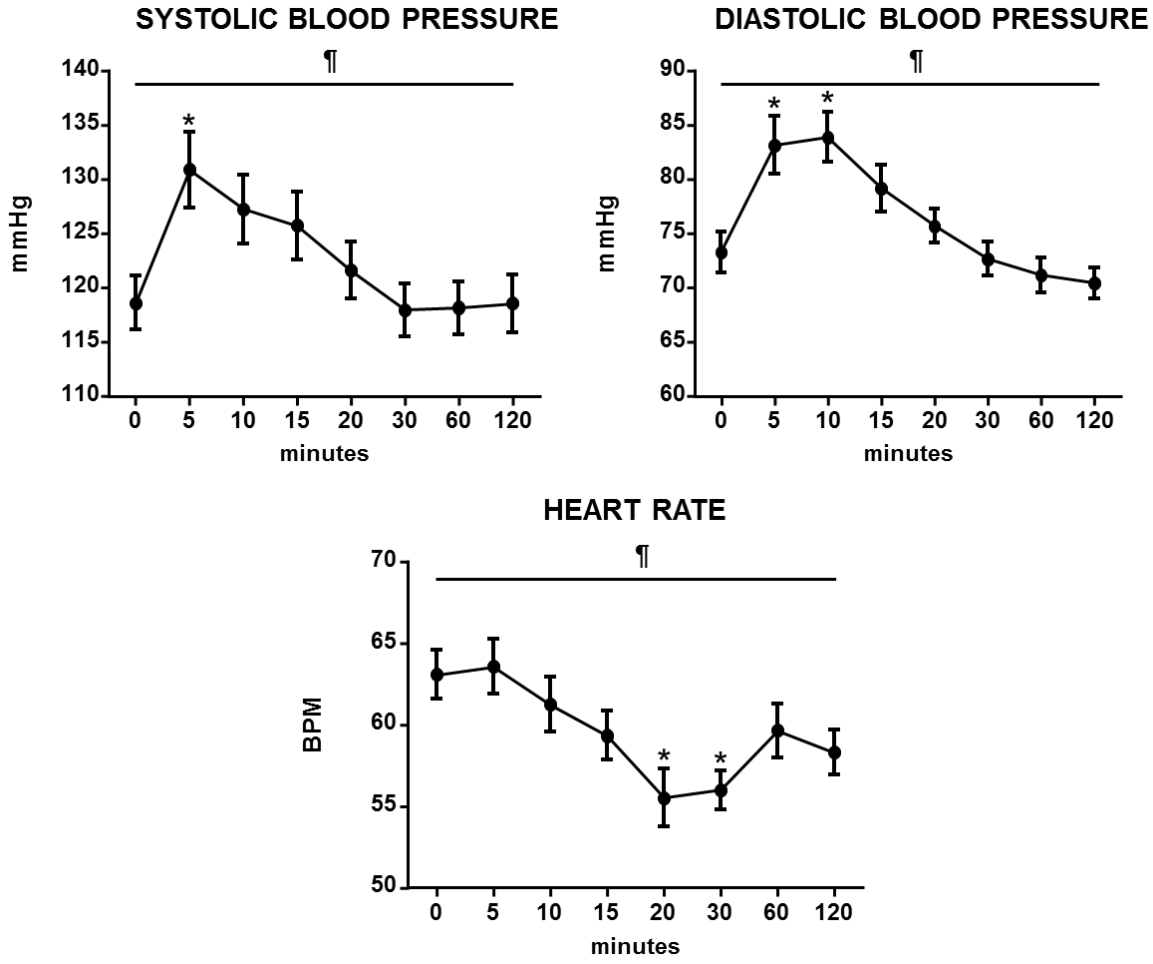


Figure 2: Autonomic response to CPS. Each dot represents the mean and the error bars represent the SEM. BPM (beats per minute). * *P*<0.05 compared to T0. ¶ ANOVA *P* value <0.05. (n=30).

6.4.3.2. Effect of CPS on psychological and pain response

CPS enhanced hand pain perception (H: 82.17; *P*<0.0001) and perceived stress levels (H: 42.48; *P*<0.0001). Multiple comparisons analysis, taking T0 as baseline time, showed that, T60 and T120 were the only different time points in SSRS (T0=2.67 *vs.* T60=1.42 and T120=1.34; *P*<0.05) while all time-points were different than T0 in hand pain measurement (T0=0.09 *vs.* T5=7.08, T10=6.62, T15=5.80, T20=3.73; *P*<0.001) as observed in figure 3.

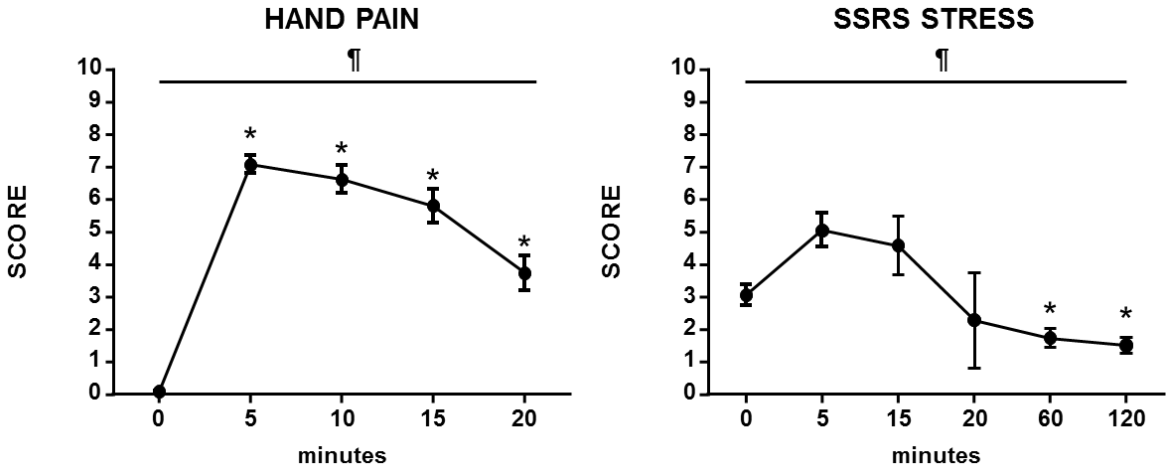


Figure 3: Hand pain and psychological response to CPS. Each dot represents the mean and the error bars represent the SEM. SSRS: Subjective stress rating scale. **P*<0.05 vs T0. ¶ ANOVA *P* value <0.05. (n=30).

6.4.3.3. Effect of CPS on hormonal response

CPS induced a hormonal response as observed by an increase in ACTH (F: 4.26; *P*=0.0462) and also in cortisol (F: 7.05; *P*=0.0126). Multiple comparisons analysis, taking T0 as baseline time, showed that T30 was the only different time point in cortisol (T0=15.79 *vs*. T30=22.58; *P*<0.001), while no significant differences were detected in ACTH as illustrated in figure 4.

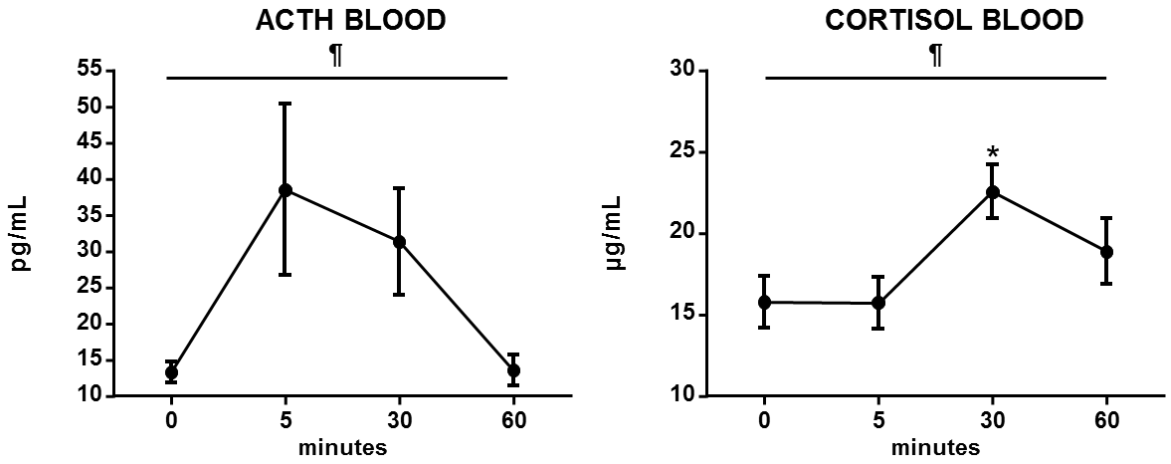


Figure 4: Blood ACTH and cortisol concentration during the experimental period. Each dot represents the mean and the error bars correspond to the SEM. ACTH: Adrenocorticotrophic hormone. **P*<0.05 vs T0. ¶ ANOVA *P* value <0.05. (n=10).

6.4.3.4. Effect of CPS on intestinal permeability

CPS did not induce any difference in individual sugar excretion, although a trend towards a reduction in mannitol absorption was observed at time 0-2h (baseline: 2,474 *vs*. stress: 1,358; *P*=0.057). CPS increased LMR in both 0-2 (baseline: 0.013 *vs*. stress: 0.020; *P*=0.007) and 2-5 h (baseline: 0.031 *vs*. stress: 0.045; *P*=0.033) periods as shown in figure 5.

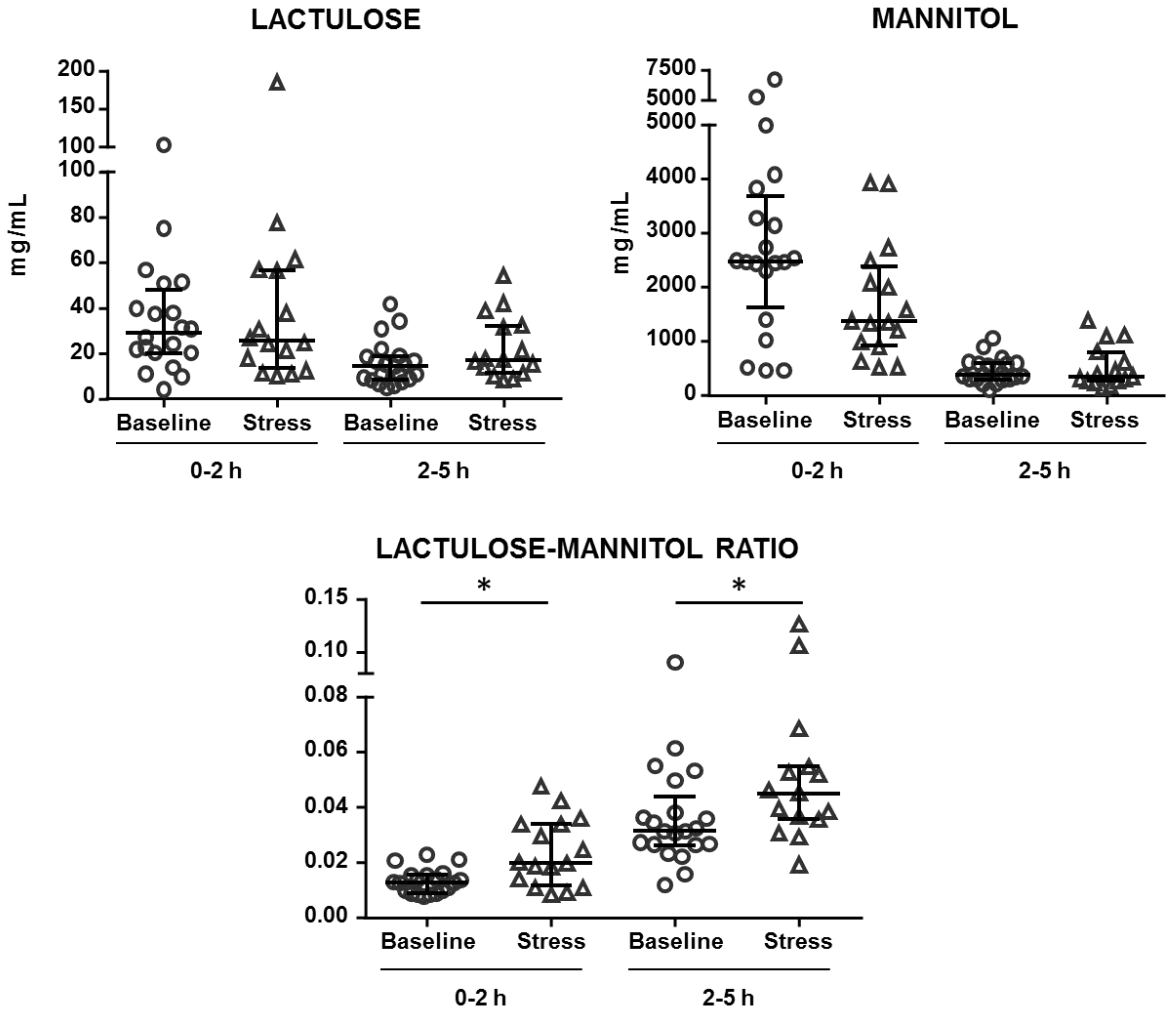


Figure 5: Intestinal permeability paired data comparison at baseline and after stress. Data are expressed as median and error bars represent interquartile range. LMR: Lactulose-mannitol ratio.* *P*<0.05 against baseline.

6.4.4. Subgroup analysis by chronic life stress and sex

Subgroup analysis by psychosocial stress did not show any differences in demographic and clinical variables, while the subgroup analysis by sex revealed differences in blood pressure and hand pain at T0. Moreover, we found a trend towards baseline differences in life stress (*P*= 0.057) between males and females. Results are summarized in table 3.

	LOW STRESS N=21	MODERATE STRESS N=9	*P*	MALE N=11	FEMALE N=19	*P*
Age, years	23 (18-58)	24 (22-47)	0.06	23 (18-25)	23 (22-58)	0.98
BMI	21 (19-28)	22 (19-26)	0.63	21 (19-28)	21 (19-26)	0.29
Bowel movements	1 (0.5-2.5)	1 (1-2.5)	0.75	1 (1-2.5)	1 (0.5-2.5)	0.052
Stool consistency	3.5 (2-5)	3 (3-4)	0.11	3.5 (3-5)	3 (2-5)	0.36
Holmes-Rahe, score	94 (25-147)	198 (157-277)	0.01*	94 (25 -160)	133 (25-277)	0.057
Systolic blood pressure T0, mmHg	119 (97-162)	119 (105-130)	0.63	123 (112 -162)	115 (97-135)	0.006*
Diastolic blood pressure T0, mmHg	71 (61-94)	73 (62 -88)	0.98	79 (66-94)	65 (61-88)	0.001*
Heart rate T0, bpm	63 (53-81)	65 (47-76)	0.87	57 (53-81)	65 (47-76)	0.11
Hand pain T0, VAS score	0.0 (0.0-0.2)	0.1 (0.0-0.5)	0.23	0.0 (0.0-0.2)	0.1 (0.0-0.5)	0.005*
SSRS stress T0, VAS score	2.5 (0.9-6.1)	2.8 (0.6-8.5)	1.00	2.5 (0.6-6.1)	2.8 (1.4-8.5)	0.55

Table 3: Subgroup analysis of demographic and clinical variables. Data are expressed as median (min-max). BMI: Body Mass Index; bpm: Beats per minute; DBP: Diastolic blood pressure; HR: Heart rate; SBP: Systolic blood pressure; SSRS: Subjective stress rating scale, VAS: Visual analogic score.*$P<0.05$.

Analysis of the intestinal permeability at baseline did not show any differences in lactulose, mannitol or LMR in the subgroup analysis by chronic life stress. On the other hand, subgroup analysis of intestinal permeability by sex showed a baseline difference in mannitol concentration between males and females at 2-5h period, as shown in table 4.

	LOW STRESS N=21	MODERATE STRESS N=9	*P*	MALE N=11	FEMALE N=19	*P*
Urine volume 0-2 h, L	0.075 (0.03-0.35)	0.103 (0.03-0.30)	0.65	0.080 (0.34-0.30)	0.075 (0.03-0.35)	0.87
Mannitol 0-2 h, mg/mL	1,706 (184-6,285)	1,846 (560-3,910)	0.48	1,708 (646-5,792)	1,821 (184-6,285)	0.76
Lactulose 0-2 h, mg/mL	46 (18-179)	54 (3-117)	0.81	56 (18-100)	40 (3-179)	0.56
LMR 0-2 h	0.029 (0.011-0.178)	0.031 (0.018-0.047)	0.81	0.029 (0.01-0.04)	0.031 (0.01-0.18)	0.34
Urine volume 2-5 h, L	0.60 (0.25-0.95)	0.68 (0.55-0.82)	0.19	0.525 (0.25-0.95)	0.60 (0.30-0.90)	0.36
Mannitol 2-5 h, mg/mL	316 (5-943)	266 (3-533)	0.36	481 (211-943)	209 (3-656)	0.004*
Lactulose 2-5 h, mg/mL	25 (6-65)	18 (9-35)	0.06	24 (15-39)	23 (6-65)	0.25
LMR 2-5 h	0.075 (0.038-4.531)	0.065 (0.031-4.045)	0.54	0.065 (0.04-0.09)	0.120 (0.03-4.53)	0.095

Table 4: Subgroup analysis of baseline permeability data. Data are expressed as median (min-max). LMR: Lactulose Mannitol Ratio.*P<0.05.

6.4.4.1. Effect of CPS on autonomic and psychological variables.

CPS induced a strong autonomic and psychological response in all subjects disregarding their chronic life stress level or sex, as illustrated in figures 6 and 7. Analysis by stress group did not show any differences between groups in any of the studied variables. Systolic and diastolic pressure and hand pain perception were different at baseline between male and female groups, subgroup analysis by sex revealed an interaction of CPS and sex in blood pressure ([SBP: F: 3.59, P=0.0012]; [DBP: F: 2.10, P=0.046]) and hand pain (F: 3.36; P=0.0125) indicating a different response in these subgroups. No differences by chronic stress or sex on heart rate or SSRS were observed.

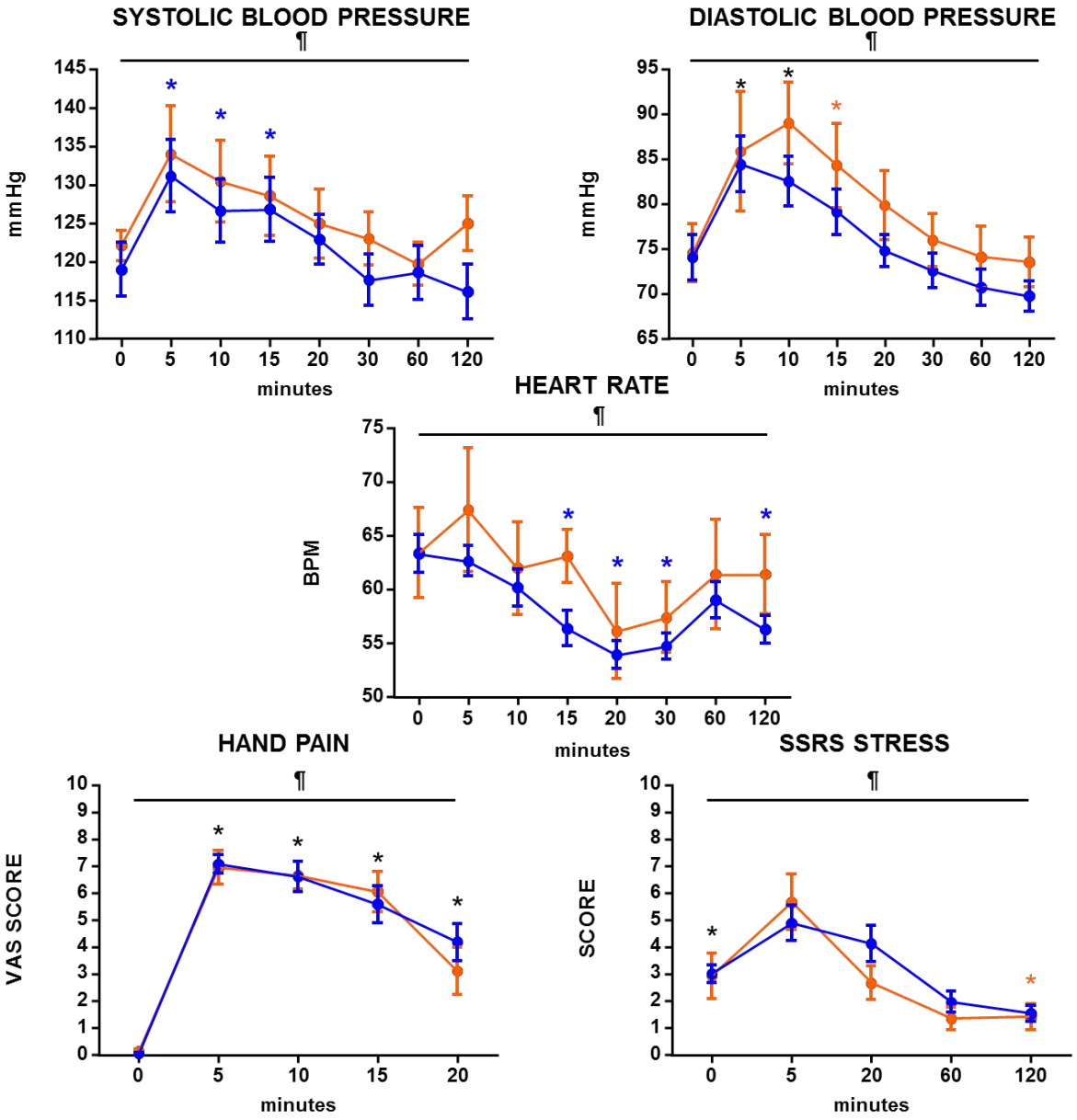


Figure 6: Systemic and psychological response to CPS stratified by stress. Each dot represents the mean and error bars represent the SEM. Blue and orange represent data from low and moderate psychosocial stress, respectively. *P<0.05 vs T0; in black time points different in all groups, in blue time points different only in low psychosocial stress group and in orange time points different only in high psychosocial stress group. ¶ ANOVA P value <0.05. (Low stress n= 21; Moderate stress n=7).

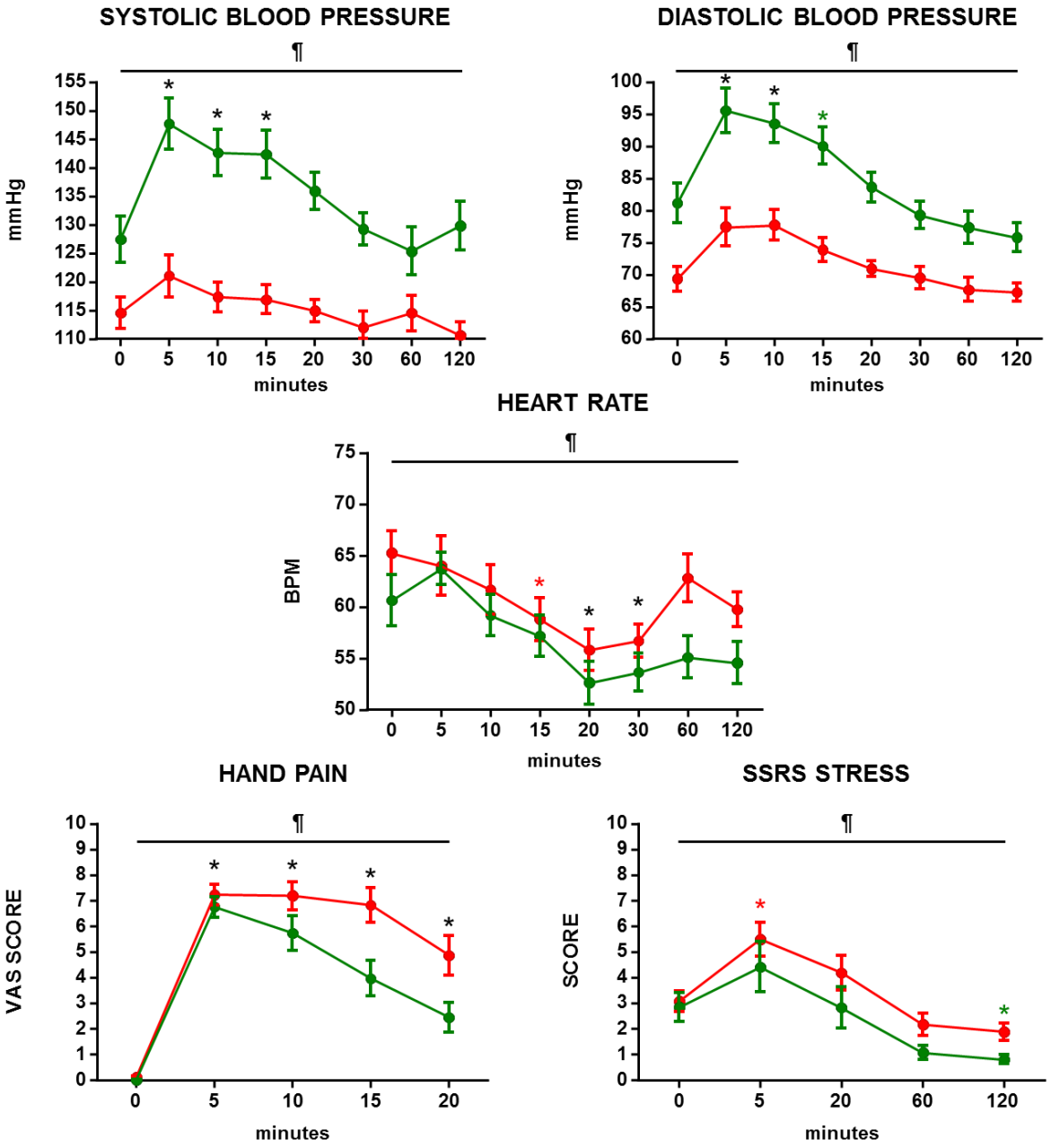


Figure 7: Systemic and psychological response to CPS stratified by sex. Each dot represents the mean and error bars represent the SEM. Green and red represent data from male and female subjects, respectively. *P<0.05 vs T0; in black time points different in all groups, in green time points different only in males and in red time points different only in females. ¶ ANOVA P value <0.05. (Male n= 11; Female n=16).

6.4.4.2. Effect of CPS on intestinal permeability.

Subgroup analysis by chronic life stress showed that subjects with moderate stress had increased lactulose excretion at 2-5h (baseline: 9.56 *vs*. stress: 26.20; *P*=0.029) as well as reduced mannitol excretion (baseline: 3273 *vs*. stress: 1375; *P*=0.030) and increased LMR ratio (baseline: 0.011 *vs*. stress: 0.025; *P*=0.022) at 0-2h period, as observed in figure 8.

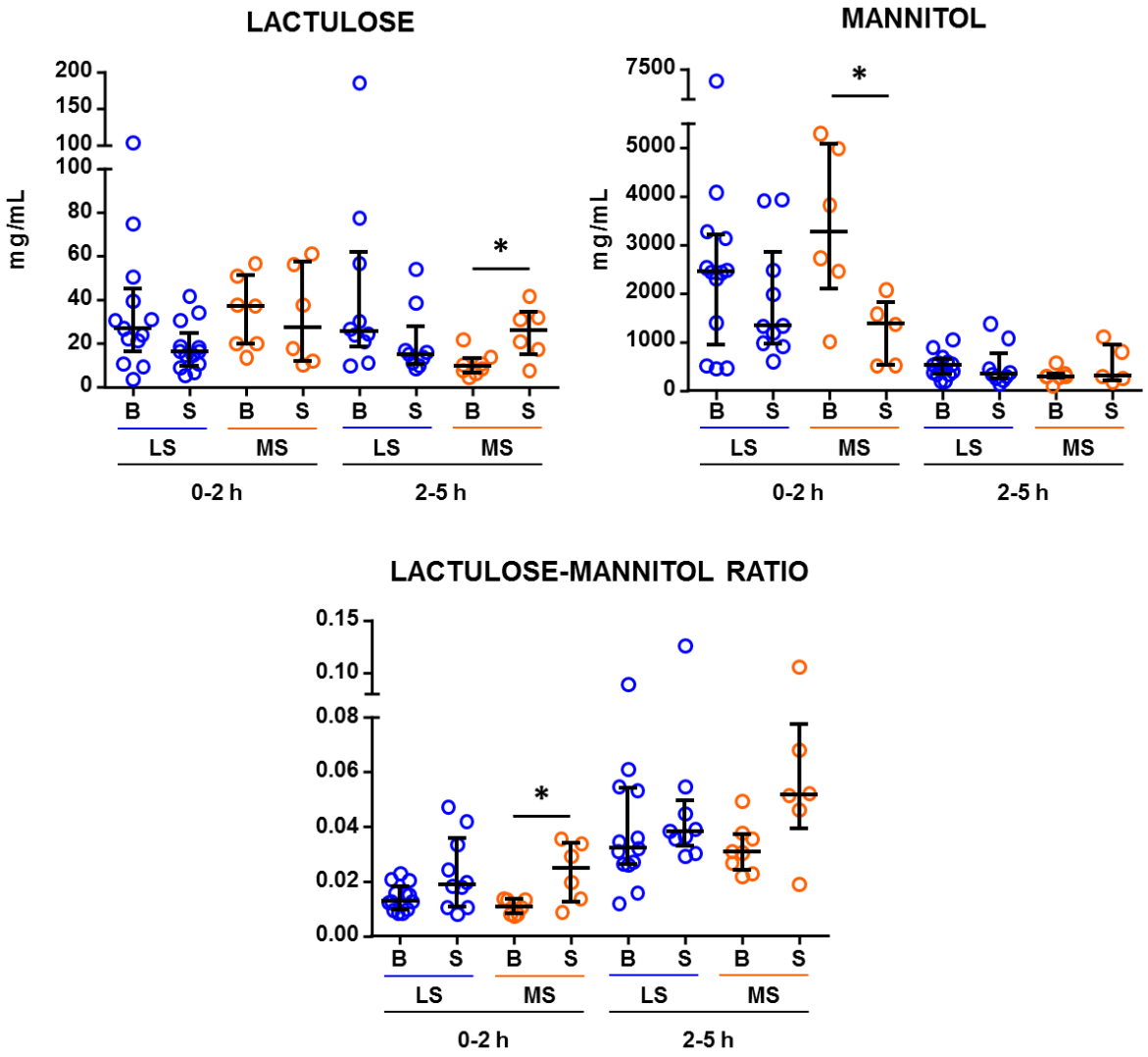


Figure 8: Subgroup analysis by stress. Data are expressed as median and error bars represent interquartile range. In blue, subjects with low psychosocial stress and in orange, subjects with moderate psychosocial stress. B; Baseline; LMR: Lactulose-mannitol ratio; LS; Low stress; MS; Moderate stress; S: Stress. * *P*<0.05 against baseline.

Analysis by sex showed that CPS did not modify lactulose or mannitol excretion. When analyzing LMR, CPS increased significantly LMR at 0-2h period only in females (baseline: 0.013 *vs*. stress: 0.020; *P*=0.046), as observed in figure 9.

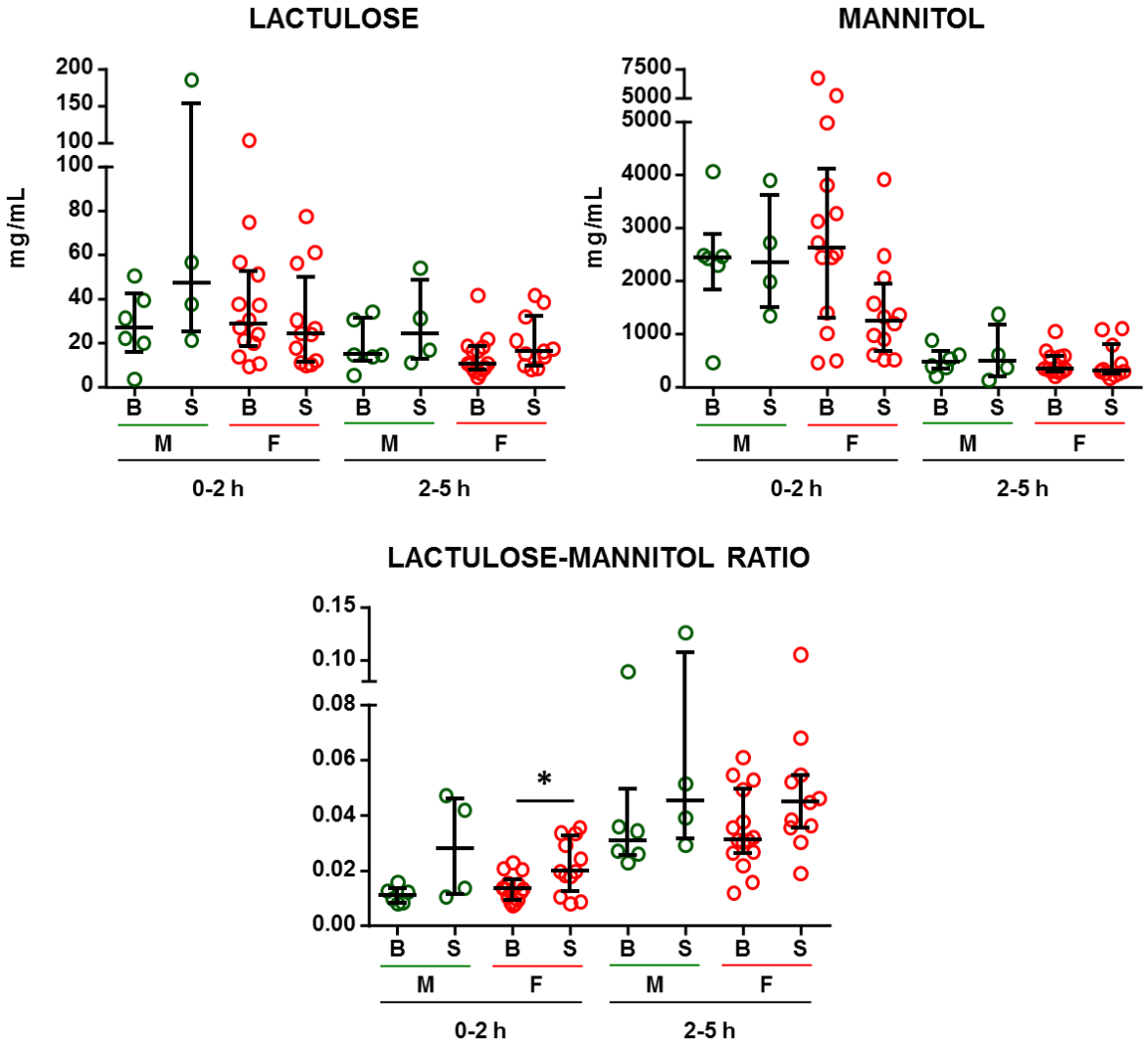


Figure 9: Subgroup analysis by sex. Data are expressed as median and error bars represent interquartile range. In blue, subjects with low psychosocial stress and in orange, subjects with moderate psychosocial stress. B; Baseline; F: Female; LMR: Lactulose-mannitol ratio; M; Male; S: Stress. * *P*<0.05 against baseline.

6.5. DISCUSSION

This is the first study demonstrating that CPS increases intestinal permeability in healthy humans, as measured by the lactulose-mannitol sugar test. Our study shows an increase in the LMR in both the small and the large bowel, with a differential response to acute stress according to sex or chronic life stress levels.

In the past few years, there has been a growing interest in deciphering the effect of stress on the human body and, especially, how it affects the gastrointestinal tract. Stress has been proposed as one of the main factors that facilitate the development of FGID and that worsen intestinal symptoms in these patients. Recent studies have shown that subjects suffering from IBS have a higher level of chronic psychosocial stress (Guilarte et al., 2007). Moreover, the intestinal epithelium of these patients have alterations at the tight junction proteins which promote a highly permeable intestine (Martínez et al., 2013; Martínez, Vicario, et al., 2012) which has been confirmed in a subgroup of IBS (Del Valle-Pinero et al., 2013; Dunlop et al., 2006; J K Marshall et al., 2004; Mujagic et al., 2014). Notably, those with impaired barrier function display significant visceral hypersensitivity (Zhou et al., 2009), indicating that intestinal barrier dysfunction plays a fundamental role in IBS pathophysiology and in symptom generation. Animal models of acute and chronic stress, have shown that stress is able to alter intestinal barrier function (Kiliaan et al., 1998; J Santos, Saunders, et al., 1999; P R Saunders et al., 1994) and those animals have more severe colitis (Collins, 2001; Qiu et al., 1999). Human studies have also demonstrated that stress has the ability to increase permeability, as measured by the lactulose-mannitol test (Vanuytsel et al., 2014), a response enhanced by nonsteroidal anti-inflammatory drugs intake. In our study, we only evaluated the effect of CPS, as a continuation of our previous studies which demonstrated, by indirect measurements, that CPS disrupts barrier function by increasing net water flux, chloride secretion and albumin output (Carmen Alonso et al., 2008). As shown previously in chapter 2 of this thesis, CPS was also able to induce molecular changes in the jejunal mucosa from healthy volunteers associated with immune, barrier and circadian rhythm functions. The present study demonstrates that CPS can be considered as a valid experimental model to determine the effect of stress in human gastrointestinal function, as it is capable of inducing an HPA axis response and also modifies intestinal function not only at a molecular level (chapter 2) but also at a functional level, reinforcing the results observed in previous studies from our group .(C. Alonso et al., 2012; Carmen Alonso et al., 2008)

It has been suggested that measuring the fractional excretion of each individual sugar could be used as a measure of intestinal permeability (M. Camilleri et al., 2009; A. S. Rao et al., 2011). This statement comes from the basis of the two sugar permeability study where mannitol, the small sugar (6.5 Å), crosses the intestinal epithelium in the villi and the crypts through the paracellular pathway; while lactulose, the large sugar (9.5 Å), has very limited paracellular permeation in the crypts (Barboza Junior, Silva, Guerrant & Lima, 1999; A. S. Rao et al., 2011). But this fact does not take into consideration two different aspects of the conditions in which this test is mainly used; one is the proinflammatory states such as IBD or celiac disease in which it is known to have a distorted mucosal architecture and, in consequence, less absorptive area, diminishing the absorption of mannitol and, due to tight junction disruption, increasing lactulose absorption (Ivana R Sequeira, Lentle, Kruger & Hurst, 2014a). The other one is the stress, as stress itself can influence intestinal transit time and also gastric emptying. This last factor could be one possible explanation, why, although we found differences in the LMR ratio, no differences were observed in the fractional excretion of individual sugars in both small intestine and large intestine.

Subgroup analysis by sex or chronic psychosocial stress revealed a differential effect of CPS on intestinal permeability, as female subjects and those with moderate stress showed an increase in LMR. This different response has also been observed in previous studies (C. Alonso et al., 2012; Carmen Alonso et al., 2008; Rodiño-Janeiro et al., 2017). Psychological stress is often presumed to negatively affect functional or other organic gastrointestinal disorders in clinical studies. Longitudinal follow-up studies showed that stress increases the risk of relapse in IBD patients in clinical remission (Bernstein et al., 2010; A Bitton et al., 2008; Alain Bitton et al., 2003). Moreover, chronic life stress is one of the multiple factors that contribute to symptom generation and severity in IBS (Whitehead, Crowell, Robinson, Heller & Schuster, 1992) and also is a strong predictor of symptom intensity variability and therefore, of the outcome in IBS patients (Bennett et al., 1998). A recent study showed that acute psychological stress (public speech) increased intestinal permeability, but it remained normal when applying a physical stress (painful electroshocks) (Vanuytsel et al., 2014). This difference was mainly due to a different activation of the HPA axis, as there was an increase in salivary cortisol in the psychological stress groups and not in the other group. We found a differential response to CPS according to sex and stress, being the female and the moderate stress groups those in which CPS was able to increase in intestinal permeability measured by the lactulose mannitol test. Despite these results, and although previous published data points

sex and stress as modifiers of stress response, we cannot state that CPS only induces permeability alterations in females but not in males or only in subjects with moderate stress. Differences found in our study could be due to low the number of subjects recruited, the inter-individual variability of lactulose and mannitol excretion, and also could be influenced by the lack of lactulose-mannitol test standardization.

The amount of lactulose and mannitol administered to evaluate intestinal permeability usually ranges from 200mg to 10g (Ivana R. Sequeira, Lentle, Kruger & Hurst, 2014b). There is a different permeability along the intestine to lactulose and mannitol (A. S. Rao et al., 2011; I. R. Sequeira, Lentle, Kruger & Hurst, 2012), thus, the ratio of the quantities of the two sugars varies with the period of time over which their excretion is determined. Hence, the LMR tend to be lower in the first two hours due to increased mannitol absorption, while the increase in lactulose absorption with the decrease of mannitol absorption at 4h, make the LMR increase at that time point (Ivana R Sequeira et al., 2014a). This trend to a higher absorption of lactulose is maintained along in the colon, were lactulose is absorbed in greater quantities than mannitol (A. S. Rao et al., 2011). A recent study demonstrated that the increase in intestinal permeability is enhanced after indomethacin administration at 4-6h collection time (Vanuytsel et al., 2014). Although we performed the study with the available information at that moment, where it was proposed that 0-2h reflects the small intestine, 2-5h reflects the distal small intestine and the proximal colon, and more than 6h reflects colonic permeability (J K Marshall et al., 2004; Piche et al., 2009; Zhou et al., 2009), recent data support that other time collection could be more suitable. Thus, part of the negative results in the fractional excretion and the lack of larger differences between our experimental groups could be due to differences in timing of urine collection. Another factor to explain this fact could also be that, although we used 2g of mannitol and 5g of lactulose, we were restrictive respect to water administration to the participants, and it has been described that sugar excretion could be influenced by urinary volume (Addobbati et al., 2014; Mattioli et al., 2011). While other studies give 200-400 mL of water (M. Camilleri et al., 2009; Ivana R Sequeira et al., 2014a; Vanuytsel et al., 2014) followed by *ad libitum* water ingestion, we considered that a controlled water intake could reduce the variability of the results, as many studies have to log transform the data in order to find differences or avoid excessive dispersion (I. R. Sequeira et al., 2012). In fact, we did obtain a good standardization as there were no differences in urinary volume at baseline or after CPS, this way minimizing urinary excretion as a confounding factor. However, these observed differences between groups could have been more notable with higher water ingestion But not only water ingestion

affects this test, there are many other factors such as alcohol intake, NSAID, smoking and vigorous exercise that could affect intestinal permeability. Despite subjects were given specific instructions previously to their inclusion in the study, we were not able to control how much subjects avoided those factors.

There is also a need to standardize not only the lactulose-mannitol protocol, sugar dose, urine time collection, water ingestion, sugar quantification method (HPLC, gas chromatography, enzymatic). Moreover, the way of expressing those results, as many studies present the data differently, makes difficult to understand or compare studies between them and can lead to false negative or false positive results.

In summary, our study confirms that acute stress impairs intestinal barrier function by increasing intestinal permeability and that this response is influenced by chronic life stress and sex. This physiological response could be influenced by different factors leading to an abnormal response to stress representing the first step towards intestinal barrier dysfunction. Therefore, the study of intestinal permeability and how stress interferes with gastrointestinal function still has to remain one of the milestones in gastrointestinal research.

DISCUSSION

7. DISCUSSION

Most of the studies in biomedical sciences are focused on studying the physiopathology of diseases in order to find better treatments and overall, improve the quality of life of people. There are fewer studies focused on the role of certain stimuli in health. The human gut maintains a functional equilibrium, named homeostasis, which depends on dynamic and complex interactions between the microbiota, the intestinal epithelium and the immune system. Stress, either physical or psychological, represents a threat to homeostasis, and triggers a coordinated multisystemic response including autonomic, endocrine, and immune activation to maintain the “normal” functioning of the intestine. This coordinated response, called ‘‘allostasis’’, provides adaptation to stress and allows the maintenance of homeostasis. However, repetitive or excessive stress exposure, negatively affect this adaptive response and could lead to the development of disease, particularly those stress-sensitive such as irritable bowel syndrome (IBS).

The present thesis provides first evidence on the molecular changes in the intestinal barrier in the jejunum in response to acute stress in healthy individuals and reinforces the conception that psychosocial stress and sex are two independent factors to take into account when studying stress-related diseases such as IBS.

IBS is one of the most prevalent gastrointestinal disorders in Western societies. Although its pathophysiology remains still not fully understood, visceral sensitivity, intestinal motility alterations and low-grade intestinal inflammation, along with brain-gut axis dysfunction have been implicated. Whether psychological stress is cause or consequence of IBS still remains unsettled; however, their association has been well stablished by experimental and epidemiological studies. The publication of the biopsychosocial model has been one of the great advances in recent years in the study of stress related disorders because it helps to understand the bi-directionality of gut alterations by central disorders such as psychosocial factors and *vice versa* (D A Drossman, 1996). Functional gastrointestinal disorders (FGID), and IBS among them, are the clinical result of psychosocial factors and gastrointestinal physiology alterations through the brain-gut axis. It has recently been published that patients with more than one FGID are more prone to suffer from anxiety and depression, emphasizing the bi-directionality of the brain-gut axis (Pinto-Sanchez et al., 2015).

Chronic psychosocial stress and psychological comorbidities have been described as differential factors between IBS and healthy subjects in previous studies from our group

and others(Bradford et al., 2012; Guilarte et al., 2007; Martínez et al., 2013; S. H. Park et al., 2016). Moreover, these alterations were related with barrier dysfunction (Martínez et al., 2013). On the first part of this thesis we performed a retrospective study with one of the largest published cohorts of IBS diarrhea (IBS-D) subjects in the world. Our analysis confirmed that psychological comorbidities are more frequent in FGID patients and that, concomitantly with dyspepsia; directly contribute to the severity of IBS symptoms. Moreover, it was striking that >40% of patients were depressed, although subjects with previously diagnosed depression were excluded and up to 50% displayed dyspeptic symptoms, when previously published studies have found that the overlap is around 20% (Enck et al., 2016).

Although huge efforts have been made to find new diagnostic tools, available biomarkers are still lacking in the clinical practice. Recently, a promising diagnostic panel test, with a combination of 34 molecules, achieved a sensibility of 81% that increased up to 93% when psychological factors where added. Moreover, this test was able to differentiate between IBS-D and IBS-C with a sensibility of 92-93% (M Camilleri et al., 2014). However, more specific tests are missing.

The biopsychosocial model suggests that a non-normal response to stress triggers symptom generation and, reciprocally, anxiety and depression are increased by abdominal symptomatology (Enck et al., 2016; Fond et al., 2014). Our study found a significant positive correlation among BDI (Beck's Depression Inventory) and abdominal pain and IBS-SSS (IBS severity scoring system) while only mild correlation between stress factors and abdominal symptoms was found.

Multiple studies have investigated the relationship between mucosal immune cells and IBS with dissimilar results (Matricon et al., 2012). This disparity of results in the number immune cells in the intestinal mucosa in conjunction with the association between IBS-D severity and the expression of tryptase (Martínez, Vicario, et al., 2012) and the differential activation of mucosal immune cells in IBS-D patients (Maria Vicario et al., 2014) when compared to controls, raised the question whether the state of activation of the immune cells, rather than their number, what contributes to IBS symptoms, as already suggested by some authors (Ohman & Simrén, 2010; Maria Vicario et al., 2014). In the first study of this thesis, no differences in the number of immune cells in the jejunal epithelium between controls and patients were found. Moreover, no association between clinical symptoms and the number of immune cells in the mucosa were detected. This results support the

hypothesis that immune mechanisms related to IBS are more dependent in the immune activation, instead of the immune infiltration in the intestinal mucosa.

IBS presents a clear female predominance that together with psychosocial stress represents an independent risk factor to develop IBS after an infectious gastroenteritis (Gwee et al., 1999). However, although psychosocial stress plays an important role in IBS development, in our study, the level of psychological stress did not affect its outcome, as we did not find any differences in clinical symptoms between IBS-D patients with low or moderate-high stress. Indicating that chronic psychosocial stress could play a more indirect role and this effect could be produced by triggering the onset; by modifying biological functions which will perpetuate the symptomatology; or by modifying other psychological factors which have been demonstrated to predispose to visceral hyperalgesia.. Moreover, patients with comorbidities such as dyspepsia and depression had more severe IBS-D symptomatology. These findings are of high clinical importance, as they implicate that clinicians have to be aware of IBS-D patients' comorbid states because they will condition their clinical outcome and overall their quality of life and resource usage.

The underlying mechanisms by which stress impairs intestinal function are still unknown. *Ex-vivo* (J Santos, Saunders, et al., 1999; Paul R Saunders, Santos, et al., 2002) and *in-vivo* (Vanuytsel et al., 2014) studies have demonstrated that stress impairs intestinal barrier function by increasing intestinal permeability and modifying gastrointestinal function. Two studies using cold pain stress (CPS) as a stress model (C. Alonso et al., 2012; Carmen Alonso et al., 2008) have shown a different intestinal response to stress according to sex and also to chronic psychosocial stress indicating again that those factors affect the stress response. Moreover, the first study of this thesis has shown that, in our population, IBS-D subjects display higher stress levels and that sex determines clinical outcome of IBS-D. Thus, a study was performed in order to determine the mechanisms underlying the mucosal response to an acute painful stress (CPS) and to determine the effect of chronic stress and sex on this response.

Interestingly, CPS was able not to only induce a strong autonomic, hormonal and psychological response in healthy volunteers, but also was able to alter the mucosal transcriptome by modifying circadian rhythm, inflammatory and epithelial barrier genes, revealing the main pathways potentially implicated in stress-induced mucosal alterations. CPS is a well validated stress model (C. Alonso et al., 2012; Carmen Alonso et al., 2008; Lovallo, 1975), that is able to induce ACTH and cortisol release, as well as psychological

factors related to stress measured by the SSRS as shown in our study. Moreover, stress is a key factor in the development of FGID (Koh et al., 2014; Rona L. Levy et al., 2006), what makes our experimental design a suitable model for the study of potential mechanisms underlying intestinal dysfunction in FGID.

CPS modified the intestinal transcriptome, affecting gene expression, mainly related with neurological disorders and immune activation. Two of the differentially expressed pathways were expected to be affected by stress, as it has been extensively described that stress alters intestinal permeability and inflammatory cells. However, the most relevant finding of this study was a differential expression of clock genes and in consequence the alteration of jejunal mucosa's circadian rhythm. Circadian rhythms are those systems that allow the organism to anticipate and prepare for precise and regular environmental changes. The circadian clock drives physiological and behavioral patterns according to light-dark cycle by organizing and generating transcriptional and biochemical rhythms in cells and tissues throughout. Its disruption has been related with increased risk of developing diseases (Golombek et al., 2013). Clock genes play a key role in human homeostasis by controlling hypothalamic-pituitary-adrenal (HPA) axis activity, controlling metabolism, sleep cycle and also there is a bidirectional relationship between the immune system and circadian timing (Castanon-Cervantes et al., 2010; Golombek et al., 2013), which has recently been related with IBS symptomatology. The regulation basis of circadian rhythm is performed by clock genes which are tightly co-regulated (Takahashi et al., 2008). Although clock genes are important in body regulation, little is known about its function and its involvement in function of the gastrointestinal tract. Its importance in gastrointestinal health has been postulated as disruption of circadian rhythm by turn-shift work has been associated with higher incidence of ulcers, development of IBD , FGIDs and also alcohol-induced intestinal hyperpermeability (Drake et al., 2004; H. I. Kim et al., 2013; Nojkov et al., 2010; Saberi & Moravveji, 2010; Segawa et al., 1987; Sonnenberg, 1990; G. Swanson et al., 2011). Moreover, clock genes could also play a role in development of IBS and also on the placebo effect and brain-gut axis dysregulation in IBS patients, as it has been shown that patients with upregulation of clock genes after placebo treatment improve their IBS-D symptoms (Lobo et al., 2013). Targeting circadian rhythm, and especially melatonin, has been suggested to be useful as adjuvant to treat colonic conditions as (Esteban-Zubero et al., 2017; Wong et al., 2015). Interestingly, the association between microbiota and clock genes (Voigt et al., 2016), emphasizes the importance of the gut-brain-microbiota axis, which is one of the major pathophysiological pathways in FGID, reinforcing the hypothesis that clock gene dysregulation plays a critical

role in these disorders. Alteration of clock genes expression by stress could affect the HPA axis and intestinal motility which are two of the factors altered in IBS. This fact could be a possible mechanism by which stress promotes IBS-D development, but further studies are needed to prove this hypothesis. In fact, our study revealed differences in immune, intestinal barrier and clock genes when stratifying groups by sex or chronic psychosocial stress, backing up the concept that these two co-factors play an important role in the response to incoming stressful stimuli.

Moreover, the study of the gene expression profile showed that CPS was able to modify genes related to intestinal barrier function and especially CLDN2 and SLC26A3 which have been related to intestinal permeability regulation and gastrointestinal diseases (Priyamvada et al., 2015; Turner, 2009). In order to determine whether these observed molecular changes were also associated to biological variations, we designed a new study in which healthy subjects were subjected to CPS and intestinal permeability was measured through the two sugar probe (lactulose-mannitol). Our study showed that CPS is able to increase intestinal permeability and that this response is influenced by psychosocial stress and by sex. This finding is important as it has been shown that subjects with IBS-D have an increased intestinal permeability (Dunlop et al., 2006; Li et al., 2016; J K Marshall et al., 2004; Piche et al., 2009), and that those with higher permeability present higher visceral hypersensitivity (Zhou et al., 2009) and also there is an impairment in tight junction genes and proteins such as CLDN2 which is upregulated (Martínez et al., 2013). Although the technical limitations to assess intestinal permeability, our study suggests that stress is a key factor in intestinal barrier disruption by increasing intestinal permeability and this could be a mechanism involved in FGIDs pathophysiology.

Sex and stress are considered two independent risk factors that could predispose to the development of IBS (C. Alonso et al., 2012; Carmen Alonso et al., 2008). It has been shown that subjects with chronic psychosocial stress or those who had early life stress events are more prone to develop IBS, especially after an episode of acute gastroenteritis, a risk that is incremented in females (Faresjö et al., 2007a; R. Spiller & Lam, 2012; Thabane, Kottachchi & Marshall, 2007). Although psychosocial stress is more prevalent in IBS-D subjects, the first study of this thesis suggests that chronic psychosocial stress is needed to develop IBS-D symptoms but, counter intuitively, does not seem to strongly influence clinical severity, as severity parameters were comparable between IBS-D with moderate-high and low stress levels. This fact is also supported by the second study presented in this thesis, as chronic psychosocial stress also evoked a different mucosal gene expression that was corroborated by an increase intestinal permeability in response

to stress performed in the third study. When stratifying by sex, IBS-D males had a higher eosinophil infiltrate in the jejunal mucosa when compared to IBS-D females or to healthy males. Male predominance is common in other eosinophilopathies of the gastrointestinal tract (Merves et al., 2014), what raises the question of a differential gender-related role of eosinophilic infiltration in gastrointestinal disorders, as shown for duodenal eosinophilic infiltration of the duodenum and dyspeptic symptoms in females (Marjorie M Walker et al., 2014). However, no other differences in the number of mucosal immune cells were observed. Interestingly, a different molecular response to stress in the in the intestinal mucosa between males and females was found.

In summary Irritable bowel syndrome pathophysiology is not fully understood which makes its diagnosis and treatment a complicated task. This thesis remarks the role of stress on the pathophysiology of this disorder. Moreover, the findings in this study open new lines of investigation as the mechanisms of intestinal circadian rhythm control are much more deeply explained in animal model studies, but much less is known about deregulation of circadian rhythm on human gastrointestinal health. The study of intestinal permeability and how stress interferes with gastrointestinal function still has to remain one of the milestones in gastrointestinal research. Thus, more studies are needed in order to unravel the interaction between stress and gastrointestinal and barrier functions. Moreover, in order to better elucidate this stress-gastrointestinal tract interaction, studies performed in in subjects suffering from stress related disorders (GI and non-GI) are needed. Better knowledge of these interactions will favor the development of new drugs and new prevention strategies to decrease the incidence of FGID or at least improve the quality of life of patients suffering from FGID. Data from this study also suggest the need to promote new treatment strategies based not only drug treatment but also on a more global patient care, taking into account biological (sex and comorbidities) and psychological (chronic psychosocial stress and depression) factors which could be influencing with its symptomatology and or treatment response.

CONCLUSIONS

8. CONCLUSIONS

The results of this thesis have generated the following conclusions:

- IBS-D severity is exacerbated by dyspepsia and depression. A looser tool consistency phenotype and bloating determine increased abdominal pain intensity.
- The number of eosinophils in the mucosa of the jejunum is higher in males than in females suffering from IBS-D independently of their atopic state.
- IBS-D patients present a higher anticipation to unknown procedures.
- Acute pain stress disrupts intestinal mucosal circadian rhythm, barrier and immune function in health. This response is determined by chronic psychosocial stress and sex.
- Acute pain stress increases intestinal permeability in both, the small and the large intestine. This response is determined by sex and chronic psychosocial stress, being higher in females and in subjects with moderate to high psychosocial stress.
- Cold pain stress is an optimal experimental acute stress model for the study of mechanisms of intestinal barrier dysfunction associated with FGID.

FUTURE INVESTIGATION

9. FUTURE INVESTIGATIONS

This thesis highlights the importance of comorbid states in subjects suffering from IBS-D. According to the results generated in this study, in order to better design research studies, IBS cohorts should be balanced by comorbid pathologies, such as dyspepsia and depression. Moreover, other studies to determine the role of other comorbidities such as anxiety are needed. According to these results and statements, in the new project designed by our group to study IBS pathophysiology and to find new biomarkers for this disorder, we not only took into account depression and dyspepsia as modifiers of severity, but also implemented new questionnaires in order to evaluate anxiety.

A role of acute stress on maintenance of intestinal barrier function is highlighted in chapters 2 and 3, and circadian rhythm and immune activation appear as the most representative mechanisms in stress-induced intestinal barrier dysfunction. Future studies should be designed to determine the effect of chronic stress, sex or the combination of both on the intestinal stress response, as our study was under powered because of the low number of subjects in some of the subgroup analysis. Actually, an ongoing study is evaluating specifically which role plays the sex in the intestinal mucosa's response to stress, by only recruiting male and female volunteers with low levels of chronic psychosocial stress.

Moreover, studies addressed to unravel how circadian rhythm disruption is modulated by stress and how this contributes to disrupt intestinal homeostasis and favours the development of functional gastrointestinal disorders are needed. To do so, a study using the same methodology applied in chapters 2 and 3 in healthy subjects who have changing or night shifts at work will be performed.

Finally, it would be interesting to perform a study to determine if stress is also able to alter circadian rhythm, immunological and intestinal barrier pathways in the intestinal mucosa of IBS-D patients. Moreover, strategies directed to modify inflammation such as mast cell stabilizers or drugs that improve the resistance of the mucosa to pathologic aggressions, such as xyloglucan could be implemented in order to revert the deleterious effects of stress in the intestinal mucosa.

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10. BIBLIOGRAPHY

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ANNEX

11. Scientific originals and reviews, book chapters and congress presentations.

11.1. ORIGINAL ARTICLES

11.1.1. Original articles from Vall d'Hebron Institut de Recerca

11.1.1.1. Decreased TESK1-mediated cofilin 1 phosphorylation in the jejunum of IBS-D patients may explain increased female predisposition to epithelial dysfunction.

- Authors: Rodiño-Janeiro BK, Martínez C, Fortea M, Lobo B, **Pigrau M**, Nieto A, González-Castro AM, Salvo-Romero E, Guagnozzi D, Pardo-Camacho C, Iribarren C, Azpiroz F, Alonso-Cotoner C, Santos J, Vicario M.

- Abstract: Disturbed intestinal epithelial barrier and mucosal micro-inflammation characterize irritable bowel syndrome (IBS). Despite intensive research demonstrating ovarian hormones modulation of IBS severity, there is still limited knowledge on the mechanisms underlying female predominance in this disorder. Our aim was to identify molecular pathways involved in epithelial barrier dysfunction and female predominance in diarrhea-predominant IBS (IBS-D) patients. Total RNA and protein were obtained from jejunal mucosal biopsies from healthy controls and IBS-D patients meeting the Rome III criteria. IBS severity was recorded based on validated questionnaires. Gene and protein expression profiles were obtained and data integrated to explore biological and molecular functions. Results were validated by western blot. Tight junction signaling, mitochondrial dysfunction, regulation of actin-based motility by Rho, and cytoskeleton signaling were differentially expressed in IBS-D. Decreased TESK1-dependent cofilin 1 phosphorylation (pCFL1) was confirmed in IBS-D, which negatively correlated with bowel movements only in female participants. In conclusion, deregulation of cytoskeleton dynamics through TESK1/CFL1 pathway underlies epithelial intestinal dysfunction in the small bowel mucosa of IBS-D, particularly in female patients. Further understanding of the mechanisms involving sex-mediated regulation of mucosal epithelial integrity may have significant preventive, diagnostic, and therapeutic implications for IBS.

- Journal: Scientific Reports 2018 Feb 2;8(1):2255. doi: 10.1038/s41598-018-20540-9.

- Impact Factor: 4.259

11.1.1.2. Downregulation of mucosal mast cell activation and immune response in diarrhoea-irritable bowel syndrome by oral disodium cromoglycate: A pilot study.

- Authors: Lobo B, Ramos L, Martínez C, Guilarte M, González-Castro AM, Alonso-Cotoner C, **Pigrau M**, de Torres I, Rodiño-Janeiro BK, Salvo-Romero E, Fortea M, Pardo-Camacho C, Guagnozzi D, Azpiroz F, Santos J, Vicario M.

- Abstract: Background and goal: Diarrhoea-predominant irritable bowel syndrome (IBS-D) exhibits intestinal innate immune and mucosal mast cell (MC) activation. MC stabilisers have been shown to improve IBS symptoms but the mechanism is unclear. Our primary aim was to investigate the effect of oral disodium cromoglycate (DSCG) on jejunal MC activation and specific innate immune signalling pathways in IBS-D, and secondarily, its potential clinical benefit. Study: Mucosal MC activation (by ultrastructural changes, tryptase release and gene expression) and innate immune signalling (by protein and gene expression) were quantified in jejunal biopsies from healthy (HS; n = 16) and IBS-D subjects after six months of either treatment with DSCG (600 mg/day, IBS-D-DSCG group; n = 18) or without treatment (IBS-D-NT group; n = 25). All IBS-D patients recorded abdominal pain and bowel habits at baseline and in the last 10 days prior to jejunal sampling. Results: IBS-D-NT exhibited significant MC activation and over-expression of immune-related genes as compared to HS, whereas in IBS-D-DSCG MC activity and gene expression were similar to HS. Furthermore, DSCG significantly reduced abdominal pain and improved stool consistency. Conclusions: Oral DSCG modulates mucosal immune activity and improves gut symptoms in IBS-D patients. Future placebo-controlled clinical trials are needed for confirmation of clinical benefit of DSCG for IBS-D.

- Journal: United European Gastroenterol J. 2017 Oct;5 (6):887-897. doi: 10.1177/2050640617691690. Epub 2017 Jan 29.

- Impact factor: 3.673.

11.1.1.3. Randomised clinical trial: the analgesic properties of dietary supplementation with palmitoylethanolamide and polydatin in irritable bowel syndrome.

- Authors: Cremon C, Stanghellini V, Barbaro MR, Cogliandro RF, Bellacosa L, Santos J, Vicario M, **Pigrau M**, Alonso Cotoner C, Lobo B, Azpiroz F, Bruley des Varannes S, Neunlist M, DeFilippis D, Iuvone T, Petrosino S, Di Marzo V, Barbara G.

- Abstract: Background: Intestinal immune activation is involved in irritable bowel syndrome (IBS) pathophysiology. While most dietary approaches in IBS involve food avoidance, there are fewer indications on food supplementation. Palmithoylethanolamide, structurally related to the endocannabinoid anandamide, and polydatin are dietary compounds which act synergistically to reduce mast cell activation. Aim: To assess the effect on mast cell count and the efficacy of palmithoylethanolamide/polydatin in patients with IBS. Methods: We conducted a pilot, 12-week, randomised, double-blind, placebo-controlled, multicentre study assessing the effect of palmithoylethanolamide/polydatin 200mg/20mg or placebo b.d. on low-grade immune activation, endocannabinoid system and symptoms in IBS patients. Biopsy samples, obtained at screening visit and at the end of the study, were analysed by immunohistochemistry, enzyme-linked immunoassay, liquid chromatography and Western blot. Results: A total of 54 patients with IBS and 12 healthy controls were enrolled from five European centres. Compared with controls, IBS patients showed higher mucosal mast cell counts (3.2±1.3 vs. 5.3±2.7%, P=0.013), reduced fatty acid amide oleoylethanolamide (12.7±9.8 vs. 45.8±55.6 pmol/mg, P=0.002) and increased expression of cannabinoid receptor 2 (0.7±0.1 vs. 1.0±0.8, P=0.012). The treatment did not significantly modify IBS biological profile, including mast cell count. Compared with placebo, palmithoylethanolamide/polydatin markedly improved abdominal pain severity (P<0.05). Conclusions: The marked effect of the dietary supplement palmithoylethanolamide/polydatin on abdominal pain in patients with IBS suggests that this is a promising natural approach for pain management in this condition. Further studies are now required to elucidate the mechanism of action of palmithoylethanolamide/polydatin in IBS. ClinicalTrials.gov number, NCT01370720.

- Journal: Aliment Pharmacol Ther. 2017 Apr;45(7):909-922. doi: 10.1111/apt.13958. Epub 2017 Feb 6.

- Impact factor: 7.286

11.1.1.4. miR-16 and miR-125b are involved in barrier function dysregulation through the modulation of claudin-2 and cingulin expression in the jejunum in IBS with diarrhoea.

- Authors: Martínez C, Rodiño-Janeiro BK, Lobo B, Stanifer ML, Klaus B, Granzow M, González-Castro AM, Salvo-Romero E, Alonso-Cotoner C, **Pigrau M**, Roeth R, Rappold G, Huber W, González-Silos R, Lorenzo J, de Torres I, Azpiroz F, Boulant S, Vicario M, Niesler B, Santos J.

- Abstract: Objective: Micro-RNAs (miRNAs) play a crucial role in controlling intestinal epithelial barrier function partly by modulating the expression of tight junction (TJ) proteins. We have previously shown differential messenger RNA (mRNA) expression correlated with ultrastructural abnormalities of the epithelial barrier in patients with diarrhoea-predominant IBS (IBS-D). However, the participation of miRNAs in these differential mRNA-associated findings remains to be established. Our aims were (1) to identify miRNAs differentially expressed in the small bowel mucosa of patients with IBS-D and (2) to explore putative target genes specifically involved in epithelial barrier function that are controlled by specific dysregulated IBS-D miRNAs. Design: Healthy controls and patients meeting Rome III IBS-D criteria were studied. Intestinal tissue samples were analysed to identify potential candidates by: (a) miRNA-mRNA profiling; (b) miRNA-mRNA pairing analysis to assess the co-expression profile of miRNA-mRNA pairs; (c) pathway analysis and upstream regulator identification; (d) miRNA and target mRNA validation. Candidate miRNA-mRNA pairs were functionally assessed in intestinal epithelial cells. Results: IBS-D samples showed distinct miRNA and mRNA profiles compared with healthy controls. TJ signalling was associated with the IBS-D transcriptional profile. Further validation of selected genes showed consistent upregulation in 75% of genes involved in epithelial barrier function. Bioinformatic analysis of putative miRNA binding sites identified hsa-miR-125b-5p and hsa-miR-16 as regulating expression of the TJ genes *CGN* (cingulin) and *CLDN2* (claudin-2), respectively. Consistently, protein expression of CGN and CLDN2 was upregulated in IBS-D, while the respective targeting miRNAs were downregulated. In addition, bowel dysfunction, perceived stress and depression and number of mast cells correlated with the expression of hsa-miR-125b-5p and hsa-miR-16 and their respective target proteins. Conclusions: Modulation of the intestinal epithelial barrier function in IBS-D involves both transcriptional and post-transcriptional mechanisms. These molecular mechanisms include miRNAs as master regulators in controlling the expression of TJ proteins and are associated with major clinical symptoms.

- Journal: Gut. 2017 Sep;66(9):1537-1538. doi: 10.1136/gutjnl-2016-311477. Epub 2017 Jan 12.
- Impact factor: 16.658

11.1.1.5. Increased humoral immunity in the jejunum of diarrhoea-predominant irritable bowel syndrome associated with clinical manifestations.

- Authors: Vicario M, González-Castro AM, Martínez C, Lobo B, **Pigrau M**, Guilarte M, de Torres I, Mosquera JL, Fortea M, Sevillano-Aguilera C, Salvo-Romero E, Alonso C, Rodiño-Janeiro BK, Söderholm JD, Azpiroz F, Santos J.

- Abstract: Background and aims: Altered intestinal barrier is associated with immune activation and clinical symptoms in diarrhoea-predominant IBS (IBS-D). Increased mucosal antigen load may induce specific responses; however, local antibody production and its contribution to IBS aetiopathogenesis remain undefined. This study evaluated the role of humoral activity in IBS-D. Methods: A single mucosal jejunal biopsy, luminal content and blood were obtained from healthy volunteers (H; n=30) and IBS-D (n=49; Rome III criteria) participants. Intraepithelial lymphocytes, mast cells, B lymphocytes and plasma cells were studied by imaging techniques. Differential gene expression and pathway analysis were assessed by microarray and PCR techniques. Blood and luminal immunoglobulins (Igs) were quantified. Gastrointestinal symptoms, respiratory atopy and stress and depression were also recorded. Results: Patients with IBS-D showed a higher number and activation of mucosal B lymphocytes and plasma cells ($p<0.05$). Mast cell density was increased in patients with IBS-D (non-atopic) and in close proximity to plasma cells ($p<0.05$). Microarray profiling identified differential humoral activity in IBS-D, involving proliferation and activation of B lymphocytes and Igs production ($p<0.001$). Mucosal humoral activity was higher in IBS-D, with upregulation of germline transcripts and Ig genes (1.3-fold-1.7-fold increase; $p<0.05$), and increased IgG(+) cells and luminal IgG compared with H ($p<0.05$), with no differences in blood. Biological markers of humoral activity correlated positively with bowel movements, stool form and depression. Conclusions: Enhanced small bowel humoral immunity is a distinctive feature of IBS-D. Mucosal Ig production contributes to local inflammation and clinical manifestations in IBS-D.

- Journal: Gut. 2015 Sep;64(9):1379-88. doi: 10.1136/gutjnl-2013-306236. Epub 2014 Sep 10.

- Impact factor: 16.658

11.1.2. Original articles from Farcombe Familiy Digestive Health Research Institute

11.1.2.1. Transplantation of fecal microbiota from patients with irritable bowel syndrome alters gut function and behavior in recipient mice.

- Authors: De Palma G[1], Lynch MD[2], Lu J[1], Dang VT[3], Deng Y[1], Jury J[1], Umeh G[1], Miranda PM[1], **Pigrau Pastor M**[1], Sidani S[1], Pinto-Sanchez MI[1], Philip V[1], McLean PG[4], Hagelsieb MG[5], Surette MG[1], Bergonzelli GE[4], Verdu EF[1], Britz-McKibbin P[3], Neufeld JD[2], Collins SM[1], Bercik P[6].

- Abstract: Irritable bowel syndrome (IBS) is a common disorder characterized by altered gut function and often is accompanied by comorbid anxiety. Although changes in the gut microbiota have been documented, their relevance to the clinical expression of IBS is unknown. To evaluate a functional role for commensal gut bacteria in IBS, we colonized germ-free mice with the fecal microbiota from healthy control individuals or IBS patients with diarrhea (IBS-D), with or without anxiety, and monitored gut function and behavior in the transplanted mice. Microbiota profiles in recipient mice clustered according to the microbiota profiles of the human donors. Mice receiving the IBS-D fecal microbiota showed a taxonomically similar microbial composition to that of mice receiving the healthy control fecal microbiota. However, IBS-D mice showed different serum metabolomic profiles. Mice receiving the IBS-D fecal microbiota, but not the healthy control fecal microbiota, exhibited faster gastrointestinal transit, intestinal barrier dysfunction, innate immune activation, and anxiety-like behavior. These results indicate the potential of the gut microbiota to contribute to both intestinal and behavioral manifestations of IBS-D and suggest the potential value of microbiota-directed therapies in IBS patients.

- Journal: Science Translational Medicine. 2017 Mar 1;9(379). pii: eaaf6397. doi: 10.1126/ scitranslmed.aaf6397.

- Impact factor: 16.796

11.1.2.2. Probiotic Bifidobacterium longum NCC3001 Reduces Depression Scores and Alters Brain Activity: A Pilot Study in Patients With Irritable Bowel Syndrome.

- Authors: Pinto-Sanchez MI, Hall GB, Ghajar K, Nardelli A, Bolino C, Lau JT, Martin FP, Cominetti O, Welsh C, Rieder A, Traynor J, Gregory C, De Palma G, **Pigrau M**, Ford AC, Macri J, Berger B, Bergonzelli G, Surette MG, Collins SM, Moayyedi P, Bercik P.

- Abstract: Background & aims: Probiotics can reduce symptoms of irritable bowel syndrome (IBS), but little is known about their effects on psychiatric comorbidities. We performed a prospective study to evaluate the effects of Bifidobacterium longum NCC3001 (BL) on anxiety and depression in patients with IBS. Methods: We performed a randomized, double-blind, placebo-controlled study of 44 adults with IBS and diarrhea or a mixed-stool pattern (based on Rome III criteria) and mild to moderate anxiety and/or depression (based on the Hospital Anxiety and Depression scale) at McMaster University in Canada, from March 2011 to May 2014. At the screening visit, clinical history and symptoms were assessed and blood samples were collected. Patients were then randomly assigned to groups and given daily BL (n = 22) or placebo (n = 22) for 6 weeks. At weeks 0, 6, and 10, we determined patients' levels of anxiety and depression, IBS symptoms, quality of life, and somatization using validated questionnaires. At weeks 0 and 6, stool, urine and blood samples were collected, and functional magnetic resonance imaging (fMRI) test was performed. We assessed brain activation patterns, fecal microbiota, urine metabolome profiles, serum markers of inflammation, neurotransmitters, and neurotrophin levels. Results: At week 6, 14 of 22 patients in the BL group had reduction in depression scores of 2 points or more on the Hospital Anxiety and Depression scale, vs 7 of 22 patients in the placebo group (P = .04). BL had no significant effect on anxiety or IBS symptoms. Patients in the BL group had a mean increase in quality of life score compared with the placebo group. The fMRI analysis showed that BL reduced responses to negative emotional stimuli in multiple brain areas, including amygdala and fronto-limbic regions, compared with placebo. The groups had similar fecal microbiota profiles, serum markers of inflammation, and levels of neurotrophins and neurotransmitters, but the BL group had reduced urine levels of methylamines and aromatic amino acids metabolites. At week 10, depression scores were reduced in patients given BL vs placebo. Conclusion: In a placebo-controlled trial, we found that the probiotic BL reduces depression but not anxiety scores and increases quality of life in patients with IBS. These improvements were associated with changes in brain activation patterns that indicate that this probiotic reduces limbic reactivity. ClinicalTrials.gov no. NCT01276626.

- Journal: Gastroenterology. 2017 Aug;153(2):448-459.e8. doi: 10.1053/j.gastro. 2017.05.003. Epub 2017 May 5.

- Impact factor: 18.392

11.1.2.3. Bead study: a novel method to measure gastrointestinal transit in mice.

- Authors: Reed DE, **Pigrau M**, Lu J, Moayyedi P, Collins SM, Bercik P.

- Abstract: *Background:* Intestinal transit assessment in mice using existing methods requires long recording periods or euthanization of animals to localize a tracer. We have developed a novel in vivo method to assess gastrointestinal (GI) transit in mice based on a clinically used 'shapes study'. Methods: Mice (n=70) were gavaged with 5 steel beads and barium 3 h before, with another dose of barium gavaged 10 min before imaging. Mice were fluoroscoped for 20-60s, and then most of them were euthanized and the GI tract removed to confirm the localization of the beads fluoroscopically. The in vivo and postmortem recordings were analyzed and each bead was scored depending on its location; a total score was calculated by adding individual bead scores. Total scores obtained from the two methods were compared. A group of mice (n=10) were examined on three occasions, before and after treatment with loperamide or prucalopride. Key results: The stomach and cecum were consistently outlined by barium, serving as reference landmarks. There was an excellent overall correlation between in vivo and postmortem transit scores (r = 0.93). Analysis of scores for individual gut segments revealed high agreement for stomach, cecum, and expelled beads, and moderate agreement for the small bowel and colon. Gastrointestinal transit scores were decreased by loperamide and increased by prucalopride compared with baseline. Conclusions & inferences: Metallic beads are reliably localized by videofluoroscopy in vivo within the GI tract. This novel imaging method enables repetitive measurements of GI transit in vivo and detects changes induced by motility-modifying agents.

- Journal: Neurogastroenterology and Motility. 2014 Nov;26(11):1663-8. doi: 10.1111/nmo.12442. Epub 2014 Sep 27.

- Impact Factor: 3.617

11.2. REVIEWS AND BOOK CHAPTERS

11.2.1. Reviews

11.2.1.1. Epithelial immunity: priming defensive responses in the intestinal mucosa.

- Authors: Pardo-Camacho C, González-Castro AM, Rodiño-Janeiro BK, **Pigrau M**, Vicario M.

- Abstract: As the largest interface between the outside and internal milieu, the intestinal epithelium constitutes the first structural component facing potential luminal threats to homeostasis. This single-cell layer is the epicenter of a tightly regulated communication network between external and internal factors that converge to prime defensive responses aimed at limiting antigen penetration and the maintenance of intestinal barrier function. The defensive role developed by intestinal epithelial cells (IEC) relies largely on the variety of receptors they express at both extracellular (apical and basolateral) and intracellular compartments, and the capacity of IEC to communicate with immune and nervous systems. IEC recognize pathogen-associated molecules by innate receptors that promote the production of mucus, antimicrobial substances, and immune mediators. Epithelial cells are key to oral tolerance maintenance and also participate in adaptive immunity through the expression of immunoglobulin (Ig) receptors and by promoting local Ig class switch recombination. In IEC, different types of antigens can be sensed by multiple immune receptors that share signaling pathways to assure effective responses. Regulated defensive activity maintains intestinal homeostasis, whereas a breakdown in the control of epithelial immunity can increase the intestinal passage of luminal content and microbial invasion, leading to inflammation and tissue damage. In this review, we provide an updated overview of the type of immune receptors present in the human intestinal epithelium and the responses generated to promote effective barrier function and maintain mucosal homeostasis.

- Journal: American Journal of Physiology-Gastrointestinal and Liver Physiology. 2018 Feb 1;314(2):G247-G255. doi: 10.1152/ajpgi.00215.2016. Epub 2017 Nov 16.

- Impact factor: 3.468

11.2.1.2. The joint power of sex and stress to modulate brain-gut-microbiota axis and intestinal barrier homeostasis: implications for irritable bowel syndrome.

- Authors: **Pigrau M**, Rodiño-Janeiro BK, Casado-Bedmar M, Lobo B, Vicario M, Santos J, Alonso-Cotoner C.

- Abstract: *Background:* Intestinal homeostasis is a dynamic process that takes place at the interface between the lumen and the mucosa of the gastrointestinal tract, where a constant scrutiny for antigens and toxins derived from food and microorganisms is carried out by the vast gut-associated immune system. Intestinal homeostasis is preserved by the ability of the mucus layer and the mucosal barrier to keep the passage of small-sized and antigenic molecules across the epithelium highly selective. When combined and preserved, immune surveillance and barrier's selective permeability, the host capacity of preventing the development of intestinal inflammation is optimized, and viceversa. In addition, the brain-gut-microbiome axis, a multidirectional communication system that integrates distant and local regulatory networks through neural, immunological, metabolic, and hormonal signaling pathways, also regulates intestinal function. Dysfunction of the brain-gut-microbiome axis may induce the loss of gut mucosal homeostasis, leading to uncontrolled permeation of toxins and immunogenic particles, increasing the risk of appearance of intestinal inflammation, mucosal damage, and gut disorders. Irritable bowel syndrome is prevalent stress-sensitive gastrointestinal disorder that shows a female predominance. Interestingly, the role of stress, sex and gonadal hormones in the regulation of intestinal mucosal and the brain-gut-microbiome axis functioning is being increasingly recognized. Purpose: We aim to critically review the evidence linking sex, and stress to intestinal barrier and brain-gut-microbiome axis dysfunction and the implications for irritable bowel syndrome.

- Journal: Neurogastroenterology and Motility. 2016 Apr;28(4):463-86. doi: 10.1111 /nmo.12717. Epub 2015 Nov 11. Review.

- Impact factor: 3.617

11.2.1.3. Role of Corticotropin-releasing Factor in Gastrointestinal Permeability.

- Authors: Rodiño-Janeiro BK, Alonso-Cotoner C, **Pigrau M**, Lobo B, Vicario M, Santos J.

- Abstract: The interface between the intestinal lumen and the mucosa is the location where the majority of ingested immunogenic particles face the scrutiny of the vast gastrointestinal immune system. Upon regular physiological conditions, the intestinal microflora and the epithelial barrier are well prepared to process daily a huge amount of food-derived antigens and non-immunogenic particles. Similarly, they are ready to prevent environmental toxins and microbial antigens to penetrate further and interact with the mucosal-associated immune system. These functions promote the development of proper immune responses and oral tolerance and prevent disease and inflammation. Brain-gut axis structures participate in the processing and execution of response signals to external and internal stimuli. The brain-gut axis integrates local and distant regulatory networks and supersystems that serve key housekeeping physiological functions including the balanced functioning of the intestinal barrier. Disturbance of the brain-gut axis may induce intestinal barrier dysfunction, increasing the risk of uncontrolled immunological reactions, which may indeed trigger transient mucosal inflammation and gut disease. There is a large body of evidence indicating that stress, through the brain-gut axis, may cause intestinal barrier dysfunction, mainly via the systemic and peripheral release of corticotropin-releasing factor. In this review, we describe the role of stress and corticotropin-releasing factor in the regulation of gastrointestinal permeability, and discuss the link to both health and pathological conditions.

- Journal: Journal of Neurogastroenterology and Motility. 2015 Jan 1;21(1):33-50. doi: 10.5056/jnm14084.

11.2.2. Book Chapters

11.2.2.1. Intestinal barrier function and the brain-gut axis.

- Authors: Alonso C, Vicario M, **Pigrau M**, Lobo B, Santos J.

- Abstract: The luminal-mucosal interface of the intestinal tract is the first relevant location where microorganism-derived antigens and all other potentially immunogenic particles face the scrutiny of the powerful mammalian immune system. Upon regular functioning conditions, the intestinal barrier is able to effectively prevent most environmental and external antigens to interact openly with the numerous and versatile elements that compose the mucosal-associated immune system. This evolutionary super system is capable of processing an astonishing amount of antigens and non-immunogenic particles, approximately 100 tons in one individual lifetime, only considering food-derived components. Most important, to develop oral tolerance and proper active immune responses needed to prevent disease and inflammation, this giant immunogenic load has to be managed in a way that physiological inflammatory balance is constantly preserved. Adequate functioning of the intestinal barrier involves local and distant regulatory networks integrating the so-called brain-gut axis. Along this complex axis both brain and gut structures participate in the processing and execution of response signals to external and internal changes coming from the digestive tract, using multidirectional pathways to communicate. Dysfunction of brain-gut axis facilitates malfunctioning of the intestinal barrier, and vice versa, increasing the risk of uncontrolled immunological reactions that may trigger mucosal and brain low-grade inflammation, a putative first step to the initiation of more permanent gut disorders. In this chapter, we describe the structure, function and interactions of intestinal barrier, microbiota and brain-gut axis in both healthy and pathological conditions.

- Book: Advanced Experimental Medicine and Biology. Microbial Endocrinology: The Microbiota-Gut-Brain-Axis. Volume: 817; Chapter 4; Pages: 73-113. Editors: Lyte M and Cryan JF. 2014, New York (USA).

11.3. CONGRESS PRESENTATIONS

11.3.1. Presentations from Vall d'Hebron Institut de Recerca

11.3.1.1. Desmosome associated genes improve predictability of ibs compared to clinical variables.

- Authors: Cristina Martinez, Jose Luis Mosquera, Bruno Kotska Rodiño-Janeiro, Marina Fortea-Guillamón, Beatriz Lobo, **Marc Pigrau**, Ana María González-Castro, Eloísa Salvo-Romero, Cristina Pardo-Camacho, Danila Guagnozzi, Beate Niesler, Fernando Azpiroz, Carmen Alonso Cotoner, María Vicario, Javier Santos.
- Name of the conference: Digestive Diseases Week 2018. Annual Meeting of the American Gastroenterological Association Institute.
- Date of event: 06/2018
- Organizing entity: American Gastroenterological Association Institute.
- Type of presentation: Poster presentation.
- Published in: Gastroenterology, May 2018; Vol. 154, Issue 6,S-501.

11.3.1.2. Acute stress triggers ibs-like mirna-mediated regulation of barrier function in the jejunum of healthy volunteers

- Authors: Bruno Kotska Rodiño-Janeiro, **Marc Pigrau**, Adoración Nieto, Eloísa Salvo-Romero, Beatriz Lobo, Ana María González-Castro, Marina Fortea-Guillamón, Cristina Pardo-Camacho, Inés de Torres, Cristina Martinez, Danila Guagnozzi, Beate Niesler, Fernando Azpiroz, María Vicario, Javier Santos, Carmen Alonso Cotoner.
- Name of the conference: Digestive Diseases Week 2018. Annual Meeting of the American Gastroenterological Association Institute.
- Date of event: 06/2018
- Organizing entity: American Gastroenterological Association Institute.
- Type of presentation: Poster presentation.
- Published in: Gastroenterology, May 2018; Vol. 154, Issue 6,S-502.

11.3.1.3. Stress regulates specific sex-related molecular alterations in epithelial barrier regulatory genes in the jejunal mucosa of healthy volunteers.

- Authors: B.K. Rodiño Janeiro, **M. Pigrau**, A. Nieto, T. Pribic, L. Hernández, E. Salvo-Romero, B. Lobo Alvarez, A.M. González-Castro, M. Fortea, M. Gallart, C. Pardo-Camacho, I. De Torres, C. Martinez, D. Guagnozzi, T. Pérez-Berezo, F. Azpiroz, M. Vicario, J. Santos, C. Alonso Cotoner.
- Name of the conference: 25th United European Gastroenterology Week 2017 Barcelona, Spain.
- Date of event: 10/2017
- Organizing entity: United European Gastroenterology.
- Type of presentation: Oral presentation.
- Published in: UEG Journal, October 2017; Volume 5, Issue 5_suppl.

11.3.1.4. Acute stress impacts clock genes and barrier integrity in the intestinal mucosa in health

- Authors: **M. Pigrau Pastor**, B.K. Rodiño-Janeiro, E. Salvo Romero, A. Nieto, L. Hernández, T. Pribic, B. Lobo-Álvarez, A.M. González-Castro, M. Fortea, C. Pardo-Camacho, D. Guagnozzi, C. Martinez, T. Pérez-Berezo, I. De Torres, F. Azpiroz, M. Vicario, C. Alonso-Cotoner, J. Santos.
- Name of the conference: 25th United European Gastroenterology Week 2017 Barcelona, Spain.
- Date of event: 10/2017
- Organizing entity: United European Gastroenterology.
- Type of presentation: Oral presentation.
- Published in: UEG Journal, October 2017; Volume 5, Issue 5_suppl.

11.3.1.5. Corticotrophin-releasing factor in activated mucosal eosinophils is associated with clinical severity in diarrhea-prone Irritable Bowel Syndrome (IBS).

- Authors: F. Azpiroz; E. Salvo Romero; C. Martínez; B. Lobo; **M. Pigrau**; A. Sánchez Chardi; A. M. González Castro; B. K. Rodiño Janeiro; M. Fortea; C. Alonso Cotoner; J. Santos; M. Vicario.
- Name of the conference: NeuroGASTRO 2017 Congress.
- Date of event: 08/2017
- Organizing entity: European Society of Neurogastroenterology and Motility.
- Type of presentation: Oral presentation.
- Published in: Neurogastroenterology and hepatology, August 2017; Volume 29, Issue S2, Pages: 1-146.

11.3.1.6. Stress induces specific gender-related molecular alterations in barrier regulatory genes in the jejunal mucosa of healthy.

- Authors: Bruno Kotska Rodiño-Janeiro**, Marc Pigrau**, Adoración Nieto, Teodora Pribic, Laura Hernández-Palet, Eloísa Salvo-Romero, Milagros Gallart, Beatriz Lobo, Ana María González-Castro, Marina Fortea-Guillamón, Cristina Pardo-Camacho, Inés de Torres, Cristina Martinez, Danila Guagnozzi, Teresa Pérez-Berezo, Fernando Azpiroz, María Vicario, Javier Santos, Carmen Alonso-Cotoner.
- Name of the conference: Digestive Diseases Week 2017. Annual Meeting of the American Gastroenterological Association Institute.
- Date of event: 05/2017
- Organizing entity: American Gastroenterological Association Institute.
- Type of presentation: Poster presentation.
- Published in: Gastroenterology, April 2017; Vol. 152, Issue 5, S720–S721.

11.3.1.7. Integrated multi-omic analysis reveals female predominance of deregulated mucosal actin depolymerization by decreased TESK1-mediated CFL1- phosphorylation in IBS-D

- Authors: Bruno Kotska Rodiño-Janeiro, Cristina Martinez, Marina Fortea-Guillamón, Beatriz Lobo, **Marc Pigrau**, Ana María González-Castro, Eloísa Salvo-Romero, Cristina Pardo-Camacho, Cristina Iribarren, Danila Guagnozzi, Fernando Azpiroz, Carmen Alonso Cotoner, Javier Santos, María Vicario.
- Name of the conference: Digestive Diseases Week 2017. Annual Meeting of the American Gastroenterological Association Institute.
- Date of event: 05/2017.
- Organizing entity: American Gastroenterological Association Institute.
- Type of presentation: Poster presentation.
- Published in: Gastroenterology, April 2017; Vol. 152, Issue 5, S721.

11.3.1.8. Acute stress impacts clock genes and barrier integrity in the intestinal mucosa in health.

- Authors: **Marc Pigrau**, Bruno Kotska Rodiño-Janeiro, Eloísa Salvo-Romero, Adoración Nieto, Laura Hernández-Palet, Teodora Pribic, Milagros Gallart, Beatriz Lobo, Ana M González-Castro, Marina Fortea-Guillamón, Cristina Pardo-Camacho, Danila Guagnozzi, Cristina Martinez, Teresa Pérez-Berezo, Cristina Iribarren, Inés de Torres, Fernando Azpiroz, María Vicario, Carmen Alonso Cotoner, Javier Santos.
- Name of the conference: Digestive Diseases Week 2017. Annual Meeting of the American Gastroenterological Association Institute.
- Date of event: 05/2017.
- Organizing entity: American Gastroenterological Association Institute.
- Type of presentation: Poster presentation.
- Published in: Gastroenterology, April 2017; Vol. 152, Issue 5, S919.

11.3.1.9. Paired transcriptomic and proteomic profiling analysis of the intestinal mucosa identifies similar biological pathways in diarrhoea-prone irritable bowel syndrome.

- Authors: B. K. Rodiño-Janeiro, C. Martínez, B. Lobo, **M. Pigrau**, A. M. González-Castro, M. Fortea, M. Casado-Bedmar, C. Pardo-Camacho, F. Azpiroz, C. Alonso-Cotoner, M. Vicario, J. Santos.
- Name of the conference: 23rd United European Gastroenterology Week 2015 Barcelona, Spain.
- Date of event: 10/2015
- Organizing entity: United European Gastroenterology.
- Type of presentation: Oral presentation.
- Published in: UEG Journal, October 2015; Volume 3, Issue 5_suppl.

11.3.1.10. Differential expression of miRNAs in the jejunal mucosa of I is involved in intestinal epithelial barrier dysfunction through modulation of specific tight junction proteins.

- Authors: C. Martinez, B. K. Rodiño-Janeiro, B. Lobo, M. Granzow, B. Klaus, C. Alonso, M. Vicario, **M. Pigrau**, R. Roeth, W. Huber, F. Azpiroz, B. Niesler, J. Santos.
- Name of the conference: 23rd United European Gastroenterology Week 2015 Barcelona, Spain.
- Date of event: 10/2015
- Organizing entity: United European Gastroenterology.
- Type of presentation: Oral presentation.
- Published in: UEG Journal, October 2015; Volume 3, Issue 5_suppl.

11.3.1.11. Down-regulation of intestinal inflammatory transcriptome after long-term treatment with disodium cromoglycate in diarrhea-predominant irritable bowel syndrome patients is associated with clinical improvement.

- Authors: B. Lobo, **M. Pigrau,** C. Martinez, A. M. González-Castro, M. Guilarte, I.de Torres, E. Salvo-Romero, B. K. Rodiño-Janeiro, M. Fortea, C. Alonso, F. Azpiroz, M. Vicario and J. Santos.
- Name of the conference: NeuroGASTRO 2015 Congress, Istanbul, Turkey.
- Date of event: 06/2015
- Organizing entity: European Society of Neurogastroenterology and Motility.
- Type of presentation: Oral presentation.
- Published in: Neurogastroenterology and hepatology, June 2015; Volume 27, Issue S2, Pages: 1-119.

11.3.1.12. Intestinal epithelial barrier dysfunction in the jejunal mucosa of IBS-D involves modulation of specific tight junction proteins by miRNAs.

- Authors: C. Martinez, B. K. Rodiño-Janeiro, B. Lobo, M. Granzow, B. Klauss, C. Alonso, M. Vicario, M. Pigrau, W. Huber, F. Azpiroz, B. Niesler and J. Santos.
- Name of the conference: NeuroGASTRO 2015 Congress, Istanbul, Turkey.
- Date of event: 06/2015
- Organizing entity: European Society of Neurogastroenterology and Motility.
- Type of presentation: Oral presentation.
- Published in: Neurogastroenterology and hepatology, June 2015; Volume 27, Issue S2, Pages: 1-119.

11.3.1.13. Gender-related differential methylation patterns of the corticotropin releasing factor gene in the intestinal mucosa may relate to female predominance in diarrhea-prone irritable bowel syndrome.

- Authors: Bruno Rodiño-Janeiro, I. Palma, M. Fortea, E. Salvo-Romero, B. Lobo, M. Pigrau, A. González-Castro, C. Martínez, F. Azpiroz, M. Vicario, J. Santos, C. Alonso-Cotoner.
- Name of the conference: NeuroGASTRO 2015 Congress, Istanbul, Turkey.
- Date of event: 06/2015
- Organizing entity: European Society of Neurogastroenterology and Motility.
- Type of presentation: Poster presentation.
- Published in: Neurogastroenterology and hepatology, June 2015; Volume 27, Issue S2, Pages: 1-119.

11.3.1.14. Jejunal Mucosal Eosinophils Show Higher Corticotropin-Releasing Hormone Content in Association With Clinical Manifestations in Diarrhea-Prone Irritable Bowel Syndrome.

- Authors: Eloísa Salvo-Romero, Cristina Martinez, Beatriz Lobo, **Marc Pigrau**, Alejandro Sanchez-Chardi, Ana María González-Castro, Bruno Kotska Rodiño-Janeiro, Marina Fortea, Fernando Azpiroz, Carmen Alonso Cotoner, Javier Santos, María Vicario.
- Name of the conference: Digestive Diseases Week 2015. Annual Meeting of the American Gastroenterological Association Institute.
- Date of event: 05/2015.
- Organizing entity: American Gastroenterological Association Institute.
- Type of presentation: Oral presentation.
- Published in: Gastroenterology, April 2015; Vol. 148, Issue 4, S38.

11.3.1.15. Clinical Benefit and Intestinal Mucosal Transcriptome Modulation After LongTerm Mast Cell Stabilization With Oral Disodium Cromoglycate in DiarrheaPredominant Irritable Bowel Syndrome (IBS-D) Patients.

- Authors: Beatriz Lobo, **Marc Pigrau**, Cristina Martinez, Ana M González-Castro, mar guilarte, ines de torres, Eloísa Salvo-Romero, Bruno Kotska Rodiño-Janeiro, Marina Fortea, Carmen Alonso Cotoner, Fernando Azpiroz, María Vicario, Javier Santos.
- Name of the conference: Digestive Diseases Week 2015. Annual Meeting of the American Gastroenterological Association Institute.
- Date of event: 05/2015.
- Organizing entity: American Gastroenterological Association Institute.
- Type of presentation: Poster presentation.
- Published in: Gastroenterology, April 2015; Vol. 148, Issue 4, S494.

11.3.1.16. A pilot randomized placebo-controlled multicenter study on the effect of palmitoylethanolamide and polydatin in patients with irritable bowel syndrome.

- Authors: C. Cremon, G. Barbara, L. Bellacosa, M.R. Barbaro, J. Santos, M. Vicario, **M. Pigrau**, C. Alonso, S. Bruley des Varannes, M. Neunlist, D. De Filippis, T. Iuvone, V. Di Marzo , R. De Giorgio, R. Corinaldesi, V. Stanghellini.
- Name of the conference: 22rd United European Gastroenterology Week 2015 Vienna, Austria.
- Date of event: 10/2014
- Organizing entity: United European Gastroenterology.
- Type of presentation: Poster presentation.
- Published in: UEG Journal, October 2014; Volume 2, Issue 1_suppl.

11.3.1.17. Female Gender Favors Activation of Gut Immune and Barrier Regulatory Networks.

- Authors: Carmen Alonso, **Marc Pigrau**, María Vicario, Beatriz Lobo, Cristina Frias, Ana María González-Castro, Eloísa Salvo-Romero, Cesar Sevillano, Cristina Martinez, Bruno Kotska Rodiño-Janeiro, Fernando Azpiroz, Javier Santos.
- Name of the conference: Digestive Diseases Week 2014. Annual Meeting of the American Gastroenterological Association Institute.
- Date of event: 05/2014.
- Organizing entity: American Gastroenterological Association Institute.
- Type of presentation: Oral presentation.
- Published in: Gastroenterology, May 2014; Vol. 146, Issue 5, S18.

11.3.1.18. Randomized Placebo-Controlled Multicenter Study on the Effect of PalmitoylEthanolamide and Polydatin on Immune Activation in Patients With Irritable Bowel Syndrome.

- Authors: Giovanni Barbara, Cesare Cremon, Lara Bellacosa, Roberto De Giorgio, Javier Santos, María Vicario, **Marc Pigrau**, Carmen Alonso, Stanislas Bruley des Varannes, Michel Neunlist, Daniele De Filippis, Vincenzo Di Marzo, Teresa Iuvone, Roberto Corinaldesi, Vincenzo Stanghellini.
- Name of the conference: Digestive Diseases Week 2014. Annual Meeting of the American Gastroenterological Association Institute.
- Date of event: 05/2014.
- Organizing entity: American Gastroenterological Association Institute.
- Type of presentation: Poster presentation.
- Published in: Gastroenterology, May 2014; Vol. 146, Issue 5, S124.

11.3.2. Presentations from Farcombe Familiy Digestive Health Research Institute

11.3.2.1. Gut microbiota-diet interactions in a humanized mouse model of IBS: the role of intestinal mast cells

- Authors: Chiko Shimbori, Giada De Palma, David E. Reed, **Marc Pigrau**, Jun Lu, Yong Zhang, YANG YU, Nestor N. Jiménez-Vargas, Jessica Sessenwein, Cintya D. Lopez Lopez, Josue O. Jaramillo Polanco, Elena F. Verdu, Alan E. Lomax, Michael Beyak, Stephen M. Collins, Stephen Vanner, Premysl Bercik.
- Name of the conference: Digestive Diseases Week 2018. Annual Meeting of the American Gastroenterological Association Institute.
- Date of event: 06/2018
- Organizing entity: American Gastroenterological Association Institute.
- Type of presentation: Oral presentation.
- Published in: Gastroenterology, May 2018; Vol. 154, Issue 6,S-182.

11.3.2.2. Gut microbiota defines host responses to dietary fermentable carbohydrates in IBS: the role of bacterial histamine.

- Authors: Giada De Palma, David E. Reed, Chiko Shimbori, Yong Zhang, **Marc Pigrau**, Yang yu, Jun Lu, Marc Louis-Auguste, Nestor N. Jiménez-Vargas, Sacha Sidani, Cintya D. Lopez Lopez, Jessica Sessenwein, Josue O. Jaramillo Polanco, Elena F. Verdu, Karen Madsen, Alan E. Lomax, Michael Beyak, Stephen M. Collins, Stephen Vanner, Premysl Bercik.
- Name of the conference: Digestive Diseases Week 2018. Annual Meeting of the American Gastroenterological Association Institute.
- Date of event: 06/2018
- Organizing entity: American Gastroenterological Association Institute.
- Type of presentation: Poster presentation.
- Published in: Gastroenterology, May 2018; Vol. 154, Issue 6,S-565.

11.3.2.3. Nutritional wheat amylase trypsin inhibitors exacerbate gluten-induced pathology and alter the gut microbiota in mice.

- Authors: Justin McCarville, Victor F. Zevallos, Alberto Caminero Fernandez, Marc Pigrau, Jennifer Jury, Joseph A. Murray, Premysl Bercik, Detlef Schuppan, Elena F. Verdu.
- Name of the conference: Digestive Diseases Week 2017. Annual Meeting of the American Gastroenterological Association Institute.
- Date of event: 05/2017.
- Organizing entity: American Gastroenterological Association Institute.
- Type of presentation: Oral presentation.
- Published in: Gastroenterology, April 2017; Vol. 152, Issue 5, S71.

11.3.2.4. Diet-microbiota interactions underlie symptoms' generation in IBS.

- Authors: Giada De Palma, David E. Reed, Marc Pigrau, Jun Lu, Sacha Sidani, Yong Zhang, Yang Yu, Nestor N. Jiménez-Vargas, Jessica Sessenwein, Cintya D. Lopez Lopez, Josue O. Jaramillo Polanco, Elena F. Verdu, Stephen M. Collins, Alan E. Lomax, Michael Beyak, Stephen Vanner, Premysl Bercik.
- Name of the conference: Digestive Diseases Week 2017. Annual Meeting of the American Gastroenterological Association Institute.
- Date of event: 05/2017.
- Organizing entity: American Gastroenterological Association Institute.
- Type of presentation: Poster presentation.
- Published in: Gastroenterology, April 2017; Vol. 152, Issue 5, S160.

11.3.2.5. The central role of the gut microbiota in chronic intestinal pseudo-obstruction.

- Authors: S. Sidani, G. De Palma, M. Pigrau, J. Lu, E.F. Verdu, N. Causada Calo, C. H. Lee, S. M. Collins, P. Bercik.
- Name of the conference: 24th United European Gastroenterology Week 2016, Vienna, Austria.
- Date of event: 10/2016
- Organizing entity: United European Gastroenterology.
- Type of presentation: Oral presentation.
- Published in: UEG Journal, October 2016; Volume 4, Issue 5_suppl.

11.3.2.6. High Salt Diet Increases Susceptibility to Experimental Colitis: A Putative Role of Gut Microbiota

- Authors: Pedro M. Miranda, Viktoria Serkis, Giada de Palma, **Marc Pigrau**, Jun Lu, Stephen Collins, Premysl Bercik.
- Name of the conference: Digestive Diseases Week 2016. Annual Meeting of the American Gastroenterological Association Institute.
- Date of event: 05/2016.
- Organizing entity: American Gastroenterological Association Institute.
- Type of presentation: Poster presentation.
- Published in: Gastroenterology, April 2016; Vol. 150, Issue 4, S583

11.3.2.7. HLA-DQ8 celiac susceptibility gene is important in the development of behavioural and motility changes associated with gluten sensitivity

- Authors: **M. Pigrau Pastor**, G. DePalma, S. Sidani, P. Miranda, J. Lu, J. McCarville, E. F. Verdu, S. M. Collins, P. Bercik.
- Name of the conference: 23th United European Gastroenterology Week 2015, Barcelona, Spain.
- Date of event: 10/2015
- Organizing entity: United European Gastroenterology.
- Type of presentation: Oral presentation.
- Published in: UEG Journal, October 2015; Volume 3, Issue 5_suppl.

11.3.2.8. Gut Microbiota and IBS: From Associations to Causality

- Authors: De Palma Giada, Lynch Michael, Lu Jun, Dang Vi, Deng Yikang, Jury Jennifer, Umeh G., Miranda Pedro, **Pigrau Mark**, Sidani Sacha, Moreno-Hagelsieb Gabo, Surette Michael, Bergonzelli Gabriela, Verdu Elena F., Britz-McKibbin Philip, Neufeld Josh D., Collins Stephen M.,Bercik Premysl
- Name of the conference: 15th International Conference on Ulcer Research (ICUR).
- Date of event: 10/2015
- Type of presentation: Oral presentation.
- Published in: Digestive diseases and sciences, September 2015; Volume 30, Issue 9, Page: 2560.