UNIVERSITA' DEL PIEMONTE ORIENTALE AMEDEO AVOGADRO

Dipartimento di scienze della salute

Scuola di Dottorato in scienze e Biotecnologie mediche XXXIV ciclo

Role of ER Stress in the pathogenesis of Celiac Disease

Candidate: Romina Monzani

Tutor: Prof. Marco Corazzari

ABSTRACT

Celiac disease (CD) is a complex inflammatory and auto-immune disorder triggered by the ingestion of gluten, a heterogeneous mixture of seed-storage proteins, such as gliadins, present in cereals as wheat, barley, rye and oats, in genetically predisposed individuals. The disease occurs in 1% worldwide population, and its onset has genetic, immunological and environmental components. Currently, the molecular mechanisms through which gliadin triggers the CD onset are not yet completely clear. The only treatment for the disease is represented by a gluten-free diet (GFD) which is not 100% effective and is difficult to adhere by patients.

In this study, we demonstrated that gliadin stimulation induces ER stress in IEC, by using both in vitro and ex vivo models. Importantly, our results indicate that ER stress has a key role in the pathogenesis of CD.

At molecular level we found that extracellular gliadin peptides interact with CXCR3 at plasma membrane level which, in turn, induces the release of IP3 through the stimulation of PLC. The interaction of IP3 with IP3R onto ER membranes results in calcium release by the ER compartment thus inducing ER stress. Of note, buffering the gliadin-induced ER stress by the chemical chaperone 4PBA completely abrogates the cytopathic effects of gliadin.

Moreover, we also show that gliadin-induced ER stress is responsible for: i) CXCR3 gene expression upregulation, through CHOP; ii) TG2 gene expression upregulation, through both canonical and non-canonical activation of NF-kB; CFTR downregulation, trough ATF6; iv) altered intestinal permeability; and v) induction and release of pro-inflammatory cytokines.

Therefore, collectively our results indicate that ER stress might represent a valuable target to design a new clinical therapeutic approach to treat CD patients.

INTRODUCTION

Celiac Disease is a chronic disease primarily affecting the gastrointestinal tract (gut) and characterized by compromised peripheral immunotolerance, dysfunction of the intestinal barrier, and gut inflammation resulting in tissue damage, altered gut permeability, malabsorption, and other systemic problems.

INTESTINAL BARRIER

The intestinal barrier represents the first line of defence of body toward the external environment and consisting of both physical and chemical components such as pathogens, toxins and unhealthy compounds. Moreover, it represents an extremely important interface with the external compartment and responsible for fundamental activity necessary to sustain the organismal healthy life such as gas and water exchange, nutrient adsorption and bidirectional communications with the gut microbiota ecosystem. Thus, this is a very complex structure, composed of physical and immunological elements, essential for its proper functions. The physical element is represented by the external mucous layer facing the commensal intestinal microbiota, by antimicrobial proteins (AMP) and by secretory immunoglobulin A (sIgA); beneath it is a single cell layer consisting of specialized epithelial cells, and deeper there is the internal lamina propria where innate and adaptive immune cells such as T cells, B cells, macrophages and dendritic cells are present (Figure 1)[1,2].

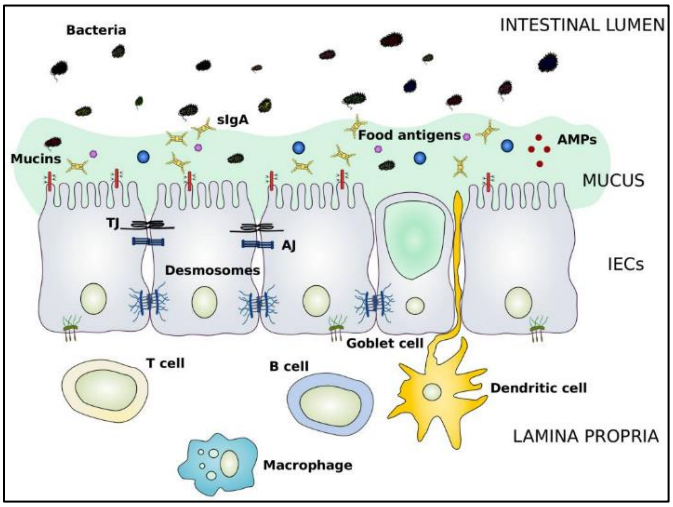


Figure 1. Schematic representation of the main components of the intestinal barrier. *The intestinal barrier absorbs essential nutrients and recognizes pathogens and bacteria. This complex function is allowed by both structural and molecular components. The outer inner is formed by mucus, composed by commensal gut microbiota, antimicrobial proteins (AMPs) and secretory immunoglobulins A (sIgA). Under mucus layer the intestinal epithelial cells form a differentiated monolayer in which cells are connected to each other by the Tight junctions (TJs), the Adherents junctions and by Desmosomes. These contribute to maintain the integrity of intestinal barrier. Under intestinal epithelial cells is present the lamina propria, which contains immune cells from innate and adaptative immune system, responsible for the immunological defence mechanisms of the intestinal barrier*[3].

STRUCTURE OF THE INTESTINAL BARRIER

The intestinal barrier, as described above, is composed by three layers, with the mucus layer representing the outermost component. This represents the first physical defence against external molecules and pathogens that reach the intestinal lumen, prevents direct contact with epithelial cells[1] and is mainly composed of glycosylated mucin proteins that cover the epithelial cell layer[4]

Under the mucus layer, the epithelial monolayer is composed of a pool of pluripotent stem cells from which absorbent enterocytes, goblet cells, enteroendocrine cells, Paneth cells and microfold cells originate[5]. These cells are physically connected to each other, thus forming a continuous and polarised monolayer. Molecular traffic within and through epithelial cells is tightly and finely regulated[1]. In particular, the intercellular junctions, such as Tight junctions (TJs), adherens junctions (AJs), and desmosomes, are deputed to the regulation of liquid and solid movements between intestinal epithelial cells[6] (Figure 2).

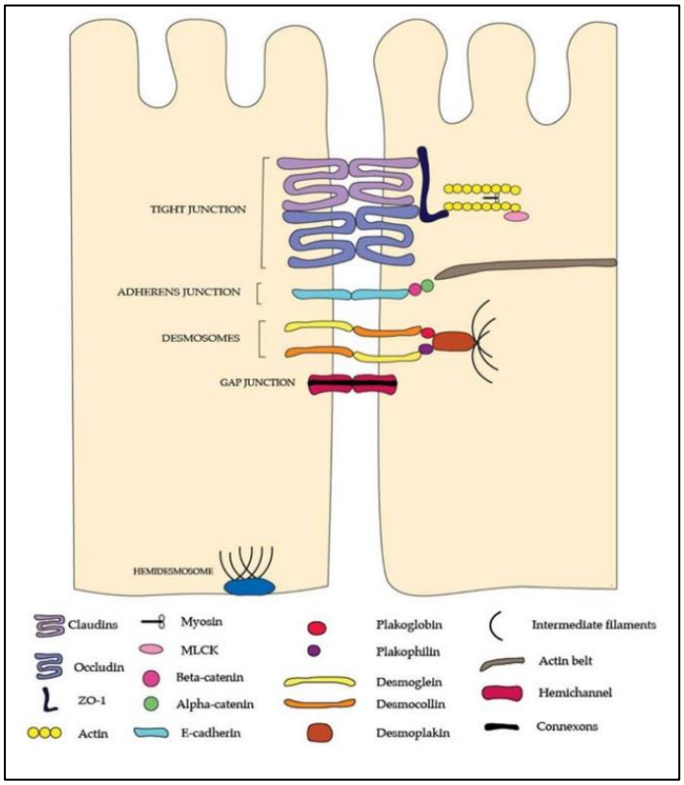


Figure 2. The junctional complexes of the intestinal barrier. *Among the junctional complexes there are the Tight junctions composed by Claudins and Occludin, which are connected to actin cytoskeleton. Then there are Adherens junction constituted by E-cadherin proteins and beta-catenin, and alpha catenin anchored to the actin cytoskeleton. The deepest junctional complexes at the basolateral end of the epithelial cells are the desmosomes and hemidesmosomes which attach epithelial cells to each other and also to the basement membrane, respectively. The main role of tight junctions is the regulation of the absorption of selective ions from the lumen of the gut. Adherens junctions, desmosomes, and hemidesmosomes give the mechanical and tensile strength to the barrier* [7].

INTESTINAL INTERCELLULAR JUNCTIONS

The functioning of the Intestinal epithelial barrier depends on the presence of intercellular junctions mentioned above. TJs and AJs are specific structures which governing both communication and adhesion between cells. The TJs are located at apical side of epithelial cells membranes and their main function is represented by a paracellular gate that limits the diffusion of molecules depending from size and charge [8]. The principal components of TJs are claudins, occludins, and junctional adhesion molecules[9]. Claudins are transmembrane proteins with different functions: sealing claudins, responsible for the tightness, and pore-forming claudins[10] (Figure 3). Sealing tight junction proteins include claudins 1, 3, 4, 5, 7, 8, 11, 14, 18, and 19[11] and allow the passage of macromolecules and ions, whereas claudins 2, 10a/10b, 15, 16, and 17 form selective pores for ions and water[12]. Moreover, claudins have also an important role in maintaining intestinal epithelial barrier in physiological ad correct form, being associated to cytoskeleton actin filaments through scaffold binding protein as ZO-1 and ZO-2[8]. Non physiological conditions leading to an imbalanced intestinal homeostasis are associated to altered claudins compositions and expression. Indeed, it has been recently reported a dysregulated expression of claudins associated with the pathogenesis of several intestinal diseases such as Celiac disease and IBD[13,14].

Figure 3 summarizes the main features of claudins and their role in human intestinal diseases[15].

Claudin	Size (kDa) (68)	Localization	Functions	Ions permeability	Interactions (68)	Role in disease
1	22.8	Ubiquitary (83)	Barrier forming	↓ Cations	It plays a general role in preventing the loss of water and macromolecules	↓ In UC (73–75), food allergy (76), liver cirrhosis (77), and IBS-D (78)
2	24.4	Intestinal crypts (79), Proximal renal tubule (80), choroid plexus (81), Human ovarian surface epithelium (82)	Pore forming	↑ Cations	It is involved in the regulation of Na^+, Cl^-, Ca^{2+} and water	↑ UC (83), celiac disease (84), IBS-D (85) ↑↓ Crohn's disease (86)
3	23.3	Human gallbladder (87) Brain capillary endothelium (88) Tighter segments of nephron (89) Liver/intestinal epithelial cells (79)	Barrier forming	↓ Cations	It is involved in the reduction of paracellular permeability of large molecules and in the formation of the blood-brain barrier	↓ Crohn's disease, acute colitis (90) ↑ Celiac disease (84)
4	22.1	Kidney and lung (68) Human gallbladder (87) Stomach, small intestine and colon (91)	Barrier forming	-	It can act as a Na^+ barrier or, interacting with claudin-8, as an anion (Cl^-) pore. In the colon strengthens tight junctions	↓ Acute colitis (73, 83) ↑ NCGS (92)
5	31.6	Endothelial tissue: endothelial cells (93) and brain capillary (94) Epithelial tissue: Human ovarian surface epithelium (82) Human colon epithelium (95)	Barrier forming	↓ Cations	It is involved in permeability of small molecules (≈800 Da)	↓ Acute colitis, Crohn's disease (96), Celiac disease (84, 96)
7	22.4	Epithelial tissue: Duodenum, jejunum, ileum, colon (97) Human palatine tonsillar epithelium (98) Nephron segments primarily at the basolateral membrane (99)	Barrier forming	↓ Anions	It plays a role in the regulation of Na^+, Cl^-, K^+ and water	↓ UC (83), Celiac disease (84, 96)
8	24.8	Epithelial tissue: Duodenum, jejunum, ileum, colon (100) Distal nephron (99)	Barrier forming	↓ Cations	It can act as a Na^+ barrier or Cl^- pore, depending on the cell type	↓ Crohn's disease (86)
12	27.1	Brain endothelial cells (94) Duodenum, jejunum, ileum, colon (97)	-	↑ Cations	It increases permeability to Ca^{2+}	↑↓ Crohn's disease (101)
15	24.4	Kidney endothelial cells (89) Intestine (duodenum, jejunum, ileum, colon) (97)	Pore forming	↑ Cations	It can act as a Na^+ channel or Cl^- barrier, depending on the cell type; it is involved in paracellular K^+ absorption and Na^+ secretion	↑ Celiac disease (84, 96)
18-2	27.7	Stomach (102) and bone cells (103)	Barrier forming	↓ Cations	It acts as selective barrier against Na^+ and H^+, protecting the epithelium from low pH	↓ Gastric cancer (104)

Figure 3. Characteristics of claudins and their expression in human intestinal diseases.

Occludin is a transmembrane protein and the first TJ protein to be identified[16], which is associated to paracellular transport regulation.

ZO-1, ZO-2, and ZO-3 are intracellular scaffold proteins[17], which bind actin filaments and serve as scaffolds linking TJ strands to the cytoskeleton[18,19], a key role in the maintenance of TJs structure and function[20].

Finally, JAMs are transmembrane proteins located to the apical cell-cell contacts and associated with TJs.

Therefore, gut barrier permeability is controlled by epithelial TJs and their disassembly results in dysregulated tissue permeability, a condition that occurs in several human diseases characterized by gut inflammation, such as Celiac Disease and in IBD[14,13].

INTESTINAL IMMUNE SYSTEM

The intestine is the largest compartment of the immune system. It constitutes a defence barrier toward bacteria and pathogens, and maintains tolerance to both diet components and commensal microbiota[21]. The majority of immunological processes take place in the mucosa, which comprises the epithelium, the underlying lamina propria and the muscularis mucosa, which is a thin muscle layer below the lamina propria. The latter contains many cells of the innate and adaptive immune system and here there is a large population of lymphoid cells[22].

Specialized epithelial M cells are found in the epithelium on Peyer's patches (PP)[23] and recognize antigens and everything passing through the intestinal lumen[24]. Antigen are taken by presenting cells (APCs), such as dendritic cells (DCs), which mature and present antigens to naïve CD4+ T cells, thus activating the antigen-specific T helper. CD4+ T cells also interact with B cells in the PP and mesenteric lymph nodes (MLN), leading to the activation and expansion of B cells to become antibody-producing plasma cells.

When CD4+ T cells and B cells are active, they migrate to the lamina propria as effector cells or antibody-producing plasma cells. Other immune cells are also present in the lamina propria, such as granulocytes; this

population together with that of migratory DCs and macrophages, has a crucial role in the adaptative immune response.

Other important immune cells in the epithelial barrier are the intraepithelial lymphocytes (IELs), specialized T cells representing the first line of defence for invading pathogens. They are classified on the basis of their phenotype into two subpopulations: induced IELs (iIELs) and natural IELs. Both have a cytolytic function and produce pro-inflammatory cytokines such as IFNγ, TNFα, IL-6 and IL-17 in response to a variety of pathogens[25].

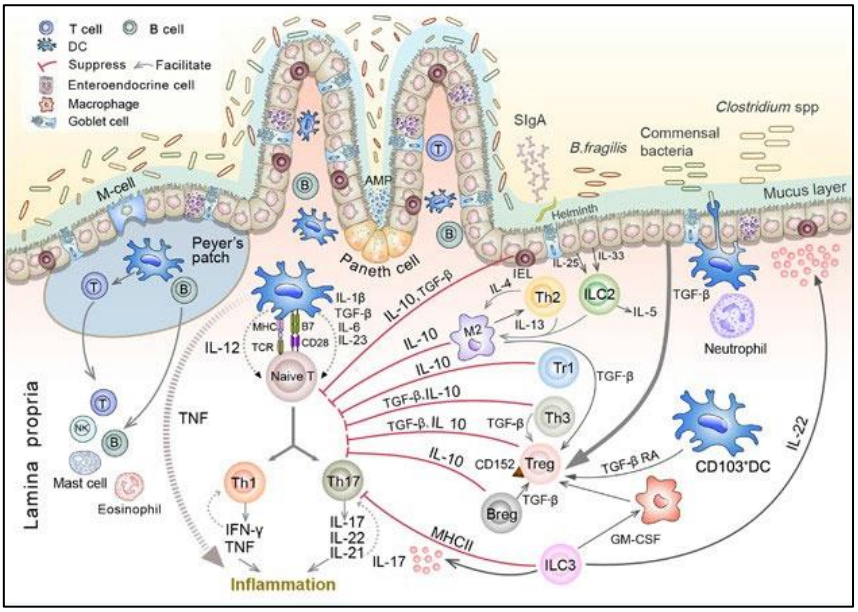


Figure 4. The immune system in the lamina propria of gut. *Schematic representation of the immune system in the intestinal compartment. The epithelial barrier is composed by TJ complexes, mucus layer, secretory immunoglobulins A, antimicrobial proteins produced by plasma cells, and Paneth cells. When pathogens are taken by APC cells, they stimulate T helper cells activation, thus producing pro-inflammatory cytokines (IFNγ, TNF, IL-17, IL-22 and IL-21)*[26].

CELIAC DISEASE

Celiac disease (CD) is an autoimmune disease characterize by a permanent intolerance to gluten proteins from wheat, rye, and barley, and occurring in about 1% of individuals worldwide[27].

In genetic predispose individuals bearing the human leukocyte antigen (HLA) DQ2/DQ8, the ingestion of gluten proteins switches tolerance toward an adaptive immune response with an autoimmune component[28,29].

CD is characterized by a chronic inflammation at the level of intestinal mucosa and this results in a damaged intestinal barrier[14]. In particular, gliadin peptides interaction with IECs results in dysregulated intestinal barrier permeability, immune cells activation, release of pro-inflammatory cytokines and TJs disassembly [30,31].

CD DIAGNOSIS

The first line for CD diagnoses is represented by serological blood tests, to reveal the presence of anti-tissue transglutaminase (tTG or TG2) immunoglobulin A (IgA). This test has a sensibility of 94% and a specificity of 97%, and is used in patients over 2 years of age[32]. In case of IgA deficiency, IgG anti-tTG and anti-deamidated gliadin peptides (DGP) tests are recommended, with the latter test recommended in patients under 2 years of age due to its better performance. Finally, analysis of anti-endomysial (EMA) IgA is used as a confirmatory test[33]. All serological tests should be performed in patients on gluten-containing diet, and they are directly connected to the level of histological lesions: CD patients with low intestinal damage may also have positive seronegative results.

The HLA-DQ2/DQ8 test can be used to confirm or exclude CD diagnosis in patients with an equivocal histopathological result or to rule out CD in other at-risk groups[34]. Patients with high suspicious of CD, with both positive or negative serology, are subjected to duodenal biopsy, which can confirm diagnosis. Generally, two biopsy specimens are collected from the duodenal bulb and four from the distal duodenum[33]. The two hallmarks in a biopsy derived from CD patients are IELs infiltration and villous atrophy, a consequence of mucosal inflammation. The histopathological changes were introduced for the first time by Marsh[35] and then modified by Oberhuber and colleagues[36] (Figure 5) as follows:

- M0: normal villous
- M1: increased IELs
- M2: increased IELs and crypt hyperplasia
- M3a: partial villous atrophy
- M3b: substantial villous atrophy

- M3c: total villous atrophy

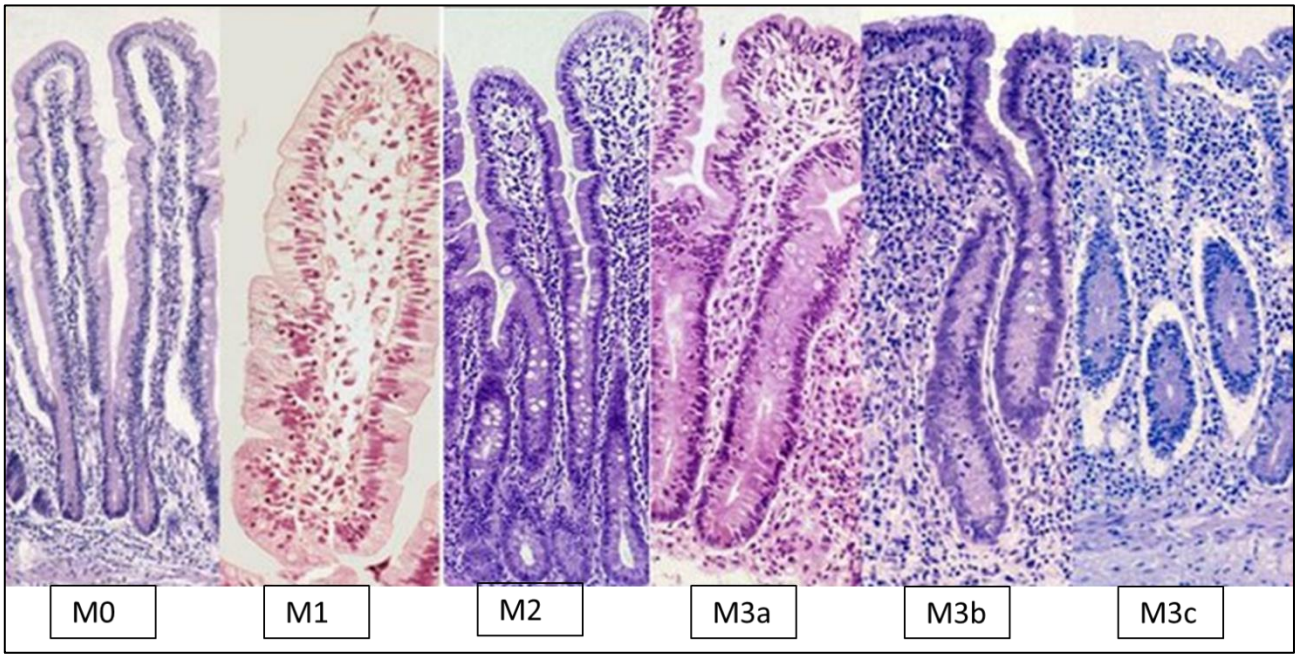


Figure 5. Marsch modified classification. *M0: normal villous, M1: increased IELs, M2: increased IELs and crypt hyperplasia, M3a: partial villous atrophy, M3b: substantial villous atrophy, M3c: total villous atrophy.*

This classification has been recently simplified by Corazza and colleagues[37] according to villous morphology, as:

- A: non-atrophic
- B1: atrophic, villous-crypt ratio <3:1
- B2: atrophic, villi no longer detectable

CLINICAL ASPECTS

In CD patients, gluten containing diet causes inflammatory damages to the mucosa of the small intestine resulting in villous atrophy[38].

Interestingly, clinical manifestations of CD can vary with age. The time from gluten exposure to the onset of symptoms is one of the criteria for the differential diagnosis of CD[39]. According to the clinical manifestation, Ludvigsson and colleagues[40] have described different types of celiac disease that can present different symptoms and can emerge at different times in a person's life:

- Typical CD: patients showing symptoms connected to malabsorption of nutrients such as chronic diarrhoea, weight and energy loss, lack of appetite, sickness and bloating. This is the most frequently encountered form of CD.
- Asymptomatic CD: patients do not manifest any common CD symptom and are not severely affected by any of the symptoms that could refer to the disease, but they present a damaged small intestine. These patients ameliorate their condition when subjected to a strict gluten-free diet.
- Atypical CD: is known as silent celiac disease, in which patients present mild gastrointestinal symptoms without clear signs of malabsorption. They may suffer from abdominal bloating and pain, with other symptoms: iron-deficiency anaemia, chronic fatigue, chronic migraine, and vitamin and mineral deficiency (including vitamin B12 and calcium), difficulty losing weight, late menarche/early menopause and unexplained infertility, dental enamel defects, depression and anxiety, and dermatitis herpetiformis, an itchy skin rash.
- Refractory CD (RCD): despite patients are under gluten-free diet, are present symptoms of malabsorption and villous atrophy. RCD can be divided in two categories: RCD1 (normal immunophenotype), that usually responds to gluten free diet and pharmacological supports (corticosteroids), and RCD2, which present an incomplete response to clinical management and poor prognosis characterized by increased risk to develop ulcerative jejunitis and enteropathy-associated T-cell lymphoma (EATL)[41].
- Latent CD: generally, refers to those patients with normal intestinal mucosa but positive serology.
- Potential CD: patients are positive to serological test, are characterized by normal intestinal mucosa but have increased risk to develop the disease. This term is also used for patients with an increased number of IELs in the villi.

CD TREATMENT

Actually, the only effective therapy consists of a gluten-free diet (GFD), containing a very low level of gluten. Only food and beverage with a gluten content less than 20 parts per million (ppm) is allowed. CD patients are

deficient in vitamins and minerals, indeed it is necessary a supplementation of vitamin B12, folate and Calcium, and this is important to prevent some complication, such as growth retardation, osteoporosis and abnormal dentition[42]. Many studies suggest that gradually introducing gluten in the diet can reduce the risk of CD development in the childhood[43].

THE PATHOGENESIS OF CELIAC DISEASE

CD is caused by environmental, genetic, and immunologic factors[44] with all these factors that are necessary for the disease to arise. The most important genetic factor connected to CD is the human leukocyte antigen (HLA) locus. The HLA-DQ2 (DQA1*0501-DQB1*0201) haplotype is expressed in 90% of patients, the DQ8 haplotype (DQA1*0301-DQB1*0302) in 5%, and 5% carry at least one of the two DQ2 alleles (usually the DQB1*0201)[45]. These alleles confer, in predisposed individual, the enhanced capacity to bind negatively charged peptides such as gliadin peptides, deamidated by TG2. The HLA antigen induces activation of T lymphocytes, whose secretion products play a key role in causing mucosal lesions[46].

Gluten is composed by two types of proteins, gliadin and glutenin, able of triggering CD, causing an immediate and transient increase in intercellular TJ permeability of intestinal epithelial cells[47]. Biochemically, gluten is rich of proline and glutamine residues, which make gluten resistant to the action of digestive enzymes, making it highly immunogenic and capable of damaging the intestinal epithelium[48]. Among peptides produced during digestion, the p31-43 leads to the upregulation of early markers of epithelial activation and leads to enterocyte apoptosis both in celiac intestine and in gluten-sensitive intestinal epithelial cell lines[49]. Furthermore, the p31-43 is the most immunogenic peptide in CD patients.

Gliadin, at level of intestinal mucosa, binds the chemokine CXC motif receptor 3 (CXCR3), leading to the activation of MYD88 and inducing the release of zonulin in the lumen. When gluten peptides cross the epithelial barrier, become a good substrate for tissue transglutaminase (TG2)[50], which plays a key role in CD pathogenesis. TG2 belongs to the transglutaminase family and carries out several enzymatic activities (Ca^{2+}-

dependent transamidating activity, GTPase/ATPase, protein disulfide isomerase, protein kinase), but also nonenzymatic functions based on its noncovalent scaffold interactions with many cellular proteins[51]. This is a multifunctional enzyme and plays a key role in several cellular processes, such as differentiation, survival, apoptosis, autophagy, and cell adhesion[52]. As reported above, TG2 recognizes undigested gluten residual peptides and removes from them an aminic group (deamination), thus generating negatively charged residues. They leads to innate immune system activation through upregulating the expression of IL-15 and promoting the CD4+ T cell adaptive immune response (figure 7)[53]. These peptides display a better ability to bind the HLA-DQ2/8 on APCs[30, 54], which present them to gluten specific CD4+ T cells. The latter cells become active and migrate to the small intestinal lamina propria, thus releasing pro-inflammatory cytokines, such as IFNγ, and metalloproteases; they also induce cryptal hyperplasia, villous atrophy, and IECs death induced by intraepithelial lymphocytes (IELs; figure 7). Moreover, active CD is also characterized by the upregulation and release of IL-15, causing the overexpression of the natural killer receptors CD94 and NKG2D by CD3+ IELs[55].

In active CD there is a continuous damage at the level of intestinal mucosa, due to autoimmune response to gluten peptides described above and mediated by cytotoxic IELs, thus causing crypt hyperplasia, which is not compensated by the regeneration activity of stem cells from the crypts[56]. Luciani and colleagues[57,58] demonstrated that the α-gliadin-derived peptide p31-43 drives an innate immune response leading to the activation of innate immunity in the intestine of CD patients and in gliadin sensitive epithelial cell lines (Caco-2 or T84 cells). Thus, gliadin peptides are able to stimulate the immune response of macrophages and dendritic cells by pattern recognition receptors (PRR), such as toll-like receptors (TLRs) 4[59]. This induces the secretion of pro-inflammatory cytokines, such as IL-1β, IL-8, TNFα, increasing the adaptative immune response for gluten and dysregulating the intestinal permeability[60].

A potential involvement of gliadin-stimulated endoplasmic reticulum stress (ER Stress) in IEC has been hypothesized by recent work from the Caputo's lab[61].

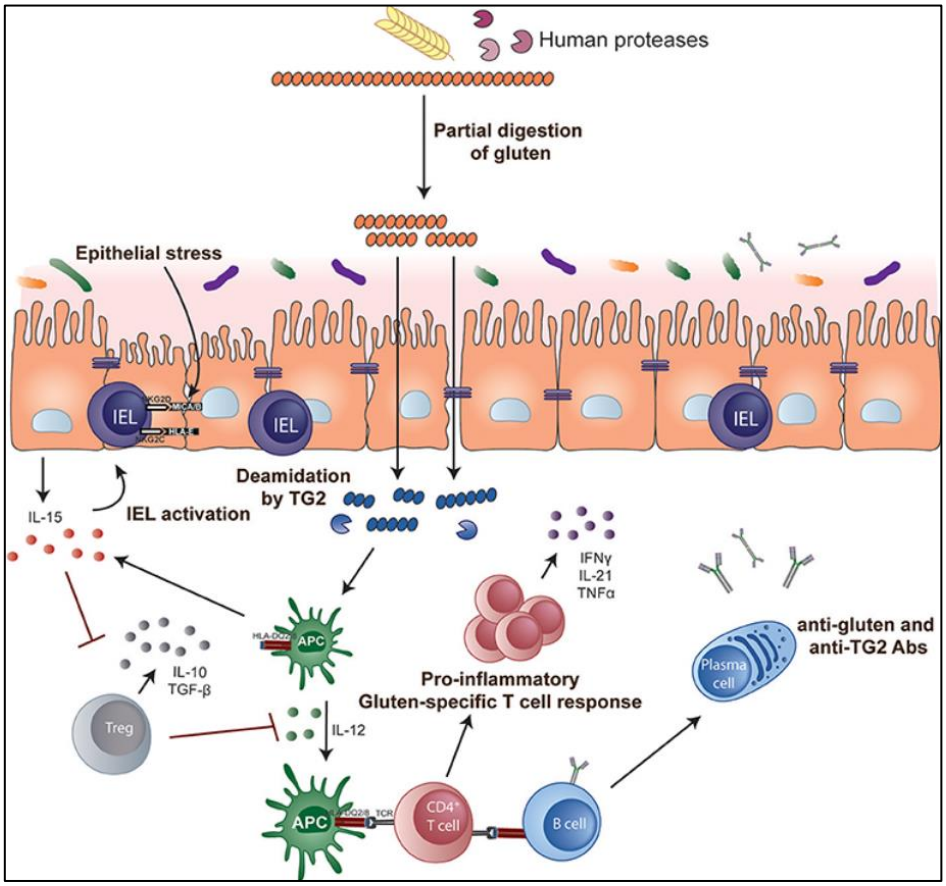


Figure 7. CD pathogenesis. *Gluten peptides are partially digested by intestinal enzymes. TG2 removes aminic group from gluten peptides, which highly recognize by HLA-DQ molecules on APCs. Activated gluten-specific CD4+ T cells secrete a variety of pro-inflammatory cytokines such as IFN-γ, TNFα and IL-21, thus promoting the intestinal damage, activation of IELs, and stimulate B-cell responses. Activated IELs differentiate in cytolytic NK-like cells responsible for further destruction of enterocytes expressing stress signals*[62].

ENDOPLASMIC RETICULUM (ER)

The ER is the largest organelle in the cell and performs many functions including protein synthesis and delivery, protein folding, lipid and steroid synthesis, carbohydrate metabolism and calcium storage[63,64]. All transmembrane proteins and lipids of cellular compartments are synthetized in the ER membranes, such as those of the Golgi apparatus, lysosomes, endosomes, secretory vesicles and the plasma membrane[65]. Moreover, the reticulum membrane participates to the formation of mitochondrial membranes and peroxisomes, producing most of their lipids.

Besides, ER has the important role in the synthesis and in the control of correct folding of newly synthetized proteins, and finally is the main intracellular Ca^{2+} store. In particular, secretory and membrane proteins are

synthesized on the rough endoplasmic reticulum thought ribosomes on the cytosolic side. When the nascent proteins are synthesized, they are concomitantly transferred into the lumen of ER, in which are N-glycosylated and folded. The latter is a process finely regulated by chaperones, which work together with protein disulphide isomerases (PDIs), contributing to ER homeostasis.

ER homeostasis can be compromised, promoting a condition known as ER stress[66]. Several stimuli lead to ER stress, and these can have physiological and pathological origins, such as hypoxia, the accumulation of unfolded proteins, glucose deficiency, viral infections, changes in calcium levels and cell differentiation. In the course of evolution eukaryotic cell has developed a conserved signalling pathway to counteract the accumulation of unfolded proteins, named Unfolded Protein Response (UPR)[66,67,68], which has the key role of restoring ER homeostasis. Grp78/BIP is the main chaperone of the ER which, in physiological conditions, given its high concentration, is able to bind both nascent or misfolded proteins and the three sensors of the UPR, PERK, ATF6 and IRE-1, keeping them in an inactive state (Figure 8)[66].

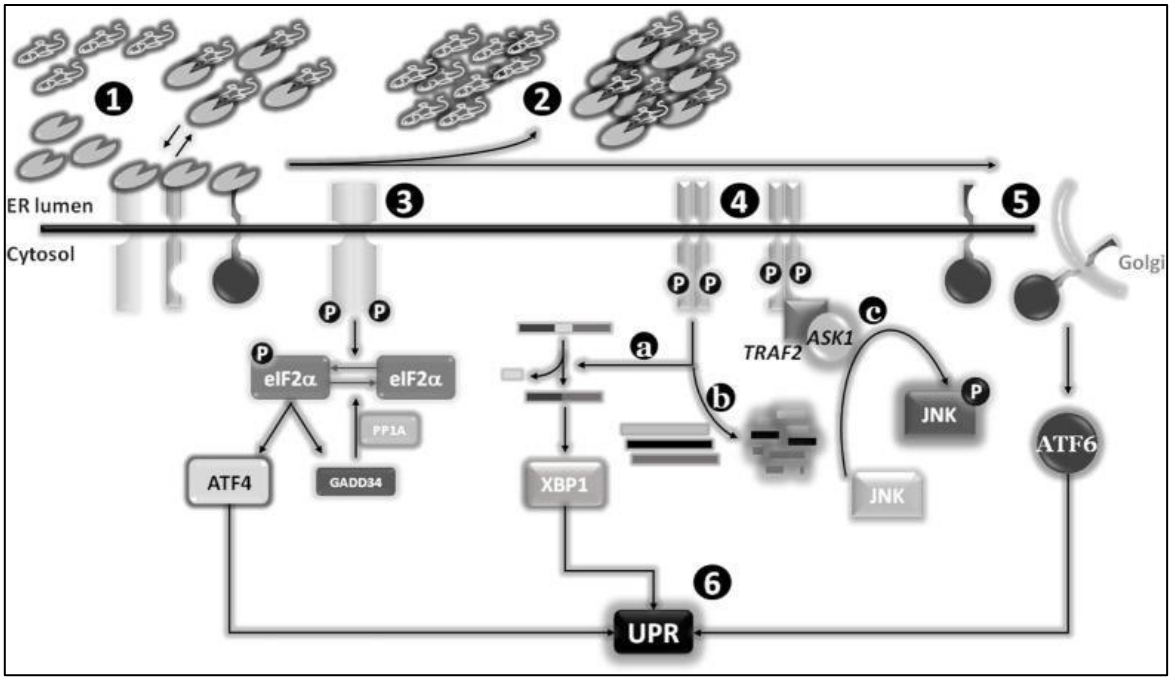


Figure 8. ER stress sensors, UPR activation, and signalling pathways. *Under physiological conditions, the ER chaperone Bip (GRP78) binds both the pool of newly synthesized proteins and the luminal domains of the three ER stress sensors PERK, IRE1, and ATF6, thus inhibiting their activity (1). Upon the increase or accumulation of unfolded or misfolded proteins into the ER lumen, the "Bip equilibrium" changes, resulting in its dissociation from the three ER stress sensors to increase the levels of "free" Bip to work as a chaperone (2). This results in the activation of the three sensors: PERK and IRE1 will form omo-dimers/multimers which after trans-phosphorylation will become active (3) and (4), whereas ATF6 will reach the Golgi apparatus were two*

proteases (SP1 and SP2) will release the cytosolic and active transcription factor (5). The activation of the three sensors will drive the "UPR" program (6) [66].

An increased presence of unfolded proteins results in the increased binding by Grp78/BIP, thus releasing of the three sensors, which become active. PERK and IRE1 homodimerize, undergo trans-phosphorylation and become active, while ATF6 translocates to the Golgi apparatus.

PERK is a type I transmembrane protein with a cytosolic serine/threonine kinase domain, and represents the most immediate sensor for ER stress[69]. Active PERK phosphorylates eukaryotic initiation translation factor 2α (eIF2α), increasing the cap-independent translation of many mRNAs, such as activating transcription factor 4 (ATF4). ATF4 activation has several effects, such as increasing the expression of antioxidant responsive genes, amino acid biosynthesis, and transport genes to sustain cell survival, but it can also promote the expression of the growth arrest and DNA damage-inducible protein 34 (Grp34), which upon interaction with PP1A dephosphorylates eIF2α, thus inducing the expression of C/EBP homologous protein (CHOP) to modulate the ER stress-dependent apoptosis. Therefore, PERK can modulate both survival or cell death based on the severity and duration of stress stimuli[70,71].

IRE1α is a type I transmembrane protein with a cytosolic serine/threonine kinase domain. It is the only sensor present in all eukaryotes and is the most conserved branch of UPR[72]. When active it promotes a kinase and endoribonuclease activity, which induces a non-conventional splicing of X-box-binding protein (XBP1) mRNA, to form a transcriptionally active mRNA, named XBP1s (spliced). XBP1 moves into the nucleus and regulates the expression of genes involved in protein folding, trafficking and the ER-associated protein degradation program (ERAD) process. XBP1s is involved in cell survival, in the expansion of the ER compartment[66], and apoptosis, promoting the activation of BIM and inhibiting the expression of Bcl-2[73].

ATF6 is a type II transmembrane protein and member of the leucine zipper family of transcription factors. When UPR is active, it translocates to the Golgi compartment, in which is cleaved by S1P and SP2 proteases, becoming a cytosolic active transcription factor. Mammals express two homologous ATF6 proteins: ATF6α is

a potent transcriptional factor driving the expression of chaperones and UPR mediators including Bip and XBP1[74], while ATF6β is involved in the inhibition of the α isoform[75,76].

Cells have developed a system to respond to stress and stimuli of different nature and can decide whether to adapt and survive or die.

Consequently, the ability to control the UPR response can be of crucial importance especially in the treatment of those pathologies characterized by an acute/chronic induction of ER stress, such as cancer, and chronic intestinal pathologies, such as celiac disease and inflammatory bowel disease.

AIMS

Celiac Disease is a multifactorial autoimmune disease characterized by chronic intestinal inflammation caused by the ingestion of gluten, in genetically predisposed individuals. Unfortunately, there are no effective treatments for CD patients to date. The only indication is based on a gluten-free diet (GDF) which has several disadvantages such as limited patient adherence and non-remission in many cases.

Therefore, it is urgent to define new potential clinical targets for designing new effective therapeutic approaches.

The aims of this project were therefore:

- To unveil the role of ER stress in the pathogenesis of CD
- To highlight the molecular mechanism(s) linking the interaction of gliadin peptides with IEC and the induction of ER stress

-

MATHERIAL AND METHODS

MATHERIAL AND METHODS

Cell lines and treatments

Human colon adenocarcinoma-derived Caco-2 cells were obtained from ATCC. Cells were maintained in T25 flasks in Dulbecco's Modified Eagle Medium (DMEM; Merck) supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine (Merck), and 1% penicillin/streptomycin (Merck). Cells were grown in 6-well plats (Sarsted) under normal condition. Cells were treated with PT (Merck) at final concentration of 1mg/ml, 4-phenylbutiric acid (4PBA, SantaCruz) 5uM, AEBSF (500 uM; Merck), GSK2606414 (5uM; Merck), or STF-083010 (60uM; Merck), BAY 11-7082 (5uM; Calbiochem), Cystamine (250uM; CMerck), Bapta-AM (10uM; Merck), U73122 (10 uM; Merck), AMG 487 (1uM; Merck).

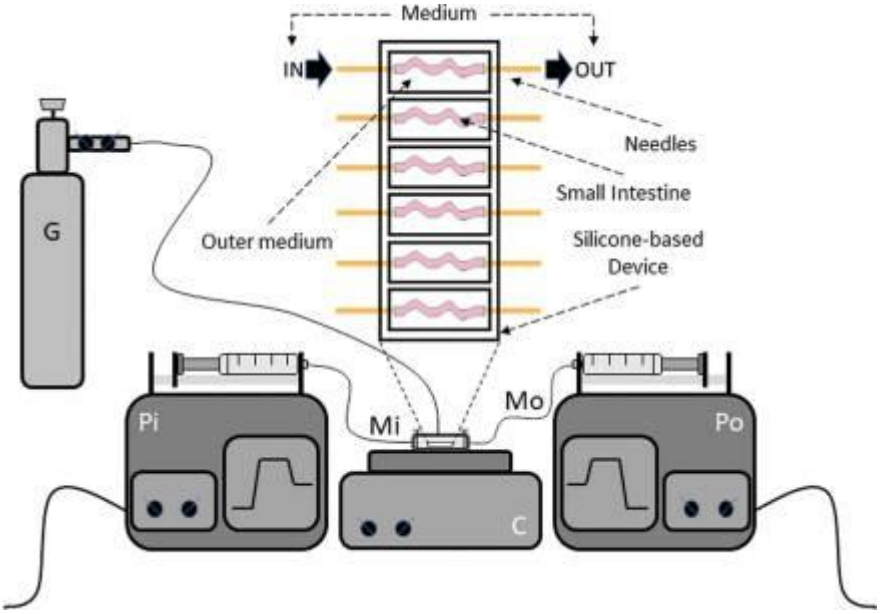
RNA interference

shCHOP (#29 or #93) and non-targeting scramble (shSCRAMBLE, used as negative control) siRNA oligonucleotides were obtained from Merck. A total of 25 x 10^4 cells/well were transfected with 1ug shRNA in six-well plates by using RNAiMAX reagent (Thermo) as recommended by the supplier. Total RNA was isolated after 48h and analysed by qPCR.

Silicone-Based Device and Organ Culture

Small intestine from 13 days old Balb/c mice, feed with a gluten-free diet for at least three-generation[14,15,81], were resected and cultivated in a silicone-based Gut-Ex-Vivo System (GEVS), as previously described[14,14]. Briefly, the custom-fabricated fluidic chip consists of a silicone-based device divided into 6 parallel isolated chambers to allocate mouse intestine (up to six). Tissue lumens were perfused with complete medium in a dynamic configuration, by using a coordinated infusing-drying pump. While the outer medium circuit is static. The temperature of the device was maintained at 37°C using a lab hot plate, and a mixture of Air/CO_2/O_2 was provided to the device from a compressed gas cylinder. Intestine were gently flushed with a sterile medium and fixed to the input and output ports. After inserting each tissue fragment into the

chamber, the device was placed on the heat-conducting adapter and sealed. A schematic representation of the system is reported below.



GEVS. *Pi infusing pump; Po sucking pump; Mi medium entering; Mo medium exit; G air/O2/CO2 cylinder.*

Small intestine Cultures and Treatments

Each intestine was infused with serum-free tissue culture medium containing IMDM, supplemented with 20% KnockOut serum replacement, 2% B-27 and 1% of N-2 supplements, 1% L-glutamine, 1% NEAA, 1% HEPES and stimulated with PT-gliadin (2 mg/ml; Merck), Thapsigargin (5.0 µg/mL, Merck), 4-phenybutirric acid (3uM, Merck), AMG (1uM or 5uM, Merck). The tissue culture medium was loaded into a 5 mL syringe and infused into the device input ports by a syringe pump (flow rate of 99 µL/h)[13]. All reagents were form EuroClone if not indicated.

PT preparation

Gliadin from wheat (Sigma) was digested, as described[77]. Briefly, whole gliadin (1 g/mL, Merck) was dissolved in 500 mL 0.2N HCl for two hours at 37° C with 1g pepsin (Merck). The peptic digest was further digested by adding 1g trypsin (Merck), and pH was adjusted to 7.4 using 2N NaOH; next, the solution was incubated at 37° C for four hours under vigorous agitation. Finally, the mixture (hereafter referred as PT) was boiled to inactivate enzymes for 30 min and was stored at -20° C.

Tissue viability assay

Tissue viability was evaluated as previously described[13], through MTS assay. Tissues were placed in a 48 well plate and incubated 4h in DMEM supplemented with 10% FBS, 2mM L-glutamine, 100 U/mL Penicillin, 0.1 mg/mL Streptomycin (Euroclone) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium solution following manufacturer's instructions. The absorbance of 100 µL of medium solution from each sample was measured by UV-VIS spectrophotometry (Victor X4, PerkinElmer), at 490 nm. Values were proportional to cell viability.

Permeability assay

Permeability test was performed by FITC-dextran (FITC D4000, Merck) released from the intestinal lumen into external medium. Briefly, medium containing 0,1ng/ml FITC-dextran was infused into intestine tissues for 16h. Medium of the outer chamber was then collected, and fluorescence was measured spectrophotometrically (SPARK Multimode Microplate Reader-TECAN) in 96-well plates (excitation: 485 nm, emission: 528 nm). FITC-dextran concentrations were calculated by using a standard curve obtained though serially diluted FITC-Dextran stock solution (0 ng/mL; 125 ng/mL; 250 ng/mL; 500 ng/mL; 1000 ng/mL; 2000 ng/mL; 4000 ng/mL; 8000 ng/mL). Then, 100 µL of each sample was transferred to a 96-well microplate. The FITC-Dextran concentration was obtained using the standard curve interpolation.

qPCR analysis

Trizol reagent (Thermo) was used to extract total RNA. ExcelRT™ Reverse Transcription Kit (Smobio) was used to produce cDNA following the manufacturer's recommendations. Quantitative PCR reactions were performed by using a CFX96 BioRad thermocycler. ExcelTaq™ 2X Fast Q-PCR Master Mix (Smobio) was used to produce fluorescently labeled PCR products during repetitive cycling of the amplification reaction and the melting curve protocol was used to check for probe specificity. Primer sets for all amplicons were designed using the IDT PrimerQuest Tool (https://eu.idtdna.com/Primerquest/Home/Index).

The sequences of human and mouse primers were as follow:

PRIMER MOUSE	FORWARD/REVERSE
CFTR	GTAGACACACCAGGAGTCTG/AGGAGGACAGGGATGATAAG
TG2	AAGAGCGAAGGGACATACT/TGAGCACAGACCCATCTT
ATF6	GATGGTGACAACCAGAAAGA/TGGAGGTGGAGGCATATAA
ATF4	GTTTAGAGCTAGGCAGTGAAG/CCTTTACACATGGAGGGATTAG
XBP1	AGTCCGCAGCAGGTG/GGTCCAACTTGTCCAGAATG
IL-15	CAGCAAGGACCATGAAGA/GGCTGAGTTCCACATCTAAC
IL-17A	CGCAATGAAGACCCTGATAG/CTTGCTGGATGAGAACAGAA
IFNγ	CCACATCTATGCCACTTGAG/CTCTTCCTCATGGCTGTTTC
GAPDH	TTCAACGGCACAGTCAAG/CCAGTAGACTCCACGACATA
ERdj4	CATCAGAGCGCCAAATCA/CCACTACCTCTTTGTCCTTTAC
GADD153	ACACGCACATCCCAAAG/ACCACTCTGTTTCCGTTTC

PRIMER HUMAN	FORWARD/REVERSE
CFTR	AAGCTGTCAAGCCGTGTTC/GATTAGCCCCATGAGGAGT
TG2	CACCCACACCTACAAATACC/CAAAGTCACTGCCCATGT
ATF6	TTTGCTGTCTCAGCCTACTGTGGT/TCCATTCACTGGGCTATTCGCTGA
ATF4	CCCGGAGAAGGCATCCTC/GTGGCCAAGCACTTCAAACC
XBP1	AGAGAAAACTCATGGCCTTGTAGTTG/CATTCCCCTTGGCTTCCG
IL-15	TACCGTGGCTTTGAGTAATG/AGATTGACTTCGACCGTAAG
IL-17A	CAAGACTGAACACCGACTAAG/CTATTACCGGGACTCCTTAC
IFNγ	TGGAGACCATCAAGGAAGA/CTTTGCGTTGGACATTCAAG
L34	GTCCCGAACCCCTGGTAATAGA/GGCCCTGCTGACATGTTTCTT
ERdj4	CATCAGAGCGCCAAATCA/CCACTACCTCTTTGTCCTTTAC
GADD153	GGAGCATCAGTCCCCCACTT/TGTGGGATTGAGGGTCACATC

Results were expressed as the threshold cycle (CT). The ΔCT is the difference between the CT for the specific mRNA and the CT for the reference mRNA, GAPDH (mouse) or L34 (human). To determine relative mRNA levels, 2 was raised to the power of ΔΔCT (the difference between the ΔCT from treated cells and the CT from untreated cells).

Immunoblot

The whole small intestine lysates were obtained by using the Cell Lytic buffer (Sigma) supplemented with a protease inhibitors cocktail (Sigma) plus phosphatases inhibitors (Na3VO4 1 mM; NaF 10 mM, Merck). Proteins were separated through SDS-PAGE electrophoresis and electroblotted onto nitrocellulose (Protran, Sigma) membranes. Membranes were incubated with indicated primary antibodies in 5% non-fat dry milk (Bio-Rad) in PBS plus 0.1% Tween20 overnight at 4°C. Primary antibodies were: anti-CFTR (clone M3A7 Abcam ab4067) 1:500, anti-TG2 (SantaCruz) 1:500, and anti-Actin (Sigma Aldrich) 1:5000. Detection was achieved using horseradish peroxidase-conjugate secondary antibodies (1:5000; Jackson ImmunoResearch)

and visualized by ECL plus (Amersham). Images were acquired by using a ChemiDoc™ Touch Imaging System (Bio-Rad) and analysed by Image Lab software (Bio-Rad), as previously described[15].

ELISA

ELISA was performed on tissue samples using standard ELISA kits (R&D Systems) for mouse IL-15, IL-17A, IFN-γ (Bio-Techne). According to the manufacturer's instructions, samples were read in triplicate at 450 nm in a microplate Reader (Bio-Rad) using Microplate Manager 5.2.1 software. Values were normalized to protein concentration evaluated by Bradford method.

Statistical analysis

All experiments were performed at least in triplicate and statistical analysis was performed using GraphPad Prism 6. The student-t test and ANOVA was used to determine statistical significance. A p-value equal to or less than 0,05 was considered significant.

RESULTS

RESULTS

1. CONFIRMING THE PT ABILITY TO INDUCE THE PATHOGENESIS OF CD USING OUR GEVS

We recently demonstrated that gliadin peptides, PT (see Mat&Met), can be efficiently used to stimulate the onset of Celiac Disease both *in vivo* and *ex vivo*, by using mice maintained on a GFD for at least three generations and a gut-*ex-vivo* system (GEVS)[14,78]. Indeed, we observed that PT stimulation results in tight junction proteins expression deregulation and TJ disassembly, compromised intestinal permeability, TG2 upregulation, pro-inflammatory cytokines upregulation and secretion, and villi erosion, all markers of human CD. Interestingly, we also recently demonstrated a link between CD and Cystic Fibrosis (CF), residing in the ability of PT to bind and drive the degradation of the Cystic Fibrosis Transmembrane Regulator (CFTR), the key factor which impaired activity is responsible for the onset of this human pathology, thus explaining, at least in part, why CD occurs at a higher frequency in patients with CF than the general population[27]. Importantly, we demonstrated that the PT-mediated CFTR protein degradation is paralleled by a gene expression modulation[78]. Finally, we also confirmed the Caputo's hypothesis that PT compromises the homeostasis of the Endoplasmic Reticulum, thus inducing a status known as ER stress, both *in vivo* and *ex vivo*[78,14]. Although this is not surprising, since ER stress has been involved in several inflammatory chronic diseases including diabetes, obesity, neurodegenerative diseases, atherosclerosis, arthritis, respiratory diseases, irritable bowel syndrome, cardiovascular diseases, cancer, and in many metabolic disorders[79-80], the molecular mechanism(s) and function in the onset and progression on CD is still unclear.

Therefore, to unveil the role of ER stress in the pathogenesis of CD at molecular level, we decided to use our Gut-*Ex-Vivo* system (GEVS), based on the cultivation of small intestine from Balb/C gluten-sensitive mice, as *ex vivo* system, and Caco-2 cells as *in vitro* system.

As shown in figure 1A and B, small intestine exposed to PT are characterized by CFTR down-regulation and TG2 up-regulation, compared to control, at both mRNA and protein levels. These alterations

were paralleled by upregulated expression of pro-inflammatory cytokines such as interleukin (IL)-15, IL-17A and interferon gamma (IFNγ) in tissue exposed to PT, compared to controls (Fig. 1C).

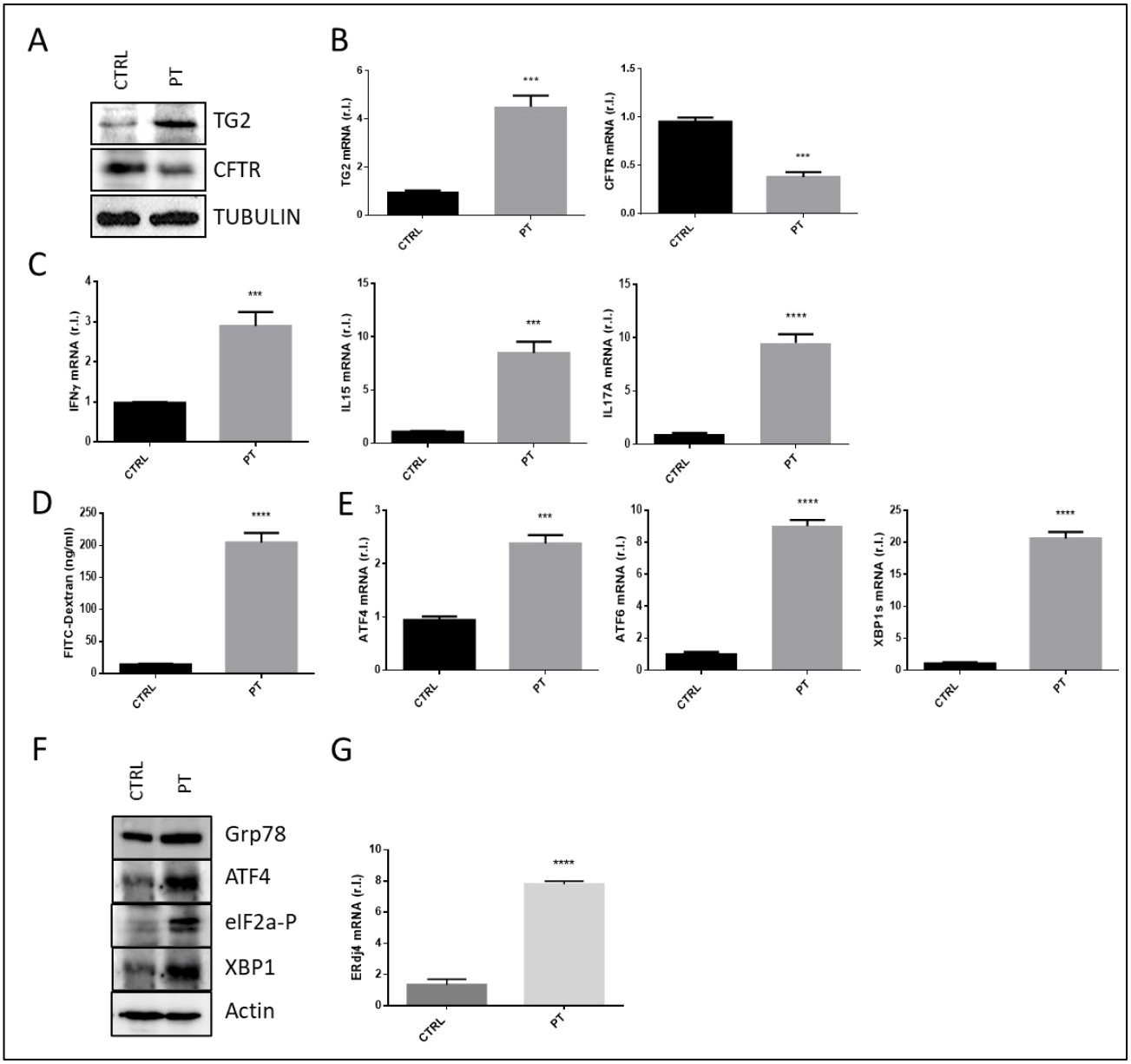


FIGURE 1. PT-induced CD markers in small intestines cultivated in a GEVS. *Small intestines from Balb/c mice were cultured 16 h in the gut-ex-vivo system (GEVS), unexposed (CTRL) or exposed to PT (2mg/ml), in complete medium. A) Immunoblot of whole tissue homogenates probed with anti-CFTR or anti-TG2 antibody; anti-Tubulin was used as loading control. B) CFTR and TG2 mRNA levels were evaluated by qPCR. C) The expression of IL-15, IL-17A and IFNγ were evaluated by qPCR. D) Small intestine permeability was evaluated by FITC-dextran release from the intestinal lumen into outer medium. E) Levels of ATF4, ATF6 and XBP1 were evaluated by qPCR. F) Immunoblot of whole tissue homogenates probed with anti-Grp78, anti-ATF4, anti-phospho-eIF2α, and anti-XBP1 antibodies; actin was used as loading control. G) ERdj4 mRNA level was evaluated by qPCR. Experiments were performed in triplicate and repeated three times. Histograms represent mean ± SD; *** p < 0.001; **** p < 0.0001.*

The compromised intestinal permeability was verified by measuring the amount of FITC-dextran released from the intestinal lumen into outer medium, as shown in figure 1D. Finally, we verified the

induction of ER stress, by measuring the expression of well-characterized markers, at both mRNA and protein levels. indeed, data reported in figure 1E clearly show the increased mRNA levels of ATF6, ATF4 and XBP1 in tissues exposed to PT, compared to untreated controls. Moreover, the increased expression of ATF4 and XBP1 was also confirmed at protein level, and the induction of ER stress was further confirmed by: the enhanced expression of Grp78 at protein level, ii) by the increased phosphorylation of eIF2α (Fig. 1F), and iii) the enhanced expression of the ATF6 downstream target ERdj4, at RNA level (Fig.1G).

2. ER STRESS INDUCTION RECAPITULATES THE KEY FEATURES OF CD

To define whether ER stress has a key role in the pathogenesis of CD or merely represents a side effect of gut exposure to gliadin peptides, we used the well-known ER stress inducer Thapsigargin (TG)[81] and evaluated the impact on the previously mentioned CD markers. To this aim, small intestine from gliadin sensitive mice were unexposed (DMSO, CTRL) or exposed to TG (5μg/ml), and tissues were recovered after 16h. Tissue viability was evaluated by an MTT assay and data reported in figure 2A (left panel) indicate that tissues were viable and that TG treatment does not significantly alter tissue viability, while the FITC-dextran assay evidenced a TG-related altered intestinal barrier function (Fig.2B, left panel). Next, total RNA was isolated after tissue homogenization, and qPCR analysis was carried out. The induction of ER Stress was evaluated by measuring the expression of the three main ER stress markers ATF4, ATF6 and XPB1. Data reported in Figure 2A clearly indicate that TG treatment efficiently induced ER Stress. Next, the TG-mediated impaired intestinal barrier function, evidenced by the FITC-dextran assay, was confirmed by the down-regulation of Occludin (OCLD) and the upregulation of both Claudin 15 (CLD15) and ZO-1 (Fig. 2B), key TJs components.

Then, we evaluated the expression of both TG2 and CFTR. Data shown in Figure 2C indicate a clear TG-dependent TG2 upregulation and CFTR downregulation. Finally, the expression and production of

the pro-inflammatory cytokines IL-15, IL-17A and IFNγ was evaluated at both mRNA (Fig.2D upper panels) and proteins (fig.2D bottom panels) levels.

Collectively these data indicate that the induction of ER Stress is sufficient to mimic the exposure of small intestine to PT, thus recapitulating the onset of CD.

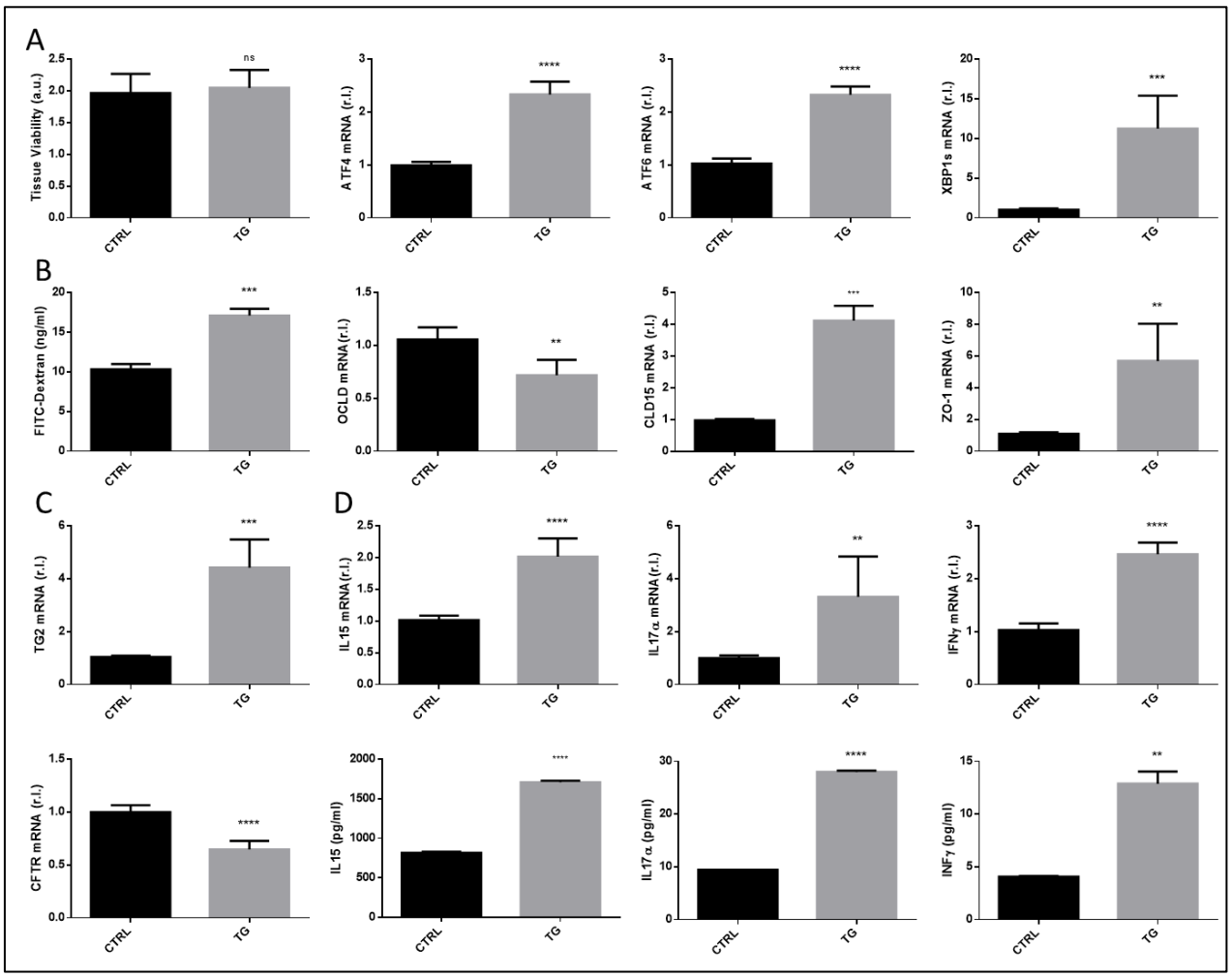


FIGURE 2. Tissue viability, Er stress, permeability, CD markers and pro-inflammatory cytokines expression in GEVS. *Small intestines from Balb/c mice were cultured 16 h in the gut-ex-vivo system (GEVS), unexposed (CTRL) or exposed to TG (5 ug/ml), in complete medium. A) Tissue viability (left) was evaluated by MTT assay while ATF4, ATF6 and XBP1 mRNA levels (left) were evaluated by qPCR. B) Intestinal permeability was evaluated by FITC-dextran release (left), while OCLD, CLD15 and ZO-1 mRNA levels were evaluated by qPCR. C) TG2 (upper panel) and CFTR (bottom panel) mRNA levels were evaluated by qPCR. D) The production of pro-inflammatory cytokines IL-15, IL-17A and IFNγ was evaluated at both mRNA (upper panels) or protein (bottom panels) levels by qRT-PCR analysis or ELISA, respectively, as indicated. Experiments were performed in triplicate and repeated three times. Histograms represent mean ± SD; ns non-significant; **p<0.01; *** p < 0.001; **** p < 0.0001.*

3. ER STRESS BUFFERING BY A CHEMICAL CHAPERONE INHIBITS THE ONSET OF CD

To further validate the above-reported data indicating a potential key role of ER stress in the pathogenesis of CD, we decided to inhibit the PT-induced ER stress by using the chemical chaperon

4-phenylbutyric acid (4PBA)[82], and evaluate the impact on CD onset. To this aim, small intestines from GS mice were cultivated in our GEVS for 16 hours in presence of complete medium (CTRL) or supplemented with PT (2mg/ml) or TG (5 ug/ml), alone or in combination with 4PBA (PT+4PBA or TG+4PBA - co-stimulation; TG or 4PBA alone were used as a control). Data reported in Figure 3A show the ability of 4PBA to prevent PT- or TG- induced ER stress, as demonstrated by the abrogation of ATF4, ATF6 and XBP1 upregulation induced by both compounds. In the same experimental condition, we also observed an abrogated PT/TG-dependent TG2 up-regulation and CFTR down-regulation, at mRNA level, in presence of 4PBA (Fig.3B left and right panels, respectively).

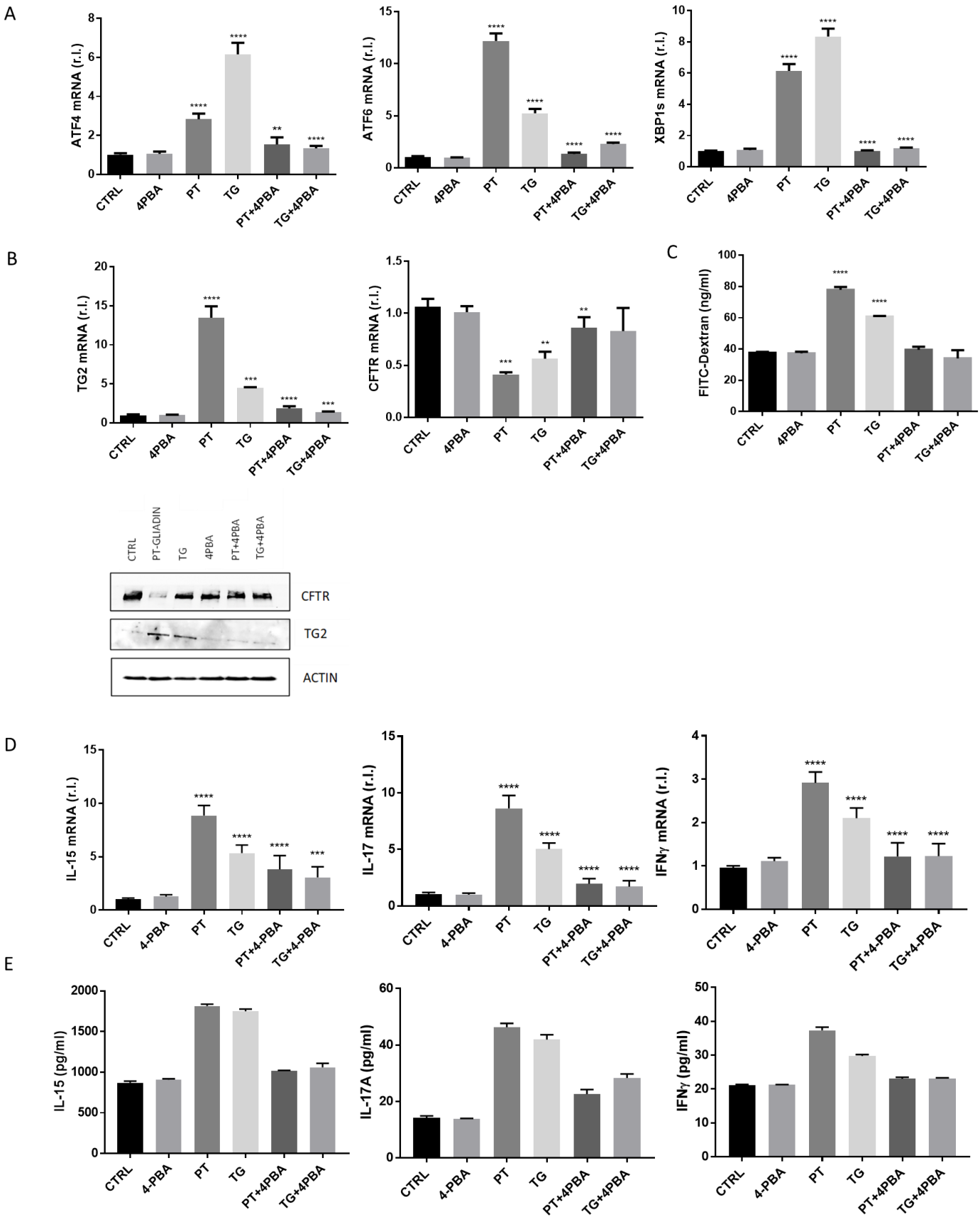

A
ATF4 mRNA (r.l.)
ATF6 mRNA (r.l.)
XBP1s mRNA (r.l.)
CTRL
4PBA
PT
TG
PT+4PBA
TG+4PBA
B
TG2 mRNA (r.l.)
CFTR mRNA (r.l.)
C
FITC-Dextran (ng/ml)
CTRL
PT-GLIADIN
TG
4PBA
PT+4PBA
TG+4PBA
CFTR
TG2
ACTIN
D
IL-15 mRNA (r.l.)
IL-17 mRNA (r.l.)
IFNγ mRNA (r.l.)
4-PBA
E
IL-15 (pg/ml)
IL-17A (pg/ml)
IFNγ (pg/ml)

FIGURE 3. ER stress buffering inhibits the impact of PT. *Small intestine cultivated in a GEVS for 16 hours and exposed to complete medium alone (CTRL) or supplemented with TG (5 ug/ml) or PT (2 mg/ml), or combined PT/TG and 4PBA (3 uM). The ER stress markers ATF4, ATF6 and XBP1 (A), TG2 and CFTR (B, upper panels) mRNA levels were evaluated by qPCR, while TG2 and CFTR protein levels were evaluated by wb analysis (B, bottom panel). Tissue permeability was evaluated by FITC-dextran release from the intestinal lumen into external medium (C). The production of pro-inflammatory cytokines IL-15, IL-17α and IFNγ were evaluated at both mRNA (D) or protein (E) levels by qPCR or ELISA, respectively. Experiments were performed in triplicate and repeated three times. Histograms represent mean ± SD; ns non-significant; **p<0.01; *** p < 0.001; **** p < 0.0001.*

Moreover, we also evidenced that 4PBA completely inhibited the PT/TG-induced enhanced expression of TG2 also at protein level (Fig.3B bottom panel). Moreover, we observed a partial restoration of physiological CFTR protein levels in samples treated with PT in combination with 4PBA, compared to PT alone (Fig.3B, bottom panel). This data further indicates that CFTR down-regulation due to gut exposure to PT results from combined PT-mediated protein destabilization and degradation[27], together with ER stress-mediated gene expression down-regulation[78].

To confirm that ER stress is directly involved also in the pro-inflammatory cytokines production, upon PT exposure, we measured both mRNA and protein levels of IL-15, IL-17A and IFNγ in small intestine from GS mice stimulated with PT or TG in presence of 4PBA, by qPCR (Fig.3D) or ELISA (Fig.3E), respectively. Data shown in Figure 3D-E clearly show that inhibiting ER stress prevents the PT/TG-induced production of pro-inflammatory cytokines, almost completely.

Finally, to evaluate whether 4PBA might also have an impact on active CD, and not only prevent the PT effects, we exposed small intestine to PT, while 4PBA was added after 4h, a time sufficient to stimulate a PT-dependent ER stress response (data not shown), and tissues were recovered after further 16h, as schematically reported in figure 4A.

Results shown in figure 4 are perfectly overlapping those of figure 3, potentially indicating the ability of 4PBA to restore intestinal physiological conditions in active CD.

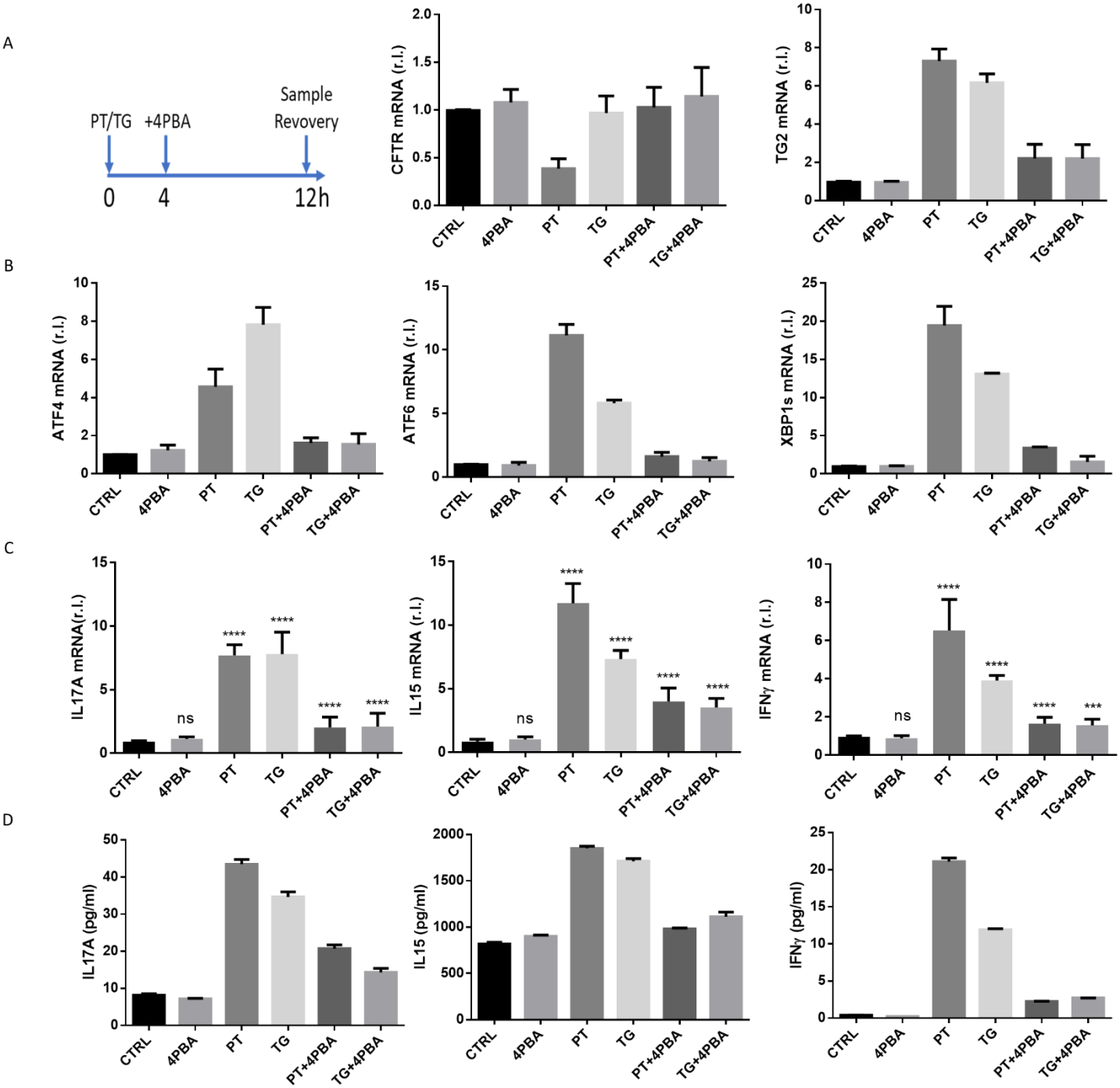


FIGURE 4. 4PBA inhibits PT activity. *A) Small intestines were cultivated in GEVS for 16 hours and infused with complete medium alone (CTRL) or supplemented with TG (5 ug/ml) or PT (2 mg/ml) alone, or stimulated with PT /TG for 4 hours and then supplemented with 4PBA (3uM), as schematically reported in panel A. CFTR and TG2 (A), ATF4, ATF6 and Xbp1 (B), IL-15, IL-17A and IFNγ (C) mRNA levels were evaluated by qPCR, while IL-15, IL-17A and IFNγ protein levels were also evaluated by ELISA(D). Experiments were performed in triplicate and repeated three times. Histograms represent mean ± SD; ns non-significant; *** p < 0.001; **** p < 0.0001.*

4. PT-STIMULATED ATF6 ACTIVATION RESULTS IN CFTR GENE EXPRESSION DOWNREGULATION

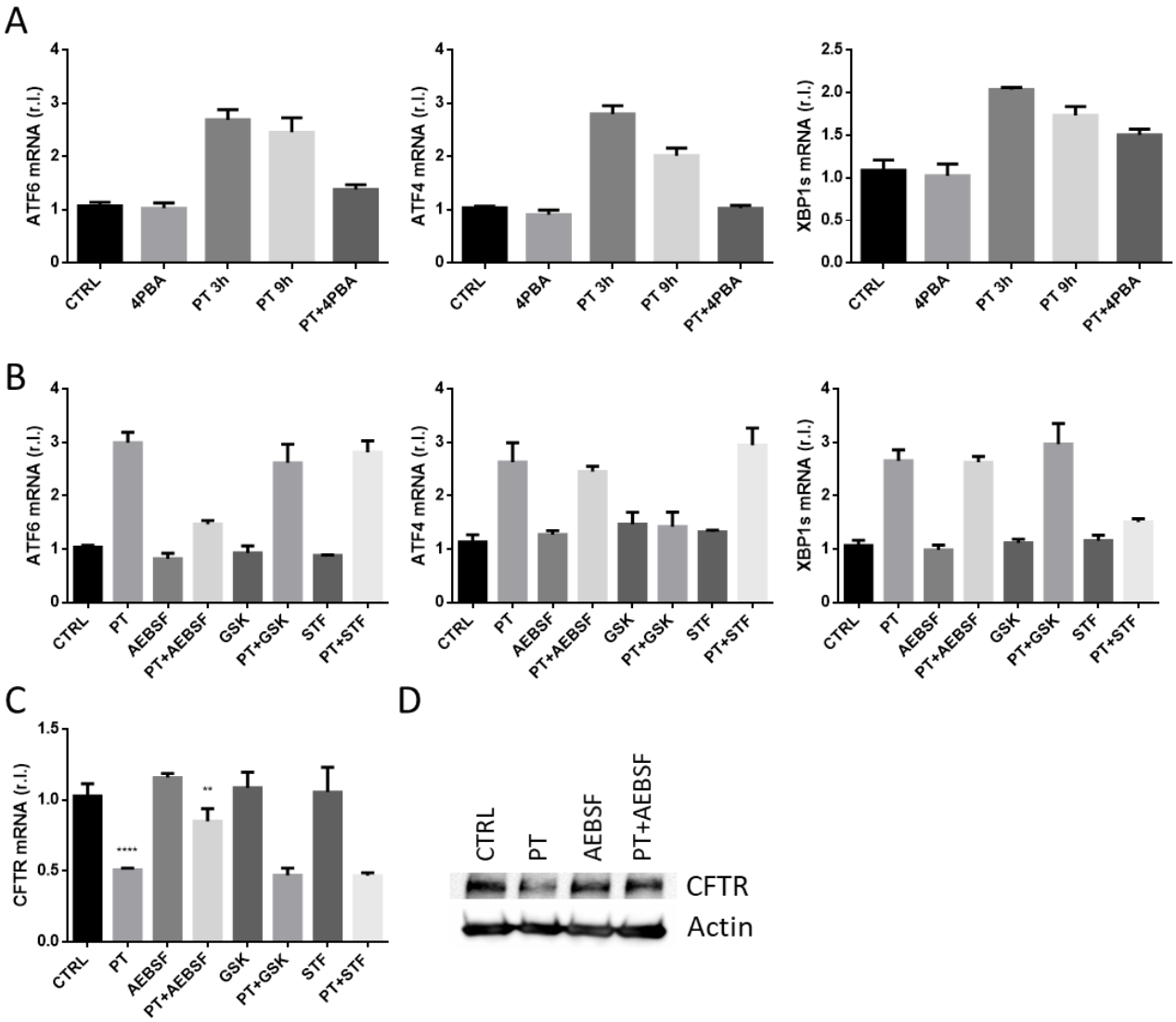


FIGURE 5. ATF6 inhibits CFTR expression. *A) Caco-2 cells were untreated or treated with PT (1 mg/ml) for 3 or 9 hours in absence or presence of 4PBA (3uM), and ATF6, ATF4 and XBP1s mRNA levels were evaluated by qPCR. B) ATF6, ATF4 and XBP1 mRNA levels were evaluated in Caco-2 cells treated 9h with PT alone or in combination with AEBSF (500 uM) or STF-083010 (60 uM) or GSK2606414 (5 uM), by qPCR. CFTR mRNA (C) and protein (D) levels were evaluated in Caco-2 cells in the same experimental conditions reported in B. Histograms represent mean ± standard deviation (SD) of triplicate sample; ns non-significant; **** $p < 0.0001$.*

To unveil the molecular mechanism responsible for CFTR downregulation upon PT exposure, we moved to an *in vitro* system consisting of Caco-2 cells, a model widely used to study the pathogenesis of CD[78,27,83,84]. To this aim we firstly evaluated the ability of PT to stimulate an ER stress response also in this *in vitro* model. We, therefore, exposed Caco-2 cells to PT for 3h or 9h in the presence or absence of 4PBA, while untreated or 4PBA (alone) treated cells were used as a control. Data in figure

5A confirm that PT treatment actives the three main ER stress pathways, as evidenced by the upregulation of the markers ATF6, ATF4 and XBP1.

Importantly, Bartoszewski and colleagues[85] have defined the transcriptional repressor role of ATF6 on CFTR expression and, in particular, indicated that the inhibition of CFTR expression under ER stress conditions leads to decreased CFTR maturation efficiency and diminished protein function. Based on these findings, we decided to selectively inhibits the three ER stress axis individually, by using specific inhibitors such AEBSF (to inhibit ATF6 processing and activation), STF-083010 (IRE1α inhibitor) and GSK2606414 (PERK inhibitor) in CaCo-2 cells treated with PT. Our results reported in figure 5B show that each inhibitor is selective for its specific pathway, without showing non-specific effect on the other ER stress signalling pathways, while data shown in figure 5C clearly confirm that AEBSF was the only effective treatment abrogating the PT-induced CFTR gene expression downregulation, almost completely. These results were confirmed by western blotting analysis demonstrating the ability of AEBSF to reduce CFTR protein decrease in presence of PT (compare lane 2 and 4 of figure 5D). It is important to note that ATF6 inhibition was not able to completely prevent CFTR protein decrease upon PT stimulation, due to the ability of PT to bind and induce CFTR protein degradation, as demonstrated by Villella and colleagues[27].

5. UPR AND CALCIUM RELEASE FROM ER UPREGULATE THE EXPRESSION OF TG2 TROUGH NF-kB

Next, we focused on the molecular mechanism responsible for TG2 upregulation induced in response to PT exposure.

To our knowledge, TG2 gene expression is modulated by the transcription factor NF-*k*B[86], which activity is regulated by the specific inhibitor I*k*Bα[87].Under physiological conditions, I*k*Bα binds and confines NF-*k*B in the cytosol, thus blocking its transcriptional activity. Upon stimulation, including cytokines, growth factors, mitogens, microbial components and stress agents[88], I*k*Bα is actively

degraded by proteasome, thus releasing NF-*k*B which moves into the nucleus to regulate the expression of targets genes, such as TG2. Importantly, two major signalling pathways have been described and responsible for I*k*Bα degradation resulting in NF*k*B activation: the canonical and non-canonical pathways. The former requires the activation of the IKK complex, composed of two catalytic subunits, IKKα and IKKβ, and the regulatory subunit 'NF*k*B essential modulator' (NEMO or IKKγ)[89,90]. Upon ER stress induction, active IRE1α recruits the tumor necrosis factor receptor (TNFR)-associated factor (TRAF)2[91,92], which in turn binds and activates IKK. The combined phosphorylation and subsequent degradation of I*k*Bα together with the inhibition of de novo synthesis of the inhibitor (I*k*Bα, characterized by enhanced protein turnover) through the general protein translation inhibition by the ER stress-mediated phosphorylation of eIF2α (through PERK activation), results in NF-*k*B activation (Fig.6A, upper panel)[93]. While, in the non-canonical pathway, the calcium-mediated TG2 activation results in direct I*k*Bα crosslink and subsequent degradation, thus activating NF-*k*B (Fig.6B, upper panel).

To evaluate which of the two signalling pathways is required for TG2 gene expression upregulation in CD, we used the Caco-2 cell model described in section 4.

To this aim, cells were exposed to PT in presence or absence of the IKK inhibitor BAY 11-7082, and mRNA levels of TG2 were evaluated by qPCR. Untreated or BAY 11-780 treated cells were used as controls. As shown in figure 6A (bottom panel), IKK inhibition consistently reduces the PT-stimulated TG2 upregulation. Next, we exposed Caco-2 cells to the TG2 specific inhibitor Cystamine, in presence or absence of PT, and the expression of TG2 was evaluated by qPCR. Untreated or PT treated cells were used as controls. Data reported in figure 6B (bottom panel) clearly show a consistent reduction of the PT-stimulated TG2 upregulation.

ER stress induction also results in ER calcium release into the cytosol, thus activating different enzymes, including TG2[94]. Indeed, TG2 is inactive at the very low Ca^{2+} concentration in the cytosol, but is rapidly activated upon increased cytosolic alcium[95]. Therefore, we prevented the activation of

this enzyme by treating cells with the cytosolic Ca^{2+} chelator BAPTA (Fig.7A). Thus, cells were exposed to PT in presence or absence of BAPTA and TG2 mRNA levels were evaluated by qPCR. Untreated or cells treated with BAPTA were used as controls. Our data reported in figure 7B confirmed a BAPTA-dependent consistent inhibition of PT-induced TG2 upregulation.

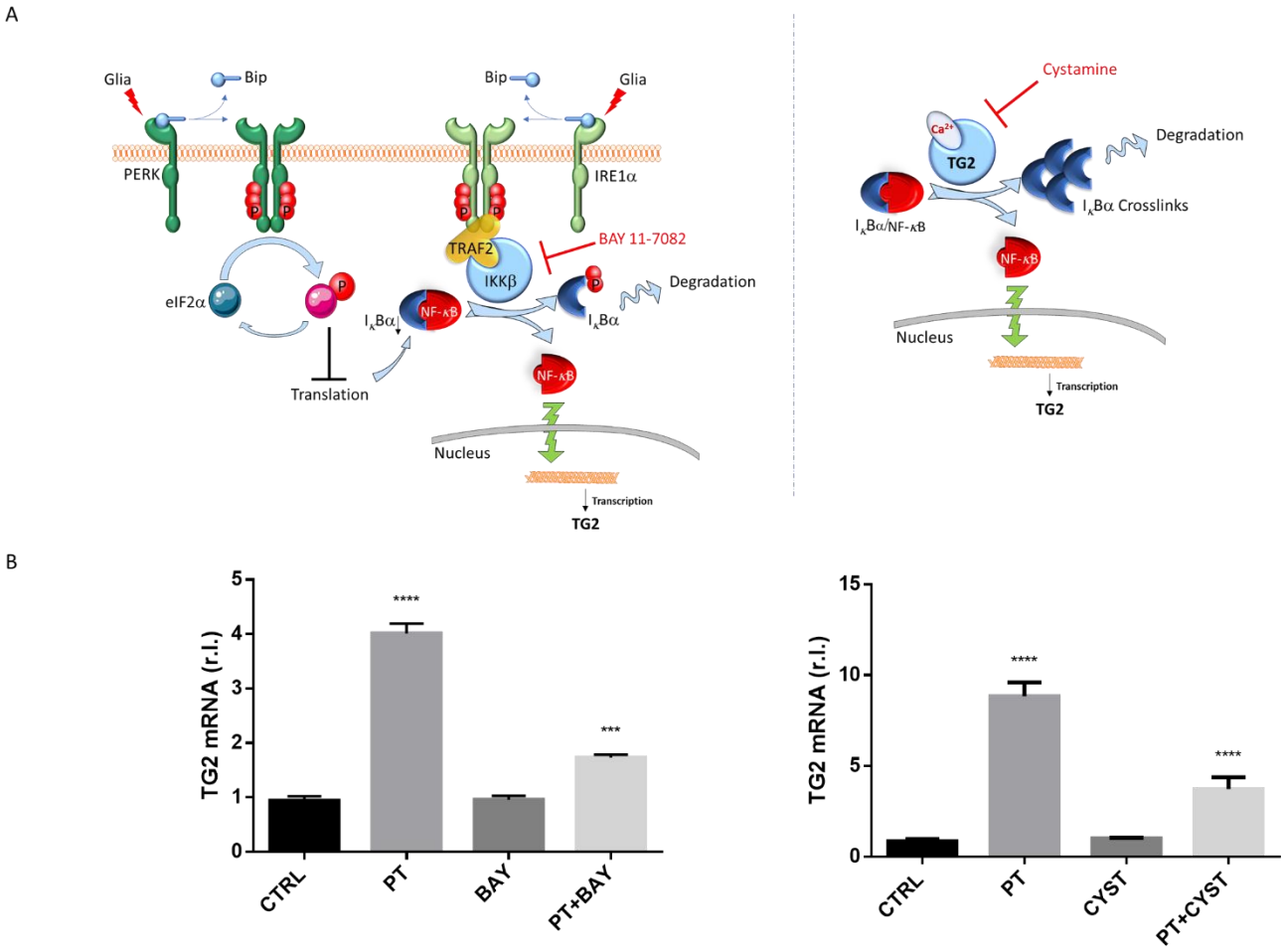


FIGURE 6. TG2 up-regulation by IKK-dependent and IKK-independent NF-kB activation in Caco-2 cells. *Schematic representation of ER stress-mediated IKK-dependent NF-kB activation and TG2 gene expression modulation (A, upper panel). Caco-2 cells were treated with PT (1mg/ml) alone for 3h or in combination with BAY 11-7082 (5uM) (1h pre-treatment) and TG2 mRNA levels were evaluated by qPCR (A, bottom panel). Schematic representation of IKK-independent NF-kB activation and TG2 gene expression modulation (B, upper panel). Caco-2 cells were treated with PT (1mg/ml) alone for 3h or in combination with Cystamine (250uM; 6h pre-treatment) and TG2 expression was evaluated by qPCR (B, bottom panel). Histograms represent mean ± standard deviation (SD) of triplicate sample; ***< p 0.001; **** p < 0.0001*

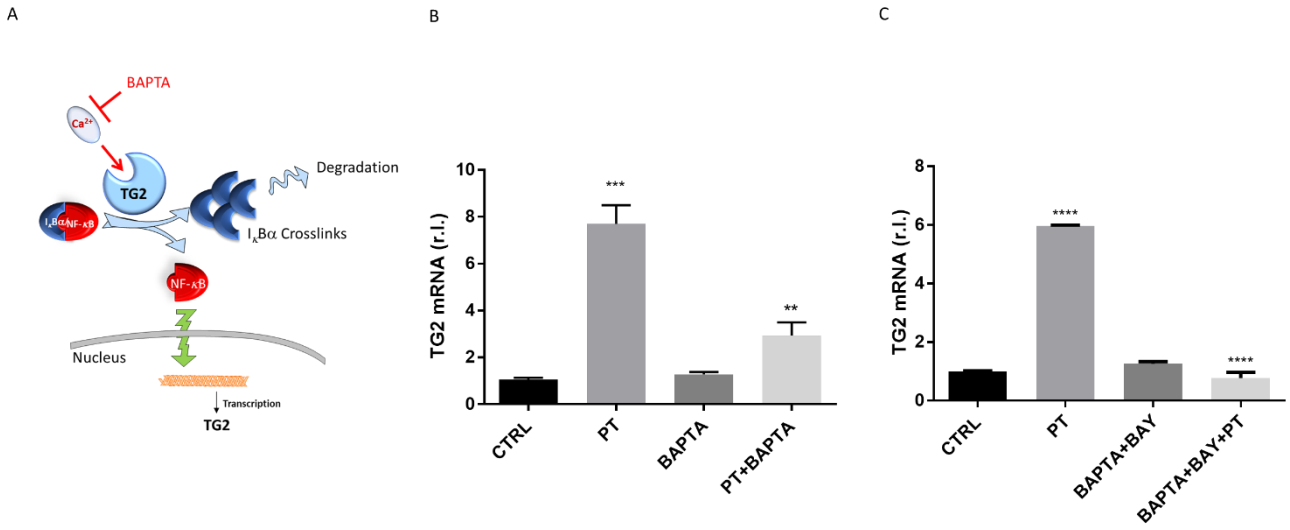


FIGURE 7. TG2 expression in Caco-2 cells treated with BAPTA and BAY. *Schematic representation of NF-kB activation by TG2 (A). TG2 mRNA levels were evaluated in Caco-2 cells treated with PT alone (1mg/ml) for 3h or in combination with BAPTA (10 uM; 1h pre-treatment). TG2 mRNA levels were evaluated in Caco-2 cells treated with PT alone (1mg/ml) or in combination with BAPTA (10uM) plus BAY (5uM) (1h pre-treatment). Histograms represent mean ± standard deviation (SD) of triplicate sample; **<p0.01; ***< p 0.001; **** p < 0.0001.*

Collectively, our data indicate that both canonical and non-canonical signalling pathways seem to be required for full activation of NF-*k*B during CD pathogenesis. To sustain this conclusion, we inhibited both signalling pathways in cells exposed to PT, by using both BAPTA and BAY 11-7082. Data reported in figure 7C clearly show a complete abrogation of TG2 upregulation in cells exposed to PT+BAY+BAPTA, compared to cells exposed to PT alone.

6. PT-INDUCED ER STRESS IS TRIGGERED BY CALCIUM RELEASE TROUGH IP3R

Endoplasmic reticulum represents the main intracellular calcium storage compartment, and calcium is actively transferred into the organelle through the ATP-consuming transmembrane calcium pump SERCA[96,97]. Although cytosolic calcium concentration is very low compared to both the extracellular and ER compartment, finely tuned oscillations are required for cell activities[98]. Indeed, increased cytoplasmic calcium is required as a second messenger in several signalling pathways regulating, among others, survival, adaptation and cell stress response[99]. On the other hand, excessive cytosolic calcium actively triggers cell death, mainly through caspase and calpain (both calcium-dependent enzymes) activation[100]. Although the molecular mechanisms regulating calcium release from the ER are still not completely clear, two ER transmembrane and inducible Ca^{2+} channels have been

characterized: Ryanodine receptors (RyR) and IP3 receptors (IP3R). The latter are activated through inositol 1,4,5-trisphosphate (IP3), a second messenger, binding to the receptors (IP3R – on ER membranes) which results in channel opening and calcium release into the cytoplasm[101]. IP3 is generated by phospholipase C (PLC), which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2) into the second messengers diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3; Fig.8A)[102].

Interestingly, as discussed in the introduction section, ER stress can be induced by massive release of calcium by the ER compartment or, alternatively, the divalent cation can be released from the organelle into the cytoplasm as a consequence of ER stress, although the precise molecular mechanism is still elusive[103].

Therefore, to test whether calcium release from ER is causative or merely a consequence of ER stress in cells exposed to PT, we inhibited the PLC/IP3/IP3R signalling pathway in Caco-2 cells exposed to PT, by using the PLC inhibitor U73122. To this aim, cells were exposed to PT in presence or absence of U73122 and the expression of the ER stress markers ATF4, ATF6 and XBP1 were evaluated by qPCR, together with the expression of TG2. Data shown in figure 8A indicate that inhibiting PLC activity completely prevents the induction of ER stress induced by PT exposure. Importantly, the experiment was replicated *ex vivo*, by using our GEVS. To this aim, small intestines from GS mice were exposed to PT in presence or absence of U73122 and ER stress markers and TG2 expression were evaluated by qPCR. Data reported in figure 8B clearly overlap with those obtained *in vitro* (8A).

Collectively these data indicate that PT stimulation triggers the PLC/IP3/IP3R signalling pathway resulting in ER stress induction.

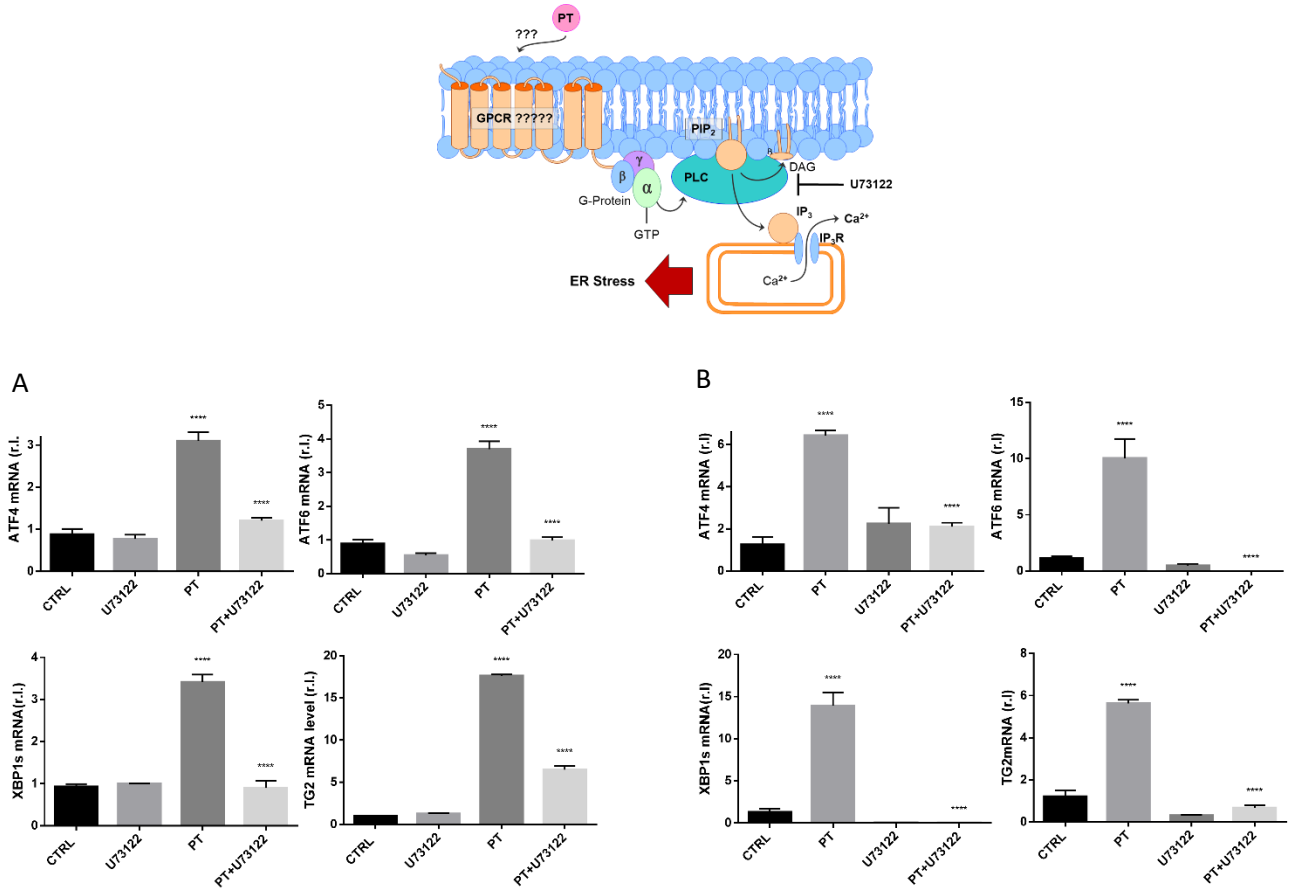


FIGURE 8. PT stimulated IP3 release. *Schematic representation of the potential signaling pathway* linking *PT stimulation and calcium release by the ER, trough the production of IP3. The expression levels (mRNA) of ATF4, ATF6, XBP1s and TG2 were evaluated by qPCR in Caco-2 cells (A) or in small intestine cultivated in a GEVS (B), exposed to PT alone (1mg/ml) for 3h or in combination with U73122 (10uM; 2h pre-treatment). Histograms represent mean ± standard deviation (SD) of triplicate sample; **** p < 0.0001.*

7. GLIADIN PEPTIDES INTERACTS WITH IEC THROUGH THE CHEMOKINE CXC MOTIF RECEPTOR 3 (CXCR3)

In 2008 Fasano and colleagues identified the chemokine CXC motif receptor 3 (CXCR3) as the potential transmembrane receptor to which gliadin peptides binds and stimulate the MyD88-dependent release of zonulin from IEC, thus increasing the intestinal permeability[104].

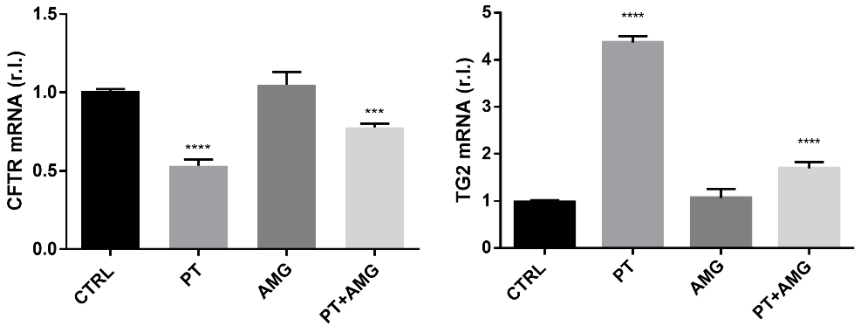
CXCR3 is a GPCR (Gαi protein-coupled receptor) of the CXC chemokine receptor family, with the three isoforms CXCR3-A, CXCR3-B and chemokine receptor 3-alternative (CXCR3-alt). CXCR3-A binds CXC chemokines CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC) whereas CXCR3-B can also bind CXCL4 in addition to CXCL9, CXCL10, and CXCL11[105]. This chemokine receptor is primarily expressed in NK and activated T cells, and some epithelial cells, such as IEC[106].

To verify the key role of CXCR3 in the gliadin-IEC communication, we inhibited the activity of the receptor both *in vitro* (Caco-2 cells) and *ex vivo* (GEVS), by using the specific and FDA approved inhibitor AMG487, and evaluated the impact of PT.

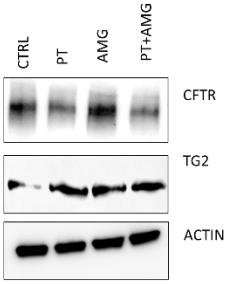
To this aim, Caco-2 cells were pre-treated with AMG (3h), then exposed to PT for 3h, and the expression of TG2 and CFTR were evaluated at both mRNA and protein levels. Data reported in figure 9A and B show that AMG did not completely restore the physiological levels of these factors, nor abrogated ER stress stimulated by PT (Fig.9C).

Therefore, we decided to pre-treat Caco-2 cells with AMG-487 for 16h, prior the addition of PT. Results reported in figure 10 clearly show that the inhibitor completely abrogated the effect of PT, thus restoring the physiological level of CFRT, TG2, and of the ER stress markers ATF4, ATF6 and XBP1, in cells exposed to PT.

A



B



C

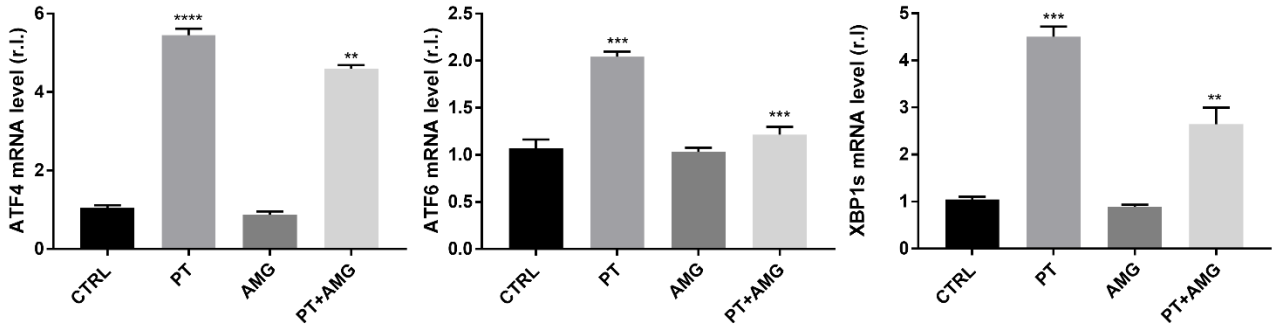


FIGURE 9. Impact of AMG-487 on PT-induced signaling pathways in Caco-2 cells. *A) CFTR and TG2 mRNA levels were evaluated in Caco-2 cells treated with PT alone (1mg/ml) or in combination with AMG-487 (1uM) (3h pre-treatment; B). Immunoblot showing CFTR and TG2 protein levels of cells treated as in A. GADPH was used as loading control. C) ATF4, ATF6 and XBP1s mRNA levels were evaluated by qPCR, in cells treated as in A&B. Histograms represent mean ± standard deviation (SD) of triplicate sample; ** p<0.01; ***p<0.001; **** p < 0.0001.*

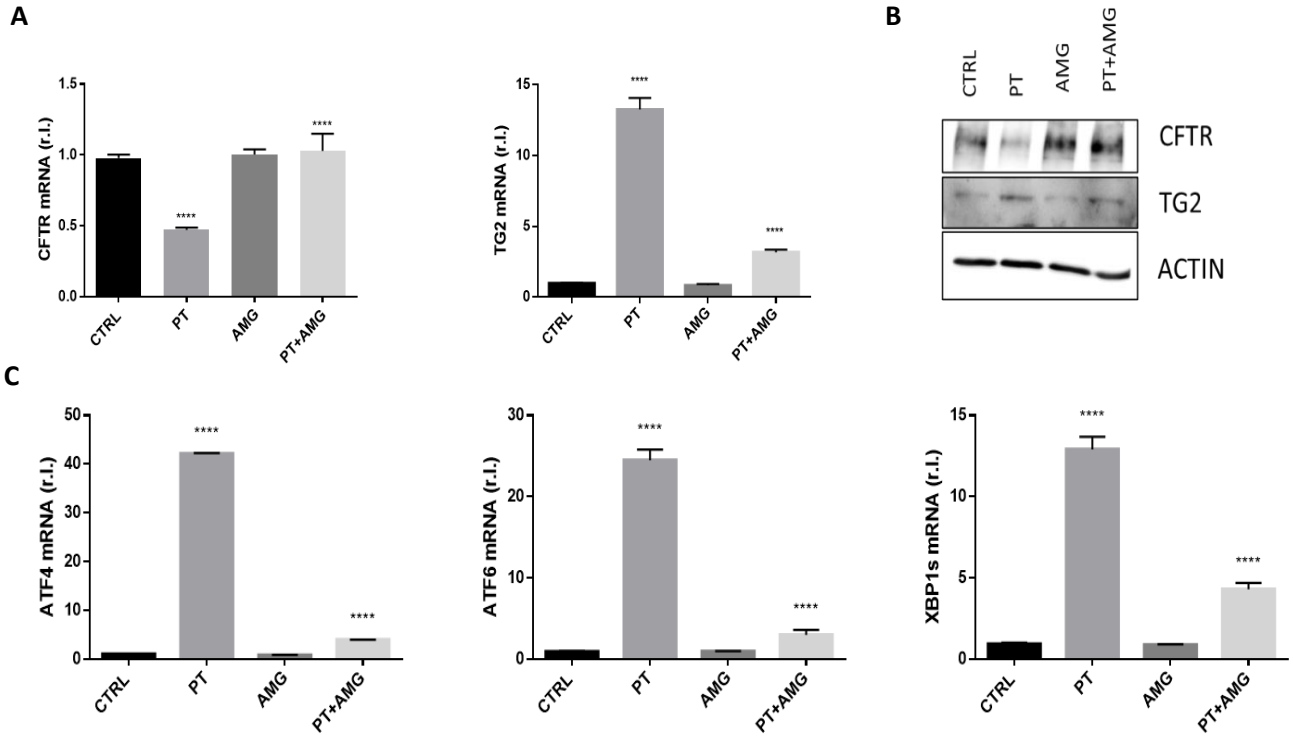


FIGURE 10. AMG-487 abrogates PT impact in Caco-2 cells. *(A) CFTR and TG2 mRNA levels were evaluated in Caco-2 cells treated with AMG-487 (1uM) for 16h and then with PT for further 3h. (B) Immunoblot with of CFTR, and TG2 protein levels of cells treated as in A. GADPH was used as loading control. (C) ATF4, ATF6 and XBP1s mRNA levels were evaluated by qPCR in cells treated as in A&B. Histograms represent mean ± standard deviation (SD) of triplicate sample; **** p < 0.0001.*

Then, we performed the same experiment by using our *ex vivo* model (GEVS). To this aim, small intestine from GS mice were pre-treated 16h with AMG and exposed to PT for the next 16h. Data shown in figure 11 clearly demonstrate that CXCR3 inhibition abrogated the effects of PT since it completely prevented the induction of ER Stress (Fig.11A), restored the physiological levels of both TG2 and CFTR (Fig.11B&C), prevented the dysregulation of intestinal permeability (Fig.11D), and abrogated the production of pro-inflammatory cytokines (Fig.11E).

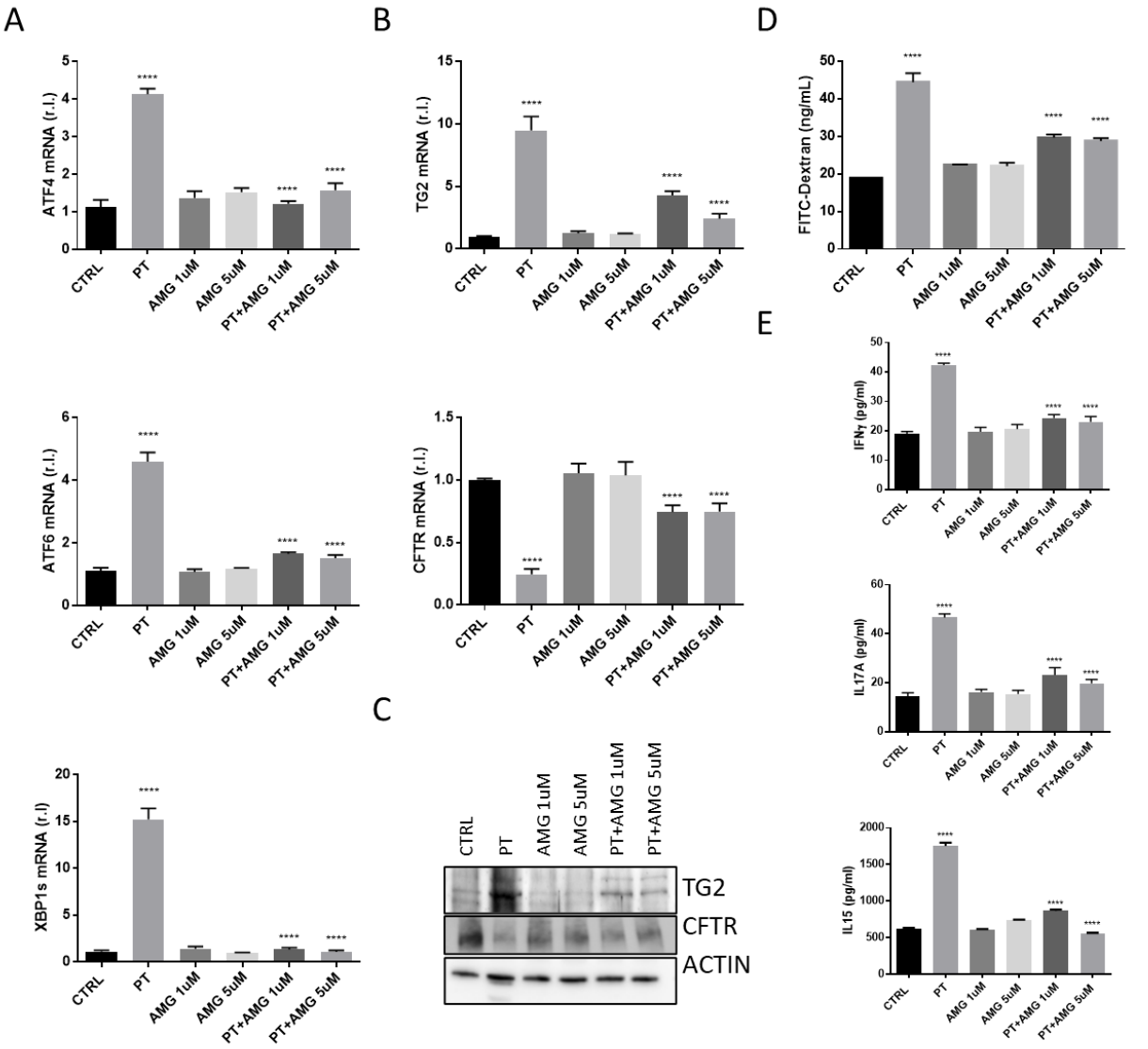


FIGURE 11. AMG-487 abrogated the effect of PT on small intestine. *(A) ATF4, ATF6 and XBP1s, (B) CFTR and TG2 mRNA levels were evaluated by qPCR in small intestine cultivated in GEVS, treated with AMG-487 16h (1uM or 5uM) and then with PT for further 16h (2,5 mg/ml). (C) TG2 and CFTR protein levels were evaluated in the same experimental condition described above, by western blotting analysis. Actin was used as loading control. (D) Intestinal permeability was evaluated by FITC-dextran release from small intestine in the experimental conditions described above. (E) The production of pro-inflammatory cytokines IL-15, IL-17α and IFNγ was evaluated by ELISA, in the same experimental conditions. Histograms represent mean ± standard deviation (SD) of triplicate sample; * p<0.1; ** p<0.01; ***p<0.001; **** p < 0.0001.*

Collectively these data indicate that blocking the activity of CXCR3 prevents the cytotoxic effects of gliadin peptides in IEC (Fig.12).

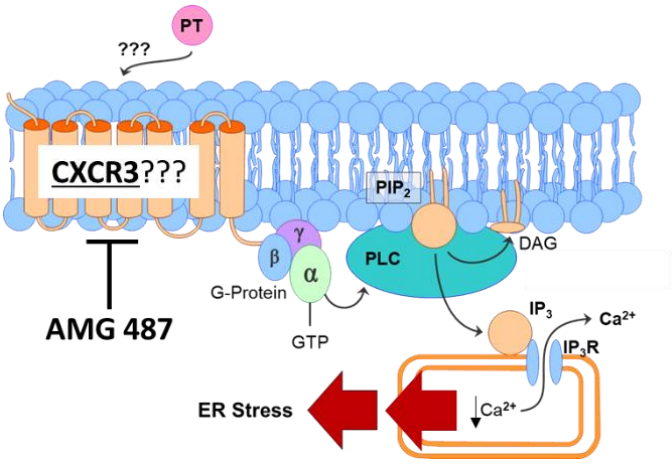


Figure 12. Schematic representation of PT interaction with IEC.

8. CXCR3 EXPRESSION IS UNDER THE CONTROL OF GADD153

It has been previously reported that CXCR3 expression is enhanced in IEC of CD patients and in cells exposed to PT[107]. Moreover, it has been suggested that CXCR3 expression is under the control of ER stress, although the molecular mechanism is still unclear[108].

To test these hypotheses, we evaluated the expression of CXCR3 at both basal levels and upon PT exposure, in Caco-2 cells and in small intestine from GS mice (GEVS). Our results indicate that the expression of the receptor was enhanced upon PT stimulation, both *ex vivo* and *in vitro*, at both mRNA and protein levels (Fig 13).

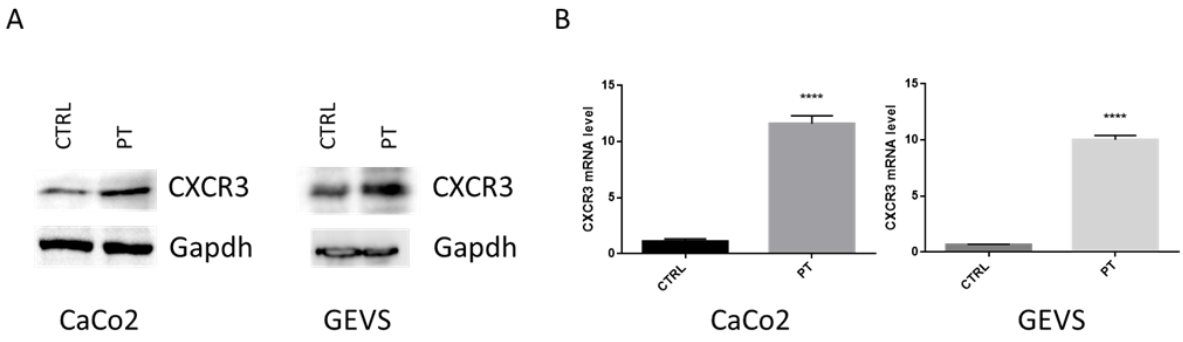


FIGURE 13. CXCR3 up-regulation by PT. *(A) Immunoblot of CXCR3 of whole lysate from Caco-2 cells (left) or small intestine (right) exposed to PT (3h, 1mg/ml and 16h, 2,5mg/ml, respectively). GAPDH was used as loading control. (B) CXCR3 mRNA levels were evaluated in Caco-2 cells (left) or small intestine (right) treated as in A. Histograms represent mean ± standard deviation (SD) of triplicate sample; **** p < 0.0001.*

To identify the transcription factor(s) (TF) potentially responsible for CXCR3 expression modulation upon PT exposure, we analysed the 1641bp sequence (Table 1) upstream the TSS of the human CXCR3 gene, by using

the online tool 'PROMO' (*http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3*), and limiting our analysis to ER Stress-related TFs.

Table 1. Putative human CXCR3 promoter region.

ACGGGCAGCCTTGCCCACTGGTAATGAGAAGAGGGCGACAGGGAGGGGAGAAGAGGGCAAAGGGGA
GAGCTGAGGGCAGGTGGGGAGAGGAATTGTTCACCATTCTCTGCCATAAAGTGACTGATGTGCTGTACC
AAGGCTGCCTTACAGCTTTTCCTTAAGATCAAGAAGTTCCTATGGCCTCCTCCCCAACCCCATCCTCCTTCT
TGCCCGAGAAGCAACTGCTTTTATGAATTTGTGGTATCTCCTGTCCCTGTTTTAAACCTCGAGAACCAACA
GTGTTATCCAAAACAACATGGTATTGATTTTGTGGTGGTTTTTAGAGTTTATGTAAGTGGCATCAAACTAT
ACATGGCATTCTGTAACTTGCTTCTTCTACCATGTGATCGTTGGCTTTTCTAGATGCATCCATGTTCAAATG
TGTAGCTTTAGTTCATTTGTTTTTAACTGCATGAATATATCATAGCTTATTCACTTAAAAGAAACTGAAGAC
AGTGTTCATGAGTAGGAAAAGGAGAATCAGACAGCCGTGGGATCAATCCTCACGCCCACCTTTAATTGCC
GTGTCTGGCGAAGGCCTGTCCCCTTTCTGAATCATCCATGCAGATAACCCCGCACGTCACAAGTGAGTTG
TGTGGTTGAAATAAGGCAGTGTCGGAGAAAGCACCTAAAGCAATGTCTATCACATAGCAAGTTCATTACT
GGTAGTTCCCTTTCTTTTTCCCCACTGATTAGCAGGGAGAGGTGGTGGTAAAAAGCTAGATTTAGTGTAA
CGGCTTTTTAAAAAAATATTTTAATTCCCTTTTTAAGAGACAGGGCCTCACTCTGTCACCAGGCTGGAGTG
CAGTGGTACAATTATATCTCAAGGCAGACTTGAACTCCCGGGCTCAAGCAATCCTCCTGTCTCAGGCTTCC
AAGTAGTTAGGACTACAGGCACATGCCACCATGCCCGGCTGGCTTTTTTTATTTTTGGTAGAGATGGAGC
CTCGCTATGCTGCCCAGGCTGGTCTCAAACTCTTGGCCTTAAGTGATCCTCTTGCCTCAGCCTCCCCAGTA
GCTGGGATTACAGGAATGAGCCACTGCACCTAGCTGAAATTTTTAAGAAGGTGGATCTCCTCTGCAGACT
TATTTCTCTACCCAGCCTTTACAATCATGCAAAGATCAACTCAAGGCCAGGACAGATGAGTTCCAATCTAT
GGAGCACTGACCCTACCAGTGCAGGCAGTAGGGTTGCCCTTCACTTCTCAGCTGCATTGCTTCCTCTTCAG
ACCTCTCTTCTCAAAGGACCTGCCCCCAGCCAGTCATCCTCTGCCCAGCTTTTCTGGTCCCAAGCTTGTAGC
CAGCTCCTCCAGAGAGGTTTCACCCAGTCACGGAAACTCTGTGGCCTGAGGTTTAGGGAGGTCTGGTAG
AGGTAACTCCCTGGAAGAGGCTGCTGCTGAGAACTGCCTGAAACTCCCACTTCCTCTGTGACTGCAGGTT
T**C**CAACCACAAGCACCAAAGCAGAGGGGCAGGCAGCACACCACCCAGCAGCCAGAGCACCAGCCCAGC
CATGGTCCTTGAGGTAAGTGCTGCTGCCCAGCCAGGCCAGGCCAGAATTCGAAGTGGAAAACCCGGGGC
ATCAGGGGAATGCCAGGGCTGGCCC

Note: The base labeled in red is the transcription starting site (TSS).

This analysis confirmed the presence of several responsive elements (RE) for the binding of C/EBPα (CCAAT/enhancer binding protein α), a known TF regulating the expression of the receptor[109]. Interestingly, our analysis also revealed the presence of a putative RE for the binding of 'members of C/EBP family'. The ER stress related TF Gadd153/CHOP is a 'C/EBP-homologous protein'. Therefore, we hypothesized that this TF might regulate the expression of CXCR3 under PT-induced ER stress. In order to test this hypothesis, we verified the expression of CHOP in cells and tissues exposed to PT. To this aim, Caco-2 cells and small intestine tissues from GS mice were exposed to PT and CHOP expression was evaluated by qPCR. Data reported in figure 14A-B confirm the upregulation of this TF upon PT exposure, both *in vitro* and *ex vivo*. Next, we verified the direct involvement of ER stress in the modulation of CXCR3 expression. Thus, Caco-2 cells or small intestine from GS mice were exposed to PT in presence or absence of 4BPA, and the expression of CXCR3 was evaluated by qPCR. Our data

indicate that inhibiting the ER stress response induced by PT completely abrogated the up-regulation of this factor, both *in vitro* (Fig.14C) and *ex vivo* (Fig.14D).

Finally, we inhibited the expression of CHOP in Caco-2 cells by means of specific shRNA sequences (Fig.14E), and evaluated the impact of PT on the expression of CXCR3. Data shown in figure 14F clearly demonstrate a complete abrogation of PT-stimulated CXCR3 upregulation in cells in which CHOP expression was silenced.

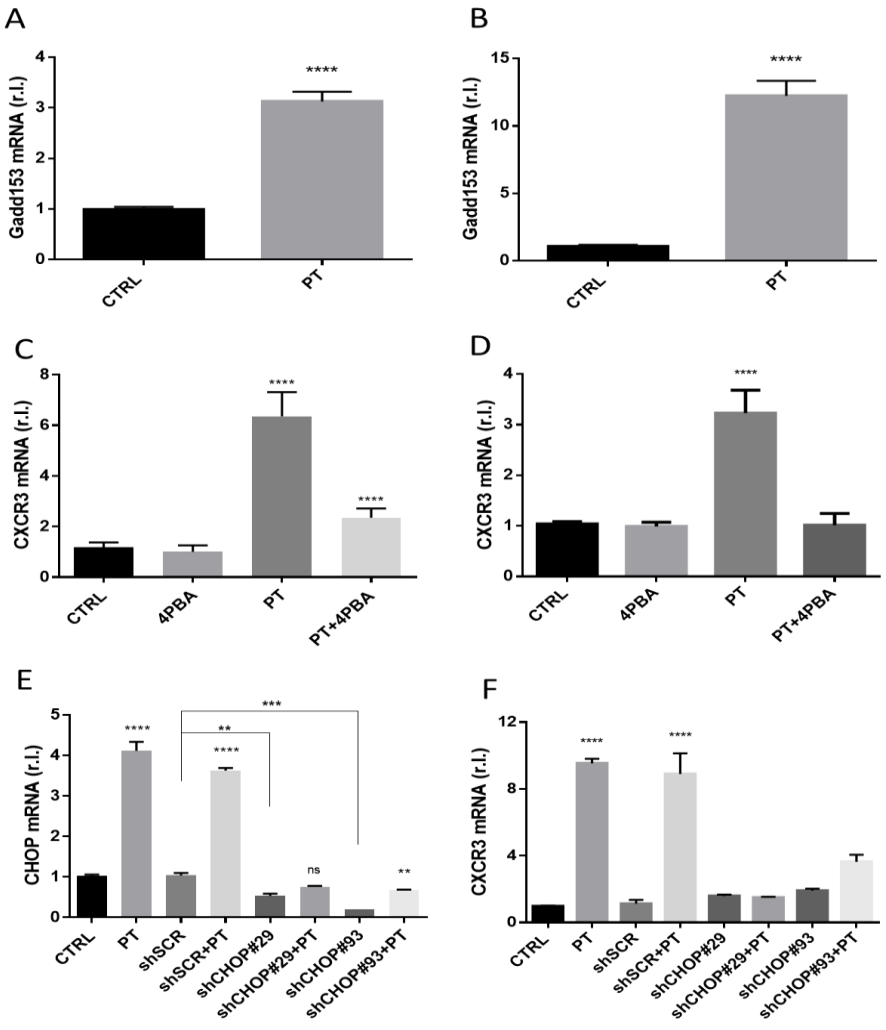


FIGURE 14. CXCR3 up-regulation by Gadd153/CHOP activation. *Gadd153/CHOP mRNA levels were evaluated in Caco-2 (A) or small intestine (B) untreated or treated with PT (1mg/ml, 3h; 2,5 mg/ml; 16h, respectively) by qPCR. CXCR3 mRNA levels were evaluated in Caco-2 cells (C) treated with PT alone (1mg/ml; 3h) or in combination with 4-PBA (5uM), or in small intestine (D) cultivated in GEVS and exposed to PT alone (2,5 mg/ml; 16h) or in combination with 4PBA (3uM). Gadd153/CHOP (E) or CXCR3 (F) mRNA levels were evaluated in Caco-2 cells in which the expression was silenced by specific shRNA and cells exposed or unexposed to PT (1mg/ml for 3h), as indicated. Histograms represent mean ± standard deviation (SD) of triplicate sample; * p<0.1; ** p<0.01; ***p<0.001; **** p < 0.0001.*

Collectively these data indicate that Gadd153/CHOP regulates the expression of CXCR3 upon gliadin stimulation (Fig.15).

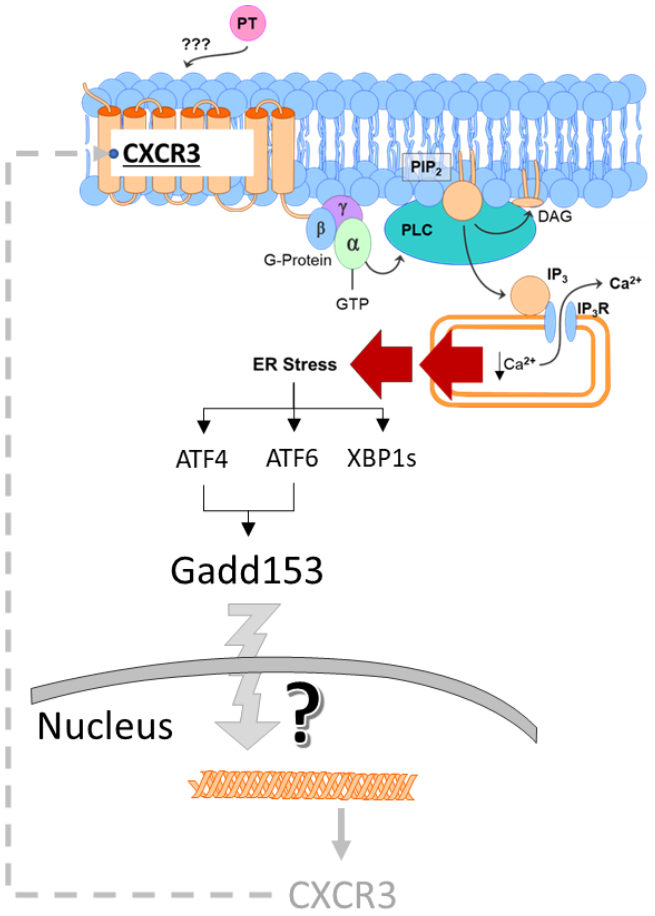


FIGURE 15. GADD153 CONTROLS THE EXPRESSION OF CXCR3*. Schematic representation of possible mechanism by which CXCR3 gene expression is regulated by Gadd153/CHOP.*

DISCUSSION

Celiac disease (CD) is a complex inflammatory and auto-immune disorder triggered by the ingestion of gluten, a heterogeneous mixture of seed-storage proteins presents in cereals such as wheat, barley, rye and oats[110]. Gluten proteins, at the level of the intestinal mucosa, cause an immune response that leads to an extensive mucosal remodelling and organ damage[111]. Besides classical intestinal manifestations of the disease (diarrhoea, malabsorption, anaemia, weight loss, growth delay, etc.), there is a wide range of possible extra-intestinal symptoms including bone, liver, skin and neurological manifestations[112].

An important hallmark of CD is represented by the development of auto-antibodies against self-proteins such as TG2[113]. Indeed, TG2 plays a key role in the pathogenesis of celiac disease (CD), primarily for its enzymatic activity that transforms common food proteins, i.e., gluten proteins contained in cereals, in unhealthy molecules for genetic predisposed individuals[114]. In particular, the causative agent in wheat is the ethanol soluble prolamin fraction, i.e., gliadins, whose toxicity remains after peptic-tryptic (PT) digestion[115].

One of the first indications that a class of gluten peptides, in particular peptide 31-43 of α-gliadin (p31-43), is the main actor in inducing an innate immune response has been reported in the work from the Maiuri's Lab[58] and then Luciani and colleagues demonstrated that it drives an innate immune response leading to TG2 upregulation in both the intestine of CD patients and in gliadin sensitive epithelial cell lines (Caco-2 or T84 cells)[57].

Recently, Caputo and colleagues investigated the effect of gliadin peptides in Caco-2 cells and demonstrated that these peptides promote the release of calcium (Ca2+) from both endoplasmic reticulum (ER) and mitochondria, the main intracellular Ca^{2+} storage compartments, thus activating both cytosolic and nuclear TG2. These authors also demonstrated a concomitant increased expression of the glucose-regulated protein (GRP)-78 and the CCAAT/enhancer binding protein-homologous protein (CHOP), thus suggesting the involvement of ER stress[61]. However, no further date about the role of ER stress in this context has been reported.

Therefore, the main objective of this study was to investigate the role of ER stress in the pathogenesis of Celiac Disease at molecular level.

In this context, we recently demonstrated that PT stimulation is able to recapitulate the molecular features of CD both *in vivo* and *ex vivo,* by using a mouse model of gluten sensitive animals and a gut-*ex-vivo* system (GEVS). Indeed, we demonstrated PT treatment can stimulate the onset of active CD, as evidenced by the upregulation of TG2,

dysregulation of intestinal permeability, and production of CD-related pro-inflammatory cytokines such as IL-15, IL-17A and IFNγ. We also confirmed the induction of ER stress and the downregulation of CFTR. The latter event has recently been described as potentially responsible for the increasing prevalence of positive serological marker of CD in Cystic Fibrosis (CF) affected patients[27,78].

Therefore, to verify that the PT-stimulated ER stress has an active and key role in the pathogenesis of CD and does not merely represent a side effect, we inhibited this signaling process by using a chemical chaperone and FDA approved 4PBA[116] .Indeed, we found that 4PBA was able to completely prevent the PT-induced ER stress, thus abrogating the downstream effects of PT, such as pro-inflammatory cytokines production and dysregulated intestinal permeability. Interestingly, we also observed the ability of PBA to consistently inhibit the effects of active CD, since its delayed administration in respect to PT resulted in the downregulation of ER stress markers, TG2 expression, pro-inflammatory cytokines, and restored the physiological intestinal permeability.

Therefore, based on our *in vitro* and *ex vivo* results, chemical chaperones might be seriously considered as new potential therapeutic treatment for CD patients, not only to prevent the onset of the diseases, but also during active CD.

Next, we investigated the missing link between the interaction between IEC and the induction of ER stress. Several reports indicated the ability of PT to penetrate inside cells and thus inducing a cellular response[117]. However, also based on data reported in the literature, we hypothesized that gliadin peptides might interact with CXCR3[104] on the extracellular side of IEC and thus, through a yet unknown pathway, stimulate an intracellular ER stress response. To explore this hypothesis, we inhibited the activity of CXCR3 by using the specific inhibitor and FDA approved AMG 487[118]. Our results indicate that inhibiting the activity of this receptor we completely prevented the intracellular and downstream effect of PT.

To fill the missing gap between CXCR3 and ER stress, we took advantage of the fact that CXCR3 is a GPCR which activity can be mediated by G proteins that produce IP3 by stimulating the activity of PLC[119]. Moreover, we previously mentioned the ER stress can be stimulated by the release of calcium from the compartment[120]. Interestingly, this condition can be the result of IP3 interaction with IP3R located onto ER membranes. Indeed, inhibiting the activity of

PLC, we completely prevented the effects of PT. Therefore, we established the PT/CXCR3/PLC/IP3/IP3R as the missing link between the interaction of IEC with extracellular PT and the induction of ER stress.

Most importantly, we demonstrated that inhibiting the activity of CXCR3 or buffering the resulting downstream ER stress, we completely abrogated the intracellular effects of gliadin peptides.

Moreover, we also demonstrated the key role of ER stress in the upregulation of CXCR3 upon IEC interaction with gliadin peptides. Indeed, the ER stress-mediated upregulation of the downstream transcription factor Gadd153/CHOP results in CXCR3 gene expression upregulation. In our opinion, this phenomenon might represent a positive loop ignited by PT to potentiate its cytopathic effects on IEC, thus resulting in villus atrophy. However, further studies are required to explore this possibility.

Although the ability of increased intracellular calcium in driving the upregulation of TG has been previously reported[121], the molecular mechanism(s) in this context is still elusive. Here we demonstrated that the activation of the transcription factor NF-*k*B is crucial and that this is achieved involving both the canonical[86] and non-canonical[122] pathway.

Collectively our data indicate that the gliadin-mediated induction of ER stress in IEC has a key role in the pathogenesis of CD and that might represent a potential new valuable target to treat these patients.

BIBLIOGRAPHY

1. Turner, J. R. Intestinal mucosal barrier function in health and disease. *Nature Reviews Immunology.*(2009). doi: 10.1038/nri2653

2. Luciana R Muniz, Camille Knosp, G. Y. Intestinal antimicrobial peptides during homeostasis, infection, and disease. *Frontiers in Immunology.* (2012). doi: 10.3389/fimmu.2012.00310

3. Maaike Vancamelbeke , S. V. The intestinal barrier: a fundamental role in health and disease. *Expert review gastroenterology hepatology .* (2017). doi: 10.1080/17474124.2017.1343143

4. Pelaseyed, T. The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. *Immunological Reviews.* (2014). doi: 10.1111/imr.12182

5. Sa'ad Y Salim. Importance of disrupted intestinal barrier in inflammatory bowel diseases. *Inflammatory Bowel Disease* (2011). doi: 10.1002/ibd.21403

6. Matthew A Odenwald. The intestinal epithelial barrier: a therapeutic target? *Nature Reviews Gastroenterology Hepatology .* (2017). doi: 10.1038/nrgastro.2016.169

7. Jessica Y Lee. Molecular Pathophysiology of Epithelial Barrier Dysfunction in Inflammatory Bowel Diseases. *Proteomes* (2018). doi: 10.3390/proteomes6020017

8. Ceniz Zihni. Tight junctions: from simple barriers to multifunctional molecular gates. *Nature Reviews Molecular Cell Biology* (2016). doi: 10.1038/nrm.2016.80

9. Citi, S. The mechanobiology of tight junctions. *Biophysical Reviews.* (2019). doi: 10.1007/s12551-019-00582-7

10. Shen, L. Tight Junction Pore and Leak Pathways: A Dynamic Duo. *Annual Reviews Physiology* (2011). doi: 10.1146/annurev-physiol-012110-142150

11. Dorothee Günzel. Claudins and other tight junction proteins. Comprehensive Physiology (2012). doi: 10.1002/cphy.c110045

12. Christina M Van Itallie. Claudins and epithelial paracellular transport. *Annual Reviews Physiology .* (2006). doi: 10.1146/annurev.physiol.68.040104.131404

13. Gagliardi, M. *et al.* A gut-ex-vivo system to study gut inflammation associated to inflammatory bowel disease (Ibd). *Biology (Basel).*(2021). doi: 10.3390/biology10070605

14. Gagliardi, M. *et al.* Gut-Ex-Vivo system as a model to study gluten response in celiac disease. *Cell Death Discovery.* (2021). doi: 10.1038/s41420-021-00430-2

15. Giovanni Barbara. Inflammatory and Microbiota-Related Regulation of the Intestinal Epithelial Barrier. *Frontiers in Nutrition. 8* (2021). doi: 10.3389/fnut.2021.718356

16. Radhakrishna Rao. Occludin phosphorylation in regulation of epithelial tight junctions. Annals of the New York Academy of Sciences (2009). doi: 10.1111/j.1749-6632.2009.04054.x

17. Kazuaki Umeda. ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. *Cell* (2006). doi: 10.1016/j.cell.2006.06.043

18. L A Jesaitis. Molecular characterization and tissue distribution of ZO-2, a tight junction protein homologous to ZO-1 and the Drosophila discs-large tumor suppressor protein. Journal of Cell Biology. (1994). doi: 10.1083/jcb.124.6.949

19. M Itoh. Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins. *Journal of Cell Biology.* (1999). doi: 10.1083/jcb.147.6.1351

20. Alan S Fanning. Isolation and functional characterization of the actin binding region in the tight junction protein ZO-1. *The FASEB Journal* (2002). doi: 10.1096/fj.02-0121fje

21. Allan M Mowat. Regional specialization within the intestinal immune system. *Nature Reviews Immunology.* (2014). doi: 10.1038/nri3738

22. O Pabst 1, A. M. M. Oral tolerance to food protein. *Mucosal Immunology.* (2012). doi: 10.1038/mi.2012.4

23. Andrea Dillon and David D. Lo*. M Cells: Intelligent Engineering of Mucosal Immune Surveillance. *Frontiers in Immunology.* (2019). doi: 10.3389/fimmu.2019.01499

24. Nobuhide Kobayashi. The Roles of Peyer's Patches and Microfold Cells in the Gut Immune System: Relevance to Autoimmune Diseases. *Frontiers in Immunology.* (2019). doi: 10.3389/fimmu.2019.02345

25. Mark Feldman. Mucosal Immunology and Inflammation. *Sleisenger and Fordtran's Gastrointestinal and Liver diseases* (2021).

26. M Sun. Regulatory immune cells in regulation of intestinal inflammatory response to microbiota. *Mucosal Immunology.* (2015). doi: 10.1038/mi.2015.49

27. Villella, V. R. *et al.* A pathogenic role for cystic fibrosis transmembrane conductance regulator in celiac disease. The *EMBO Journal*, (2019). doi: 10.15252/embj.2018100101

28. Bertrand Meresse. Celiac disease: an immunological jigsaw. *Immunity* (2012). doi: 10.1016/j.immuni.2012.06.006

29. Ludvig M Sollid 1, B. J. Triggers and drivers of autoimmunity: lessons from coeliac disease. *Nature Reviews Immunology.* (2013). doi: 10.1038/nri3407

30. Elin Bergseng. Different binding motifs of the celiac disease-associated HLA molecules DQ2.5, DQ2.2, and DQ7.5 revealed by relative quantitative proteomics of endogenous peptide repertoires. *Immunogenetics* (2015). doi: 10.1007/s00251-014-0819-9

31. H Sjöström. Identification of a gliadin T-cell epitope in coeliac disease: general importance of gliadin deamidation for intestinal T-cell recognition. *Scandinavian Journal of Immunology* (1998). doi: 10.1046/j.1365-3083.1998.00397.x

32. Giacomo Caio. Celiac disease: a comprehensive current review. *BMC Medicine* (2019). doi: 10.1186/s12916-019-1380-z

33. Alberto Rubio-Tapia. ACG clinical guidelines: diagnosis and management of celiac disease. *Am. J. Gastroenterology.* (2013). doi: 10.1038/ajg.2013.79

34. KatriKaukinen. HLA-DQ typing in the diagnosis of celiac disease. *Official journal of the American College of Gastroenterology.* (2002). doi: 10.1111/j.1572-0241.2002.05471.x

35. Marsh, M. N. Marsh MN. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum. *Gastroenterology* (1992).

36. G Oberhuber. The histopathology of coeliac disease: time for a standardized report scheme for pathologists. *European Journal of Gastroenterology & Hepatology.* (1999). doi: 10.1097/00042737-199910000-00019

37. Corazza G R. Coeliac disease. *Journal of Clinical Pathology* (2007). doi: 10.1136/jcp.2004.023978

38. Richard J Farrell 1, C. P. K. Celiac sprue. *The New England Journal of Medicine.* (2002). doi: 10.1056/NEJMra010852

39. Detlef Schuppan. Non-celiac wheat sensitivity: differential diagnosis, triggers and implications. *Best Practice & Research Clinical Gastroenterology* (2015). doi: 10.1016/j.bpg.2015.04.002

40. Jonas F Ludvigsson. The Oslo definitions for coeliac disease and related terms. *Gut* (2013). doi: 10.1136/gutjnl-2011-301346

41. Abdul R Rishi. Refractory celiac disease. *Expert Review of Gastroenterology & Hepatology .* (2016). doi: 10.1586/17474124.2016.1124759

42. Freeman, H. J., Chopra, A., Clandinin, M. T. & Thomson, A. B. Recent advances in celiac disease. *World Journal of Gastroenterology.* **17**, 2259–2272 (2011). doi: 10.3748/wjg.v17.i18.2259

43. Ilaria Parzanese. Celiac disease: From pathophysiology to treatment. *World Journal of Gastrointestinal Pathophysiology.* (2017). doi: 10.4291/wjgp.v8.i2.27

44. M F Kagnoff 1. Celiac disease. A gastrointestinal disease with environmental, genetic, and immunologic components. *Gastroenterology Clinics of North America* (1992).

45. Valérie Abadie. Integration of genetic and immunological insights into a model of celiac disease pathogenesis. *Annual Reviews Immunology.* (2011). doi: 10.1146/annurev-immunol-040210-092915

46. Edwin Liu. Risk of celiac disease according to HLA haplotype and country. *The New England Journal of Medicine.* (2014). doi: 10.1056/NEJMc1409252

47. Sandro Drago. Gliadin, zonulin and gut permeability: Effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. *Scandinavian Journal of Immunology.* (2006). doi: 10.1080/00365520500235334

48. Atul Munish Chander. Cross-Talk Between Gluten, Intestinal Microbiota and Intestinal Mucosa in Celiac Disease: Recent Advances and Basis of Autoimmunity. *Frontiers in Microbiology* (2018). doi: 10.3389/fmicb.2018.02597

49. Maria Vittoria Barone. Gliadin Peptide P31-43 Localises to Endocytic Vesicles and Interferes with Their Maturation. *PLoS One* (2010). doi: 10.1371/journal.pone.0012246

50. Willemijn Vader. The gluten response in children with celiac disease is directed toward multiple gliadin and glutenin peptides. *Gastroenterology* (2002). doi: 10.1053/gast.2002.33606

51. Benedict Onyekachi Odii 1, P. C. 1. Biological functionalities of transglutaminase 2 and the possibility of its compensation by other members of the transglutaminase family. *The Scientific World Journal.* (2014). doi: 10.1155/2014/714561

52. H Tatsukawa. Transglutaminase 2 has opposing roles in the regulation of cellular functions as well as cell growth and death. *Cell Death Disease.* (2016). doi: 10.1038/cddis.2016.150

53. Fangming Tang. Cytosolic PLA 2 is required for CTL- Mediated immunopathology of celiac disease via NKG2D and IL-15. *Journal of Experimental Medicine.* (2009). doi: 10.1084/jem.20071887

54. O Molberg. Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. *Nature Medicine.* (1998). doi: 10.1038/nm0698-713

55. Bana Jabri, V. A. IL-15 functions as a danger signal to regulate tissue-resident T cells and tissue destruction. *Nature Reviews Immunology.* (2015). doi: 10.1038/nri3919

56. Peter H R Green, B. J. Coeliac disease. The *Lancet* (2003). doi: 10.1016/S0140-6736(03)14027-5

57. Luciani, A. Lysosomal accumulation of gliadin p31-43 peptide induces oxidative stress and tissue transglutaminase-mediated PPARgamma downregulation in intestinal epithelial cells and coeliac mucosa. *Gut* (2010). doi: 10.1136/gut.2009.183608

58. Maiuri, L. Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease. The *Lancet* (2003). doi: 10.1016/S0140-6736(03)13803-2

59. Jeroen Visser. Tight Junctions, Intestinal Permeability, and Autoimmunity Celiac Disease and Type 1 Diabetes Paradigms. *Annals of the New York Academy of Sciences* (2009). doi: 10.1111/j.1749-6632.2009.04037.x

60. Detlef Schuppan. Celiac disease: from pathogenesis to novel therapies. *Gastroenterology* (2009). doi: 10.1053/j.gastro.2009.09.008

61. Caputo I. Gliadin peptides induce tissue transglutaminase activation and ER-stress through Ca2+ mobilization in Caco-2 cells. *PLoS One* (2012). doi: 10.1371/journal.pone.0045209

62. Jason A Tye-Din. Celiac Disease: A Review of Current Concepts in Pathogenesis, Prevention, and Novel Therapies. *Frontiers in Pediatrics.* (2018). doi: 10.3389/fped.2018.00350

63. David W Reid. Diversity and selectivity in mRNA translation on the endoplasmic reticulum. *Nature Reviews Molecular Cell Biology.* (2015). doi: 10.1038/nrm3958

64. L M Westrate. Form follows function: the importance of endoplasmic reticulum shape. *Annual Review of Biochemistry.* (2015). doi: 10.1146/annurev-biochem-072711-163501

65. Bruce Alberts. Molecular biology of cell. (2002).

66. Corazzari M. Endoplasmic Reticulum Stress, Unfolded Protein Response, and Cancer Cell Fate. *Frontiers in Oncology.* (2017). doi: 10.3389/fonc.2017.00078.

67. Corazzari M. ER Stress & Autophagy in Cancer: Contenders or Partners in Crime? *American Journal of Molecular Biology.* (2013).

68. SANO. ER stress-induced cell death mechanisms. Biochimica et Biophysica Acta (BBA) (2013). doi: 10.1016/j.bbamcr.2013.06.028

69. Jingjing Huang. Unfolded protein response in colorectal cancer. *Cell & Bioscience.* (2021). doi: 10.1186/s13578-021-00538-z

70. Giulia Mearini. Ubiquitin-proteasome system in cardiac dysfunction. Biochimica et Biophysica Acta (BBA) (2008). doi: 10.1016/j.bbadis.2008.06.009

71. David Ron. Translational control in the endoplasmic reticulum stress response. *European Journal of Clinical Investigation.* (2002). doi: 10.1172/JCI16784

72. Aya Uemura. Unconventional splicing of XBP1 mRNA occurs in the cytoplasm during the mammalian unfolded protein response. *Journal of Cell Science.* (2009). doi: 10.1242/jcs.040584

73. Ying-Chun Chen. Rutaecarpine analogues reduce lipid accumulation in adipocytes via inhibiting adipogenesis/lipogenesis with AMPK activation and UPR suppression. *ACS Chemical Biology* (2013). doi: 10.1021/cb4003893

74. Robert F Hillary. A lifetime of stress: ATF6 in development and homeostasis. *Journal of Biomedical Science.* (2018). doi: 10.1186/s12929-018-0453-1

75. K Haze. Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. *Molecular Biology of the Cell* (1999). doi: 10.1091/mbc.10.11.3787

76. Jingshi Shen. ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. *Developmental Cell (*2002). doi: 10.1016/s1534-5807(02)00203-4

77. Naiyana Gujral. Effect of anti-gliadin IgY antibody on epithelial intestinal integrity and inflammatory response induced by gliadin. *BMC Immunology* (2015). doi: 10.1186/s12865-015-0104-1

78. Ferrari, E. *et al.* Probiotics supplements reduce er stress and gut inflammation associated with gliadin intake in a mouse model of gluten sensitivity. *Nutrients* (2021). doi: 10.3390/nu13041221

79. Pahl, H. L. & Baeuerle, P. A. A novel signal transduction pathway from the endoplasmic reticulum to the nucleus is mediated by transcription factor NF-κB. The *EMBO Journal.* (1995). doi: 10.1002/j.1460-2075.1995.tb07256.x

80. Tom Verfaillie. Targeting ER stress induced apoptosis and inflammation in cancer. *Cancer Letters* (2013). doi: 10.1016/j.canlet.2010.07.016

81. Gagliardi, M. Aldo-keto reductases protect metastatic melanoma from ER stress-independent ferroptosis. *Cell Death Disease* (2019). doi: 10.1038/s41419-019-2143-7

82. Corazzari M. Oncogenic BRAF induces chronic ER stress condition resulting in increased basal autophagy and apoptotic resistance of cutaneous melanoma. *Cell death Differentiation.* (2015). doi: 10.1038/cdd.2014.183

83. G., I. Structural analysis and Caco-2 cell permeability of the celiac-toxic A-gliadin peptide 31-55. *Journal of Agricultural and Food Chemistry* (2013). doi: 10.1021/jf3045523

84. Antonella Capozzi. Modulatory Effect of Gliadin Peptide 10-mer on Epithelial Intestinal CACO-2 Cell

Inflammatory Response. *PLoS One* (2013). doi: 10.1371/journal.pone.0066561

85. Bartoszewski, R. The mechanism of cystic fibrosis transmembrane conductance regulator transcriptional repression during the unfolded protein response. *Journal of Biological Chemistry.* (2008). doi: 10.1074/jbc.M707610200

86. Kevin D Brown. Transglutaminase 2 and NF-κB: an odd couple that shapes breast cancer phenotype. *Breast Cancer Research Treatment* (2013). doi: 10.1007/s10549-012-2351-7

87. Atul Munish Chander, Hariom Yadav , Shalini Jain, Sanjay Kumar Bhadada, D. K. D.Cross-Talk Between Gluten, Intestinal Microbiota and Intestinal Mucosa in Celiac Disease: Recent Advances and Basis of Autoimmunity. *Frontiers in Microbiology* (2018). doi: 10.3389/fmicb.2018.02597

88. Alain Israël. The IKK complex, a central regulator of NF-kappaB activation. *Cold Spring Harbor Perspectives in Biology* (2010). doi: 10.1101/cshperspect.a000158

89. Andrea Oeckinghaus. The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harbor Perspectives in Biology* (2009). doi: 10.1101/cshperspect.a000034

90. M Karin. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annual Review of Immunology* (2000). doi: 10.1146/annurev.immunol

91. Masayuki Kaneko. Activation signal of nuclear factor-kappa B in response to endoplasmic reticulum stress is transduced via IRE1 and tumor necrosis factor receptor-associated factor 2. *Biological and Pharmaceutical Bulletin* (2003). doi: 10.1248/bpb.26.931

92. Ping Hu. Autocrine tumor necrosis factor alpha links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1alpha-mediated NF-kappaB activation and down-regulation of TRAF2 expression. *Molecular and Cellular Biology.* (2006). doi: 10.1128/MCB.26.8.3071-3084.2006

93. Arvin B Tam. ER stress activates NF-κB by integrating functions of basal IKK activity, IRE1 and PERK. *PLoS One* (2012). doi: 10.1371/journal.pone.0045078

94. Der-Yen Lee. Methylglyoxal in cells elicits a negative feedback loop entailing transglutaminase 2 and glyoxalase 1. *Redox Biology (*2014). doi: 10.1016/j.redox.2013.12.024

95. Stefania Martucciello. Interplay between Type 2 Transglutaminase (TG2), Gliadin Peptide 31-43 and Anti-TG2 Antibodies in Celiac Disease. *International Journal of Molecular Sciences.* (2020). doi: 10.3390/ijms21103673

96. Saverio Marchi. The endoplasmic reticulum-mitochondria connection: one touch, multiple functions. *Biochimica et Biophysica Acta* (2013). doi: 10.1016/j.bbabio.2013.10.015.

97. Arun Raturi. Where the endoplasmic reticulum and the mitochondrion tie the knot: the mitochondria-associated membrane (MAM). *Biochimica et Biophysica Acta* (2012). doi: 10.1016/j.bbamcr.2012.04.013

98. Rafaela Bagur. Intracellular Ca 2+ Sensing: Its Role in Calcium Homeostasis and Signaling. *Molecular Cell* (2017). doi: 10.1016/j.molcel.2017.05.028

99. Entaz Bahar. No TitleER Stress-Mediated Signaling: Action Potential and Ca(2+) as Key Players. *International Journal of Molecular Sciences.* (2016). doi: 10.3390/ijms17091558

100. P, Pinton. Calcium and apoptosis: ER-mitochondria Ca2+ transfer in the control of apoptosis. *Oncogene* (2008). doi: 10.1038/onc.2008.308

101. Zhang, S. Inositol 1,4,5-trisphosphate receptor subtype-specific regulation of calcium oscillations. *Neurochemical Research.* (2011). doi: 10.1007/s11064-011-0457-7

102. Kazunori Kanemaru. Calcium-dependent N-cadherin up-regulation mediates reactive astrogliosis and neuroprotection after brain injury. *Proceedings of the National Academy of Sciences* (2013). doi: 10.1073/pnas.1300378110

103. Djalila Mekahli. Endoplasmic-reticulum calcium depletion and disease. *Cold Spring Harbor Perspectives in Biology* (2011). doi: 10.1101/cshperspect.a004317

104. Karen M. Lammers. Gliadin Induces an Increase in Intestinal Permeability and Zonulin Release by Binding to the Chemokine Receptor CXCR3. *Gastroenterology* (2008). doi: 10.1053/j.gastro.2008.03.023

105. Ryuma Tokunaga. No TitleCXCL9, CXCL10, CXCL11/CXCR3 axis for immune activation - A target for novel cancer therapy. *Cancer Treatment Reviews* (2018). doi: 10.1016/j.ctrv.2017.11.007

106. Joanna R Groom. CXCR3 in T cell function. *Experimental Cell Research.* (2011). doi: 10.1016/j.yexcr.2010.12.017

107. Y H Yuan. Chemokine receptor CXCR3 expression in inflammatory bowel disease. *Inflammatory Bowel Disease* (2001). doi: 10.1097/00054725-200111000-00001

108. Y Ha. Endoplasmic reticulum stress-regulated CXCR3 pathway mediates inflammation and neuronal injury in acute glaucoma. *Cell Death Disease.* (2015). doi: 10.1038/cddis.2015.281

109. Bao-Chun Jiang. Promoted Interaction of C/EBPα with Demethylated Cxcr3 Gene Promoter Contributes to Neuropathic Pain in Mice. *Journal of Neuroscience* (2017). doi: 10.1523/JNEUROSCI.2262-16.2016

110. Herbert Wieser. Chemistry of gluten proteins. *Food Microbiol.* (2007). doi: 10.1016/j.fm.2006.07.004

111. L M Sollid. Molecular basis of celiac disease. *Annual Reviews Immunolology* (2000). doi: 10.1146/annurev.immunol.18.1.53

112. Daniel A Leffler. Leffler D.A, 2015. *Nature Reviews Gastroenterology & Hepatology .* (2015). doi: 10.1038/nrgastro.2015.131

113. W Dieterich. Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nature Medicine* (1997). doi: 10.1038/nm0797-797

114. L M Sollid. Celiac disease and transglutaminase 2: a model for posttranslational modification of antigens and HLA association in the pathogenesis of autoimmune disorders. *Current Opinion in*

Immunology (2011). doi: 10.1016/j.coi.2011.08.006

115. H J Cornell. The toxicity of certain cereal proteins in coeliac disease. *Gut* (1974). doi: 10.1136/gut.15.11.862

116. Pedro Ayala. Attenuation of endoplasmic reticulum stress using the chemical chaperone 4-phenylbutyric acid prevents cardiac fibrosis induced by isoproterenol. *Experimental and Molecular Pathology.* (2012). doi: 10.1016/j.yexmp.2011.10.012

117. Maria Vittoria Barone. Gliadin Peptides as Triggers of the Proliferative and Stress/Innate Immune Response of the Celiac Small Intestinal Mucosa. *International Journal of Molecular Sciences.* (2014). doi: 10.3390/ijms151120518.

118. Kirk R Henne. Sequential metabolism of AMG 487, a novel CXCR3 antagonist, results in formation of quinone reactive metabolites that covalently modify CYP3A4 Cys239 and cause time-dependent inhibition of the enzyme. *Drug Metabolism & Disposition* (2012). doi: 10.1124/dmd.112.045708

119. Martine J Smit. CXCR3-mediated chemotaxis of human T cells is regulated by a Gi- and phospholipase C-dependent pathway and not via activation of MEK/p44/p42 MAPK nor Akt/PI-3 kinase. *Blood* (2003). doi: 10.1182/blood-2002-12-3945

120. Djalila Mekahli. Endoplasmic-Reticulum Calcium Depletion and Disease. *Cold Spring Harbor Perspectives in Biology.* (2011). doi: 10.1101/cshperspect.a004317

121. Keiko Morotomi-Yano. Calcium-dependent activation of transglutaminase 2 by nanosecond pulsed electric fields. *FEBS Open Bio.* (2017). doi: 10.1002/2211-5463.12227

122. Santosh Kumar. Tissue Transglutaminase Constitutively Activates HIF-1α Promoter and Nuclear Factor-κB via a Non-Canonical Pathway. *PLoS One* (2012). doi: 10.1371/journal.pone.0049321