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# The potential role of the epithelial cells and the mesenchymal stem cells in Crohn's disease fibrosis : from bedside to bench and back

Sophie VIEUJEAN

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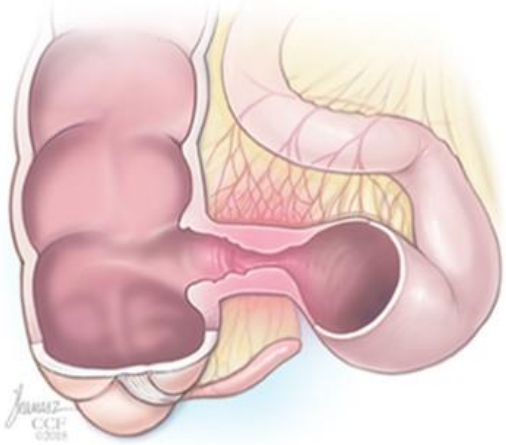
Doctor in Medical Sciences



Promotor: Prof. Edouard Louis

Laboratory of Translational Gastroenterology

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## List of abbreviations

| | |
|---|---|
| α-SMA | Alpha-smooth muscle actin |
| AIEC | Adherent-invasive Escherichia coli |
| AGR2 | Anterior gradient protein 2 homolog |
| ANOVA | Analysis of variance |
| ATF4 | Activating transcription factor 4 |
| ATF6 | Activating transcription factor 6 |
| ATG16L1 | Autophagy related 16-like 1 |
| BiP | Binding-immunoglobulin protein |
| CAFs | Cancer-associated fibroblasts |
| CARD15 | Caspase recruitment domain-containing protein 15 |
| CD | Crohn's disease |
| CDAI | Crohn's Disease Activity Index |
| CDOS | Crohn's Disease Obstructive Score |
| CHOP | C/EBP Homologous Protein |
| COL1A1 | Collagen alpha-1(I) chain |
| COL3A1 | Collagen alpha-1(III) chain |
| CRP | C-reactive protein |
| CT | Computed tomography |
| CTGF | Connective tissue growth factor |
| DAMPs | Damage-associated molecular patterns |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DSS | Dextran sulfate sodium |
| EBD | Endoscopic balloon dilatation |
| ECM | Extracellular matrix |
| EGF | Epidermal growth factor |
| eIF2α | Eukaryotic translation initiation factor 2α |
| ELISA | Enzyme-linked immunosorbent assay |
| EMT | Epithelial-to-mesenchymal transition |
| EndoMT | Endothelial-to-mesenchymal transition |
| ER | Endoplasmic reticulum |
| ERAD | Endoplasmic reticulum-associated degradation |
| ERP44 | Endoplasmic reticulum resident protein 44 |
| EVs | Extracellular vesicles |
| FBS | Foetal bovine serum |
| FFPE | Formalin-fixed paraffin-embedded |
| FN | Fibronectin |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GWAS | Genome-wide association study |
| GRP78 | Glucose-regulated protein 78 |
| HGF | Hepatocyte growth factor |
| H&E | Haematoxylin and eosin |
| HPRT | Hypoxanthine-guanine phosphoribosyltransferase |
| IBD | Inflammatory bowel disease |
| ICAM | Intercellular adhesion molecule |
| IEC | Intestinal epithelial cell |
| IF | Immunofluorescence |

| | |
|---|---|
| IFN-γ | Interferon gamma |
| Ig | Immunoglobulin |
| IHC | Immunohistochemistry |
| IL | Interleukin |
| IRE1 | Inositol-requiring enzyme 1 |
| JNK | c-Jun N-terminal kinase |
| LAP | Latency-associated peptide |
| LLC | Large latent complex |
| LTBP | Latent-transforming growth factor beta-binding protein |
| MAPK | Mitogen-activated protein kinase |
| MDP | Muramyl dipeptide |
| MMP | Matrix metalloproteinase |
| MRI | Magnetic resonance imaging |
| MSCs | Mesenchymal stem cells |
| MUC2 | Mucin-2 |
| MVs | Microvesicles |
| NF-κB | Nuclear factor-kappa B |
| NLR | NOD-like receptor |
| NOD2 | Nucleotide-binding oligomerization domain-containing protein 2 |
| PAMPs | Pathogen associated molecular patterns |
| PBA | 4-phenylbutyric acid |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PDI | Protein disulfide isomerase |
| PDIA6 | Protein disulfide-isomerase A6 |
| PERK | Protein kinase related-like endoplasmic reticulum kinase |
| PDGF | Platelet-derived growth factor |
| PRR | Pattern recognition receptor |
| RNA | Ribonucleic acid |
| RIDD | Regulated IRE1-dependent decay |
| RT-qPCR | Reverse transcription quantitative polymerase chain reaction |
| SEMS | Self-expanding metal stents |
| SLC | Small latent complex |
| sXBP1 | Spliced X-box binding protein 1 |
| TBS-T | Tris-buffered saline with tween |
| TC | Transitional cells |
| TGF-β | Transforming growth factor beta |
| TLR | Toll-like receptor |
| TIMPs | Tissue inhibitors of metalloproteinases |
| Tm | Tunicamycin |
| TME | Tumor microenvironnement |
| TNBS | 2,4,6-trinitrobenzene sulfonic acid |
| TNF-α | Tumor necrosis factor alpha |
| TRAF2 | TNF receptor-associated factor 2 |
| TUDCA | Tauroursodeoxycholic acid |
| UC | Ulcerative colitis |
| UPR | Unfolded protein response |
| US | Ultrasound |
| VCAM | Vascular cell adhesion protein |
| WB | Western blot |

# GENERAL INTRODUCTION

# 1. GENERAL INTRODUCTION

## 1.1. Crohn's disease

### 1.1.1. Crohn's disease: epidemiology

Inflammatory bowel disease (IBD) is a major problem of public health with a sustained increasing incidence[1]. This encompasses two main entities which differ by their location and the depth of intestinal wall damage: ulcerative colitis (UC) and Crohn's disease (CD). The latter, described for the first time by Dr. Burrill B. Crohn in 1932, is characterised by a submucosal or transmural inflammation and affects any segment of the gastrointestinal tract in a non-continuous way while UC is characterised by a mucosal inflammation and is limited to the colon[2]. Although during the 20$^{th}$ century, IBD was mainly a westernised countries disease (Europe, North America and Oceania), it became, at the turn of the 21$^{st}$ century, a global pandemic with an increased incidence in newly industrialised countries (Asia, the Middle East and South America) secondary to the westernisation of cultures and societies in these parts of the world[3–7]. IBD affects all ethnic groups, with a higher incidence in the Ashkenazi Jews population[8]. In Europe, it is generally estimated that 1.3 million people (0.2% of the European population) suffer from IBD with a north-south, a west-east and an urban-rural reported gradient[3,9,10]. Figure 1 shows the incidence and prevalence of CD in Europe in the 21$^{st}$ century as maps[6]. According to recent data, the CD incidence ranged from 0.4 to 22.8 per 100 000 person-years and the prevalence from 1.5 (Romania) to 331 (Netherlands) per 100 000, both varying greatly according to countries and studies[11]. The latest Belgian epidemiological IBD studies, performed in the 90's in Brussels and Liège, reported a CD incidence of 4.1 and 5.5 cases per 100 000 inhabitants per year, respectively[12–14]. The disease can occur at any age but mainly affects young adults, with a disease onset between 20 and 40 years[3]. A second less prominent peak has been reported in the elderly population between 60 and 80 years, but this remains an inconsistent finding[3,15,16]. There is no sex-specific distribution in adult CD[17].

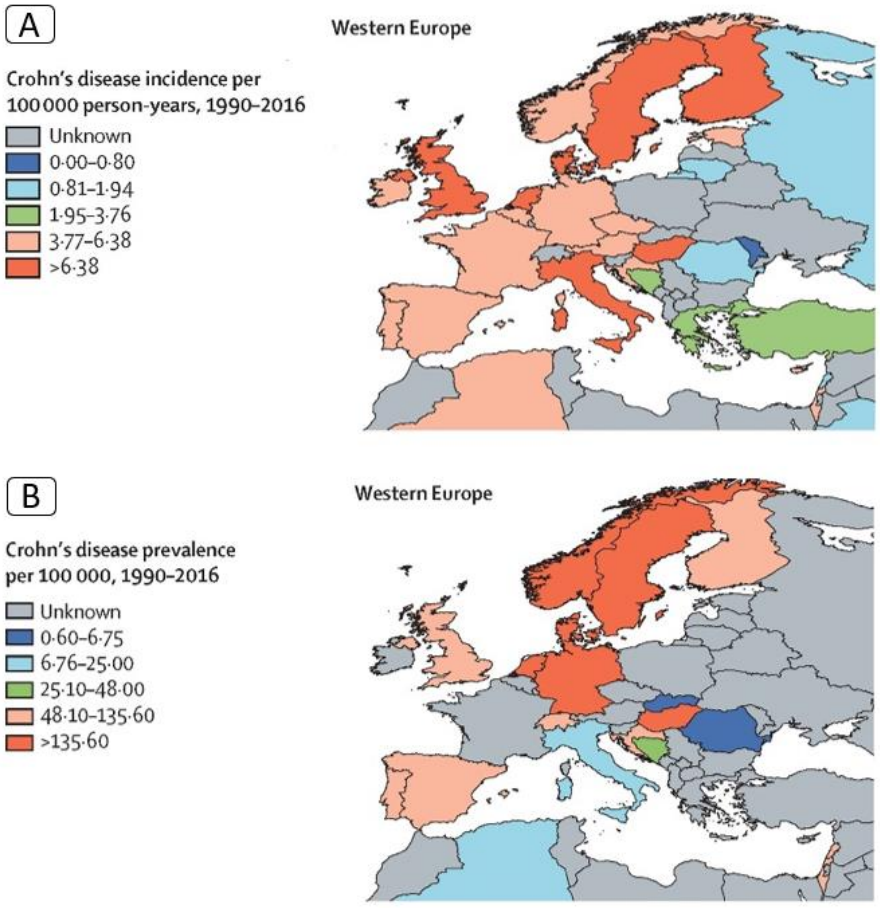



**Figure 1. European incidence [A] and prevalence [B] of Crohn's disease**[6]

### 1.1.2. Crohn's disease: clinical, endoscopic and histological presentation

CD is a chronic inflammatory condition characterised by a relapsing-remitting course[18,19]. This inflammation may affect, in a segmental manner, any part of the gastrointestinal tract, from the mouth to the anus with a predilection for the terminal ileum, colon or ileocecal region[20]. The CD clinical presentation is heterogeneous and can be insidious, depending on the disease location, disease activity and the presence or not of complications. Symptoms commonly include chronic diarrhoea, abdominal pain, weight loss, fatigue as well as blood and/or mucus in the stool especially in patients with a colonic involvement[21–24]. Patients may also experience extraintestinal manifestations affecting joints (peripheral arthropathies or axial arthropathy), skin (erythema nodosum, pyoderma gangrenosum, aphthous stomatitis, Sweet syndrome…), and eyes (uveitis, episcleritis, scleritis) as well as the biliary tract, pancreas, lungs or kidneys[25]. Beside these systemic symptoms, the disease can also manifest by symptoms secondary to a local complication such as perianal fistulas (present in 4-10% of patients at diagnosis)[23,26], the presence of strictures, fistulas or intra-abdominal abscesses.

Coupled to the physical examination (which may reveal abdominal tenderness and/or masses as well as fissures, fistulas or abscesses at perianal examination), the laboratory findings (including anaemia, thrombocytosis, hypoalbuminemia, elevated C-reactive protein (CRP) or erythrocyte sedimentation rate, vitamins deficiencies and presence of anti-Saccharomyces cerevisiae antibodies IgA), the elevated faecal calprotectin, as well as the cross-sectional imaging findings, it is generally the ileocolonoscopy with biopsies which remains the gold standard to confirm CD diagnosis[17,22,27,28]. Typical endoscopic CD hallmarks (Figure 2) include patchy distribution of inflammation with skip lesions occurring on a normal adjacent mucosa. These lesions may be small aphthous ulcers, which may coalesce over time, to become linear or large deep serpiginous ulcers. These ulcers, if deep, discrete, and separated by oedematous mucosa islands may give rise to the classical so-called cobblestone appearance. The endoscopic examination may also reveal strictures and fistulas, resulting from the transmural impairment, but also scaring lesions such as ulcers scars typically depressed, as well as inflammatory pseudopolyps[29]. CD may also be diagnosed after a surgical resection. The inflamed segment may have an external hyperaemic appearance as well as a mesenteric fat wrapping, known as creeping fat[29].

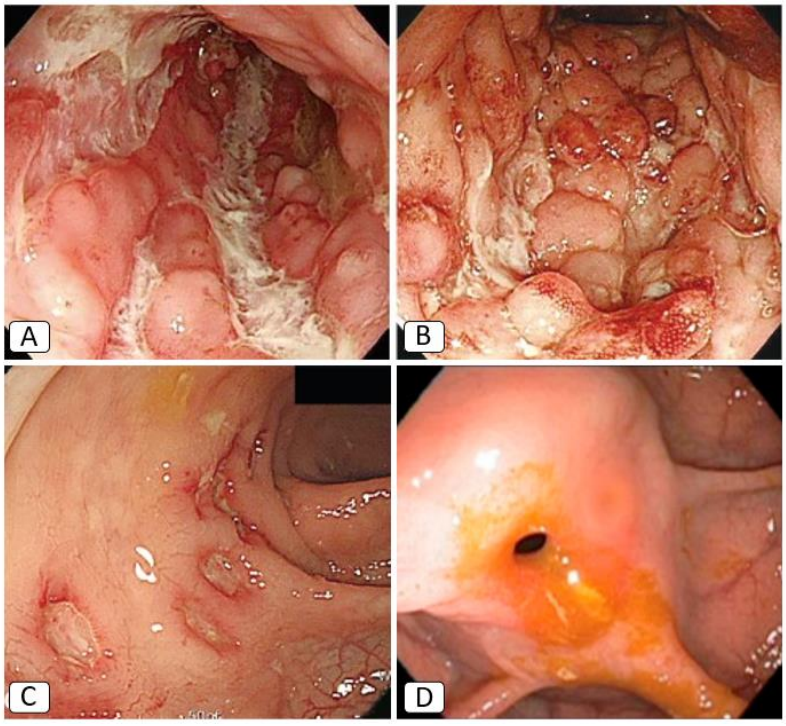



**Figure 2. Typical endoscopic features of Crohn's disease** (adapted from Lee JM et al., 2016[30] and Keihanian S et al., 2016[31])
[A] Longitudinal ulcers; [B] cobblestone appearance; [C] aphthous ulcers showing longitudinal array; [D] stricture.

Several histological features may help in making the CD diagnosis (Figure 3). Generally, a focal (discontinuous) chronic inflammation, characterised by an increase of lymphocytes and plasma cells in the lamina propria, can be find[32]. Mucosal architecture is commonly altered and this may present irregularities of the villi (in small bowel) or of the crypts (in colon or in small bowel), described as crypt distortion, crypt branching or crypt shortening as well as crypt abscesses[33]. Finally, epithelioid and gigantocellular granulomas without caseous necrosis can be present, which are characteristic of CD, arising from the accumulation of epithelioid cells, as well as macrophages and lymphocytes which can be found in both inflamed and normal mucosa[29].

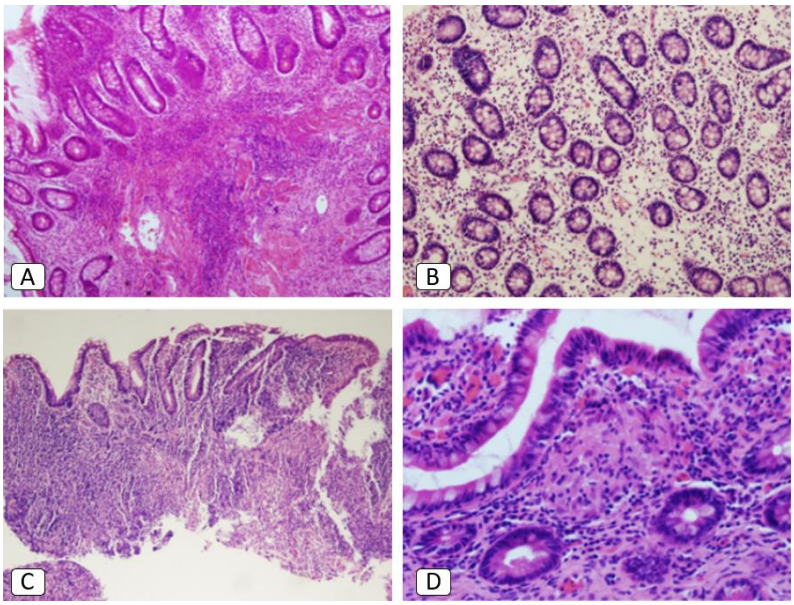



**Figure 3. Microscopic features of Crohn's disease** (adapted from Cui Y et al., 2019[32]) [A] Inflammation involving the submucosa and basal plasmacytosis. Haematoxylin and eosin (H&E) 100x. [B] Aberrant crypt structure: distorted, non-parallel, and irregular. H&E 200x. [C] Villi changes (shortening, widening, and blunting) and decrease in intra-epithelial mucus. H&E 100x. [D] Non-caseating epithelioid granuloma. H&E 400x.

### 1.1.3. Crohn's disease: pathophysiology

The pathogenesis of IBD is not fully understood but the most commonly accepted hypothesis is an inappropriate gut mucosal immune response towards the constituents of the gut microbiota which cross an impaired epithelial barrier, in genetically predisposed individuals and under the influence of environmental factors (Figure 4)[34,35].

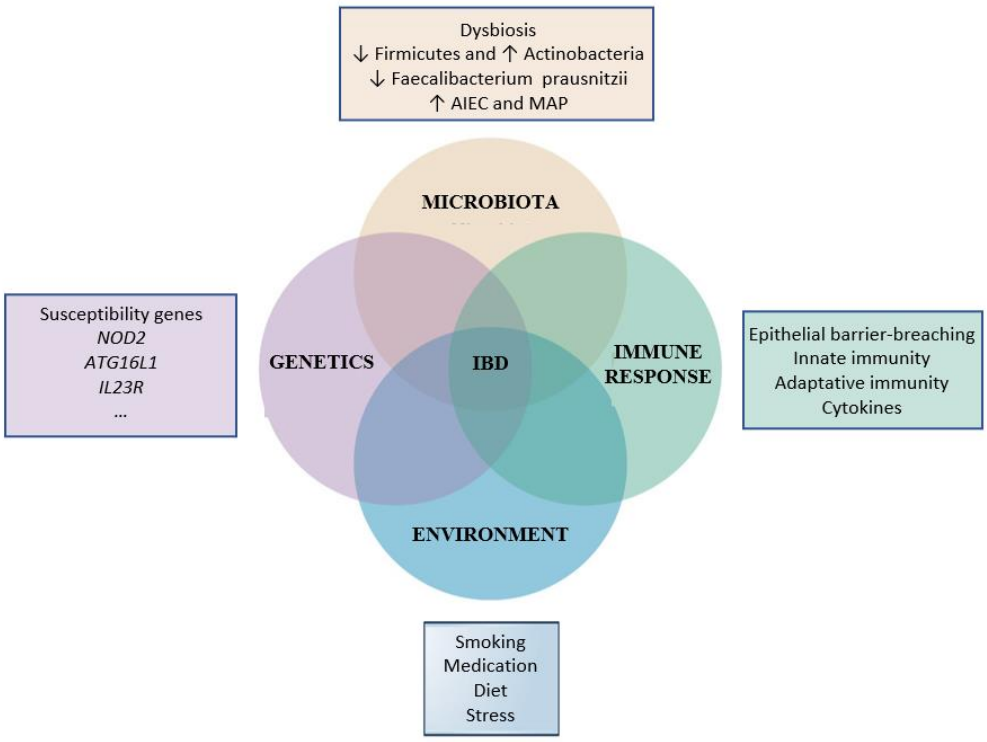



**Figure 4. The interplay mechanisms involved in IBD pathogenesis** (adapted from Sartor RB et al.[37], 2006 and Ramos et al., 2019[38])
AIEC, Adherent-invasive Escherichia coli; *ATG16L1, Autophagy related 16-like 1*; IBD, Inflammatory bowel disease; *IL23R, Interleukin 23 receptor*; MAP, Mycobacterium avium subspecies paratuberculosis*; NOD2, Nucleotide Binding Oligomerization Domain Containing 2*.

### *Genetic*

The existence of a higher prevalence of IBD in certain ethnics groups and of familial clusters, the concordance among monozygotic twins as well as the identification of specific disease susceptibility genes are evidences for a genetic predisposition in IBD[34,36–39]. To date, genome-wide association studies (GWASs) identified 240 IBD susceptibility loci[40] (including 37 CD-specific[41,42]) allowing to provide major insights about key cellular pathways involved in IBD immunopathogenesis (Figure 5), such as innate microbial sensing and mucosal defences (*NOD2 gene*), autophagy (*ATG16L1*, *IRGM genes*), ER stress (*XBP1* gene) or Th17-cell function (*IL23R* gene[43]). The most strongly CD-associated gene is *NOD2/CARD15* (nucleotide-binding oligomerization domain containing 2/caspase recruitment domain-containing protein 15) encoding for a member of NOD-like receptor (NLR) family, one of the five major classes of PRRs (pattern recognition receptors), dedicated to quickly recognize intracellular pathogens and alert the immune system. NOD2 was initially described as cytoplasmic sensor of muramyl dipeptide or MDP (a fragment of bacterial peptidoglycan)[44,45],

but once activated, it is also able to activate the nuclear factor-kappa B (NF-κB) pathway and the upstream inflammatory cascade[46]. This gene is associated with a family history of CD, an earlier age of onset, more frequent ileal involvements and fibrostenotic disease[47]. Although a considerable progress has been made in susceptibility loci identification, this component still only explains a little more than 20% of the CD heritability, which, together with the relatively low concordance rates in monozygotic twins, emphasize the importance of environmental factors[48].

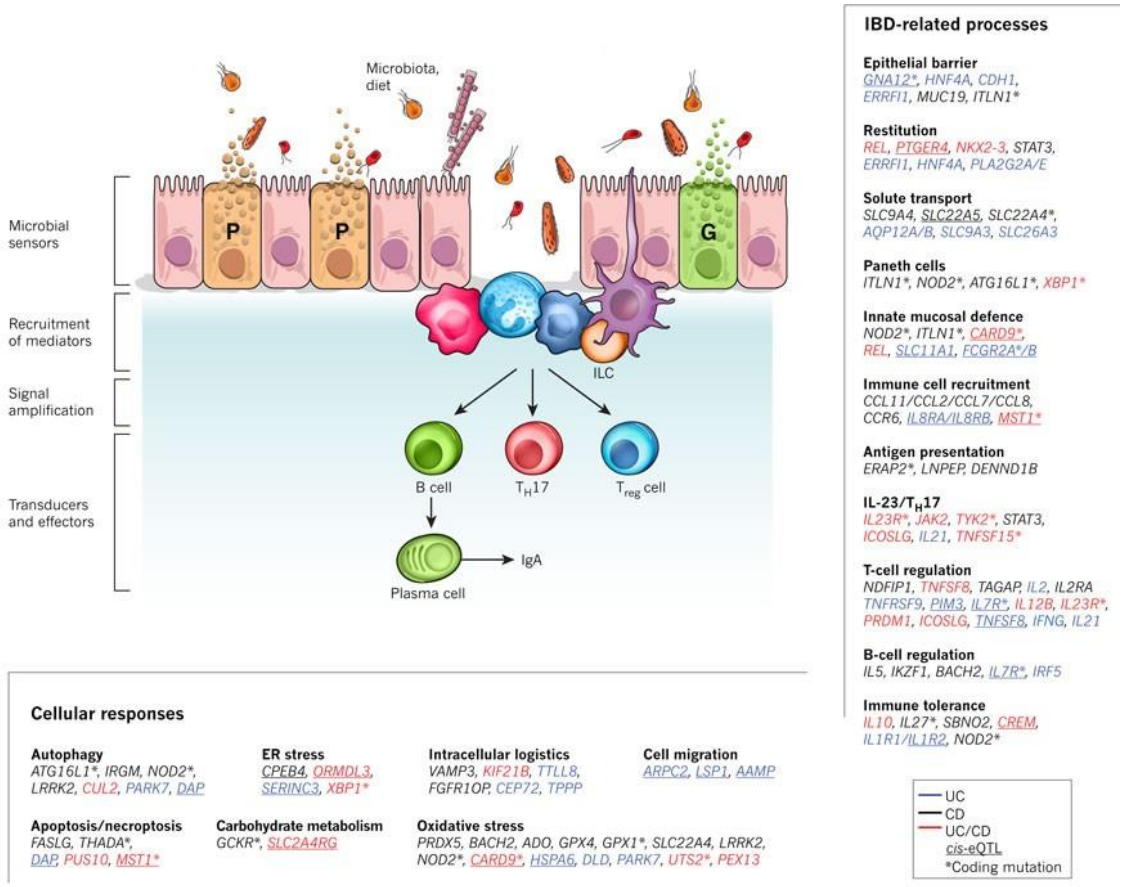



**Figure 5. A model for IBD pathways based on genome-wide association studies[49]**
CD, Crohn's disease; IBD, Inflammatory bowel disease; UC, Ulcerative colitis.

### *Environmental factors*

The best-studied environmental factor is smoking, which confers a relative risk of developing CD that is twice as high in smokers as in non-smokers and which is responsible for an aggravated disease course (including the occurrence of strictures and fistulas) and a poorer response to immunosuppressive treatments[50,51]. In addition to this major risk factor, there are others, more minor ones. The use of some medications such as antibiotics exposure in childhood, oral contraceptive, aspirin, and non-steroidal anti-inflammatory drugs has also been

incriminated[52–54]. Western diet most likely plays a role in the genesis of these diseases, by the presence of emulsifiers and through a low-fiber diet and high in saturated fats, as well as stress (however, most studies associating the onset of IBD with the occurrence of stress are retrospective, which limits their relevance)[55–57].

### *Microbiota*

These genetic and environmental factors contribute to the pathogenesis of IBD in part through the changes in the microbiota they induce, characterised by a bacterial, viral, and fungal dysbiosis. The most recognised changes include a reduced commensal biodiversity, a decrease in Firmicute bacteria and an increase in Actinobacteria[58]. These modifications lead to an imbalance between anti-inflammatory bacteria (such as *Faecalibacterium prausnitzii*, which are reduced in IBD[59,60]) and pro-inflammatory bacteria (such as adherent-invasive *Escherichia coli* and *Mycobacterium avium subspecies paratuberculosis*, which are observed in greater numbers in Crohn patient's mucosa)[61–63].

### *Immune response*

In IBD, the immune defence against pathogens from the intestinal microbiota (Figure 6) is deficient at two levels. On one hand, the intestinal barrier (which will be discussed further below) is damaged, promoting contact between pathogens and the cells of the underlying immunity. On the other hand, the innate and adaptative immune responses initiated by these contacts are impaired, contributing to an aberrant inflammatory response. The innate response represents a first line of non-specific defence against invading pathogens, aiming to quickly recognise them and alert the immune system. Next to epithelial cells, a variety of other cell types contributes to this innate response including stromal cells, neutrophils, dendritic cells, monocytes, macrophages and natural killer cells[64]. These cells sense invading pathogen-associated molecular patterns or PAMPs (including lipopolysaccharide, MDP, bacterial DNA, and double-stranded RNA) thanks to their PRRs, which may be on the surface cell (such as Toll-like receptors – TLRs) or in the cytoplasm (such as NOD-like receptors – NLRs, including NOD2)[65]. In IBD, where there is an altered expression and function of these PRRs, these innate immunity cells respond in an inappropriate way to microbial stimuli and secrete a serie of cytokines and chemokines promoting inflammation as well as the recruitment of other inflammatory cells. Located at the interface between epithelial cells (thanks to their

transepithelial dendrites[66]) and T cells, dendritic cells are antigen presenting cells responsible of T cells activation and differentiation, playing a key role in the crosstalk between innate and adaptative response, which confers a more specific and long-lasting immunity. In CD, the ability of dendritic cells to induce, from naive CD4+ LT cells (Th0), tolerogenic regulatory T cells or Tregs (secreting interleukin (IL)-10, transforming growth factor β or TGF-β or IL-35) is reduced in favour of differentiation into Th1 and Th17 effector T-cells, known to contribute to the excessive inflammatory response (through secretion of tumour necrosis factor-α (TNF-α), IL-17, IL-22 and interferon γ (IFN- γ))[34,51,67–70].

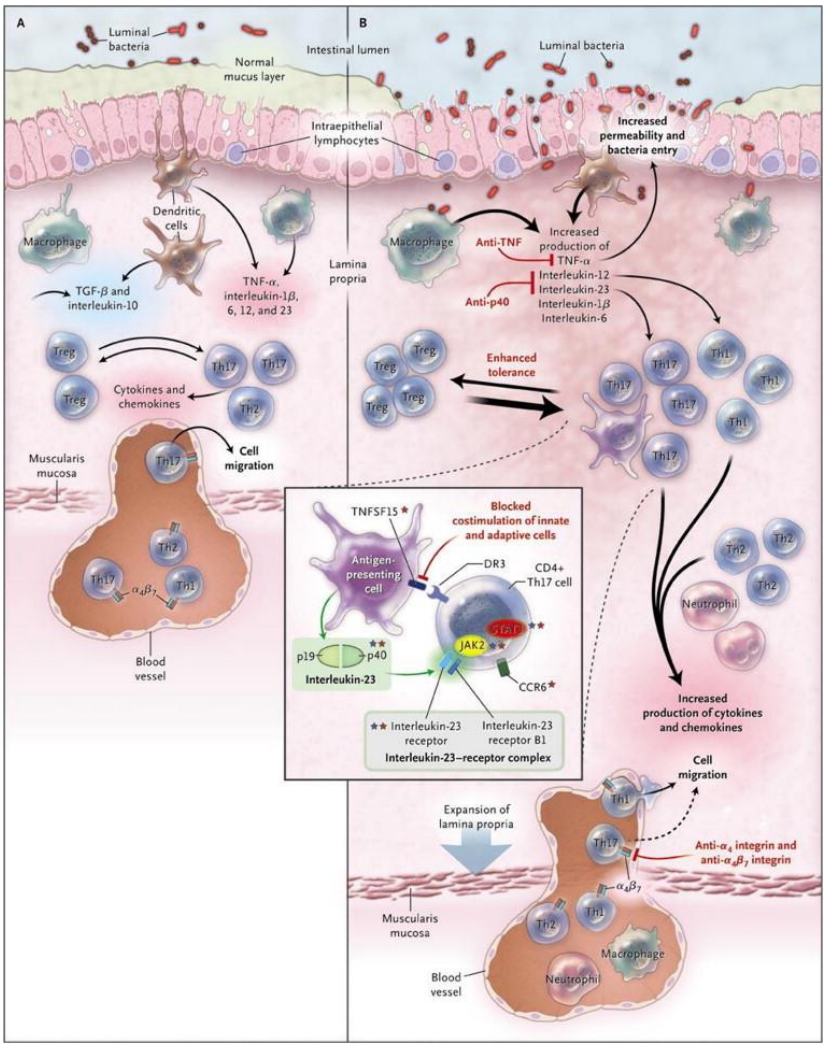



**Figure 6. The intestinal immune system in healthy subject [A] and in Crohn's disease [B][2]**
In the absence of inflammation [A], the lamina propria contains immune cells that secrete anti-inflammatory cytokines (such as TGF-β or IL-10) but also immune cells that secrete pro-inflammatory factors, limiting the entry of pathogens into the mucosa. This state is also characterized by a balance between Treg and Th1, Th2 and Th17 effector T-cells. In the case of inflammation [B], immune cells produce an excess of TNF-α as well as pro-inflammatory interleukins, promoting the differentiation of naive CD4+ LT cells (Th0) into pro-inflammatory Th1 and Th17 effector T-cells. These cells present an increased production of cytokines and chemokines, promoting additional leukocytes recruitment, sustaining the inflammatory process[2].

***Epithelial barrier-breaching***

The epithelial barrier represents the first line of defence against invading pathogens, but its integrity is defective in CD, favouring their interactions with the inappropriate and exaggerated immune system in this condition[71]. This epithelial barrier is composed of a single layer of epithelial cells covered by a mucus biofilm, and, in addition to its role of physical barrier, the epithelium also fulfils key roles in immunological function and in nutrients absorption[71]. The different cell types composing it (Figure 7) derive from a common intestinal stem cell[71,72]. Enterocytes, are the most common intestinal epithelial cells (IECs), absorb nutrients, produce digestive enzymes, and reabsorb bile salts to redirect them to the portal vein[73]. Between these enterocytes are interposed the goblet cells, which produce the mucus that lines the epithelium and protects it from the intestinal microflora[74]. In addition to these two cell types that migrate upwards along the crypt-villus axis[75–77], Paneth cells are a third type of cell (which mainly reside at the crypts' bottom unlike the others) and secrete anti-microbial peptides (such as α-defensins and lysozyme) as well as factors such as Wnt (playing a role in cells' differenciation[78]), epidermal growth factor (EGF) or TGF-β playing a key role for regeneration of the intestinal epithelium from the stem cell niche[79]. More scarce within the epithelium, enteroendocrine cells secrete a variety of hormones such as cholecystokinin and YY peptide[80,81]. Finally, other cells have a less characterised role, such as the M cells, which capture antigens by endocytosis in order to present them to the other immune system cells[82], and Tuft cells which could act as sensors of luminal agents and trigger a secretion of biologically effective molecules[83]. Under homeostatic conditions, this barrier is kept tightly sealed by intercellular junctions (tight junctions, adherens junctions, gap junctions and desmosomes)[84], by the renew of epithelial cells as well as mucus secretion[85].

The mucus lining the epithelium, whose production depends mainly on goblet cells[74], acts as a chemical shield due to the presence of Paneth cells anti-microbial peptides, IgA immunoglobulins[86,87] but also acts as a physical shield thanks to its viscoelastic properties, depending on the characteristics of the glycoproteins of which it is mainly composed, mucin MUC2[85]. This glycoprotein has an extremely complex structure, and its formation highly depends on an endoplasmic reticulum (ER)-resident protein, anterior gradient protein 2 homolog (AGR2) protein[88]. For instance, in the absence of AGR2, mice with a null mutation in the *Agr2* gene (*Agr2*−/− mice) produce less intestinal mucin and may develop spontaneous

terminal ileum and colonic inflammation, depending on genetic background and/or environmental factors, similar to what is seen in human CD[89–91].

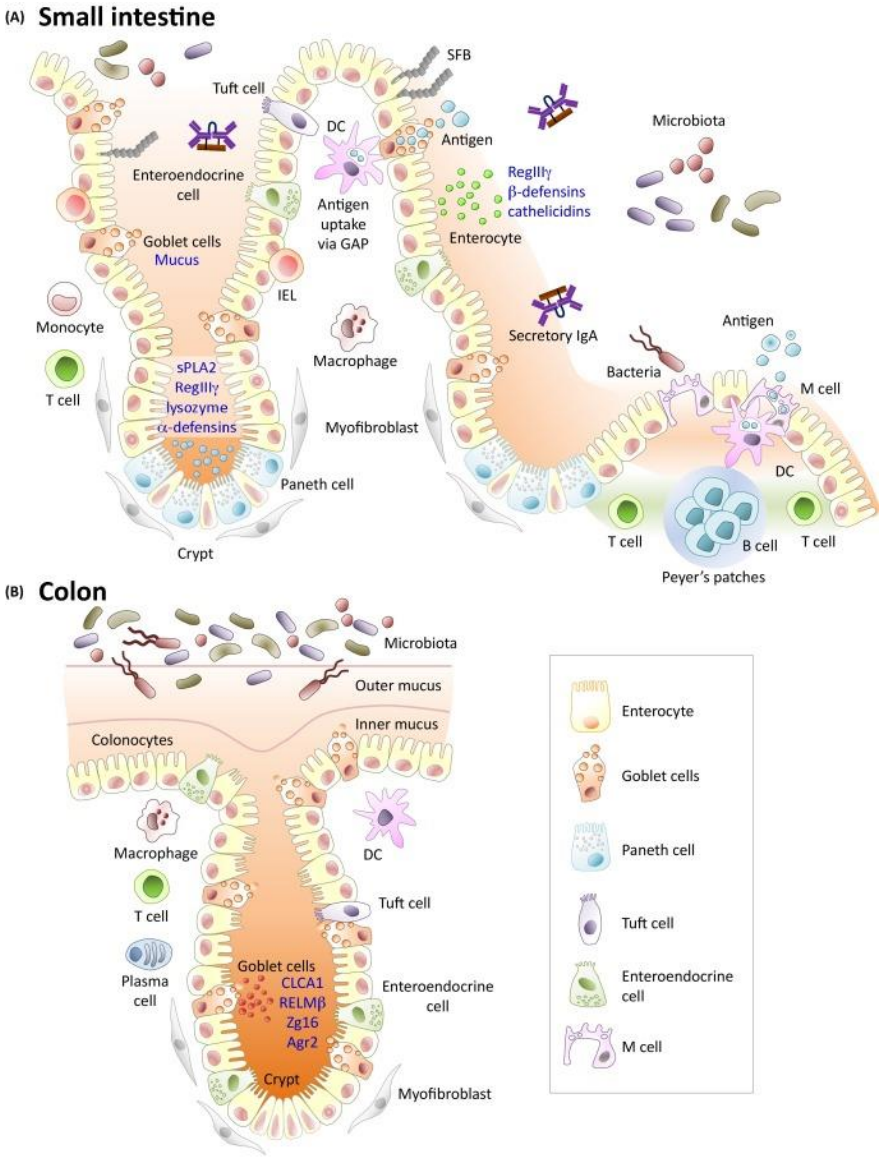



**Figure 7. Small bowel [A] and colonic [B] intestinal epithelial barrier[92]**
This barrier forms crypts and villi in the small intestine but only crypts, called Lieberkühn crypts, in the colon. Intestinal stem cells are found at the base of the crypts and can differentiate into 6 different types of intestinal cells, each with their own functions: enterocytes (referred as colonocytes in colon), goblet cells, enteroendocrine cells, Tuft cells, as well as 2 cell types specific to the small intestine: Paneth cells and M cells[92].

In IBD, as a result of changes in mucus production and composition[93], environmental factors[55] or defect of the intercellular junctions, holding the epithelial cells together[94], an epithelial barrier-breaching can occur. This is accompanied by a translocation of intraluminal microorganisms into the gut mucosa resulting, in addition to the activation of immune cells and

the related inflammatory cascade, in the activation of epithelial cellular defence mechanisms, including ER stress and autophagy[95].

***Endoplasmic reticulum stress, autophagy and inflammation***

In IECs, which have a highly secretory function (anti-microbial peptides, mucin…)[96], the ER plays a key role in proteins synthesis and maturation, achieving notably post-translational modifications such as N-glycosylation but also formation of disulfide bonds allowing proteins to acquire their 3D conformation to perform their function. When the IECs are subject to stress, including the intracellular pathogens entry, misfolded or unfolded proteins can accumulate in the ER lumen and trigger an ER stress. IECs then elicit a homeostatic adaptative response which aim at resolving this ER stress, known as unfolded protein response (UPR)[97]. This is mediated by three ER transmembrane sensors (Figure 8), IRE1 (inositol-requiring enzyme 1), PERK (Protein Kinase Related-like ER kinase) and ATF6 (Activating transcription factor 6; α and β isoforms), which cooperate with the chaperone binding immunoglobulin protein (BiP, also known as 78-kDa glucose regulated protein, GRP78, encoded by *Hspa5*) to sense misfolded proteins.

When inactive, these 3 ER transmembrane sensors are bound to BiP. Upon the accumulation of misfolded proteins in the ER lumen, the BiP chaperone preferentially binds to these (and directs them to the Endoplasmic-Reticulum Associated Degradation or ERAD pathway for degradation by the proteasome) and dissociates from the transmembrane sensors allowing their transition into an active state[98].

Once activated, PERK phosphorylates the translation initiation factor eIF2α (eukaryotic initiation factor 2α), making it inactive, and downregulates the translation of most mRNAs (and thus synthesis of overall proteins), but specifically upregulates ATF4 (activating transcription factor 4). This transcriptionally upregulates UPR target genes[99] including CCAAT/enhancer-binding protein (C/EBP) homologous protein (*CHOP*), which under conditions of prolonged unresolved ER stress promotes apoptosis[100].

The second arm of the UPR is mediated by IRE1α , which, upon sensing misfolded proteins, dimerizes and auto-phosphorylates, activating its endoribonuclease activity[101] which unconventionally splices X-box binding protein-1 (*Xbp1*) mRNA[102] to produce a functional

sXBP1 transcription factor of UPR target genes. The nonspecific endonuclease activity of IRE1 contributes to the mRNA degradation by a process known as regulated IRE1-dependent decay (RIDD)[103,104], also reducing their translation and the load of proteins entering the secretory pathway[105].

The third arm is initiated by ATF6 which, once activated, translocates to the Golgi apparatus where it is cleaved by the resident site-1 and 2 proteases (S1P and S2P)[106]. This cleavage allows the release of an active amino terminus domain of ATF6 (ATF6 p50) which translocates to the nucleus and binds to the ERSEs (ER stress response element) upstream of a subset of UPR-associated genes (partially overlap genes induced by IRE1α and PERK), including chaperones, involved in protein maturation[107].

Beside apoptosis (only triggered if adaptative mechanisms aimed at resolving ER stress fail), the main function coded by these UPR-associated genes are (1) to attenuate translation, which prevent an accumulation of unfolded proteins; (2) to strengthen the ERAD ability to clear unfolded proteins and send them to the cytoplasm for proteasome degradation and (3) to increase the protein folding capacity by upregulating ER-chaperones and folding enzymes, such as protein disulfide isomerase or PDIs (which rearrange the disulfide bonds of the proteins), including the above-mentioned AGR2 protein.

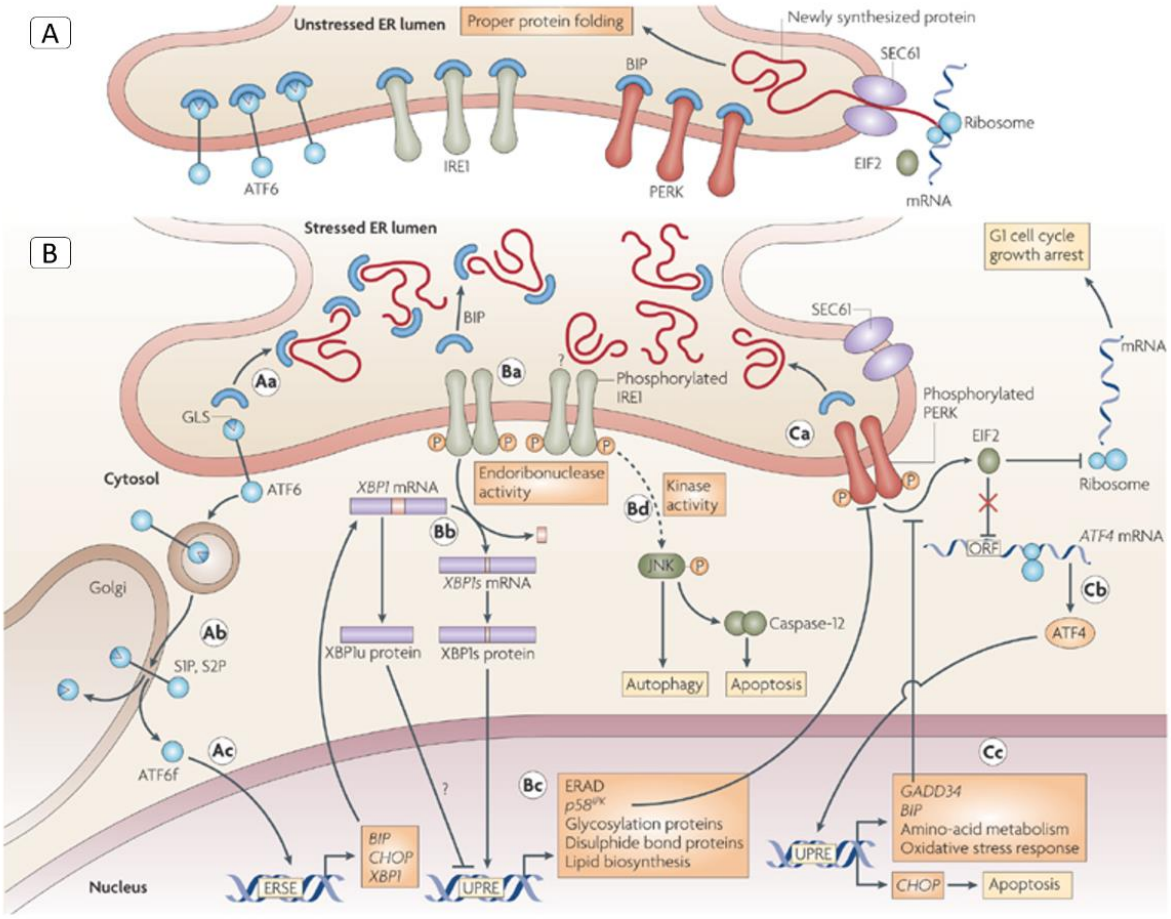



**Figure 8. Endoplasmic reticulum stress induces the unfolded protein response[108]**
In the absence of ER stress, when protein folding is correct [A], the 3 ER transmembrane sensors (PERK, IRE1 and ATF6) are retained inactive, bounded to BiP. Upon accumulation of misfolded proteins in the ER lumen and thus ER stress [B], BiP preferentially binds these misfolded or unfolded proteins, dissociating of the 3 ER transmembrane sensors, which become active. The first arm of the UPR to get going is PERK which attenuate translation, thus preventing the accumulation of new unfolded proteins in the ER lumen. IRE1/sXBP1 axis then induces the expression of a number of UPR target genes such as genes coding for ERAD (Endoplasmic-Reticulum Associated Degradation) pathway proteins or PDIs. The nonspecific endonuclease activity of IRE1 contributes also to the mRNA degradation. Finally, the ATF6 arm promotes transcription of chaperone proteins like BiP, PDIs and enables transcription of the XBP1 factor, which is then cleaved by IRE1[108].
ATF4, Activating Transcription Factor 4; ATF6, Activating Transcription Factor 6; CHOP, C/EBP Homologous Protein; eIF2α, eucaryotic Initiation Factor 2α; ERSE, ER stress-response element; GRP, Glucose-regulated Protein; IRE1α, Inositol Requiring Enzyme 1α; PERK, PKR-like Endoplasmic Reticulum Kinase; UPRE, Unfolded protein response element; XBP-1, X-box Binding Protein 1.

ER stress can also trigger autophagy, a cellular process allowing the clearance of intracellular unwanted microorganisms by the formation, sequestration and degradation of intracytoplasmic vacuoles[109]. Several pathways have been identified such as PERK-EIF2α-ATF4-CHOP and IRE1α Janus kinase (JNK)-dependent pathways[110]. ER stress and autophagy pathways are

particularly interlinked and some authors suggest that they are both necessary for the development of ileal inflammation[111].

Interestingly, the way in which the ER stress (notably triggered by the presence of invading bacteria in the cell[112]) promotes inflammation is partially orchestrated by the NOD2 protein (which is also a major regulator of autophagy[95,113,114]). Indeed, the activation of IRE1α (a branch of the UPR) promotes recruitment of TRAF2 (or TNF receptor-associated factor 2), which activates the NOD1/2-RIPK2 (Receptor-interacting serine/threonine-protein kinase 2) pathway resulting in NF-κB[115] and JNK-AP1 (protein-1 activator) activation[116,117]. This promotes the transcription of pro-inflammatory cytokines such as IL-6[98] (Figure 9). The mechanism whereby CD risk-conferring NOD2 variants interfere with this ER stress-mediated inflammation is still unresolved[46,118].

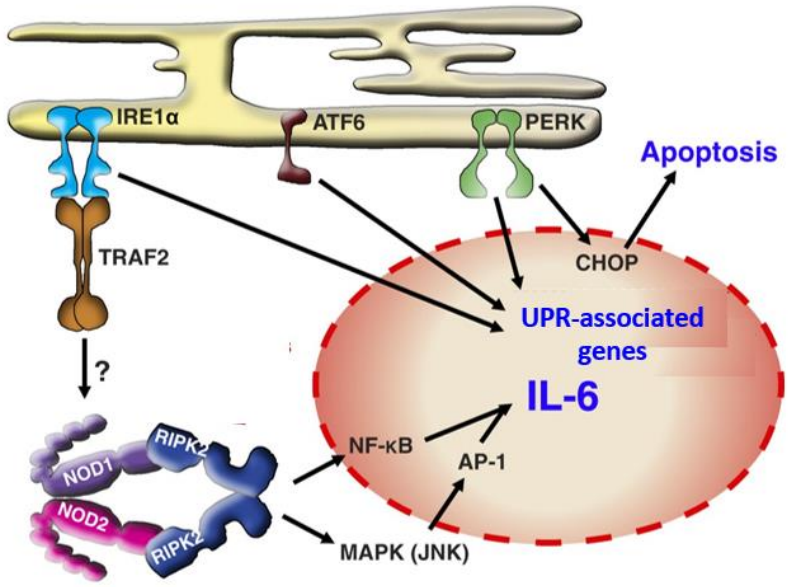



**Figure 9. Model of how ER stress induces a NOD1/2 dependent pro-inflammatory response** (adapted from Stafford C et al.[117])

The ER stress can also promote inflammation independently of NOD2 through different ways, depending on PERK, IRE1α and ATF6, which are summarised in Figure 10[119]. PERK has been shown to mediate inflammation through several processes[119]: (1) the PERK-eIF2α ER stress pathway up-regulates the pro-inflammatory factors IL-1β, TNF-α, and MCP-1 (monocyte chemoattractant protein 1) via ATF4/NF-κB pathway[119,120] but also IL-6, MCP-1 and CCL20 independently of ATF4[119]; (2) PERK/eIF2α also activates autophagy and inflammation through the PI3K/AKT/mTOR (phosphoinositide 3-kinase/Protein kinase B/mammalian target of rapamycin) signaling pathway[119,121]; (3) PERK additionally activates the p38/ERK

(extracellular signal-regulated kinase) axis promoting the secretion of IL-6 and IL-8[119,122]; (4) PERK induce an inflammatory response by activating the NOD1-dependent NF-κB pathway[119,123]; and finally (5) PERK promotes production of IL-6 through the JAK1/STAT3 pathway[119,124].

The pro-inflammatory mechanisms mediated by the IRE1α pathway include[119]: (1) the induction of expression of a range of inflammatory factors such as IFN-α, IFN-β, TNF-α, IL-6 by the transcription factor sXPB1; (2) the activation, by the complex formed by activated IRE1α and TRAF2, of JNK-AP1 and NF-κB, promoting IL-6 and TNFα production[119]; (3) the nonspecific endonuclease activity of IRE1 contributes to the mRNA degradation by RIDD into antiviral sensor RIG-1 (retinoic acid–inducible gene I) ligand, which triggers an inflammatory response through MAVS (mitochondrial antiviral signaling protein)[119,125,126]; (4) the activation of GSK-3β (glycogen synthase kinase-3β) promotes IL-1β transcription[119,127].

The role of ATF6 in inflammatory response remains to be further characterized[119]. ATF6 enhances the expression of sXBP1 by specifically binding to the XBP1 promoter and could play a role in the secretion of IL-6 and IL-1β.

In addition, UPR can also activate NLRP3 inflammasome, whose effector protein-caspase-1 converts pro-inflammatory cytokine IL-1β in its active form and promotes an inflammatory form of cell death named pyroptosis[128,129].

To sum up, some authors suggest that the trigger for CD could be ER stress, which can be induced by pathogens, and that this ER stress promotes chronic inflammation[118,130].

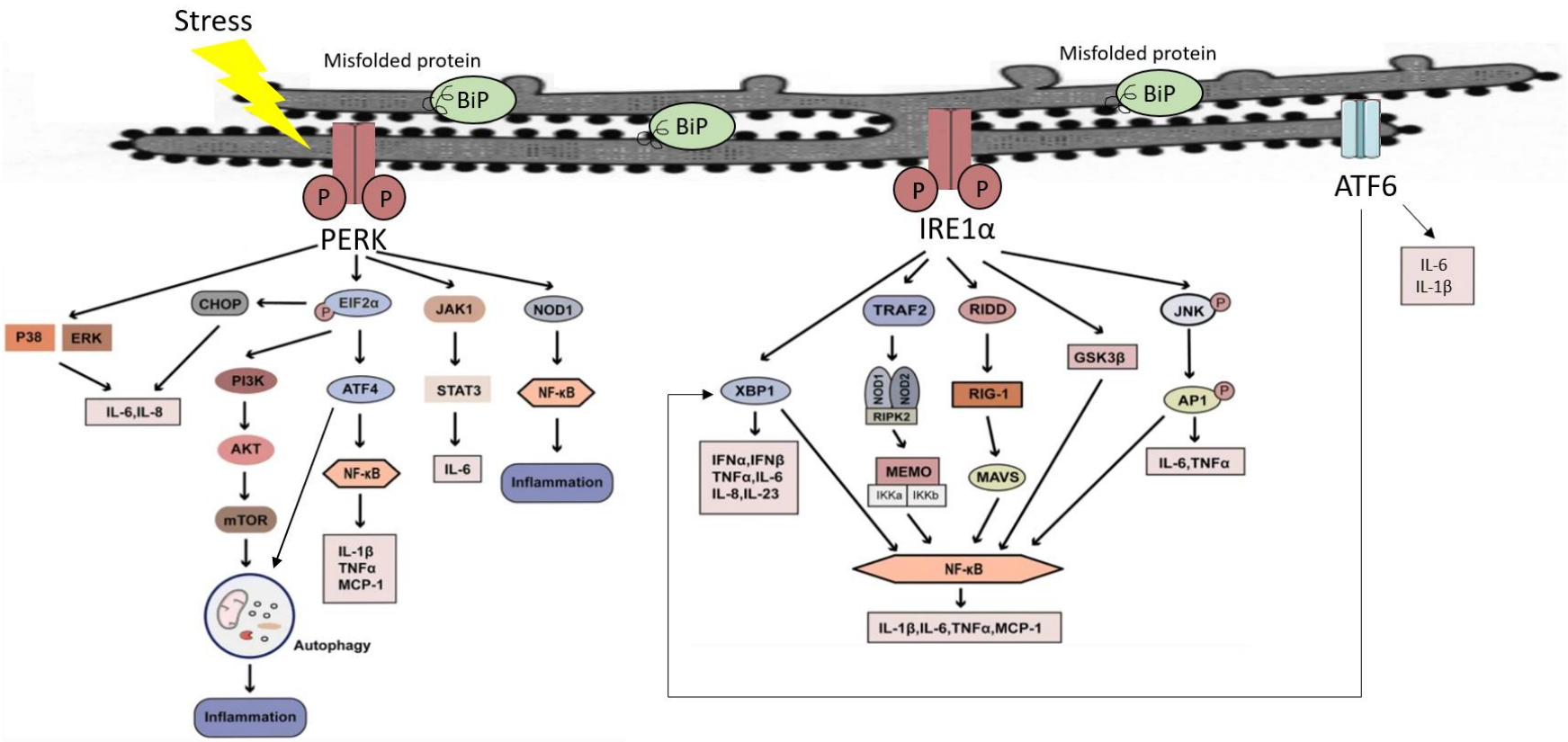



**Figure 10. PERK, IRE1α and ATF6 mediated inflammation** (adapted from Li W et al., 2020[119])

ER stress contributes to inflammation through the 3 arms of the UPR: IRE1α, PERK and ATF6. The NOD2-dependent pathway is activated via IRE1α/TRAF2/ NOD1/2-RIPK2. In addition to this NOD2-dependent pathway, many others pathways contribute to the inflammation independently of NOD2, including the promotion of NF-kB pathways and a range of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-8[119]. AP1, protein-1 activator; AKT, Protein kinase B; ATF4, Activating transcription factor 4; EIF2α, eukaryotic initiation factor 2α; ERK, extracellular signal-regulated kinase; IFN, Interferon; JAK1, Janus kinase 1; JNK, c-Jun N-terminal kinases; MAVS, mitochondrial antiviral signaling protein; MCP1, monocyte chemoattractant protein 1; mTOR, mammalian target of rapamycin; NF-κB, Nuclear factor-kappa B; PERK, Protein Kinase Related-like ER kinase; PI3K, phosphoinositide 3-kinase; RIDD, Regulated IRE1-dependent decay; RIG-1, retinoic acid–inducible gene; STAT3, Signal transducer and activator of transcription 3; TNF, Tumor necrosis factor.

### 1.1.4. Crohn's Disease: complications

This chronic inflammation leads to 2 types of complications. On one hand, it promotes carcinogenesis and IBD patients have a 2 to 6 times more likely to develop colorectal cancer[131] as well as an even greater relative risk to develop ileal cancer[132]. One the other hand, this chronic transmural inflammation initiates a wound healing process, which may, however, be pathological and can lead to penetrating complications (such as fistula or abscess formation) if it is insufficient or lead to stricturing complications if it is exaggerated. Indeed, in this situation, the excessive deposition of extracellular matrix (ECM) (characteristic of the intestinal fibrosis) contributes to lead to a progressive wall thickening and stricture formation resulting in a bowel lumen narrowing. Whereas, in UC, the ECM deposition is limited to the mucosa/submucosae and may contribute to colon shortening or stiffening, in CD, fibrosis can be transmural (as the inflammation) explaining the occurrence of these strictures[133,134]. As this intestinal fibrosis follows distribution and location of the inflammation, it is generally accepted that the local chronic inflammation is necessary to initiate the fibrosis process, but seems to subsequently play a minor role for its perpetuation and worsening[135,136]. Indeed, once ECM deposition initiate, it evolves on its own, independently of the inflammation, suggesting that the mechanisms regulating fibrosis are distinct from those regulating inflammation[137,138], and explaining why patients can develop strictures when they are in remission (Figure 11)[139].

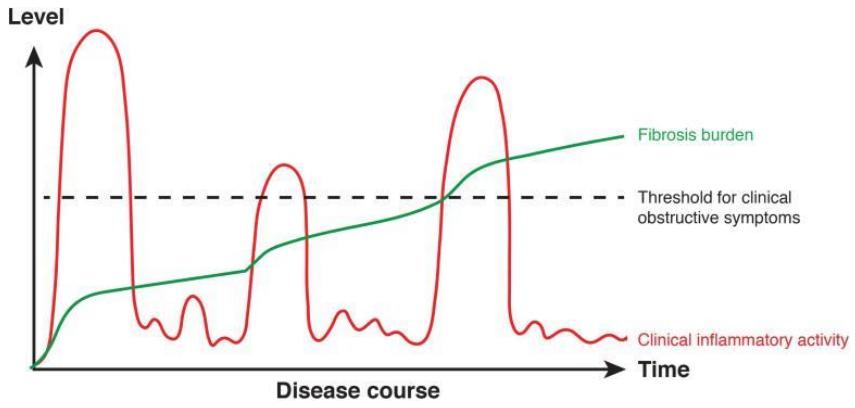



**Figure 11. Progression of the fibrosis burden and the inflammatory activity in a CD patient[136]**
The incidence of the intestinal fibrosis is probably underestimated due to the subclinical accumulation of extracellular matrix over time.

According to the population studies, most IBD patients present an inflammatory phenotype (nonstricturing, nonpenetrating) at diagnosis and it is estimated that only 5-28% have a stricturing disease[140–142]. However, 50% of patients develop stricturing or penetrating

complications within the first 10 years after diagnosis[141,143]. Up to 75% of CD patients require surgical resection during the disease course, for native stricture, for a large proportion of these[144]. This surgery is associated to a high rate of stricture recurrence (a so-called anastomotic stricture by opposition to the native) which frequently needs to repeat surgery[145].

## 1.2. Intestinal fibrosis

### 1.2.1. Intestinal fibrosis: clinical and histological considerations

Beside IBD, other enteropathies can lead to intestinal fibrosis (including the formation of intestinal stricture and occlusions) such as radiation enteropathy, ischemia, graft-versus-host disease, collagenous colitis, eosinophilic enteropathy, drug-induced enteropathy, cystic fibrosis, intra-peritoneal fibrotic adhesions or desmoplastic reactions in gastrointestinal tumors[146]. However, among all these enteropathies, the main cause of intestinal fibrosis remains IBDs[136,146,147].

#### *Clinical considerations*

While some CD patients with severe inflammation never develop strictures during their lifetime, others develop strictures quite early and may relapse with a stricturing disease even after surgical resection, suggesting the existence of predisposing factors[147,148]. Table 1 summarizes all these clinical, environmental, endoscopic, genetic and serological factors[136,140,148,149]. However, these risk factors are not specific to fibrostenosis, and are rather predictive of a 'complicated' or 'debilitating' disease *per se* (including stricture formation)[140,148]. In the absence of specific clinical predictive factors, as well as accurate diagnostic tools for intestinal fibrosis, strictures are generally diagnosed when they become symptomatic (and that a critical degree of lumen narrowing has been reached) or fortuitously (during a colonoscopy or at the time of a cross-sectional imaging)[148].

| **Predictors of fibrostenosing Crohn's Disease** | |
|---|---|
| *Clinical* | Age of diagnosis < 40 years[150,151]<br>Need for steroids during the first flare[150,151]<br>Perianal disease at diagnosis[150,151]<br>Small bowel disease location[152]<br>Early use of azathioprine or anti-TNF[152] |
| *Environment* | Smoking[152–154] |
| *Endoscopic* | Deep mucosal ulceration[155] |
| *Genetic* | NOD2/CARD15[156]<br>MMP3[157]<br>rs1363670[158]<br>Increased amount of rick alleles for IBD5, DLG5, ATG16L1, and IL23R[136,159–161] |
| *Serological* | Anti-microbial antibodies (presence of ASCA)[162]<br>ECM molecules (Fibronectin, collagen propeptides, laminin)[163,164]<br>Growth factors (YKL-40, bFGF)[165,166] |

**Table 1. Predictors of fibrostenosing Crohn's Disease[136,160]**

ATG, autophagy-related protein 16-1; ASCA, anti-Saccharomyces cerevisiae; bFGF: basic fibroblast growth factor; DLG, discs large; ECM, extracellular matrix; IBD: inflammatory bowel disease; IL23R, interleukin 23 receptor; MMP, matrix metalloproteinase; NOD-2, nucleotide-binding oligomerization domain containing 2; YKL-40, tyrosine lysine leucine-40.

Native intestinal strictures are generally located in the ileocolonic region (40-55%), in terminal ileum (25-40%) and in the colon (15-25%) but can also occur in the upper gastrointestinal tract (10%)[140]. When it becomes symptomatic, these patients will usually present obstructive symptoms with crampy abdominal pain, nausea, vomiting, inability to pass gas or stool and postprandial bloating[167]. The development of these symptoms usually leads to the realization of a diagnostic work-up either by endoscopy or by cross-sectional imaging. Endoscopy allows to evaluate the stricture and the local inflammation, to classify it as passable or not passable with an adult colonoscope as well as to perform a therapeutic procedure on it (which will be discussed later) at the same time if indicated. However, if this technique is very sensitive to highlight surface mucosa changes, it does not allow to evaluate disease transmural character, which is better evaluated through cross-sectional imaging, including computed tomography or CT (with 89% sensitivity and 99% specificity), magnetic resonance imaging or MRI (with 89% sensitivity and 94% specificity) and in a slightly less efficient way, ultrasound or US (with 79% sensitivity and 92% specificity)[149,168]. Radiologically, a stricture is defined by the presence of at least 2 out of the 3 following criteria: a bowel wall thickening, a localized luminal narrowing, and a pre-stenotic dilatation[169,170]. Figure 12 summarizes the proposed ranges for these 3 key items, used in US, CT or MRI, to diagnose strictures[170]. In addition to assess the disease

transmural character, these imaging techniques allow to better differentiate the fibrotic and the inflammatory strictures components and thus to guide the therapeutic strategy[171,172].

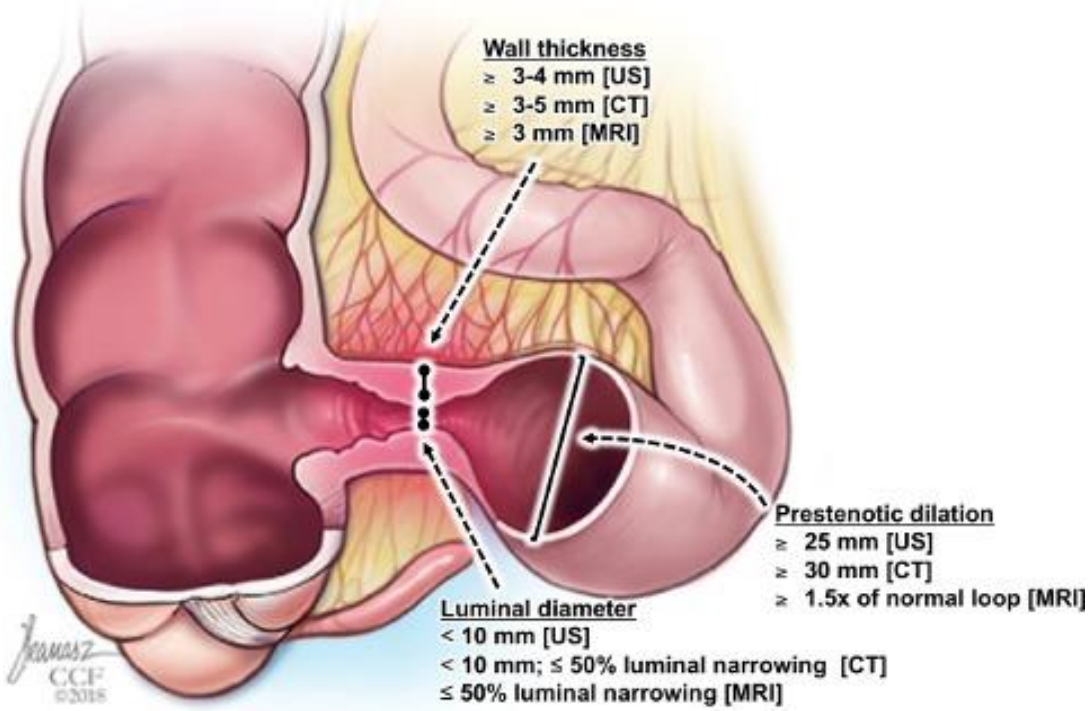



**Figure 12. Proposed ranges for key items used to diagnose stricture by cross-sectional imaging modalities[170]**
CT, computed tomography; MRI, magnetic resonance imaging; US, ultrasound.

***Histological consideration***

Strictures frequently have a mixed pattern and are characterised by varying degrees of inflammation and fibrosis (Figure 13). The stricture inflammatory component is characterised by both elements of acute inflammation (such as neutrophilic infiltration, ranging from erosion, to ulcer, cryptitis and crypt abscess[133,173–177]) and elements of chronic inflammation (such as infiltrates by lymphocytes[178], lymphoplasmacytic cells[179] and mononuclear cells[180,181]). Epithelioid granulomas may be found in all layers of the bowel wall[182]. In addition to these cellular infiltrates, other criteria indicating the presence of an inflammation may be present including Paneth cell hyperplasia[183], a reduction in the number of goblet cells and mucin depletion[181,184], pyloric glands metaplasia[185], oedema[176,185–187] as well as crypts or villous atrophy[185,188]. Finally, an inflammation of Auerbach's and Meissner's plexuses could be present (and is associated to early postoperative CD recurrence)[173,189–191]. The majority of scores used to evaluate inflammation severity are based on the degree and depth of the inflammation, which can be transmural and can be associated with fistulas[173].

The stricture fibrotic component is essentially characterised by an expansion of the smooth muscle layers and an ECM excessive deposition, which can undertake all bowel wall layers. Muscularis mucosa can be marked by a hyperplasia of the smooth muscle cells[176], which can be disrupted by this excessive ECM deposition. Together, these changes can lead to a certain degree of muscularis thickening which can lead to a complete obliteration of the submucosa (the so-called "obliterative muscularization of the submucosa")[187]. In addition, this submucosa can also present an arteries and veins fibromuscular hyperplasia as well as a nerves hypertrophy[187]. Muscularis propria can also be characterised by a hyperplasia and hypertrophy of smooth muscle cells and collagen septae but which does not disorganize the muscle fibers[133,187,192], and this significant fibrosis may extend into the subserosa[188,193–195]. Finally, the mesenteric fat can extend along the affected segment anti-mesenteric border and dissects the subserosa to reach the external muscle layers[177]. This creeping fat can secrete factors which can affect wall layers and contribute to stricture development[177]. Most of the histological scores evaluating the fibrosis severity are based on the depth of fibrosis as well as on the muscularis propria involvement but do not take into account the collagen deposition, which is however a major histological characteristic of intestinal fibrosis[196].

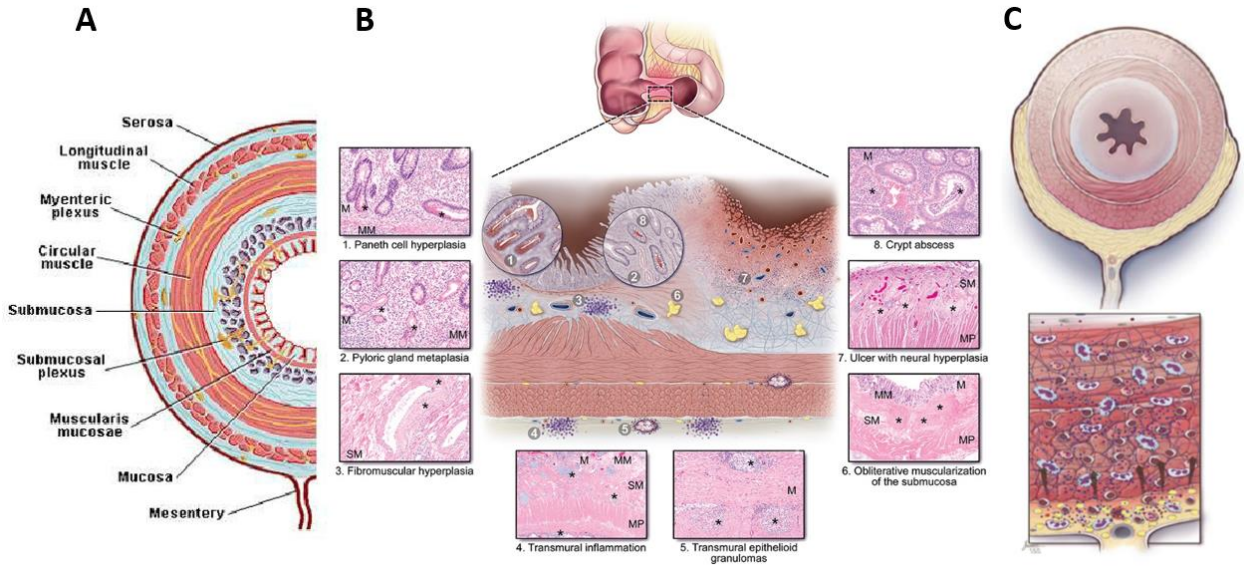



**Figure 13. Typical histopathologic features found in CD associated strictures[173,177]**
[A] Schema of the different layers that compose the intestinal wall (picture from www.lib.mcg.edu); [B] Histological abnormalities found in CD stricture; [C] The creeping fat dissecting the subserosa and reaching the external muscle layers.
M, mucosae; MM, muscularis mucosae; SM, submucosa; MP, muscularis propria.

### 1.2.2. Intestinal fibrosis: pathophysiology

One of the main actors of this pathophysiological process is the fibroblast which accumulates and undergoes differentiation into activated myofibroblast (characterised by the intracellular increase of the alpha-smooth muscle actin (α-SMA) marker), which is the cell secreting this ECM[197,198]. Whereas in other organs, the source of these ECM-producing myofibroblasts is limited to a few cell types, in the gut, multiple cells may become activated myofibroblasts (Figure 14)[147,199–202]. These cells derive from resident mesenchymal cells (fibroblasts, sub-epithelial myofibroblasts, smooth muscle cells), stellate cells, pericytes, circulating bone marrow mesenchymal stem cells (MSCs) but also from the de-differentiation of epithelial and endothelial cells (via the epithelial [EMT]/endothelial-mesenchymal transition [EndoMT] process)[145,203].

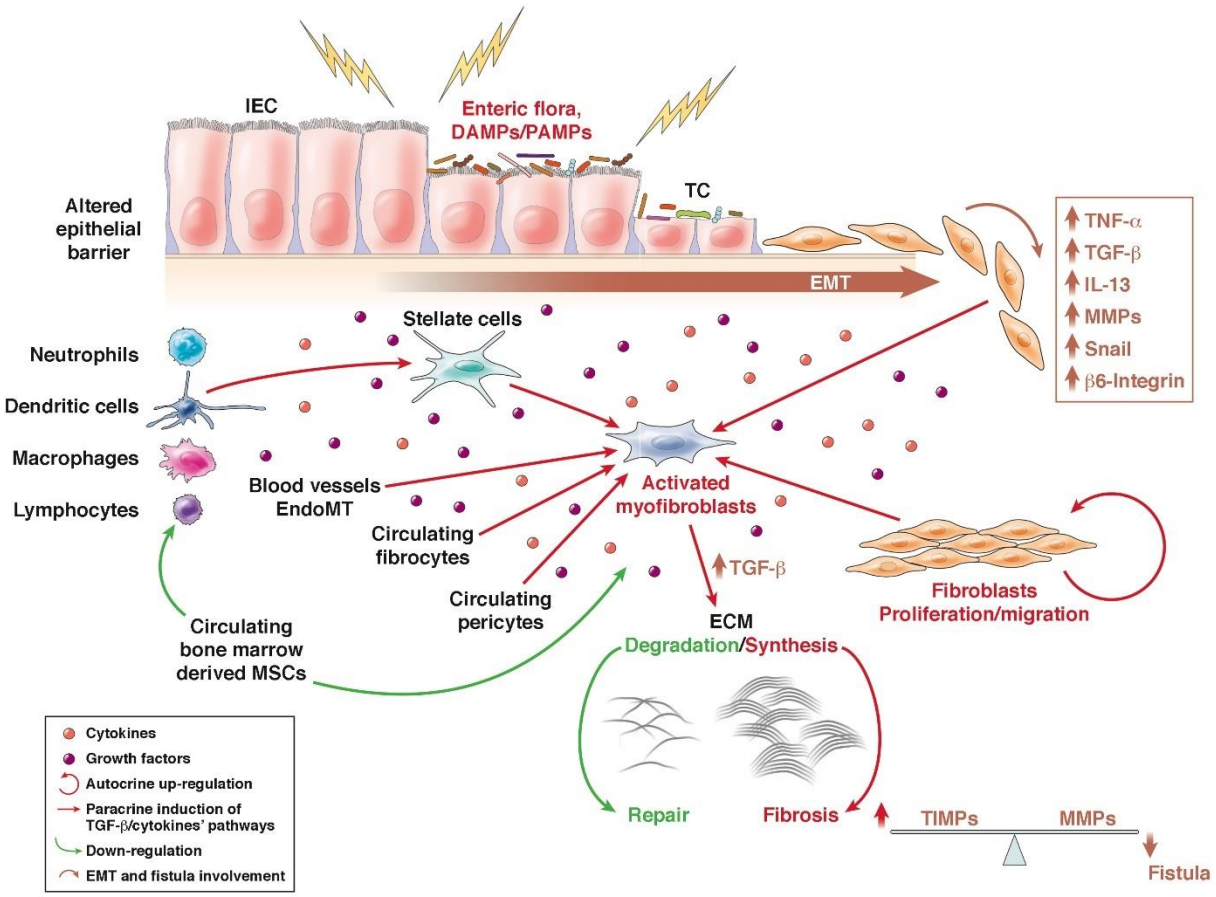



**Figure 14. Pathophysiology of intestinal fibrosis**[204]
ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; IEC, intestinal epithelial cell; IL, interleukin; MMPs, matrix metalloproteases; MSCs, mesenchymal stem cells; TC, transitional cells; TGF-β, transforming growth factor β; TIMPs, tissue inhibitors of metalloproteinases; TNF-a, tumour necrosis factor α.

This differentiation of mesenchymal progenitors into myofibroblasts is elicited by a whole series of factors among which transforming growth factor-β1 (TGF-β1) seems to play a key role[205]. Among the 3 isoforms of TGF-β (TGF-β1, TGF-β2 and TGF-β3), TGF-β1 is the isoform predominantly involved in fibrosis[206,207]. This cytokine is synthesized as a precursor form composed by : (1) a short carboxy-terminal fragment, which is the mature (or active) TGF-β peptide, (2) a large amino-terminal fragment which is the latency-associated peptide or LAP and (3) a signal peptide to direct TGF-β into the ER (Figure 15)[208,209]. After removal of the signal peptide, the remaining complex (TGF-β associated to LAP, also called "small latent complex" or SLC) translocates into the ER lumen where it dimerizes, folds and links by disulfide bonds to another protein called latent TGF-β binding protein (LTBP)[210–212]. This TGF-β dimer is subsequently cleaved from its LAP pro-peptide in the Golgi by furin enzymes but remains strongly associated to it via noncovalent interactions forming the "large latent complex" or LLC, which is then secreted[210–212]. This TGF-β is thus secreted as a latent form (and cannot interact with TGF-β receptor) and the LTBP (which belongs to the superfamily of ECM proteins) binds various matrix fibers that sequester latent TGF-β until its release by an activator. Several specific molecules such as MMPs, plasmin, thrombospondin as well as integrins $\alpha_V\beta_8$[213] and $\alpha_V\beta_6$[214,215] (whose expression is promoted by inflammation as well as tissue injury[214,216]) can activate this complex and release the active form of TGF-β[213–218].

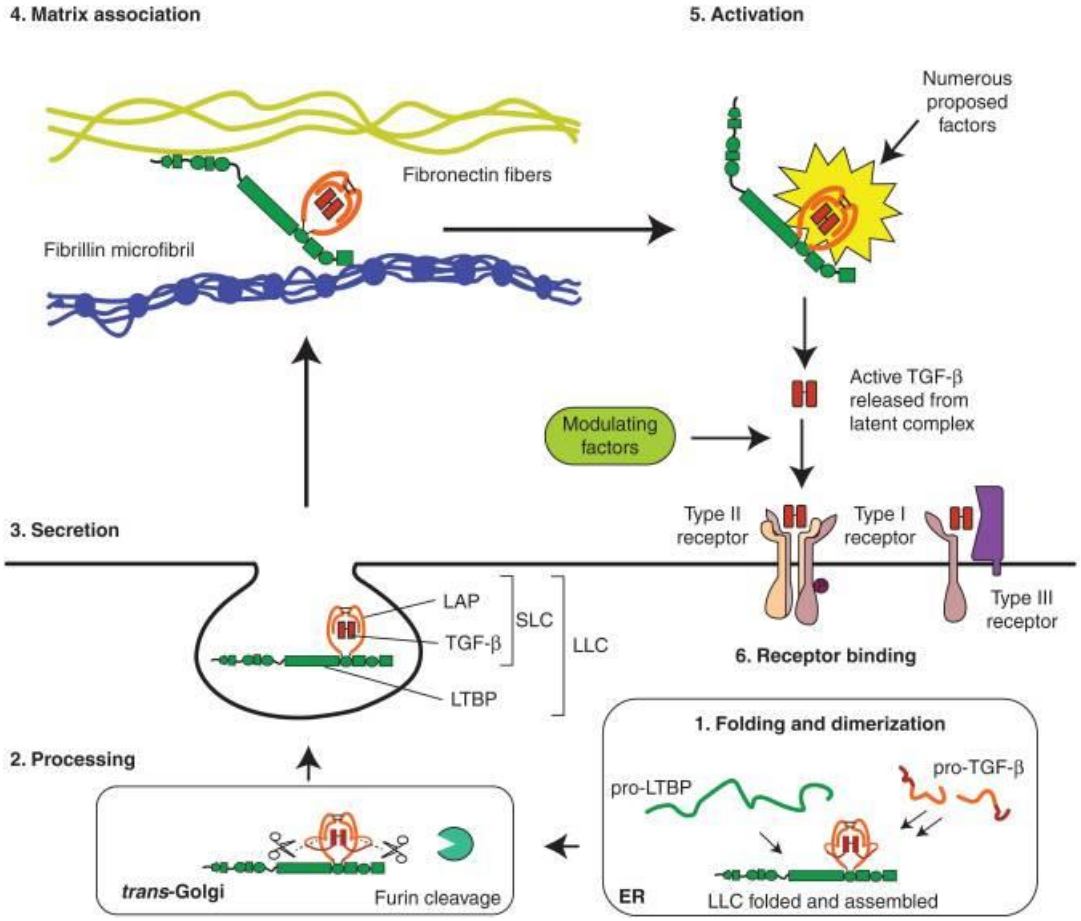



**Figure 15. Synthesis and secretion of TGF-β[219]**
[1] The pro-protein of TGF-β (TGF-β associated to latency-associated peptide or LAP) and pro-protein of LTBP (latent TGF-β binding protein) are directed to the endoplasmic reticulum (ER) *in bottom right*. Once there, pro-TGF-β dimerizes and is linked to LTBP by disulfide bonds between LTBP and LAP. [2] The peptide is then directed to the trans-Golgi network (*in bottom left)*, were TGF-β dimer is cleaved from LAP by furin enzymes but remains strongly associated to it via noncovalent interactions forming the "large latent complex" or LLC, which is secreted [3] *in middle left*. Once secreted, the LTBP binds to the extracellular matrix [4] which sequesters TGF-β in latent form (*at the top left*). [5] When TGF-β is activated (there are several possible mechanisms) *at the top right*, mature TGF-β is released and can activate TGF-β receptors on the cell surface [6] *in the middle right*[219].
LAP, latency-associated peptide; LLC, large latent complex; LTBP, latent TGF-β binding protein; SLC, small latent complex; TGF-β, transforming growth factor β.

Once activated, TGF-β1 binds to receptors (TGF-β-R1 and 2) and subsequently induce the canonical Smad-dependant signalling pathway. The TGF-β receptor phosphoryles Smad2 and Smad3 proteins which interact with Smad4. This Smad2/3-Smad4 complexes translocate from the cytosol to the nucleus, resulting in myofibroblasts activation and excessive production of ECM[218,220,221]. In addition to this Smad-dependant signalling pathways, TGF-β can activate Smad-independent signalling pathways including the p38 and Jun N-terminal kinase (JNK)

mitogen-activated protein kinase (MAPK) pathways, the PI3K-AKT-mTOR pathway, the small GTPases Rho, Rac, and Cdc42, and the Ras-Erk-MAPK pathway[222,223].

Beside TGF-β, other pathways have been reported to be implicated in this mesenchymal progenitor differentiation including hedgehog (Hh)–Gli, platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), EGF, Wnt and Notch (Figure 16)[224].

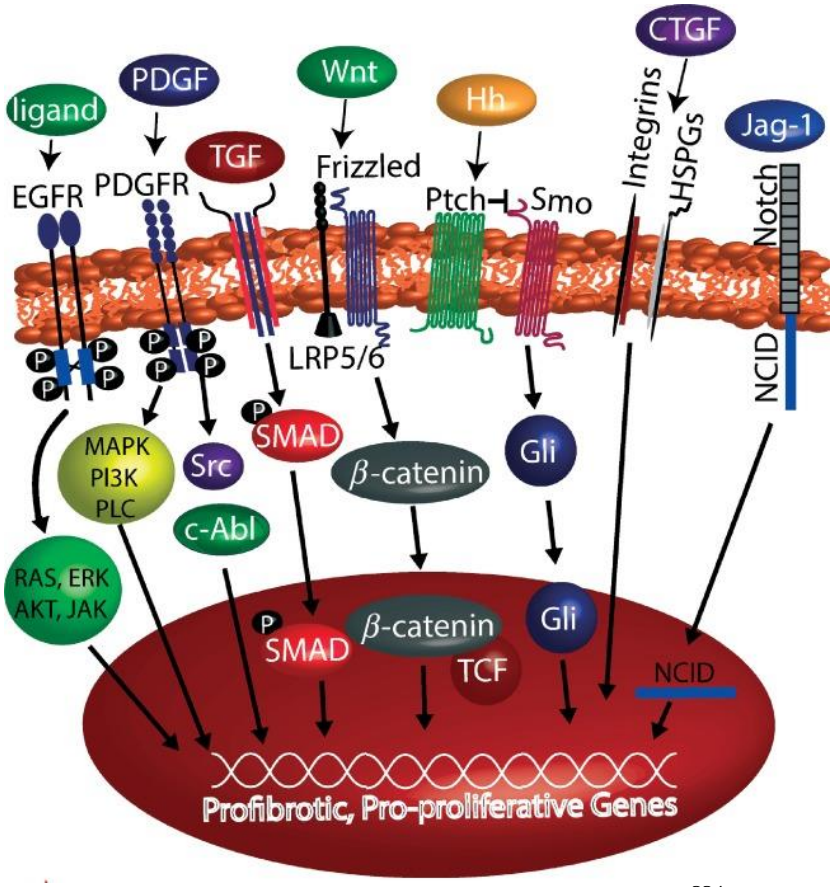



**Figure 16. Key regulatory pathways driving transdifferenciation**[224]
PDGF, platelet-derived growth factor; TGF-β, Transforming growth factor-β; Hh, Hedgehog; CTGF, connective tissue growth factor; EGFR, epidermal growth factor receptor.

Once these myofibroblasts are activated, they secrete the ECM, composed of collagen proteins (fibrillar including collagens I, III, V and non-fibrillar as type IV), glycoproteins (including laminin, entactin/nidogen, vitronectin, as well as peptidoglycan (glycosaminoglycans as hyaluronic acid, heparan sulfate, chondroitin sulfate, perlecan)[225]. In conditions of homeostasis, this ECM is degraded by matrix metalloproteinases (MMPs)[226]. These MMPs can be induced by the hepatocyte growth factor or HGF (which has an anti-fibrotic action) and inhibited by the tissue inhibitors of metalloproteinases (TIMPs)[147,227]. In intestinal fibrosis, the fine balance between MMPs and TIMPS appears to be altered with a downregulation of some MMPs and

an overexpression of TIMPs, contributing to this excessive ECM deposition and fibrogenesis[228,229].

Finally, this ECM acts as a reservoir of cytokines, chemokines and pro-fibrotic factors which act on these ECM-producing cells and sustain this whole pathophysiological process. Table 2 lists the fibrogenic and anti-fibrogenic factors involved in the intestinal fibrosis process.

| Fibrogenic | Anti-fibrogenic |
|---|---|
| Transforming growth factor-β (TGF-β) | Peroxisome Proliferator Activator Receptor-γ (PPAR-γ) |
| Smad2/3 proteins | Interferon-α (IFN-α) |
| Activin A | Interferon-γ (IFN-γ) |
| Connective tissue growth factor (CTGF) | IL-7, IL-10, IL-12 |
| Platelet derived growth factor (PDGF) | Smad7 protein |
| Insulin-like growth factors (IGF-I and II) | PGE2 |
| Epidermal growth factor (EGF) | Hepatocyte growth factor (HGF) |
| Basic fibroblast growth factor (bFGF) | Adiponectin |
| Cytokines (IL-1β, IL-4, IL-6, IL-13, IL-17, IL-21, IL-22, IL-23, IL-33, TNF-α) | Klotho |
| | Nitric Oxide (NO) |
| CC- and CXC-chemokines (CCL2, CCL3, CCL4, CCL20) | Relaxin |
| Reactive Oxygen Species (ROS) | Matrix Metalloproteinases (MMPs) |
| Integrins ($\alpha_V\beta_6$, $\alpha_V\beta_8$) | |
| Mammalian Target of Rapamycin (mTOR) | |
| PAMPs and TLRs (TLR2&4 ligands) | |
| DAMPs (DNA, RNA, ATP, HMGB1, uric acid, fragments of ECM) | |
| Hedgehog (Hh) signalling pathway | |
| Wnt–β-catenin signalling pathway | |
| Advanced glycation endproducts receptor (RAGE) | |
| Notch signalling pathway | |
| MicroRNAs (miRNAs) | |
| Endoplasmic reticulum (ER) stress | |
| Vascular endothelial growth factor (VEGF) | |
| Endothelins (ET-1) | |
| Angiotensin Converting Enzyme (ACE) | |
| Angiotensin-II (AT-II) | |
| Norepinephrine | |
| Thrombospondin-1,2 | |
| Leptin | |
| Tissue Inhibitor of Metalloproteinases (TIMPs) | |

**Table 2. Molecules involved in intestinal fibrosis**[147]

### 1.2.3. Management of intestinal strictures in Crohn's disease

The management of fibrostenosing CD complications should ideally be discussed by a multidisciplinary team comprising gastroenterologist(s), colorectal surgeon(s), radiologist(s), pathologist(s), as well as dietician(s), who can together propose medical, endoscopic or surgical strategies.

#### *Drug therapies*

The response to medical treatment will mainly depend on the inflammatory and fibrotic components of the stricture and it is usually in inflammatory CD-strictures that a benefit from

medical treatment can be expected. If anti-inflammatory treatment with corticosteroids can be effective on the short term, it does not improve long-term outcomes of stricturing CD patients[230]. An alternative short-term treatment strategy could be exclusive enteral nutrition (EEN)[231,232], also known for anti-inflammatory properties (by altering gut microbiota, modulating stem cells differentiation and promoting epithelial healing)[233–235] but is not a long term appropriate treatment[230]. Immunosuppressive agents (such as thiopurines, cyclosporine and methotrexate) were not able to reduce neither intestinal complications of CD nor the need for surgery[230,236–238]. Finally, data regarding the efficacy of anti-TNF in patients with established CD strictures are controversial[140]. To better define the place of this therapy in this condition, the GETAID (Groupe d'Études Thérapeutiques des Affections Inflammatoires du tube Digestif) conducted a prospective study (CREOLE) to identify clinical and imaging (evaluated by MRE or contrast-ultrasound) predictive factors of anti-TNF (Adalimumab) success in symptomatic small bowel stricture (see Table 3)[239]. Patients with 4 or more predictors had 87% chance of short-term anti-TNF success. However, although data are lacking for vedolizumab (an anti-integrin $\alpha_4\beta_7$ antibody) and ustekinumab (an antagonist of the p40 subunit of interleukin-12 and interleukin-23)[240], whatever the treatment, it improves the inflammatory component of the stricture as well as related symptoms but does not have a direct anti-fibrotic effect[241]. Several potential anti-fibrotic agents have shown promising results in animal models and are summarized in Table 4[242]. However, to date, no clinical trials evaluating efficacy of anti-fibrotic agents in stricturing CD patients has been performed and further investigations are required[149,242].

| |
|---|
| Combination of immunosuppressive treatments |
| Crohn's Disease Obstructive Score (CDOS) > 4* |
| Duration of obstructive symptoms < 5 weeks |
| Length of stricture < 12 cm |
| Maximal small bowel diameter proximal to stricture on MRE of 18-29 mm |
| Marked enhancement on delayed MRE T1-weighted sequence |
| Absence of intestinal fistula |

**Table 3. Clinical and radiological predictors of anti-TNF success**[239]

MRE, magnetic resonance enterography. *CDOS > 4 corresponding to severe abdominal pain for more than 1 week, mild to moderate abdominal pain every day that was associated with nausea, vomiting, abdominal cramps for >3 days or hospitalisation during the 8 previous weeks.

| | Reference and year | Model system | Mechanism | Prevention or Reversal of Fibrosis | Effects |
|---|---|---|---|---|---|
| TGF-β1 vaccination | Ma Y, et al.[243] 2010 | Murine chronic TNBS induced colitis | TGF-β1 antagonism | Both preventive and reversal (from week 0 or 2 of TNBS colitis) | Reduced collagen deposition and decreased levels of TGFβ1, IL-17 and IL-23 |
| Smad7 specific antisense oligonucleotide | Izzo R, et al.[244] 2018 | Murine chronic TNBS induced colitis | TGF-β1 signalling modulator | Reversal (from week 5 or 9 of TNBS colitis) | Reduced signs of colitis, reduced collagen deposition and diminished fibrosis |
| Pirfenidone | Li G, et al.[245] 2016 | Murine chronic DSS-induced colitis | TGF-β, TNF-α and NF kappaB modulator | Both preventive and reversal (from day 0 or 14 of DSS colitis) | Reduced collagen deposition, suppressed the mRNA expression of COL1A2, COL3A1, and TGF-β. |
| IL-10 | Yuan C, et al.[246] 2013 | Spontaneous colitis of IL10 knock-out mice | IL-10 administration | Preventive (from week 12 of age) | Decreased tissue fibrosis, decreases inflammatory cytokines and TGF-β1 |
| IL-13 Receptor α2 specific small interfering RNA | Fichtner-Feigl S, et al.[247] 2007 | Murine chronic TNBS induced colitis | IL-13 inhibitor | Therapeutic (from day 35 of TNBS colitis) | Alleviated fibrosis and TGFβ1 production |
| IL-36 receptor antibody | Scheibe K, et al.[248] 2019 | Murine chronic DSS- and TNBS induced colitis | IL-36 inhibitor | Preventive (from day 1 of DSS or TNBS colitis) | Reduced fibrosis score, submucosal thickness and αSMA positive cells |
| AMA0825 | Holvoet T, et al.[249] 2017 | Murine chronic TNBS induced colitis and adoptive Tcells transfer colitis | local ROCK inhibitor | Both preventive and reversal (from week 2 of T-cell transfer or week 7 of DSS colitis) | Inhibited myofibroblast accumulation, expression of pro-fibrotic factors, and accumulation of fibrotic tissue; reversed established fibrosis |
| GED-0507-34 Levo | Speca S, et al.[250] 2016 | Murine chronic DSS-induced colitis | 5-ASA analogue (mimicked by PPAR-γ) | Preventive (from day 12 of DSS colitis) | Improved macroscopic and microscopic intestinal lesions, reduced the profibrotic gene expression of ACTA2, COL1A1 and FN1, reduced protein levels of α-SMA and collagen I/II, TGF-β/Smad pathway components, IL-13 and CTGF |
| GED-0507-34 Levo | Di Gregorio J, et al.[251] 2017 | Murine chronic DSS-induced colitis | 5-ASA analogue (mimicked by PPAR-γ) | Preventive (from day 12 of DSS colitis) | Reduced the expression of the main fibrosis markers (αSMA, collagen I-III and fibronectin) as well as the pivotal pro-fibrotic molecules IL-13, TGF-β and Smad3, increased the anti-fibrotic PPAR-γ |
| LOX inhibitor | de Bruyn JR, et al.[252] 2008 | Murine chronic DSS-induced colitis | LOX inhibitor | Preventive (from day 0 of DSS colitis) | Reversed the ECM contraction and MMP3 activity in stenotic myofibroblasts grown in fibrotic environment |
| Captopril | Wengrower D, et al.[253] 2004 | Murine chronic TNBS induced colitis | ACEI | Preventive (from day 0 of TNBS colitis) | Reduced the score of macroscopic and histologic lesions, colonic tissue levels of collagen α1, hydroxyproline, Ang II and TGF-β1 proteins, and TGF-β1 mRNA |
| Losartan | Wengrower D, et al.[254] 2012 | Murine chronic TNBS induced colitis | ARB | Preventive (from day 0 of TNBS colitis) | Improved macro- and microscopic scores of intestinal fibrosis and reduced TGF-β1 concentration |
| Simvastatin | Abe Y et al.[255] 2012 | Murine chronic TNBS induced colitis | HMG-CoA reductase inhibitor | Preventive (from day 2 of TNBS colitis) | Attenuated intestinal fibrosis by lowering CTGF and inducing apoptosis of fibroblasts and myofibroblasts |

**Table 4[242]. Preclinically tested approaches for intestinal fibrosis in animal models**

5-ASA, 5-aminosalicylic acid; Ang, angiotensin; ARB, angiotensin II receptor blocker; α-SMA, α-smooth muscle actin; CTGF, connective tissue growth factor; DSS, dextran sulfate sodium; ECM, extracellular matrix; HMG-CoA, 5-hydroxy-3-methylgultaryl-coenzyme A; IL, interleukin; LOX, lysyl oxidase; MMP, matrix metalloproteinase; NF, necrosis factor; PPAR, peroxisome proliferator activator receptor; ROCK, Rho-associated coiled-coil forming protein kinase; TGF, transforming growth factor; TNBS, 2,4,6-trinitrobenzenesulfonic acid solution; TNF, tumour necrosis factor

When anti-inflammatory therapies are not successful to alleviate symptoms or if these recur within a short interval, endoscopic treatment, stricturoplasty, or intestinal resection should be considered[136,256]. The stricture location, its length or angulation, the presence of associated complications (such as phlegmon, abscess, dysplasia) and patient's preference are the elements that will guide the procedure choice[136,256].

***Endoscopic therapies***

Endoscopic management of CD-associated strictures include the following modalities: endoscopic balloon dilatation (EBD), intralesional injection of steroids or anti-TNF agent, stents placement and endoscopic stricturotomy.

**Endoscopic balloon dilatation (EBD)** is indicated for short endoscopy-accessible strictures (< 5 cm in length), non-angulated, without complication (such as the presence of fistula, abscess or malignant disease)[256–258]. Most commonly, through-the-scope balloons (TTS) are used to reach and pneumatically dilate strictures, allowing a direct endoscopic visualization during the dilation procedure[140]. Although most authors report a graded approach (consisting in a gradual increase in the balloon size) with a 1-min dilation and balloon sizes of 18–20 mm for the colon and 12–15 mm for small bowel strictures, in routine clinical practice, the endoscopist individually tailors the size of the balloon, time of dilation, and a graded versus non-graded type of dilation depending on the size, length, and anatomy of the stricture[259–261]. This procedure is associated with a technical success rate (defined by the ability to pass an adult colonoscope through the non-passable stricture) of 89%, a clinical efficacy rate (defined by the improve of sub-occlusion symptoms) of 80.8% and a complications rate of 2.8% (mainly bleeding and perforations)[257]. The only characteristic able to predict a need for surgery after EBD was a stricture length of 5 cm in a multivariate analysis[257]. In contrast, the presence of active disease at the site of the stricture, the naive versus anastomotic nature of the stricture, or any technical features did not affect short-term or long-term outcomes[257]. This long-term efficacy (defined either by the time before symptom recurrence or by the time passed before the need for surgery or re-intervention) has proved to be more challenging, as many patients do require repeated balloon dilatations (73.5% of patients in a 2-year follow-up) or surgery (42.9% in a 2-year follow-up) following a successful dilatation[257].

To improve these outcomes, the **intralesional injection** of steroids (betamethasone[262] or triamcinolone[263–267]) following EBD has been proposed. If this technique has proven to be effective in strictures occurring in other gastrointestinal conditions (such as peptic, corrosive or anastomotic strictures or fibrosis post-radiotherapy)[268–270], the results of the 2 randomized controlled trials conducted in IBD were conflicting[271]. While intra-stricture triamcinolone injection after EBD in a single-centre paediatric CD cohort prolonged the time to re-dilation or surgery[264], intralesional injection of corticosteroids in ileocolonic anastomotic strictures of adult Crohn's did not reduce the time to re-dilation and a trend toward a worse outcome was reported[265]. In a systematic review of observational studies, no outcome differences were noted regardless of steroids injection[257] and, corticosteroid injection after EBD cannot be therefore currently recommended in clinical practice. There were some case reports showing success of injection of anti-TNF agents in CD-associated strictures but larger prospective studies are required before intralesional injection of anti-TNF could be recommended in clinical practice[272,273].

The successful use of **endoscopic metallic stents** in oesophageal and colonic malignant strictures[274,275] has allowed extension of their use in CD related strictures. Two types of stents are available: self-expanding metal stents (SEMS) and biodegradable stents. SEMS appear to be effective with an estimated technical success rate of 92% and a clinical efficacy rate of 65%[276] but with frequent complications (67%), including stent migration, perforation or fistulisation[277]. Currently, there is still a lack of data on the recommended type of stent (fully covered stents, partially covered or uncovered ones), the length of time it should remain in place as well as the long-term efficacy and safety of this technique[276]. The use of biodegradable stents may be an emerging alternative, but data are limited to case reports[278,279] or retrospective cases[280] series and no definitive conclusion could thus be made.

Finally, **endoscopic needle knife stricturotomy** is a new promising technique to treat strictures in IBD patients[281]. The procedure was initially tested for the treatment of strictures of the upper GI tract and distal common bile duct[282,283]. It consists of a radial incision, performed on the rim of the stenosis, with a knife catheter (either a regular needle knife or an electrosurgical IT knife), under direct vision[282]. The reported immediate technical success rate was 100% with a procedure complication rate of 3.7% (perforation and bleeding)[281,284]. In a study comparing endoscopic stricturotomy with ileocolonic resection for the treatment of ileocolonic anastomotic strictures, Lan et al. found a comparable surgery-free survival, but a significant

decreased morbidity for the endoscopic stricturotomy group[284]. Although it is technically feasible for short and non-angular strictures, endoscopic needle knife stricturotomy remains a procedure whose success highly depends on the operator's expertise and should be the subject of future studies[149].

***Surgery***

If the place of surgery is frequently questioned by the emergence of new drug therapy, and by the constant progress of instrumental techniques, it nevertheless remains a treatment of choice of refractory intestinal strictures, after failure of medical therapy and EBD or inability to perform EBD. Two common surgical procedures are recognised in the management of CD stricture: resection and strictureplasty[285].

**Resection** is recommended in cases of long strictures where EBD is not technically feasible, in cases of complications such as fistula, abscess, phlegmon, dysplasia, or malignancy[256] and is also preferred in colonic CD strictures (where there is a higher risk of underlying malignancy)[286]. Laparoscopic surgery is possible in cases of ileal or caecal strictures, with better postoperative comfort than laparotomy, but is not recommended for complex cases[287,288]. The risk of post-operative recurrence is approximatively 25% per year[289], and the cumulative risks of second and third resections are 31% and 28% at 10 years and 48% and 54% at 20 years, respectively[290]. These iterative resections can lead to short bowel syndrome over time[291] and therefore, resections should be as limited as possible[140].

To preserve the bowel length, **strictureplasty** emerged as an option to widen the strictured area without shortening the bowel, which consists in a stricture enlargement plasty[140,240]. Indications for this procedure include presence of multiple strictures over an extensive length of bowel, previous significant small bowel resections (>100 cm), early recurrence of strictures within 12 months of previous surgery, short bowel syndrome, as well as anastomotic stricture (particularly ileorectal or ileocolonic) and duodenal strictures[136,286,292,293]. Contraindications for this approach include the presence of complication (such as fistula, abscess, phlegmon, bleeding) at intended strictureplasty site, small bowel perforation, suspicion for carcinoma in the stricture or poor nutritional status (serum albumin <2.0 g/dL) because of the fistula risk[136,286,292,293]. The choice between the strictureplasty approaches is based on the stricture length. For short strictures (<10 cm), the Heineke–Mikulicz procedure is usually indicated[294];

intermediate strictures (10–25 cm) may benefit from the Finney procedure[295], while non-conventional strictureplasties such as Michelassi technique are performed for long (>25 cm) or multiple strictures[286,296]. The overall reported complication rate of strictureplasties is 13%, including anastomotic leakage, abscess, fistula, sepsis, early postoperative gastrointestinal bleed[297–299].

Although considerable progresses have been made in the treatment of IBD in the last 25 years, the incidence of bowel strictures has not significantly decreased and to date, no CD anti-inflammatory drugs nor any other treatment, in particular, anti-fibrotic, exists to reverse or prevent CD-associated strictures[238,256,300]. Patients consequently require multiples balloon dilatations and/or repeated surgeries, procedures associated with higher morbidity, significantly impaired quality of life and costs for the health care system[301]. The progression to a stricturing behaviour is still an issue for clinicians and represents an unmet medical need to date[149]. Better understanding of the mechanisms involved in intestinal fibrogenesis to find appropriate anti-fibrotic agents is imperative and is a worldwide challenge today.

# RATIONALE FOR THE CHOICE OF THE TWO AXES OF WORK FOR THIS THESIS ON INTESTINAL FIBROSIS

## 2. RATIONALE FOR THE CHOICE OF THE TWO AXES OF WORK FOR THIS THESIS ON INTESTINAL FIBROSIS

The role played by the epithelium, which is the first to encounter many diet toxins and bacterial agents, at the interface between the host and the "outside" lumen, has been poorly studied and is probably underestimated. In this context, our laboratory performed a pilot proteomic study to highlight pathways and potential epithelial proteins involved in CD fibrosis. The over-expression of some candidate proteins in fibrotic tissues was next confirmed by immunochemistry (IHC). The first part of this thesis is a mechanistic study in which we have investigated the functional role of a major protein highlighted (by this proteomic/IHC study) in intestinal fibrosis.

Secondly, as the mesenchymal stem cells (MSCs) are known to have anti-fibrotic properties[302–304], in particular by their impact on the epithelium (in radiation-induced fibrosis for example) [305–307], we conducted a pilot clinical study to evaluate the safety and efficacy of local MSCs injections in CD strictures. Indeed, whereas clinical studies have demonstrated the safety and the variable efficacy of intravenous MSCs administration to treat luminal CD[308] and local MSCs administration to treat perianal CD fistulas[309–311], MSCs injection in CD stricture had never been tested so far to our knowledge.

# THE ROLE OF EPITHELIUM IN INTESTINAL FIBROSIS, AND IN PARTICULAR OF EPITHELIAL ENDOPLASMIC RETICULUM STRESS AND THE ANTERIOR GRADIENT PROTEIN 2 HOMOLOG

# 3. THE ROLE OF EPITHELIUM IN INTESTINAL FIBROSIS, AND IN PARTICULAR OF EPITHELIAL ENDOPLASMIC RETICULUM STRESS AND THE ANTERIOR GRADIENT PROTEIN 2 HOMOLOG

## 3.1. INTRODUCTION

### 3.1.1. Intestinal epithelium involvement in Crohn's Disease fibrosis

Under recurrent or persistent injuries, epithelial cells produce damage-associated molecular patterns (DAMPs) including a wild range of products such as DNA, RNA, ATP, high-mobility group box proteins (HMGB), microvesicles, IL-1α, IL-18, IL-33, TGF-β which promote recruitment and activation of the mesenchymal cell precursors in activated myofibroblasts[138,203,312,313]. Furthermore, after tissue injuries, IECs can also over-express $\alpha_V\beta_6$ integrin, which plays a role in local activation of latent TGF-β1, a key driver of the transformation of epithelial cells in mesenchymal cells, through EMT process, and of the transformation of mesenchymal cells into myofibroblasts[314,315].

EMT is involved in fibrosis of several organs including heart, lung, kidney, liver[316–320] and several evidences suggest its involvement in intestinal fibrosis[321–323]. The mechanism by which EMT contribute to the fibrogenic process could be two-fold: on one hand, these IECs, in trans-differentiating in mesenchymal cells, expand the pool of ECM-producing activated myofibroblasts, and on the other hand, loss of epithelial cells associated contribute to the parenchyma destruction and loss of epithelial integrity[324]. These cells acquire a newly mesenchymal phenotype, characterised by the acquisition of a fibroblast-like elongated morphology, a loss of cell-cell contacts and apical-basal polarity, enhanced invasive capacity allowing them to migrate into the submucosa and initiate secretion of ECM[325,326]. They present a progressive up-regulation of genes typically expressed by mesenchymal cells (such as the EMT-orchestrating transcription factors *Twist*, *Snail*, *Slug* and *ZEB1/2* as well as *α-SMA*, *vimentin*, *fibronectin* and *collagens*) together with a downregulation in the expression of epithelial hallmarks such as adherens and tight junctions (E-cadherin, Zonulae Occludentes and claudins) and cytoskeleton proteins (cytokeratins)[326] as detailed in Figure 17. Between these 2 cell types, the cell experience a so-called transitional state which still retains gap junctions and basement membrane-like structures[327]. Several factors can promote this trans-differentiation, most prominently TGF-β, but also IGF-1 and 2, EGF, FGF-2, IL-1β and TNFα[199,321]. In contrast, HGF and bone-morphogenetic protein-7 (BMP-7) prevent the mesenchymal transformation of these epithelial cells[199,317,321,328].

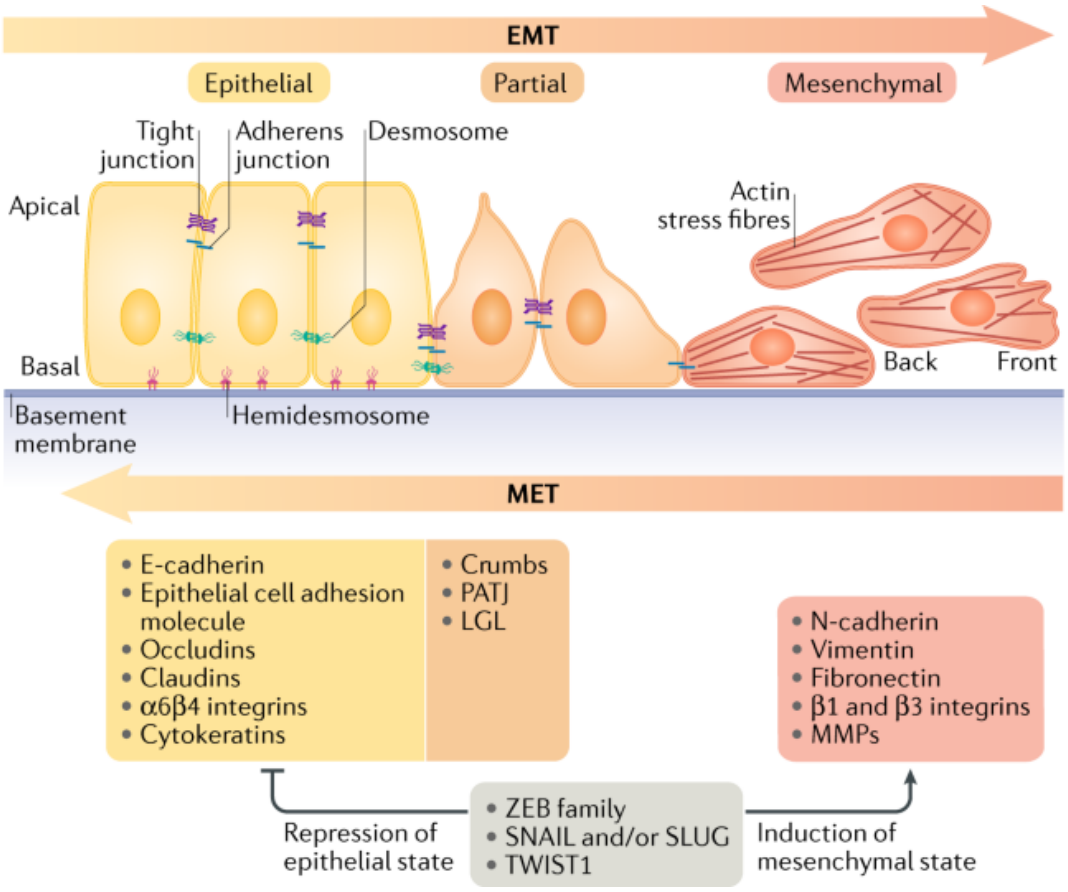



**Figure 17. The epithelial-to-mesenchymal transition[333]**
Epithelial cells are characterised by a cuboidal appearance and apicobasal polarity. They are held together by cellular junctions (tight junctions, adherens junctions and desmosomes) and connected to the basement membrane by hemidesmosomes. They express a range of epithelial markers such as E-cadherin, Occludins and Claudins. During the EMT process, transcription factors such as ZEB, SNAIL, SLUG and TWIST, repress genes associated with the epithelial phenotype and stimulate the expression of genes associated with the mesenchymal phenotype such as N-cadherin, vimentin and fibronectin. The transition can be partial (partial EMT), and the cell has a concomitant loss of some epithelial markers and a gain of some mesenchymal markers. The transition can also be complete, and the cells will acquire a spindle-like shape, increased contractility capacity, become motile and increase matrix deposition.
EMT, Epithelial-to-mesenchymal transition; LGL, Lethal giant larvae, MET, mesenchymal-epithelial transition; MMP, Matrix metalloproteinase; PATJ, PALS1-associated tight-junction protein; SLUG, Snail family zinc finger 2; SNAIL, Snail family zinc finger 1; TWIST, Twist family bHLH transcription factor 1; ZEB, Zinc finger E-box binding.

The death of IECs (and in particular, the mode of death cell), as a result of injuries, affects tissue restitution and, at term, the intestinal fibrosis risk[329]. During homeostasis, dead IECs are replaced by intestinal stem cells (assuring the integrity of the intestinal epithelial barrier) and the death of IECs occur in an appropriate death mode depending on the context: apoptosis for old cells located at crypt-base, anoikis for epithelial cells at the tip of the villi, and pyroptosis for infected IECs[329]. CD patients have a dysregulated IECs death with an increased level of

cells' apoptosis, contributing, with EMT, to the loss of epithelial barrier integrity[329]. This contributes to the production of the above-mentioned molecules (which recruit and activate the mesenchymal cell precursors) in response to injuries, to which these cells are more vulnerable due to epithelial barrier breaching, leading to a vicious cycle[329]. Beside this mechanism, epithelial cells (in particular, Paneth cells) can die through a process called necroptosis (pro-inflammatory lytic mode of death), leading to DAMP release leading to a pro-inflammatory polarization of activated macrophages releasing cytokines such as IL-36, acting on fibroblasts activation[329].

While epithelial ER stress has been shown to be involved in fibrosis in other organs[330,331], few data confirms its involvement in intestinal fibrosis[332]. ER stress could promote fibrotic remodelling through promotion of inflammatory responses, induction of EMT and activation of pro-apoptotic pathways[330].

### 3.1.2. Preliminary work performed in the laboratory of translational gastroenterology GIGAi3, U Liège

Apart from these few data, the role played by the surface epithelium in CD intestinal fibrosis remains poorly studied. In this context, we performed, in our laboratory, a preliminary work in order to highlight potential epithelial proteins involved in CD fibrosis. We first performed a pilot proteomic study to identify the surface epithelium proteome changes in CD strictures. In a second time, we selected, from this experiment, proteins of interest and evaluated them by immunohistochemistry (IHC). Supplemental Tables of this thesis are available at https://dox.uliege.be/index.php/s/EHWMJfGMA4jHzxG and can be accessed with the following password: 13122021.

***Patient enrolment and selection for this preliminary work***

This project was approved by the Ethics Committee of the university hospital of Liège in 2014 (reference: B707201421402). Selected patients were searched retrospectively through our pathology database from 2009 to 2017 and their formalin-fixed paraffin-embedded (FFPE) tissues were obtained from our institution biobank. We first reviewed CD patients that underwent surgery and we selected patients with evidence of strictures and fibrosis in medical or histological records (n=44). Secondly, we searched for CD patients with no evidence of fibrostenosing disease (pure inflammatory CD, based on endoscopic and imaging evaluations), and who were biopsied during endoscopy in inflammatory area or in normal mucosa (normal CD tissues) (n=29). Thirdly, non IBD patients who underwent surgery between 2012 and 2017 were included for analysis of ileal and colonic normal tissues extracted at the surgical margin (n=40).

The pilot proteomic study was performed on 5 patients out of the 44 stricturing CD patients (also studied in the IHC study). Among these patients, 3 (60%) were male, the median (range) age was 33 years (22-41), the median (range) disease duration was 8.2 years (4.4-12.3) and 60% were active smokers. Regarding treatments, one patient was treated by infliximab, three patients were treated by adalimumab (in monotherapy for one patient and in a combination therapy with azathioprine for one and with corticoids for the third one) and the last patient was treated by corticoids alone.

Immunochemistry experiments were performed on the 73 CD and the 40 control subjects. The clinical information of the 73 CD studied patients (44 stricturing CD and 29 pure inflammatory CD) are detailed in Table 5. The 40 non IBD controls included 50% of males with a median age of 71 years (range: 32-92 years) and underwent surgery for colonic adenocarcinoma (n=14), diverticular diseases (n=23), volvulus (n=2) and colonic perforation (n=1).

| | | **CD patients (n=73)** | |
|---|---|---|---|
| | | ***Fibrostenosing CD patients (n=44)*** | ***Non fibrostenosing CD patients (n=29)*** |
| Age (median, range) years | | 31 (17-72) | 43 (21-68) |
| Male gender (n ; %) | | 22; 50.0 | 12; 41.4 |
| Disease duration (median, range) years | | 8.7 (0.2 - 51.5) | 10.19 (0.07-40.42) |
| CD treatment (*stopped before surgery) | *Infliximab* (n)* | 5 | 3 |
| | *Adalimumab* (n)* | 15 | 6 |
| | *Vedolizumab (n)* | 1 | 0 |
| | *Azathioprine (n)* | 10 | 5 |
| | *Mercaptopurine (n)* | 3 | 5 |
| | *Methotrexate (n)* | 2 | 2 |
| | *Corticoids (n)* | 7 | 3 |
| | *5ASA (n)* | 0 | 5 |
| | *None (n)* | 11 | 9 |
| Smoking | *Active smokers (n)* | 16 | 11 |
| | *Former smokers (n)* | 8 | 6 |
| | *Non smoker (n)* | 19 | 11 |
| | *Unknown (n)* | 1 | 1 |

**Table 5. Clinical information of the studied CD patients**

***Histopathologic evaluation and classification of tissues***

All FFPE tissues were graded for inflammation (I) and fibrosis (F). Histopathologic inflammation evaluation of the tissue specimens was done on H&E-stained sections[334]. An expert gastrointestinal pathologist (N.B.) graded separately chronic and acute inflammation, according to the neutrophil and lymphoplasmacytic infiltrates, respectively, using a classical four grades scale ranging from none (N), mild (I1), moderate (I2) to severe (I3)[327,335–337]. Crypt micro-abscesses and cryptitis were considered as hallmarks of the severe acute inflammation grade as acknowledged by others[188,338,339]. Histopathologic intestinal fibrosis evaluation of

tissue specimens was done after Masson's trichrome staining (Merck Millipore, GE) performed according to the manufacturer's recommendations. A four grades scale was used as previously performed[181,340,341] ranging from none (N; no architectural distortion, no abnormal MT staining), mild (F1; no architectural distortion, mild abnormal MT staining in mucosa/submucosa), moderate (F2; substantial abnormal mucosal/submucosal MT staining with modest distortion of architecture) to severe (F3; transmural fibrosis with abnormal MT staining in all layers, transmural architectural distortion).

#### 3.1.2.1. Pilot proteomic study

***Patients and tissue selection***

For the pilot proteomic experiment, we selected 5 CD patients who had, in the same ileal stricture surgical resection sample, one area with high grade of fibrosis (F2-3), one area with low grade of fibrosis (F1) and, except for 2 patients, one area with normal aspect (without any sign of fibrosis) (N), allowing a paired comparison of the surface epithelium proteomic profiles according to the fibrosis grade. Thus, 13 samples have been analysed for the proteomic study.

***Laser capture microdissection, samples preparation and proteomics by LC-MS/MS***

Before the proteomic analysis, we collected, by laser capture microdissection, the surface epithelial cells (~ 20 000) of the different areas selected for each patient tissues (5 F2-3, 5 F1 and 3 N) using a Leica LMD 7000 (Leica Microsystem, GmBh, GE) and treated them for protein extraction according to a standardised procedure[342] (see Supplementary data for details). The samples were analysed by ultra-performance liquid chromatography/electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS). This system consists in a 2D nanoAcquity chromatography (Waters) coupled online with a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer (Thermo Fisher Scientific), equipped with a nano-electrospray source operated in positive ion mode (see Supplementary data for details).

***Proteins identification and differential analysis***

The analysis of the raw spectra was performed using MaxQuant software 1.5.5.1 for proteins identification and label-free quantification (LFQ) (see Supplementary data for search

parameters) [343]. Raw data files have been deposited to the ProteomeXchange Consortium via the PRIDE[344] partner repository (http://proteomecentral.proteomexchange.org, identifier PXD022214). We used Perseus 1.6.0.7 for differential analysis with log2 transformed LFQ values[345,346]. Only proteins detected and quantified in at least 3 out of 5 replicates for fibrotic tissues and in at least 2 out of 3 replicates for normal tissues were included in the differential the analysis.

First, four comparisons were applied to compare epithelial proteome in the different areas (N, F1, F2-3): N versus F1 versus F2-3 (unpaired analysis using ANOVA test), N versus F1 (unpaired analysis using Welch's t-test), N versus F2-3 (unpaired analysis using Welch's t-test) and F1 versus F2-3 (paired analysis using t-test). We determine the p-value associated to the difference in abundance between areas (significant if $p \leq 0.05$). Second, we searched for the "absent" proteins (below the limit of quantitation) in one specific area and "present" in at least 3/5 or 2/3 samples of the two other tissue types.

***Gene Ontology, pathways enrichment analyses, functional annotation, and binding partner identifications***

Beside differential analysis, the proteins found significantly increased or "absent" (or inversely decrease or "present") in one or several groups in the different comparisons were submitted to Gene Ontology using different tools: DAVID™ (bioinformatics version 6.8; https://david.ncifcrf.gov/) for functional enrichment and annotations[347], STRING (consortium 2019; https://string-db.org/) for network and cluster analysis and the BioGRID database (https://orcs.thebiogrid.org/) for identification of the gene interactors of the proteins of interest (interactors gene list downloaded in November 2019).

***Result of label free proteomic analysis***

Proteomics identified and quantified 1287 proteins (Supplemental Table 1) among which 1153 were detected in, at least, 3/5 (for F1/F2-3) or 2/3 (for N) patient samples and were subjected to the differential analysis. Supplemental Table 2 includes these proteins, their relative distributions and-statistics. We found 203 proteins with significant different abundances in the four comparisons applied (Figure 18 shows Venn diagram with these different comparisons and Supplemental Table 3 shows results of these differences). Supplemental Table 4 details results

of the second analysis concerning the research of absent proteins in one specific area and detected in at least 3/5 or 2/3 samples of the two other tissue types.

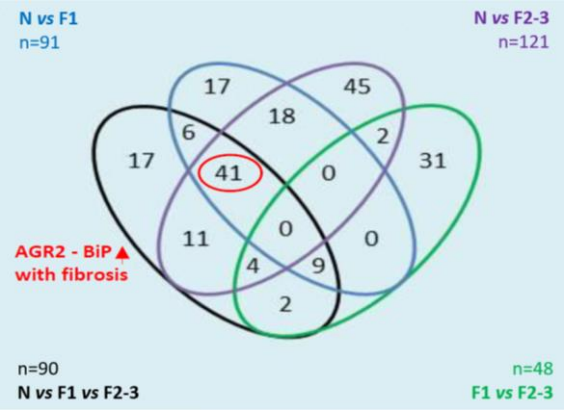



**Figure 18. Venn diagram with group comparisons**
Four group comparisons were applied using the proteomic results and the significant numbers of proteins shared or unique to each comparison are illustrated.

When classifying proteins according to the p-value of the ANOVA test comparing normal ileum (N) to ileum with low grade (F1) or high grade (F2-3) of fibrosis, we found 5 PDIs among the 25 most differentially distributed proteins according to the fibrosis grade (Table 6), the first being AGR2. BiP, a well-known other ER stress marker followed the distribution of AGR2 in all performed comparisons. Figure 19A illustrate the volcano plots of proteins involved in ER stress comparing N vs F1 and N vs F2-3 tissues.

Search for EMT involved proteins was done for 70 proteins of the EMT process[316,348] but only 32 could be identified. Epithelial and mesenchymal proteins highlighted were not differentially expressed in the surface epithelium surrounding fibrotic and normal tissues in the differential analysis (Figure 19B). Many single apoptosis specific proteins were found differentially expressed in epithelium harbouring fibrotic compared to N tissues (Supplemental Table 2). Some negative regulator of apoptosis (as Ribosomal protein S6 kinase alpha-3 (*RPS6KA3*) or Fatty acid-binding protein 1 (*FABP1*)[349,350], were found decreased in fibrosis areas. Fibrotic areas exhibited higher expression of proteins involved in cell differentiation, a decrease of the normal oxidation-reduction process, a decrease of some extracellular matrix components in surface epithelium as well as a decrease of some mitotic checkpoint proteins compared to normal tissues.

| Protein Ids | Majority protein IDs | Protein names | Gene names | Peptides | Razor + unique peptides | Unique peptides | MS/MS count | N vs F1 vs F2-3 ANOVA test p-value | N vs F1 Welch's t-test p-value | N vs F2-3 Welch's t-test p-value | F1 vs F2-3 Student's t-test p-value | Gene names for BiP (*HSPA5*) interactors (n=123) | Gene names for AGR2 Interactors (n=435) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **O95994** | **O95994** | **Anterior gradient protein 2 homolog** | ***AGR2*** | **10** | **10** | **9** | **470** | **0,00010293** | **0,0148937** | **0,0107909** | **0,231551** | ***AGR2*** | ***AGR2*** |
| Q02818 | Q02818 | Nucleobindin-1 | *NUCB1* | 14 | 14 | 14 | 96 | 0,00217074 | 0,0075531 | 0,0001661 | 0,546601958 | *NUCB1* | |
| P62906 | P62906 | 60S ribosomal protein L10a | *RPL10A* | 4 | 4 | 4 | 63 | 0,00530649 | 0,0217258 | 0,0007105 | 0,154768024 | | *RPL10A* |
| **Q15084** | **Q15084** | **Protein disulfide-isomerase A6** | ***PDIA6*** | **12** | **12** | **12** | **264** | **0,00612205** | **0,0079761** | **0,007557** | **0,867628874** | ***PDIA6*** | ***PDIA6*** |
| Q8N4A0 | Q8N4A0 | Polypeptide N-acetylgalactosaminyltransferase 4 | *GALNT4* | 3 | 3 | 3 | 16 | 0,00703411 | 0,0156691 | 0,0045735 | 0,770988572 | | |
| P50914 | P50914 | 60S ribosomal protein L14 | *RPL14* | 3 | 3 | 3 | 49 | 0,00708882 | 0,0141703 | 0,0005672 | 0,481432554 | | *RPL14* |
| P21399 | P21399 | Cytoplasmic aconitate hydratase | *ACO1* | 7 | 7 | 7 | 25 | 0,00870719 | 0,2308691 | 1,0000001 | 1 | | *ACO1* |
| Q13510 | Q13510 | Acid ceramidase;Acid ceramidase subunit alpha;Acid ceramidase subunit beta | *ASAH1* | 2 | 2 | 2 | 26 | 0,00940029 | 0,0021152 | 0,0101158 | 0,199985878 | | |
| Q13228 | Q13228 | Selenium-binding protein 1 | *SELENBP1* | 14 | 14 | 14 | 94 | 0,00988778 | 0,0451073 | 0,0277982 | 0,491179663 | | *SELENBP1* |
| P48735 | P48735 | Isocitrate dehydrogenase [NADP], mitochondrial | *IDH2* | 16 | 16 | 15 | 378 | 0,01003183 | 0,0934848 | 0,0898935 | 0,941017216 | | *IDH2* |
| P35606 | P35606 | Coatomer subunit beta | *COPB2* | 13 | 13 | 13 | 89 | 0,01012208 | 0,0587076 | 0,008697 | 0,177188178 | | *COPB2* |
| P63000; P15153; P60763 | P63000; P15153; P60763 | Ras-related C3 botulinum toxin substrate 1; Ras-related C3 botulinum toxin substrate 2; Ras-related C3 botulinum toxin substrate 3 | *RAC1; RAC2; RAC3* | 2 | 2 | 2 | 25 | 0,01115215 | 0,1930587 | 0,1433346 | 0,77293185 | | |
| P18085 | P18085 | ADP-ribosylation factor 4 | *ARF4* | 5 | 3 | 3 | 55 | 0,01166479 | 0,0998687 | 0,3392676 | 0,072652382 | | *ARF4* |
| P30041 | P30041 | Peroxiredoxin-6 | *PRDX6* | 7 | 7 | 7 | 30 | 0,01226217 | 0,176258 | 0,1077234 | 0,106492953 | | *PRDX6* |
| **P13667** | **P13667** | **Protein disulfide-isomerase A4** | ***PDIA4*** | **15** | **15** | **15** | **331** | **0,01287307** | **0,0171605** | **0,0013301** | **0,313325643** | | ***PDIA4*** |
| **Q9BS26** | **Q9BS26** | **Endoplasmic reticulum resident protein 44** | ***ERP44*** | **7** | **7** | **7** | **105** | **0,01365964** | **0,011489** | **0,0018995** | **0,989554185** | | |
| P53992 | P53992 | Protein transport protein Sec24C | *SEC24C* | 3 | 3 | 3 | 16 | 0,01368429 | 0,1832328 | 0,0019216 | 0,144700414 | | |
| P80404 | P80404 | 4-aminobutyrate aminotransferase, mitochondrial | *ABAT* | 5 | 5 | 5 | 13 | 0,01436063 | 0,0418549 | 0,4883502 | 0,008794984 | | |
| P08758 | P08758 | Annexin A5 | *ANXA5* | 12 | 12 | 12 | 120 | 0,01443147 | 0,500855 | 0,0104712 | 0,051350087 | | *ANXA5* |
| **P11021** | **P11021** | **78 kDa glucose-regulated protein** | ***HSPA5*** | **24** | **24** | **23** | **661** | **0,01453538** | **0,0462898** | **0,047508** | **0,815414069** | ***HSPA5*** | ***HSPA5*** |
| P04843 | P04843 | Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1 | *RPN1* | 12 | 12 | 12 | 160 | 0,01456857 | 0,0074703 | 0,0042961 | 0,805619356 | *RPN1* | *RPN1* |
| O75340 | O75340 | Programmed cell death protein 6 | *PDCD6* | 3 | 3 | 3 | 30 | 0,01477223 | 0,0024878 | 0,0100022 | 0,60108642 | | |
| **Q8NBS9** | **Q8NBS9** | **Thioredoxin domain-containing protein 5** | ***TXNDC5*** | **10** | **10** | **10** | **85** | 0,01599924 | 0,0651848 | 0,044914 | 0,533377197 | | *TXNDC5* |
| O94826 | O94826 | Mitochondrial import receptor subunit TOM70 | *TOMM70A* | 4 | 4 | 4 | 18 | 0,01811076 | 0,0955309 | 0,9500943 | 0,041834749 | | *TOMM70A* |
| P09110 | P09110 | 3-ketoacyl-CoA thiolase, peroxisomal | *ACAA1* | 8 | 8 | 8 | 34 | 0,01892842 | 0,0229383 | 0,0271681 | 0,11526454 | | *ACAA1* |

**Table 6. Proteomic results - Identification and quantification of the 25 most differentially expressed proteins according to the fibrosis grade, classified according to the p-value of the ANOVA test comparing normal ileum (N) to ileum with low grade (F1) or high grade (F2-F3) of fibrosis with the results of the differential analyses for other comparisons.** All the proteins identified and quantified by MaxQuant were annotated with the different results of the statistical analysis performed on Log2 LFQ intensity used to calculate the p value associated to the differences in distribution between the compared groups: N vs F1 vs F2-3 (in black), N vs F1 (in blue), N vs F2-3 (in purple) and F1 vs F2-3 (in green). PDIs, including AGR2, PDIA6, PDIA4, ERP44 and TXNDC5, as well as BiP are highlighted in bold. Razor peptides are peptides found in

several protein sequences sharing at least partial identity of sequence. Unique peptides are peptides that only match with one protein sequence. MS/MS count: number of times that the considered protein entry was associated to a MS/MS identification (or a Peptide Spectrum Match). The "gene name for BiP (HSPA5) Interactors" column details the proteins identified as such using BioGrid according to their gene names (and not their protein name or ID). The "gene name for AGR2 Interactors" column details the proteins identified as such using BioGrid according to their gene names (and not their protein name or ID).

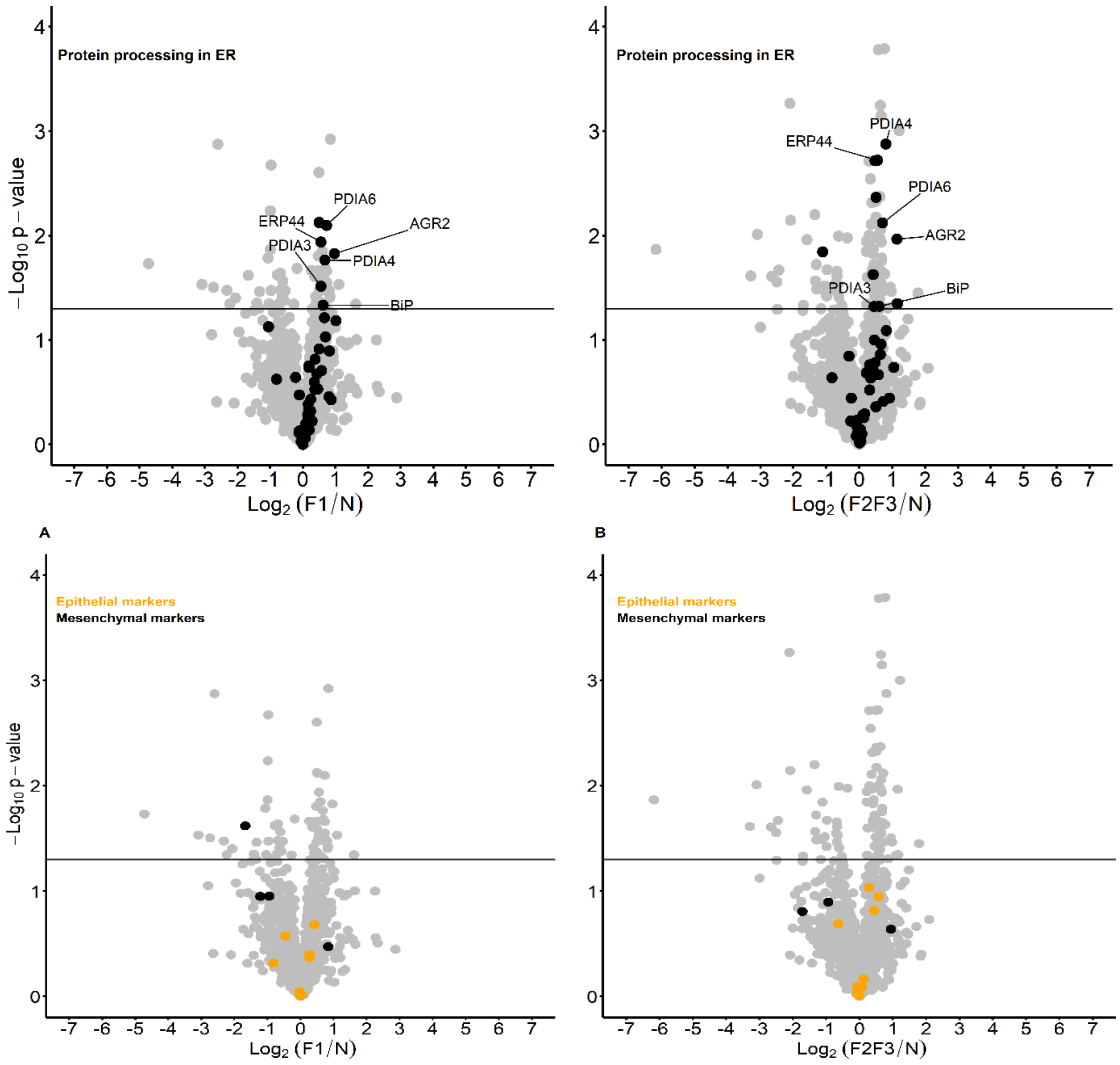



**Figure 19. Endoplasmic reticulum stress and epithelial-mesenchymal transition proteins**
Proteins were represented in volcano plots for the comparison of low grade of fibrosis [F1] to normal tissue [N] and the comparison of high grade of fibrosis [F2-3] to normal tissue [N]. Differential abundance (F1 vs N and F2-3 vs N) of proteins was represented by plotting the $Log_{10}$ p-value against the $Log_2$ of F1/N and F2-3/N. The horizontal line represents the significance threshold (p-value = 0.05).
[A] The proteins involved in "Proteins processing in ER" were selected according to the KEGG pathway database hsa04141 in which two proteins were manually added (AGR2: Anterior gradient protein 2 homolog; ERP44: Endoplasmic reticulum resident protein 44). Among the proteins involved in this pathway and significantly increased in fibrosis, 5 out 7 (N vs F1) and 5 out of 11 (N vs F2-3) are protein disulfide isomerases (PDIs): AGR2, ERP44, Protein disulfide-isomerase A6 (PDIA6), Protein disulfide-isomerase A4 (PDIA4), Protein disulfide-isomerase A3 (PDIA3). Binding immunoglobulin Protein (BiP) was also highlighted.
[B] The proteins involved in EMT were selected according to the literature[36,348] and are listed in Supplemental Table 6. Epithelial and mesenchymal proteins (highlighted in the figure) were not differentially expressed in epithelium surrounding fibrotic and normal tissues.

***Gene Ontology, pathways enrichment analyses, functional annotation, and BioGRID interactor identifications based on proteomic results***

Supplemental Table 5 contains a compilation of the enrichment's charts obtained with each list of sets of proteins identified in our experiment (Supplemental Tables 3 and 4) analysed using DAVID$^{TM}$. The identified pathways included the metabolism of proteins from production to translation and maturation (*ribosomal proteins, translation control, protein maturation, protein transport, protein involved in the spliceosomal complex assembly, mitotic nuclear division, antigen processing and presentation*), membrane proteins, but also confirmed the results of our differential analysis with the presence of proteins processing in ER and especially in response to *ER stress* including many PDIs and several of their complex binding partners. The *EMT* process was not found to be enriched neither the *apoptotic* process (Supplemental Table 5).
Figure 20 illustrates the STRING network of proteins significantly increased in high grade fibrosis compared to N and proteins detected only in fibrotic tissues, highlighting ER stress, unfolded protein response (UPR) and structural interconnected proteins.
Based on the BioGRID database, 435 out of 840 (51.79%) known genes coding for AGR2 protein partners and 123 out of 563 (21.85%) BiP interactors could be identified.

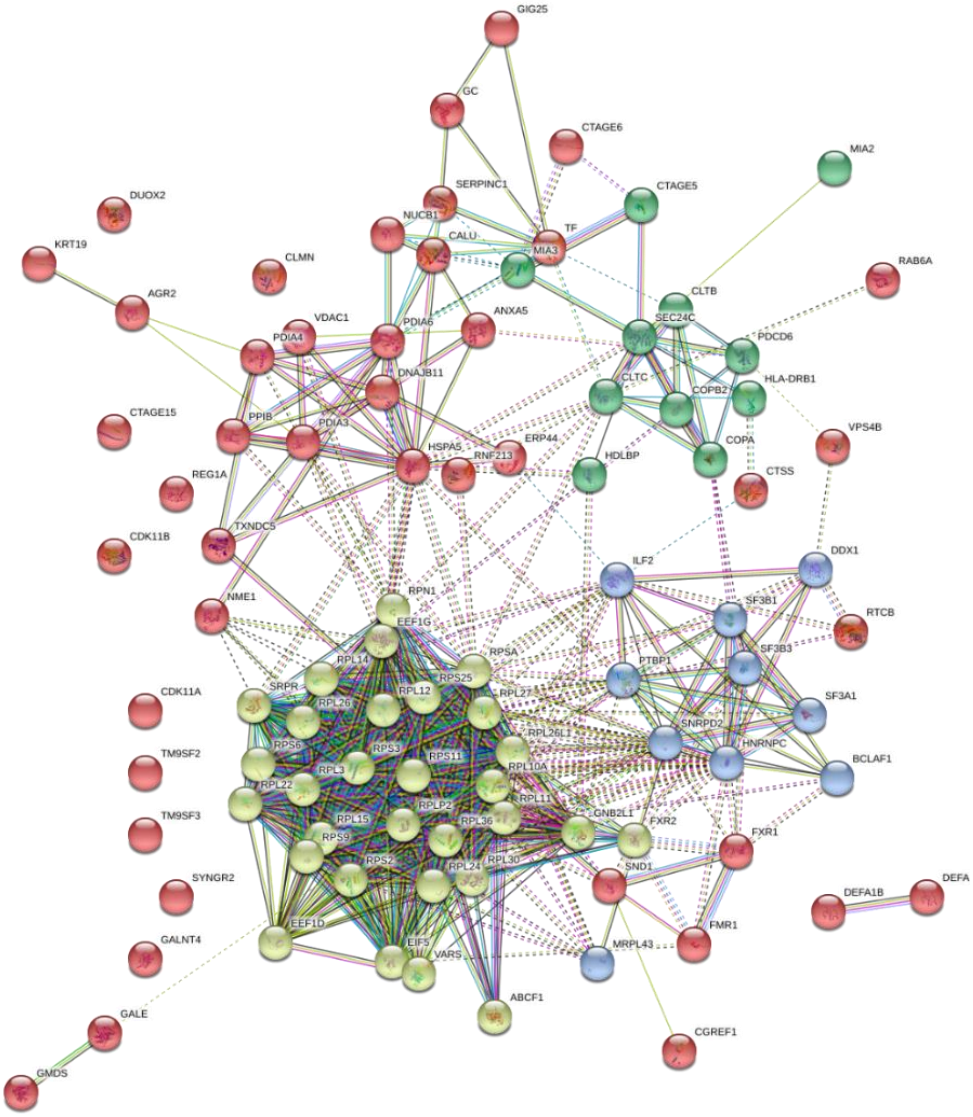



**Figure 20. STRING network of proteins established with the proteins found significant in F2-3 > N and absent in N**

Ribosomal proteins (light green), proteins involved in transcription and alternative splicing control (blue) and proteins implicated in peptides or protein transport (ER-Golgi) or endosomal route (dark green) are highlighted. The proteins not belonging to any of these 3 clusters are by defaults represented in red and most of these are annotated in the STRING database as ER metabolism proteins and are PDIs. The optional k-mean clustering fixed to 3 clusters.

### 3.1.2.2. Immunochemistry confirmatory study

***Proteins selection strategy for IHC confirmation***

As ER stress was found as a predominant pathway in the epithelium of CD stricture, both in the differential analysis and in the pathways enrichment analyses, as the differential analysis highlighted several PDIs, in particular AGR2, among the most discriminating proteins between tissues of different grades of fibrosis, as a pro-fibrotic role of one PDI has already been

demonstrated in renal fibrosis[351] and as AGR2 has a known action on the ECM in oncology[352], we decided to focus the IHC confirmation study on AGR2. BiP was also studied as well-known ER stress marker.

***IHC characterisation***

Immunochemistry experiments were performed on FFPE tissue sections (from the 73 CD and the 40 control subjects), which were treated as previously described[353] (see Supplementary data for details).

A total of 80 tissue sections were collected from the 40 controls subjects (tissue referenced as "N not IBD"; i.e. normal tissue from a non-IBD patient) including 34 sections of ileal tissue (17 that were used for AGR2 staining and 17 for BiP staining) and 46 colonic tissue sections (23 that were used for AGR2 staining and 23 for BiP staining). On these tissue sections, the staining of AGR2 and BiP in the surface epithelium and crypt epithelium was separately studied.

For tissues of CD patients (including 44 fibrostenosing CD patients and 29 non fibrostenosing CD patients), a total of 172 ileal and 158 colonic tissue sections were collected and prepared. The grade of inflammation and fibrosis on each of these tissue sections was characterised as described above. Due to the limited number of samples presenting the same inflammation and fibrosis grades, we decided to group tissue sections combining all positive inflammatory and/or fibrosis grades together in four groups: normal (N), purely inflammatory (I1-3 referenced as I), fibro-inflammatory (I1-3F1-3 referenced as IF) and purely fibrotic (F1-3 referenced as F). Tissue sections characterised as fibro-inflammatory or purely fibrotic tissues (n=130) were obtained only from 44 fibrostenosing CD patients whereas the tissues characterised as normal or purely inflammatory (n=200) were obtained from the 73 CD patients but then collected in normal or purely inflammatory areas at a distance from the fibrosis areas. Approximately half of these FFPE tissue sections were used for AGR2 staining and the other half for BiP staining, and again, on each tissue section, both the surface epithelium and the crypt epithelium were analysed. In some samples, due to ulceration associated to fibrotic area, only the crypt epithelium could be scored (for example, for purely inflammatory ileal tissues, AGR2 staining in the crypt epithelium could be analysed on 14 tissue sections whereas AGR2 staining in the surface epithelium could only be assessed on 13 tissue sections).

For each IHC tissue sections, 3 fields (3 for surface epithelium and 3 for crypt epithelium) were scored independently by 3 observers (M.-A.M., C.M. and C.S.), blinded to the degree of fibrosis and inflammation. AGR2 and BiP immuno-stained sections were scored positive if epithelial cells showed specific staining in the cytoplasm. A semi-quantitative IHC score was determined by estimating the global signal intensity detected in the surface and the crypts epithelium. The following staining scores were used: 0 (none), 1 (weak), 2 (medium), 3 (strong), and 4 (very strong). The final semi-quantitative score attributed for the surface and crypt epithelium for each tissue section was the average value of the three observers in the different analysed fields (n=3)[353]. When for a given segment of intestine of an individual patient several equal inflammation and fibrosis grades sections of tissue were available, only the averaged IHC score obtained for these redundant tissue sections was considered. The statistics were performed using GraphPad Prism 5. IHC scores of the proteins of interest according to inflammation and fibrosis grades (N, I, IF, F) were compared with ANOVA.

***Confirmation of AGR2 and BiP distribution by immunohistochemistry***

Figure 21 illustrates the isotype antibody (Figure 21 A) and positive controls performed as well as the staining scores used (Figure 21 B). AGR2 and BiP appeared within the cytoplasm of epithelial cells, as previously observed on FFPE tissues[354] and were also detected in some blood vessel endothelial cells as well as in some smooth muscle cells. No *lamina propria* IHC signal for AGR2 has been observed and studied. The *lamina propria* staining for BiP was positive (in nucleus and cytoplasm) in 83.1% of the tissue sections evaluated whatever group considered and segment location. The results of the IHC semi-quantitative characterisation for AGR2 and BiP in the ileum and the colon epithelium, according to the tissue classification, are summarised in Figure 22 and Figure 23, respectively. In the ileum, AGR2 was not significantly increased in I tissues but it was the case in the IF tissues, suggesting an association with the fibrotic process.

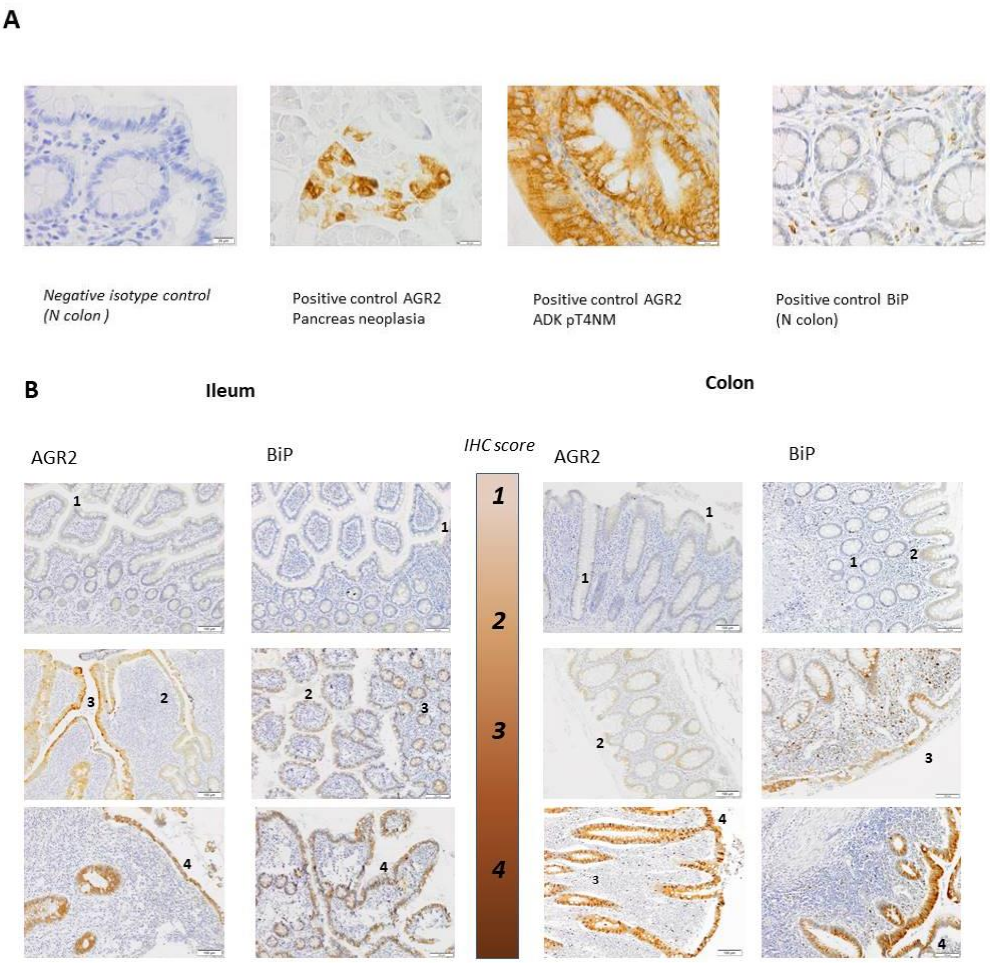



**Figure 21. Illustrative pictures of immunohistochemistry scores**

[A] Negative isotype and positive controls used for AGR2 and BiP IHC protein detection: picture of the negative isotype control is provided for a normal (N) colon. Positive controls were tested on pancreas neoplasia, colorectal adenocarcinoma (ADK) at pT4NM stage and on a normal colon extracted at the surgical margin of diverticular disease.

[B] Illustrative pictures of IHC scores: the 4-grade scale is illustrated for AGR2 and BiP staining performed on ileal and colonic FFPE tissues.

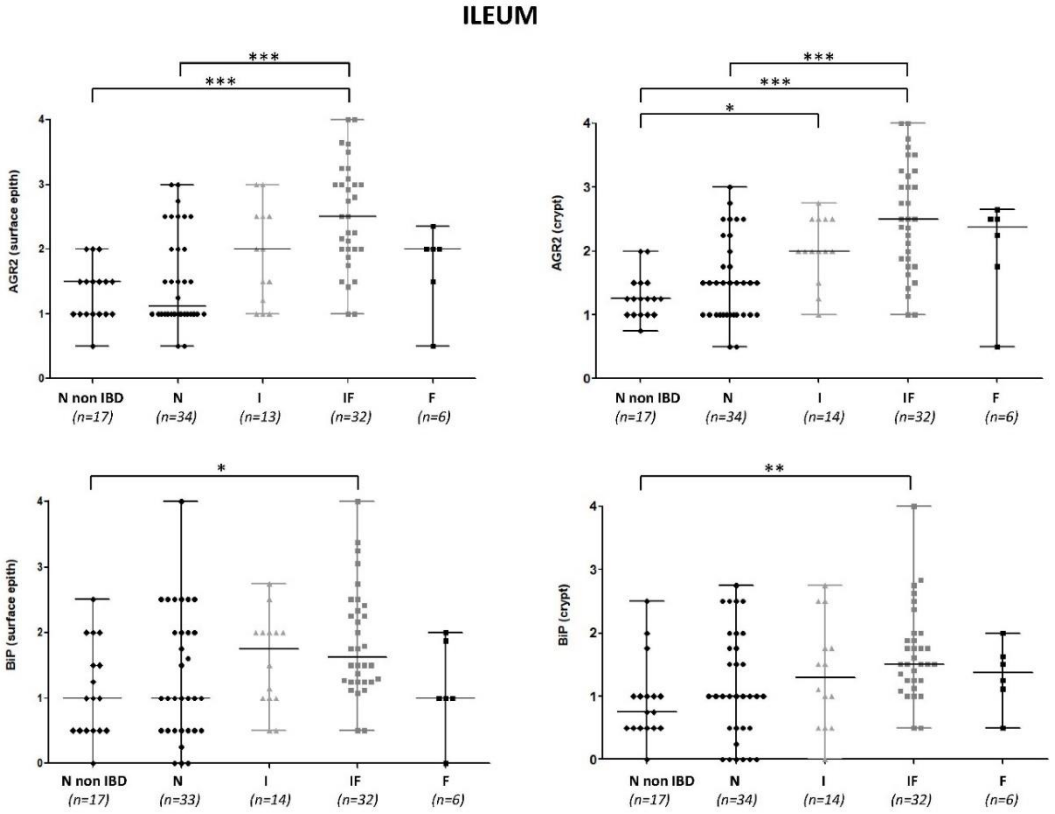



**Figure 22. Distribution of AGR2 and BiP IHC scores for ileal tissues**
Distribution of AGR2 and BiP IHC scores for the CD patients groups (N, I, IF, F) and non IBD controls in the surface and crypt epithelium for ileal tissues. The ANOVA test was significant for AGR2 and BiP in the surface epithelium ($p<0.0001$ and $p<0.05$, respectively) and in the crypt epithelium ($p<0.0001$ and $p<0.001$, respectively). Horizontal black lines highlight the significant two by two comparisons of groups obtained by Dunn's Multiple Comparison Test. *$p<0.05$, **$p<0.001$, ***$p<0.0001$.

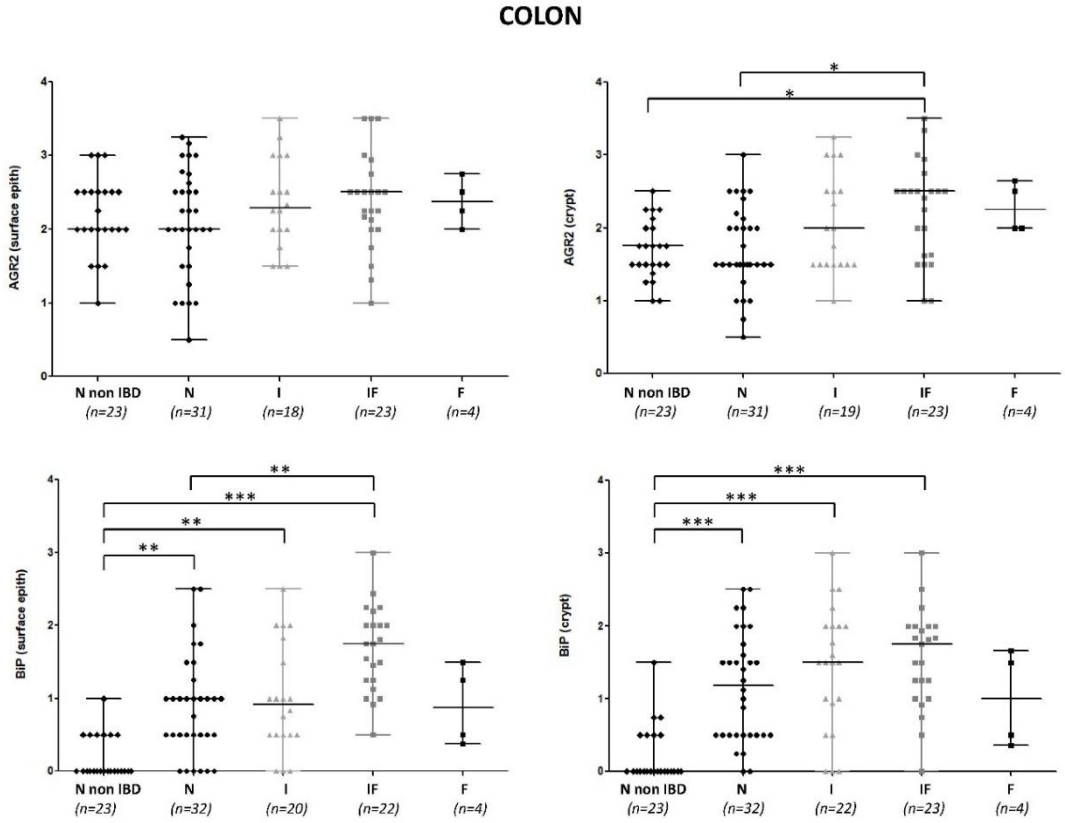



**Figure 23. Distribution of AGR2 and BiP IHC scores for colonic tissues**
Distribution of AGR2 and BiP IHC scores for the CD patients groups (N, I, IF, F) and non IBD controls in the surface and crypt epithelium for colonic tissues. The ANOVA test was significant for AGR2 in the crypt epithelium ($p<0.001$) as well as for BiP in the surface ($p<0.0001$) and crypt ($p<0.0001$) epithelium but was not significant for AGR2 in the surface epithelium. Horizontal black lines highlight the significant two by two comparisons of groups obtained by Dunn's Multiple Comparison Test. *$p<0.05$, **$p<0.001$, ***$p<0.0001$.

Correlations between AGR2 and BiP IHC score and inflammation (N, I1, I2, I3) or fibrosis grades (N, F1, F2, F3) were also performed (using the Spearman correlation analyses). We found weak but significant correlation between AGR2 and fibrosis or acute inflammation grades in the ileum, but not with chronic inflammation grade, for the surface and crypt epithelium (Table 7).

| *Correlation between* | Fibro-inflammatory CD tissues | | | |
|---|---|---|---|---|
| | **Colon** (tissues: n=63) | | **Ileum** (tissues: n=106) | |
| | **r** | ***p-v*** | **r** | ***p-v*** |
| AGR2 surface- fibrosis grade | -0,14 | *2,66E-01* | **0,22** | ***2,08E-02*** |
| AGR2 surface - acute inflammation grade | 0,06 | *6,65E-01* | **0,23** | ***1,97E-02*** |
| AGR2 surface- chronic inflammation grade | -0,06 | *6,36E-01* | 0 | *9,60E-01* |
| AGR2 crypt - fibrosis grade | 0 | *9,76E-01* | **0,23** | ***1,83E-02*** |
| AGR2 crypt - acute inflammation grade | 0,05 | *6,80E-01* | **0,29** | ***2,24E-03*** |
| AGR2 crypt- chronic inflammation grade | -0,01 | *9,11E-01* | 0,07 | *4,58E-01* |
| BiP surface - fibrosis grade | -0,03 | *8,71E-01* | 0,12 | *2,14E-01* |
| BiP surface- acute inflammation grade | -0,02 | *8,67E-01* | 0,13 | *1,70E-01* |
| BiP surface- chronic inflammation grade | -0,16 | *2,40E-01* | 0,14 | *1,57E-01* |
| BiP crypt - fibrosis grade | -0,18 | *1,71E-01* | **0,27** | ***5,72E-03*** |
| BiP crypt - acute inflammation grade | -0,03 | *8,04E-01* | **0,32** | ***8,59E-04*** |
| BiP crypt - chronic inflammation grade | -0,05 | *7,01E-01* | **0,33** | ***6,26E-04*** |

**Table 7. Correlations analysis between IHC score and fibrosis, acute and chronic inflammation grades.** Correlation results (r and associated p-value) are provided for fibro-inflammatory CD tissues, originated from the ileum or the colon. For this correlation, we used individually all tissues samples studied and analysed (colon; n=63; ileum, n=106).

### 3.1.3. Anterior gradient protein 2 homolog in Crohn's Disease

Originally characterized in *Xenopus laevis*[355], AGR2 is an evolutionarily highly conserved protein, present in multiple mammalian cells, which contributes to cell proliferation, adhesion, motility, developmental cell fate and inhibition of apoptosis[89,355–362]. Human *AGR2* gene is localized on chromosome 7p21.3, which is an IBD susceptibility region[363], and *AGR2* genetic variants that decrease the protein expression are associated with an increased risk of both CD and UC[363,364]. AGR2 is one of the PDI superfamily members[88]**,** which are proteins facilitating the folding and the multimerization of specific proteins into their bioactive conformations (by catalyzing formation of intrachain and interchain disulfide bonds) before their secretion or membrane association. AGR2 has therefore the necessary characteristics to be labelled as a PDI: an ER localization[365] and a functional thioredoxin-like domain with a CXXS motif[365,366]. Composed of 175 amino acids (for a molecular mass of 17 kDa)[367], AGR2 is a multidomain protein (Figure 24A) which contains (1) a NH2-terminal cleavable sequence (amino acids or AA 1-21) which directs AGR2 into the ER[368]; (2) a domain for cell adhesion (AA 21–44)[369]; (3) a domain for dimerization (AA 60–64)[370]; (4) a single thioredoxin-like domain for its PDI activity (AA 81–84)[365]; (5) a peptide binding loop domain (AA 104-111)[367] and (6) a COOH-terminal ER-retention sequence (AA 172-175), containing tetrapeptide sequence of lysine (K)–

threonine (T)–glutamic acid (E)–leucine (L) abbreviated as KTEL[371] (which is a non-conventional variant of the classic ER localizing motif, whose classical form is KDEL[372])[367].

In the ER, AGR2 can exist as monomeric and homodimeric forms[369,373,374]. Indeed, AGR2 monomers (thanks to their dimerization domains AA 60-64), can form dimers in which the E60 of each monomer form salt bridges with the K64 of the other monomer, stabilizing the dimer (Figure 24B)[374]. When AGR2 is in its homodimeric form, the dimerization interface is opposite to its CXXS motif (which accepts substrat proteins for disulfide bonds formation), allowing the protein to keep its PDI activity[374]. There are cellular regulators of AGR2 dimerization including AGR2 homodimer enhancers (including TMED2 protein, which interacts simultaneously with both monomers of the AGR2 dimer) and AGR2 homodimer inhibitors[374].

In the absence of ER stress, AGR2 homodimer enhancers (like TMED2) are present in abundance and AGR2 exists mainly as a homodimer[374]. When AGR2 is in this form, the homodimer plays a role in productive protein folding and interacts with Golgi export components[374]. When ER stress occurs, there is an increase in AGR2 homodimer inhibitor. In this case, AGR2 homodimer dissociates and AGR2 is then found in monomeric form[374]. In experimentally induced ER stress models, this dissociation of AGR2 dimers appears to be dose-dependent regardless of the ER stress inducer used (tunicamycin, thapsigargin or dithiothreitol, which are 3 ER stress inducers)[374]. During ER stress, AGR2 monomeric manages misfolded proteins and forms functional complexes with ERAD machinery to clear the misfolded proteins from the ER[374].

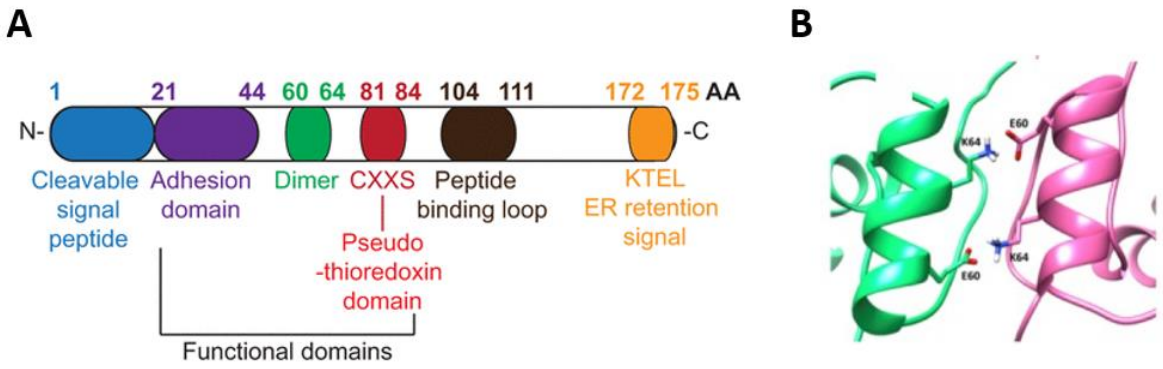



**Figure 24[367,374]. AGR2 protein domains and structure (adapted from Delom et al.[362] and Maurel et al.[369]).**
[A] Primary structure of the AGR2 protein. Colored boxes indicate the identified functional domains and amino acids involved in the regulation of AGR2 function; [B] Homodimer formation. AA, amino acid; ER, endoplasmic reticulum; K—Lysine; T—Tyrosine; E—Glutamic acid; L—Leucine.

*AGR2* is expressed in all regions of the small intestine and the colon[89], especially in secretory IECs such as Paneth cells, goblet cells and enteroendocrine cells as well as $MSII^+$ (Musashi-1) intestinal progenitors[88,361,375]. Although mainly located in the ER of these cells (where it is held back by its carboxyl-terminal KTEL sequence[376]), this protein is also known to be secreted in the intestinal mucus[73,88,361,377,378], suggesting that AGR2 has still unknown extracellular functions in the intestine[73].

In view of its pattern of expression, its multiple domains but also the fact that these PDIs have multiple substrates, AGR2 was rapidly supposed to play diverse roles in IECs[89]. In goblet cells, the single cysteine residue of the AGR2 thioredoxin-like domain forms mixed disulfide bonds with Mucin 2 (MUC2) allowing its correct folding and secretion[365]. In the absence of AGR2, *Agr2*−/−mice decrease their intestinal mucus production and develop severe spontaneous granulomatous ileocolitis, closely similar to human CD[88,89]. The presence of AGR2 in non-mucin producing cells (such as Paneth cells and enteroendocrine cells)[361] suggests that AGR2 may interact with proteins other than MUC2 but further works will be required to identify the full range of AGR2 substrates[88,379]. AGR2 also contributes to the Paneth cell's homeostasis[89] and is important for their appropriate migration away from the crypt base into the upper crypt and villi[364]. Although in *Agr2* deficiency mice, Paneth cells anomalies preceds the decrease of intestinal mucus production and the development of intestinal inflammation, the role of AGR2 in the folding and secretion of antimicrobial peptides has not, to our knowledge, been studied to date[89]. As the deficit of AGR2 was also reported to be associated to an increase of the ER stress protein level (such as BiP and sXBP1) in goblet cells, Paneth cells' lineage and MSI1-positive intestinal stem/early progenitor cells[89], AGR2 was supposed to play a role in the ER stress alleviation in IECs and contributes to UPR (where it is controlled by the IRE1α and ATF6α arms)[89,365]. This protein also prevents apoptosis of IECs and contributes to maintain their proliferation[89], and assists the holding of the intestinal epithelial barrier function[380].

In the mucosa of IBD patients, the gene expression of *AGR2*, but also *XBP1*, *HSPA5* (encoding the BiP protein) mRNA level were significantly higher than in non IBD patients[381]. However, in these IBD patients, there is no difference between inflammatory and non-inflammatory mucosa[382], consistent with what we found at the protein level in the previously performed IHC study. Although there is no difference between inflammatory and non-inflammatory tissues, AGR2 has been shown to play a role in intestinal inflammation. Indeed, some authors suggest that intracellular AGR2 could mitigate TNF-α-induced intestinal epithelial barrier injury by

reducing the permeability of the epithelial barrier, by increasing the expression of the tight junction proteins (zonula-occludens-1, claudin-1 and occluding) and by stabilizing the cytoskeletal F-actin (through inhibition of NF-κB p65 mediated MLCK/p-MLC pathway), also implicated in intestinal epithelial barrier permeability.

More recently, Maurel et al. demonstrated that the role of AGR2 in intestinal inflammation may depend on the modulation of AGR2 dimer formation (Figure 25)[374]. They showed that, in normal condition (in the absence of IBD), there is a certain ratio between the monomeric and dimeric forms of AGR2 in the ER (the dimeric form being predominant), and this ration contributes to the sensing of ER homeostasis[374]. In this case, AGR2 mainly interacts with Golgi export components and thus contributes to secretory pathways[374]. The demonstrated that in IBD patients, and especially in CD[374], the expression of a range of AGR2 dimer modulators (including TMED2 but not only), was dysregulated, which may contribute to the perturbation of AGR2 dimerization (and of the AGR2 monomer/dimer ratio). They found that, in CD patients with quiescent disease, the homodimeric enhancer of AGR2 called TMED2 is found in smaller quantities than under normal conditions, resulting in a reduction of AGR2 dimers (AGR2 is then essentially in the form of a monomer). This state leads to the secretion of the AGR2 monomer, which contributes to the induction of a pro-inflammatory signal (explained below)[374]. In active CD, however, there is an overexpression of TMED2, resulting in an increase in the formation of AGR2 homodimer. This state leads to the uncontrolled autophagy-dependent release of an aberrant entity of AGR2 into the extracellular medium, contributing to inflammation[374]. Indeed, they demonstrated the role of this extracellular AGR2 in the pro-inflammatory cells chemoattraction in an in vitro model[374]. They exposed peripheral blood mononuclear cells (PBMCs) purified from three independent healthy donors to supernatant of cells, in which, different modulations of AGR2 expression were induced and shown that chemoattraction of PBMCs was only observed when AGR2 was found in the extracellular medium (chemoattraction impede after the use of an AGR2 blocking antibody), highlighting the extracellular gain-of-function of AGR2 as a pro-inflammatory chemokine[374].

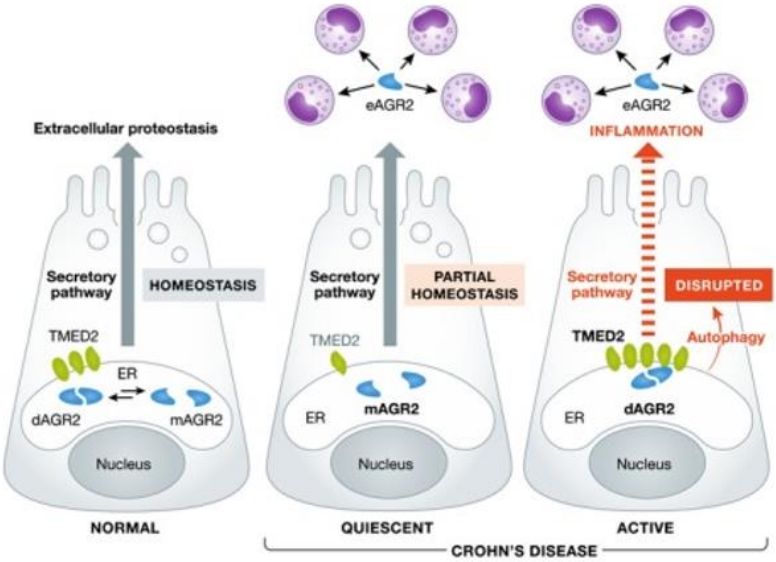



**Figure 25. Schematic representation of the role of AGR2 dimer alteration in IECs under basal conditions or in CD[374]**

In normal condition (in the absence of IBD), there is a certain ratio between the monomeric and dimeric form of AGR2 (the dimeric form being predominant). This ratio between the two forms contributes to the sensing of homeostasis in the ER[374]. In these conditions, AGR2 mainly interacts with Golgi export components and thus contributing to secretory pathways[374]. In patients with quiescent CD, TMED2 levels appear to be lowered, resulting in a reduction of AGR2 dimers and leading to the secretion of AGR2 monomer, which acts as a pro-inflammatory signal[374]. In active CD, there is an overexpression of TMED2, which leads to overexpression of AGR2 dimers, resulting in the secretion of an autophagy-dependent aberrant form of AGR2 into the extracellular environment, also associated with inflammation[374].

Although the role of AGR2 begins to be described in CD inflammation[374], it has never been reported to be implicated in intestinal fibrosis to date. Preliminary studies performed in our laboratory highlighted a significant increase of AGR2 in fibrotic tissues of CD patients (while it was not significantly increased in pure inflammatory tissues) and its expression was correlated to fibrosis grade in IHC. Although these observations do not allow us to make it a protein purely related to fibrosis neither to ensure that the observed increase is not partly the reflect of chronic inflammation, this work is the first, to our knowledge, to show a more specific implication of AGR2 in CD fibrosis, and the link between them deserves to be studied.

### 3.1.4. Experimental models to study Crohn's disease fibrosis

Experimental models used to explore intestinal fibrosis are either *in vitro* or *in vivo*, each with their advantages and disadvantages. *In vitro* models correspond to culture of cells in nutrient media outside the body. These models can involve primary cells or immortalised cell lines. Primary cells (including fibroblasts, myofibroblasts, smooth muscle cells or endothelial

cells[147]) or tissue (explants) can be directly collected from IBD patients and can be cultivated[383]. While these cells have characteristics of in vivo cells, they lose them during divisions, limiting the life span of these models. On the contrary, immortalised cell lines, derived from cancer cells harvested in a patient or from normal cells transformed by genetic engineering, have not in vivo cells characteristics, but have the advantage of multiplying infinitely, retaining their original characteristics[384]. In fibrosis, these *in vitro* models generally focus on the transformation of fibroblast (which is the key cell in this pathophysiological process) in myofibroblast, using fibrogenic cytokine stimulation such as TGF-β[385–388]. While these experimental models based on cells culture have allowed significant advances in the understanding of the molecular mechanisms implicated in the fibrogenic process, this remains too complex to be studied only in vitro and experimental animal models are essential, allowing the validation of new therapeutic approaches that can then be extrapolated to humans[389].

In contrast to *in vitro* models, *in vivo* models are carried out in living organisms. Several *in vivo* animal models have been developed to understand the intestinal fibrosis pathogenesis including: (1) spontaneous models (such as SAMP1/Yit mouse[390], a model of chronic intestinal inflammation which represents the best model to understand the induction and perpetuation of inflammation-induced intestinal fibrosis); (2) chemical-induced models (by 2,4,6-trinitrobenzenesulphonic acid or TNBS[391], by dextran sodium sulphate or DSS, or by peroxynitrite); (3) bacteria-induced models (by peptidoglycan-polysaccharide or PG-PS, or by gut microbiota or infection by Salmonella or Escherichia Coli); (4) immune and (5) radiation-induced models; (6) post-operative model; as well as (7) gene knockout and transgenic models (IL-10 deficiency, TGF-β1 overexpression, MCP-1 overexpression)[147,389]. Although useful for studying a range of parameters relating to fibrosis, to date, none of these in vivo models recapitulates all of the pathogenic and clinical features of human intestinal fibrosis[320].

More recently developed, organoids, an intermediate experimental model between the *in vitro* and *in vivo* models (sometimes referred as «*ex vivo* model») could be even more interesting to study intestinal fibrosis[392–395]. Organoids are generated from normal human pluripotent stem cells, which, thanks to a culture medium containing the appropriate growth and differentiation factors, form a 3D cellular structure with similar architectures and functionalities than organs from which they derived[396–398]. The use of this model to study intestinal fibrosis is particularly interesting because the lumen is surrounded by epithelium (with villi and crypts), but above all supported by a layer of mesenchymal cells, which are key cells in fibrosis and therefore enables

the study of multi-lineage human cells, while being amenable to higher-throughput screening than in vivo models to test anti-fibrotic drugs for example and without sacrificing animals.

While it is true that, compared to *in vitro* models, *in vivo* and *ex vivo* models better reflect the 3D complex intestinal architecture as well as the multitude of specialised intestinal fibrosis cells, immortalised cell lines are readily available and easy to manipulate, does not create ethical concerns, allow quick and cost-effective results, with good reproducibility and have been widely used in mechanistic studies[384].

## 3.2. RESEARCH STRATEGY AND OBJECTIVES

Our pilot proteomic study showed that the surface epithelium from ileal fibrotic CD strictures exhibits signatures of ER stress, UPR and likely apoptosis. Importantly, we revealed an increase of several PDIs including AGR2 in fibrotic tissues, leading us to study, by IHC, links between AGR2 but also BIP and the fibrosis on a larger cohort of CD patients. In the ileum, a significant increase of AGR2 was confirmed by IHC in tissues with a fibrotic component, while it was not significant in pure inflammatory samples, highlighting the association of this protein with the fibrostenosing process.

The objective of this part of my doctoral thesis was to perform functional assays, with a two cell lines model (intestinal epithelial cells (IEC) and intestinal fibroblasts), to understand the capacity of this ER stress in IECs to promote fibrosis, and more specifically, its capacity to induce a fibroblast to myofibroblast differenciation[250], as well as to preliminary assess the potential modulation of this ER stress.

Our detailed and specific aims were to:

- Reproduce the ER stress proteins expression, as observed by proteomics and IHC, in IECs using tunicamycin (Tm), a known ER-stress inducer, and compare basal and Tm-induced expression of these proteins in different intestinal epithelial cells lines to choose the most appropriate one to study, in particular, the AGR2 protein

- Evaluate if ER stress in IECs persists after Tm removal and if these cells, after Tm-induced ER-stress secrete PDIs, especially AGR2, to see if these proteins could be potential paracrine pro-fibrotic factors

- Assess, by using a two cell lines model, if IECs, under ER stress, can induce a fibroblast to myofibroblast differentiation by a paracrine action

- Investigate the specific pro-fibrotic role of AGR2 by studying the capacity of recombinant AGR2 to induce a fibroblast to myofibroblast differentiation and the impact of AGR2 blockade

- See if these IECs, under induced ER stress, release TGF-β1, known to play a key role in the myofibroblastic differentiation, using specific TGF-β1 ELISA

- Check whether the presence of an ER stress in IECs could contribute to fibrosis also by inducing an EMT in these cells

- Assess the effect of prototypical pro-fibrotic (TGF-β1), pro-inflammatory (TNF-α) inducers or both on IECs to see if these cytokines, predominantly present in CD, contribute to the induction of ER stress.

-Assess the alleviation of ER stress using TUDCA which is known to improve ER stress[399]

## 3.3. MATERIAL AND METHODS

### 3.3.1. Cell lines and culture conditions

The human colon adenocarcinoma epithelial cell line HT-29 (ATCC® HTB-38™), the human colon carcinoma epithelial cell line HCT 116 (ATCC® CCL-247™), human colon epithelial cells HCEC-1CT (Evercyte) and the normal human intestinal fibroblast cell line CCD-18Co (ATCC® CRL-1459™) were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, USA) supplemented with 10% foetal bovine serum (FBS) (R&D systems, Minneapolis, USA) and 1% penicillin/streptomycin (Sigma, St Louis, USA). All cell lines were maintained in humidified incubator at 37°C under a 5% $CO_2$.

#### 3.3.1.1. Induction of an endoplasmic reticulum stress in intestinal epithelial cells

HT-29, HCT 116 and HCEC-1CT cells were seeded in six-well plates ($3x10^6$ cells/well) and were grown during 48 h before treatments. Then, ER stress induction was performed using 2 to 10 µg/mL of tunicamycin (Tm, solubilised in DMSO) (Sigma) and Tm treatment was applied for 2, 4, 6, 8, 18 and 24 h at the selected dose (Figure 26). DMSO was used as control condition (at the same final concentration as in the Tm treated condition).

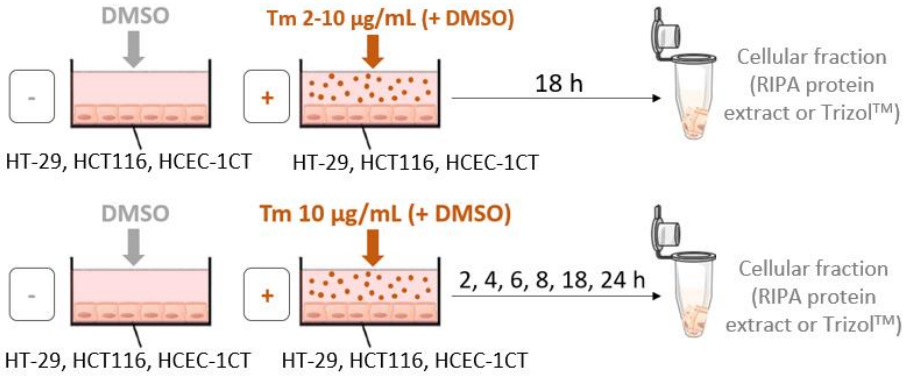



**Figure 26. Induction of an ER stress in IECs using increasing doses and timing of tunicamycin**
DMSO, Dimethyl sulfoxide; Tm, Tunicamycin

#### 3.3.1.2. Sensitivity of CCD-18Co to tunicamycin

CCD-18Co cells were seeded in six-well plates ($1x10^6$ cells/well) and grown for 48 h before treatments. CCD-18Co were subjected to Tm stimulation (10 µg/mL during 18 h) to assess the

sensitivity of these cells to Tm (Figure 27). DMSO was used as control condition (at the same final concentration).

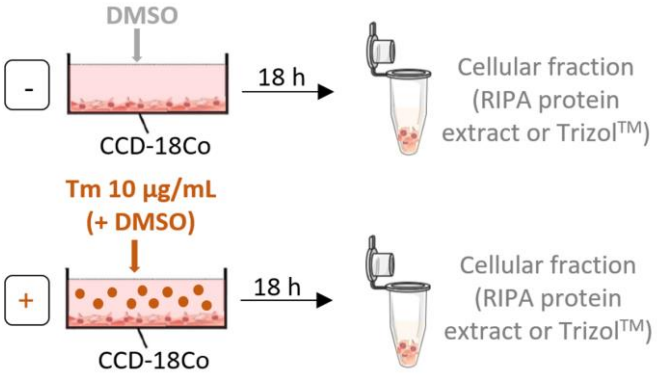



**Figure 27. Sensitivity of CCD-18Co to Tm**
DMSO, Dimethyl sulfoxide; Tm, Tunicamycin

### 3.3.1.3. Persistent endoplasmic reticulum stress and protein disulfide isomerases secretion in HT-29 cells after tunicamycin removal

To assess if ER stress persists after stimulus (Tm) removal, HT-29 supernatant was changed after 18 h of Tm treatment (10 µg/mL) for the stimulated conditions and the relevant control conditions (Figure 28). Cell extracts and matching supernatants were collected 2, 4, 8, 24 and 32 h after the media change.

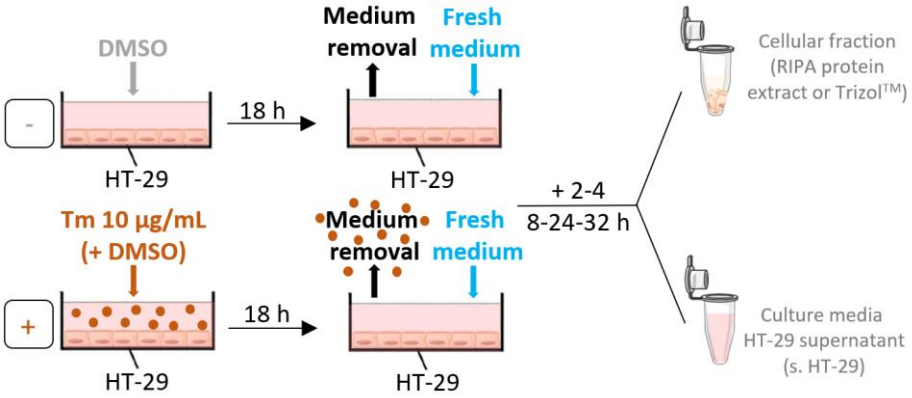



**Figure 28. Persistence of HT-29 cells ER stress and PDIs secretion after tunicamycin removal**
DMSO, Dimethyl sulfoxide; Tm, Tunicamycin.

### 3.3.1.4. CCD-18Co fibroblast to myofibroblast differentiation induced by HT-29 supernatants

The CCD-18Co differentiation positive control was obtained using a 48 h treatment with 10 ng/mL of TGF-β1 (R&D systems) added in the media of CCD-18Co (after 24 h of serum starvation and addition of 1% FBS at the time of stimulation).

The supernatants of HT-29 cells, pre-conditioned or not by Tm (thus, subject or not to a transient ER stress) and collected 8, 24 and 32 h after the media change, were applied on CCD-18Co, as inducing conditions, for further 48 h of incubation. The HT-29 supernatant was used without any freezing cycle (Figure 29).

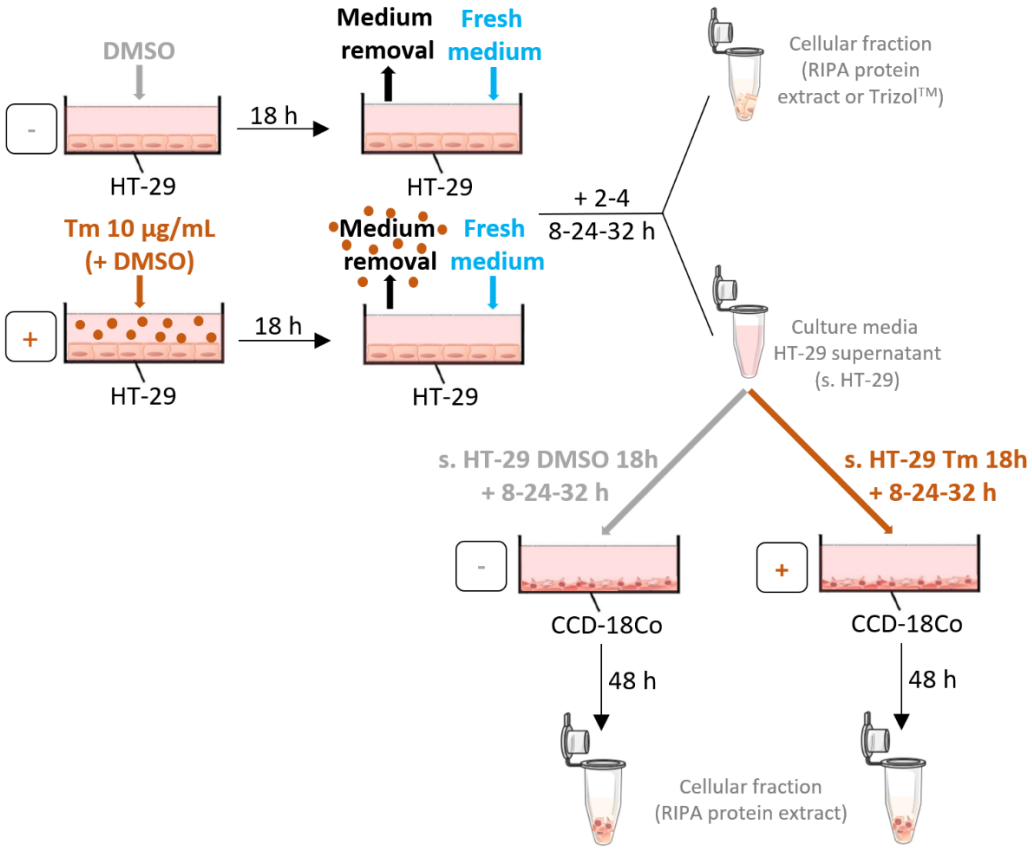



**Figure 29. CCD-18Co fibroblast to myofibroblast differentiation induced by HT-29 supernatants pre-conditioned or not by Tm**
DMSO, Dimethyl sulfoxide; s. HT-29, supernatant of HT-29 cells; Tm, Tunicamycin.

### 3.3.1.5. Investigation of the specific pro-fibrotic role of anterior gradient protein 2 homolog

The capacity of recombinant AGR2 (rAGR2) to induce fibroblast to myofibroblast differentiation was investigated by application of 40 ng/mL of rAGR2 (Bio-Connect BV, OPCD01192) in the media of CCD-18Co (after 24 h of serum starvation and addition of 1% FBS at the time of stimulation) as well as in the supernatant of HT-29 cells, collected in control condition and applied on CCD-18Co (Figure 30). The control being the same condition without rAGR2 supplementation. The impact of AGR2 blockade, using an anti-AGR2 antibody (rabbit, 1.936 µg/mL, ab76473, Abcam), at the dose of 40 ng/mL, supplemented to the conditions with rAGR2 or to the HT-29 supernatant pre-conditioned by Tm, was also evaluated (Figure 31).

The anti-AGR2 antibody was applied to CCD-18Co concomitantly to rAGR2 or to HT-29 supernatant pre-conditioned by Tm.

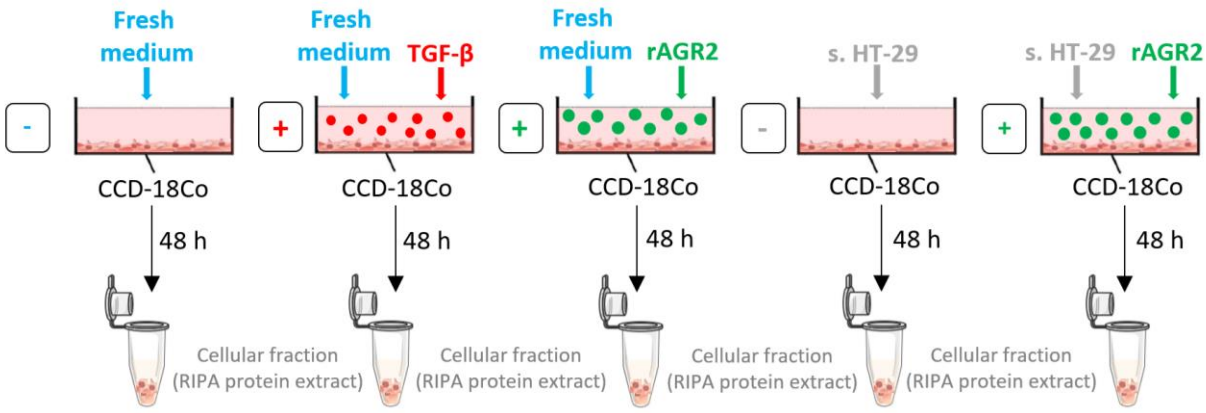



**Figure 30. Effects of recombinant AGR2 on myofibroblast differentiation**
rAGR2, recombinant AGR2; s. HT-29, supernatant of HT-29 cells; TGF-β, Transforming growth factor β.

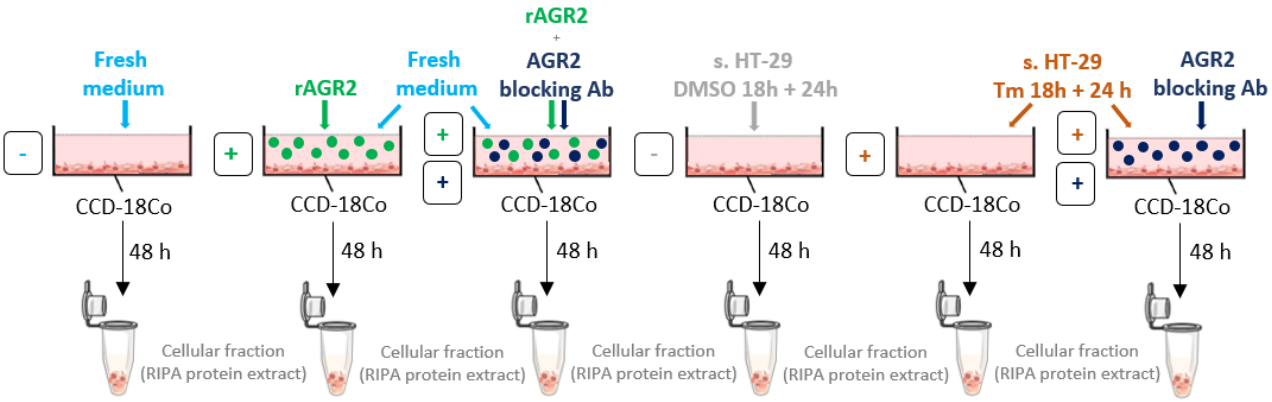



**Figure 31. Impact of AGR2 blockage, using anti-AGR2 antibody, supplemented to the conditions with rAGR2 and to the HT-29 supernatant pre-conditioned by Tm, on myofibroblast differentiation. The anti-AGR2 antibody was applied on CCD-18Co concomitantly to rAGR2 and concomitantly to HT-29 supernatant.**
Ab, antibody; DMSO, Dimethyl sulfoxide; rAGR2, recombinant AGR2; s. HT-29, supernatant of HT-29 cells; Tm, Tunicamycin.

### 3.3.1.6. Study of transforming growth factor β1 secretion and epithelial-to-mesenchymal transition induction in response to endoplasmic reticulum stress in intestinal epithelial cells

In the same experiment as the one performed to see if ER stress persists after removal of tunicamycin, we analysed, in the cell extracts, different EMT markers, and in the supernatant, the secretion of TGF-β1 by ELISA (Figure 28).

### 3.3.1.7. Impact of transforming growth factor β1 or/and tumor necrosis factor α on epithelial endoplasmic reticulum stress

HT-29 cells were seeded in six-well plates ($3x10^6$ cells/well) and they were grown during 48 h before treatments. After 24 h of serum starvation, addition of 1% FBS was applied together with stimulation using TNF-α (Pepro tech, London, UK) and/or TGF-β1 (R&D systems, Minneapolis, USA), both at 10 ng/mL, for 48 or 72 h incubation (Figure 32).

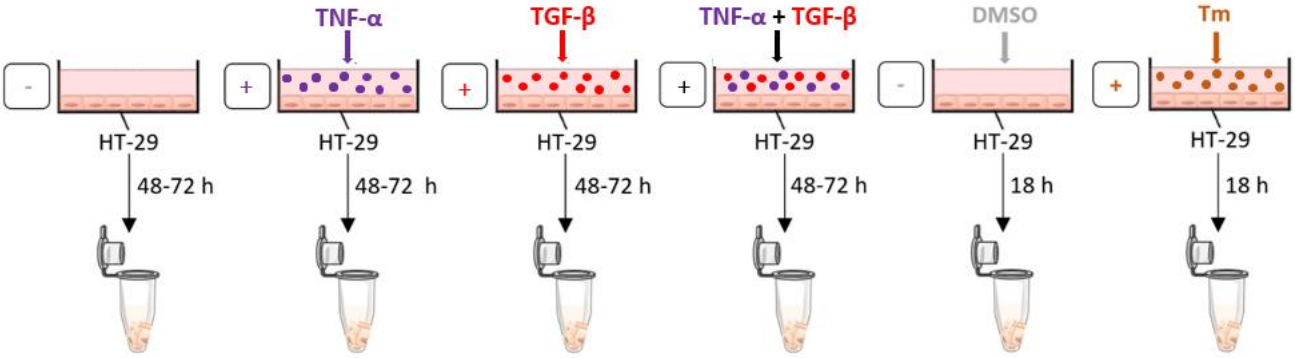



**Figure 32. Impact of TGF-β or/and TNF-α on epithelial ER stress**
DMSO, Dimethyl sulfoxide; TGF-β, Transforming growth factor β; Tm, Tunicamycin; TNF-α, Tumor necrosis factor α.

### 3.3.1.8. Alleviation of endoplasmic stress and protein disulfide isomerases secretion using tauroursodeoxycholic acid

The ability of TUDCA (Sigma) to alleviate ER stress in IEC with Tm-induced ER stress was tested using increasing doses of TUDCA (Figure 33). We also looked at how TUDCA treatment concomitant to induced ER stress impacts PDIs, and particularly the AGR2 secretion.

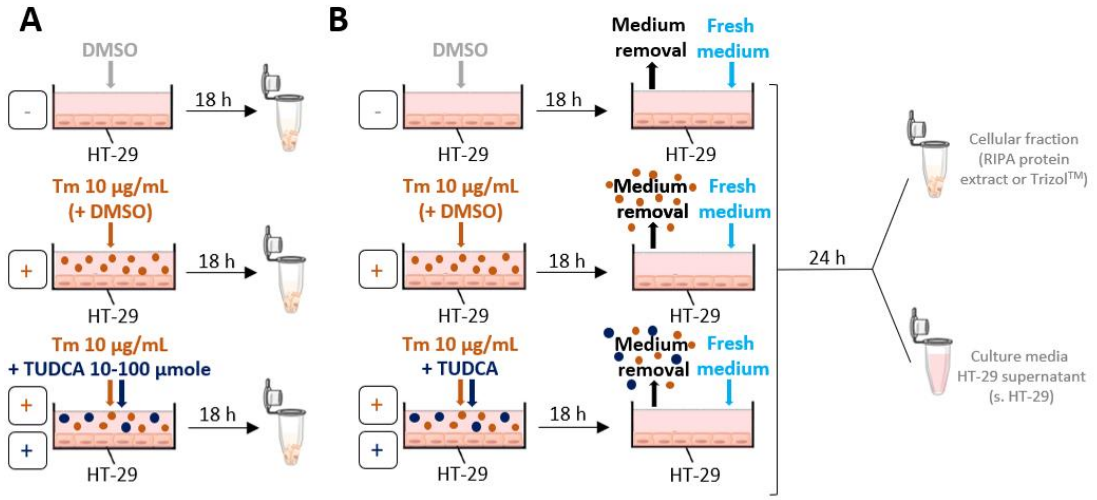



**Figure 33. Alleviation of ER stress by TUDCA**
[A] Alleviation of ER stress in IECs using increasing doses of TUDCA; [B] Impact of TUDCA treatment applied concomitantly to induced ER stress in IECs, on intracellular and extracellular PDIs. DMSO, Dimethyl sulfoxide; s. HT-29, supernatant of HT-29 cells; Tm, Tunicamycin; TUDCA, Tauroursodeoxycholic acid.

The HT-29 and CCD-18Co cells were harvested and treated for either total protein extracts (stored at -20°C) or for RNA extractions (stored at -80°C). HT-29 supernatants collected for analysis were stored at -20°C. For all reported *in vitro* experiments, at least three independent assays were performed with triplicate samples.

### 3.3.2. Technical aspects

#### 3.3.2.1. Western Blot

Total proteins were extracted on ice using RIPA buffer (hepes 25 mM, NaCl 150 mM, triton 0.5%, glycerol 10%, DTT 1 mM, sodium orthovanadate 1 mM, beta-glycerophosphate 25 mM, sodium fluorure 1 mM (Sigma Merk) containing the protease inhibitor complete™ EDTA-*free* (Roche, Mannheim, GE). After centrifugation, proteins cell extracts and supernatants were quantified, samples were separated by SDS-PAGE (10 µg for protein extracts and 40 µg for supernatants), transferred onto poly-vinylidene difluoride (PVDF) membranes, blocked with 10% non-fat milk and incubated with mouse anti-AGR2 (1:2000, clone 1C3, H00010551-M03, Abnova), rabbit anti-BiP (1:500, C50B12, Cell Signaling Technology, USA), rabbit anti-GAPDH (1:12000, D16H11, Cell Signaling Technology), rabbit anti-PDIA6 (1:1000, HPA034652, Sigma), rabbit anti-ERP44 (1:2000, D17A6, Cell Signaling Technology), rabbit anti-ERP57 (1:1000, G117, Cell Signaling Technology) and rabbit anti-ERP72 (1:2000, D70D12, Cell Signaling Technology) in 5% non-fat milk for 2 h. HRP-conjugated-Ab (Cell Signaling Technology) at a 1:1000 dilution was used for 1 h as secondary antibody before revelation using the Electrochemiluminescence Pierce™ ECL Western Blotting Substrate reagent (Thermo Fisher Scientific).

#### 3.3.2.2. Ribonucleic acid extraction and reverse transcription quantitative polymerase chain reaction

Total RNA was extracted using TRI Reagent® (Sigma) according to the manufacturer's instructions. The RNA quantity and purity were assessed with Nanodrop® spectrophotometer (Isogen, NL). Reverse transcription was performed with the Revertaid H Minus First Strand cDNA kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Real-time PCR experiments were done on a light cycler 480 instrument (Roche Life Science) using the following conditions: 3 min at 95°C, followed by 45 cycles of 3 s at 95°C and 20 s at 60°C. Reactions were performed in triplicate in a 10 µL reaction volume containing 4.8 µL of Kapa

SYBR Fast (Sigma), 0.1 µL of each primer (200 nM final concentration), and 5 µL of cDNA. Primers sequences are presented in Table 8. Melting curves were systematically analysed to verify the specificity of the amplification. Analysis was done using the $2^{-\Delta CT}$ method with *HPRT* as a reference gene.

| Gene name | Symbol | NCBI reference | Forward | Reverse |
|---|---|---|---|---|
| Actin, Alpha 2, Skeletal Muscle | *ACTA2* | NM_001613.2 | CGTGTTGCCCCTGAAGAGCAT | ACCGCCTGGATAGCCACATACA |
| Anterior gradient protein 2 homolog | *AGR2* | NM_006408.3 | TACCACAGTCAAACCTGGAGC | GTGGGCACTCATCCAAGTGA |
| Connective tissue growth factor | *CTGF* | NM_001901.2 | TTGGCAGGCTGATTTCTAGG | GGTGCAAACATGTAACTTTTGG |
| DNA damage-inducible transcript 3 | *CHOP* | NM_004083.5 | CTGGCTTGGCTGACTGAGGAG | CGGGCTGGGGAATGACC |
| E-cadherin | *CDH1* | NM_004360.4 | AGCCTCAGGTCATAAACATCATTG | GATAGATTCTTGGGTTGGGTCG |
| Fibronectin | *FN* | NM_212474.2 | CTGGCCGAAAATACATTGTAAA | CCACAGTCGGGTCAGGAG |
| Fibroblast-specific protein 1 | *FSP1* | NM_019554.3 | CCACAAGTACTCGGGCAAAG | GTCCCTGTTGCTGTCCAAGT |
| Heat Shock Protein Family A (Hsp70) Member 5 | *GRP78* | NM_005347.4 | CATCACGCCGTCCTATGTCG | CGTCAAAGACCGTGTTCTCG |
| Homo sapiens collagen type I alpha 1 chain | *COL1A1* | NM_000088.3 | AGTTCGAGTATGGCGG | CAGTGACGCTGTAGGT |
| Hypoxanthine-guanine phosphoribosyltransferase | *HPRT* | NM_000194.2 | TGACACTGGCAAAACAATGCA | GGTCCTTTTCACCAGCAAGCT |
| Transforming growth factor beta | *TGF-ß1* | NM_000660.6 | AACCCACAACGAAATCTATGACAAG | AGAGCAACACGGGTTCAGGTA |
| Type III collagen | *COL3A1* | NM_000090.3 | GCGGTTTTGCCCCGTATTAT | TGCAGTTTCTAGCGGGGTTT |
| Vimentin | *VIM* | NM_001017921 | CGCCAAGGACCGTGAGATTG | GGGCTGCAACTGCCTAATGA |
| Snail Family Transcriptional Repressor 1 | *SNAI1* | NM_005985.4 | GGACAGAGTCCCAGATGAGC | GCGAGCTGCAGGACTCTAAT |
| sliced from of X-box binding protein 1 | *sXBP1* | NM_001079539.1 | TGCTGAGTCCGCAGCAGGTG | GCTGGCAGGCTCTGGGGAAG |

**Table 8. List of specific primer couples used for RT-qPCR experiments**

### 3.3.2.3. Immunofluorescence

Cells grown on cover slips were fixed using 4% paraformaldehyde (PFA) for 10 min and permeabilized with 0.1% Triton. After blocking with 1% BSA and 5% FBS in PBS, cells were incubated with the primary antibody overnight at 4°C. Cells were thereafter washed and incubated with Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488; Abcam, Cambridge, UK) for 1 h at RT. DAPI (Sigma) staining was used to visualize the nucleus. Confocal analysis was performed using confocal Leica SP5 imaging system (Leica microsystem GmBh, GE).

### 3.3.2.4. Enzyme-linked immunosorbent assay

The TGF-β1 concentration in cell supernatants (collected and preserved at -20°C) was measured using the commercial ELISA kits (R&D Systems DY240) following the manufacturer's instructions. The level of bioactive TGF-β1 and total TGF-β1 was measured. The measure of bioactive TGF-β1 was done without prior dilution of the samples. The measure of total TGF-β1 was done after a 1:5 dilution of the samples and their acidification. Indeed, as TGF-β1 is secreted in a latent form (where it is linked to LAP), acidification of samples were performed to dissociate TGF-β1 from $LAP^{210–212,400}$ and measure both free TGF-β1 and dissociated latent TGF-β1. Samples were then acidified using 20 µl of activation solution or

1M HCl per 100µl of culture supernatant and incubated at room temperature for 10 min. The samples were then neutralized by 20µl of neutralisation solution. The absorbance was measured at 450 nm using spectrophotometer (Molecular Devices, SpectraMax Plus 384). The limit of sensitivity was 15.4 pg/mL.

### 3.3.3. Statistics

One-way ANOVA test was used to assess differences between conditions in RT-qPCR, as well as the Turkey's multiple comparison test when appropriate. All results were considered significant if associated to a p-value $\leq 0.05$.

## 3.4. RESULTS

### 3.4.1. Choice of epithelial cell line

In order to select the most appropriate epithelial cell line to study the role of epithelial ER stress and in particular AGR2 protein in fibrosis, we first assess the basal and the Tm-induced ER stress expression of this protein in different epithelial cell lines (HT-29, HCT 116 and HCEC-1CT). Compared to HCT 116 and HCEC-1CT, HT-29 showed a higher production of AGR2, both in the basal state and in response to Tm, leading us to choose this epithelial cell line (Figure 34).

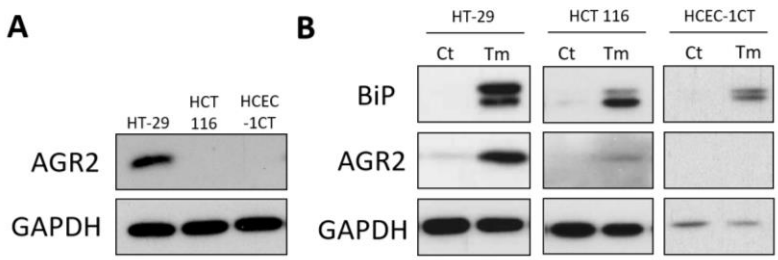



**Figure 34. Basal and Tm-induced expression of AGR2 protein in different epithelial intestinal cells**
[A] Western blot analysis of basal expression of AGR2 protein in different epithelial cell lines (HT-29, HCT 116 and HCEC-1CT). [B] Western blot analysis of AGR2 and BiP proteins in different cell lines (HT-29, HCT 116 and HCEC-1CT), in control condition (DMSO) and after Tm (10 µg/mL) stimulation. The GAPDH was used as loading control.

### 3.4.2. Induction of endoplasmic reticulum stress and anterior gradient protein 2 homolog expression in intestinal epithelial cells

We then assessed, in HT-29, the expression of AGR2, as well as other ER stress markers such as BiP, CHOP and sXBP1 in response to increasing doses and durations of Tm, to determine the optimal dose and timing necessary to achieve a maximal AGR2 intracellular induction. The AGR2 maximal induction (at transcript level) was reached after 18 h of Tm treatment at the dose of 10 µg/mL (Figure 35).

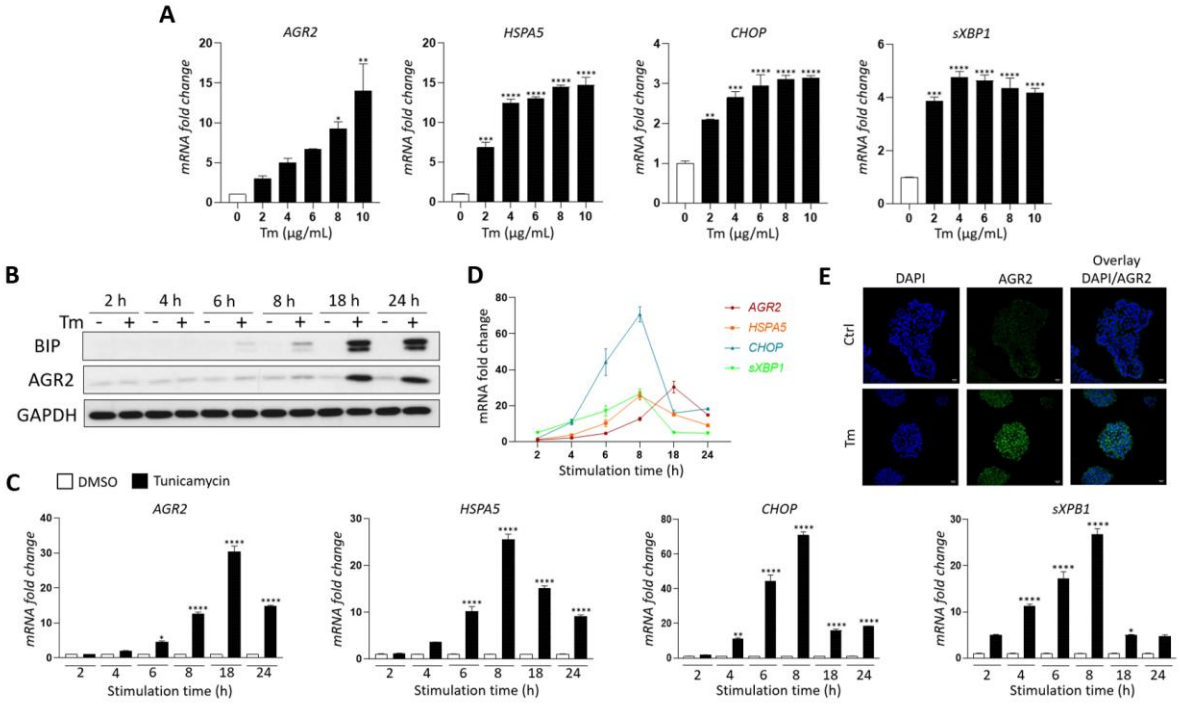



**Figure 35: Increase of ER stress markers in HT-29 in response to Tm stimulation**
[A] RT-qPCR expression fold change of *AGR2*, *HSPA5*, *CHOP* and *sXBP1* in HT-29 stimulated by Tm during 18 h at different doses. [B] Western blot analysis of AGR2 and BiP in HT-29 under Tm (10 µg/mL) stimulation time course. The GAPDH was used as loading control. [C, D] RT-qPCR expression fold change of *AGR2*, *HSPA5*, *CHOP* and *sXBP1* in HT-29 under Tm treatment time course (in black) compared to time-control conditions with DMSO (in white). [E] IF analysis of AGR2 in HT-29 after 18 h of Tm treatment at 10 µg/mL and time-control DMSO. *p<0.05, **p<0.01, ***p<0.001, ****p <0.0001.

### 3.4.3. Sensitivity of CCD-18Co to tunicamycin

Afterwards, as our aim was to assess the role of epithelial ER stress on fibroblast, we checked if CCD-18Co fibroblasts were sensitive to Tm (our ER stress inductor). As shown in Figure 36, ER stress was induced in CCD-18Co when exposed to Tm.

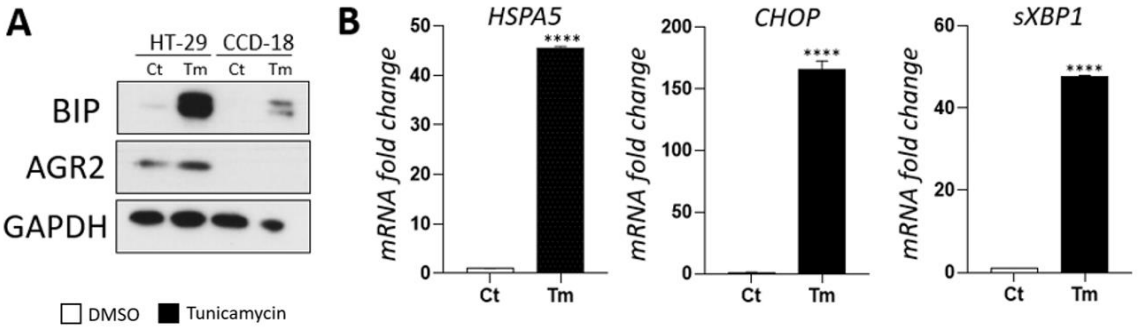



**Figure 36. CCD-18Co sensitivity to Tm**
[A] WB analysis of AGR2 and BiP in HT-29 and CCD-18Co stimulated by Tm during 18 h and compared to time-control with DMSO for 18 h (Ct). AGR2 is expressed in HT-29 but not in CCD-18Co fibroblasts. The increase of BiP in CCD18-Co in response to Tm treatment is nevertheless a piece of evidence of the induction of an ER stress in these cells when in contact with this ER stress inducer. [B] RT-qPCR expression fold change of *HSPA5*, *CHOP* and *sXBP1* in CCD-18Co after ER stress induction.

### 3.4.4. Persistent endoplasmic reticulum stress and protein disulfide isomerases secretion in HT-29 cells after tunicamycin removal

This result led us to assess if Tm-induced ER stress persists in HT-29 after stimulus (Tm) removal. To this end, we studied AGR2, ER stress markers and PDIs expression during recovery of HT-29 cells in fresh media. At the same time, we evaluated the extracellular expression of PDIs (especially AGR2), by collecting the corresponding supernatants, in response to Tm-induced ER stress and after Tm removal. A sustained intracellular production of most of the ER stress markers and the tested PDIs persisted up to 32 h after the medium change and the maximal intracellular increase of AGR2 was reached 24 h after the transient ER-stress (Figure 37). Several PDIs (AGR2, ERP44 and PDIA6) were secreted and found in the supernatant of HT-29, pre-conditioned by Tm, especially 24 h and 32 h after media change (Figure 37).

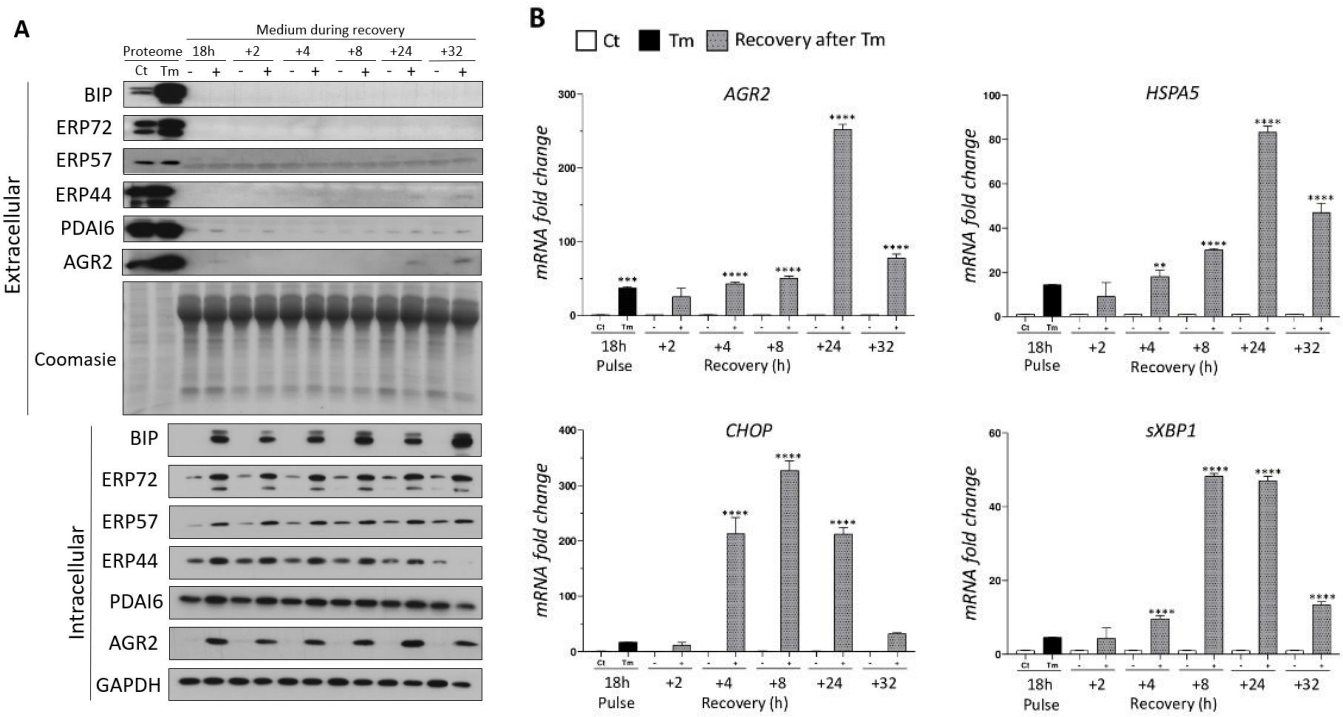



**Figure 37. ER stress markers and PDIs persistence in HT-29 during cell recovery in fresh media after a 18 h transient treatment with Tm**

[A] WB analysis of AGR2, PDIA6, ERP44, ERP57, ERP72 and BiP in the intracellular protein extracts and in the supernatant of HT-29 cells, during cell recovery, after transient ER stress induced by Tm (pulse) or not (DMSO treatment; Ct) and collected 2 to 32 h after the medium change. GAPDH was used as loading control for the intracellular proteins and Coomasie blue coloration of the gel was used for normalization of the extracellular proteins loading quantities. [B] RT-qPCR expression fold change of *AGR2*, *HSPA5*, *CHOP*, *sXBP1* in HT-29 cells treated by 18 h of Tm induction (black) or in time control condition with DMSO (Ct) (white) as well as after 2, 4, 8, 24, 32 h of recovery in fresh media (grey). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

### 3.4.5. CCD-18Co fibroblast to myofibroblast differentiation induced by HT-29 supernatants

Next, as we aimed to assess the role of ER stress in IECs on the differentiation of fibroblast to myofibroblast, we first verified that we were able to induce this previously described myofibroblastic differentiation (to use it as positive control) thanks to TGF-β1 stimulation (known to play a key role in this differentiation) [250]. After stimulation by TGF-β1, we observed an increased expression of α-SMA (which is the marker of fibroblast to myofibroblast differentiation) by WB, IF together with an increased expression of *ACTA2*, collagen proteins (*COL1A1* and *COL3A1*), fibronectin (*FN*) and connective tissue growth factor (*CTGF*) by RT-qPCR (Figure 38).

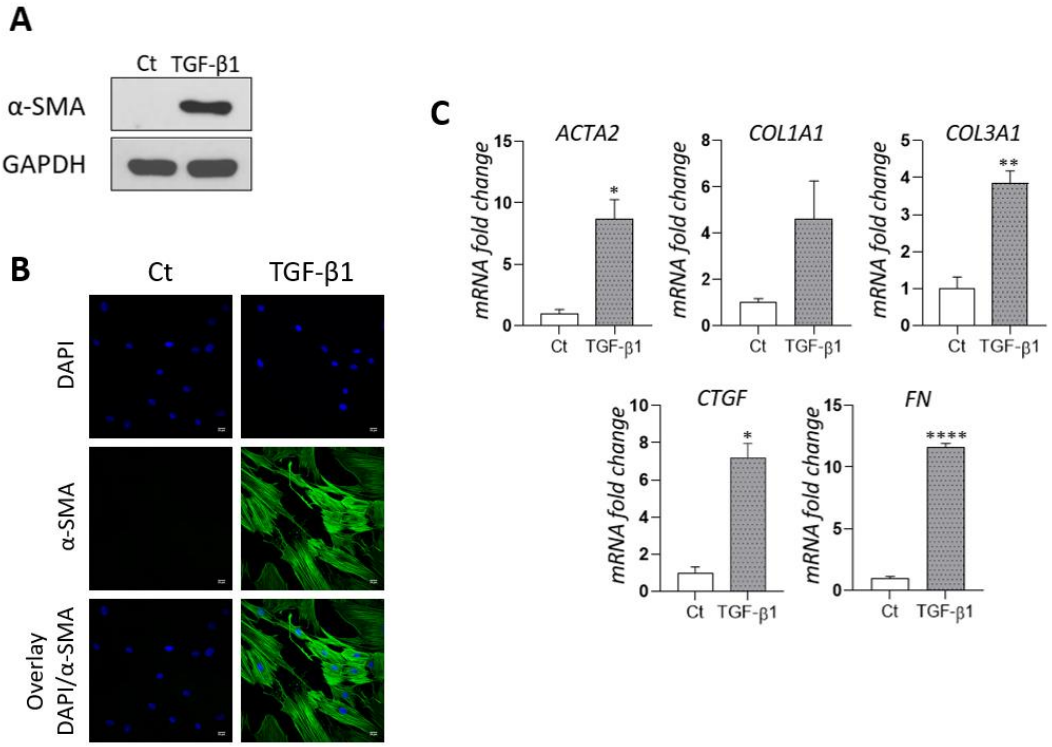



**Figure 38. Fibroblast to myofibroblast differentiation obtained by TGF-β1**
[A,B] WB and IF analysis of α-SMA in CCD-18Co with TGF-β1 treatment as positive control of the myofibroblastic differentiation. [C] RT-qPCR expression fold change of *ACTA2*, *COL1A1*, *COL3A1*, *CTGF* and *FN* in CCD18-Co under TGF-β1 treatment (grey) compared to time control conditions (Ct) (white).

Then, we tested the capacity of supernatant of HT-29 cells, pre-conditioned by tunicamycin or not, to induce a fibroblast to myofibroblast differentiation when applied on the CCD-18Co. Supernatant of HT-29 pre-conditioned by Tm and collected 8, 24 and 32 h after media change induced CCD-18Co differentiation after 48 h of treatment as illustrated by the increased expression of α-SMA obtained by WB and IF (Figure 39) but not by RT-qPCR (data not shown).

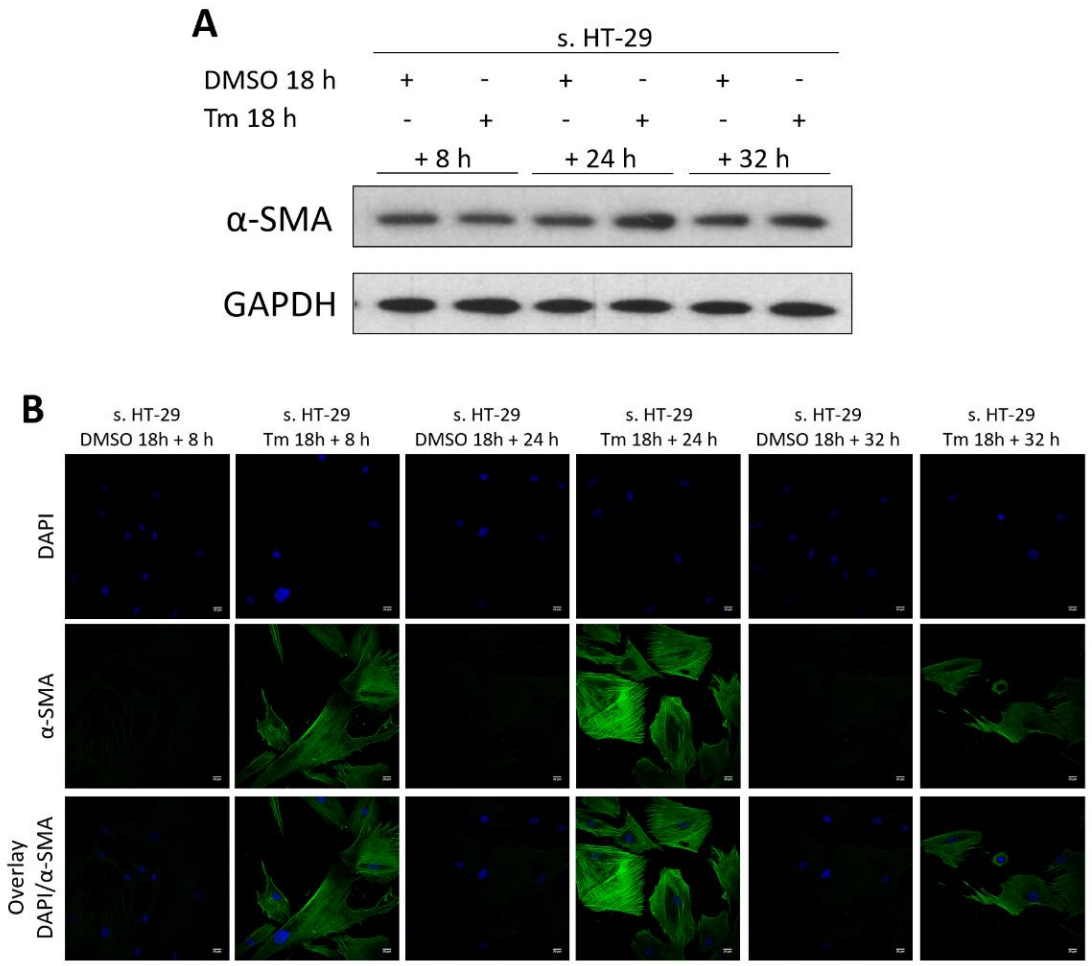



**Figure 39. Fibroblast to myofibroblast differentiation obtained with supernatant of HT-29 cells pre-conditioned by tunicamycin**

[A] WB and IF [B] analysis of α-SMA in CCD-18Co after application of supernatant of HT-29 (s. HT-29) either pre-conditioned by Tm or treated with DMSO as control condition and collected 8, 24 and 32 h after media change.

### 3.4.6. Investigation of the specific pro-fibrotic role of anterior gradient protein 2 homolog

To study the specific pro-fibrotic role of AGR2, we tested the capacity of recombinant AGR2 (rAGR2) to induce this fibroblast to myofibroblast differentiation and observed an increased expression of α-SMA by WB and IF (Figure 40) but not by RT-qPCR (data not shown).
Blocking AGR2 by using an anti-AGR2 antibody in the supernatant of HT-29 pre-conditioned by Tm or in the condition with rAGR2 showed a mitigation of the increased expression of α-SMA in the CCD-18Co cells (Figure 41).

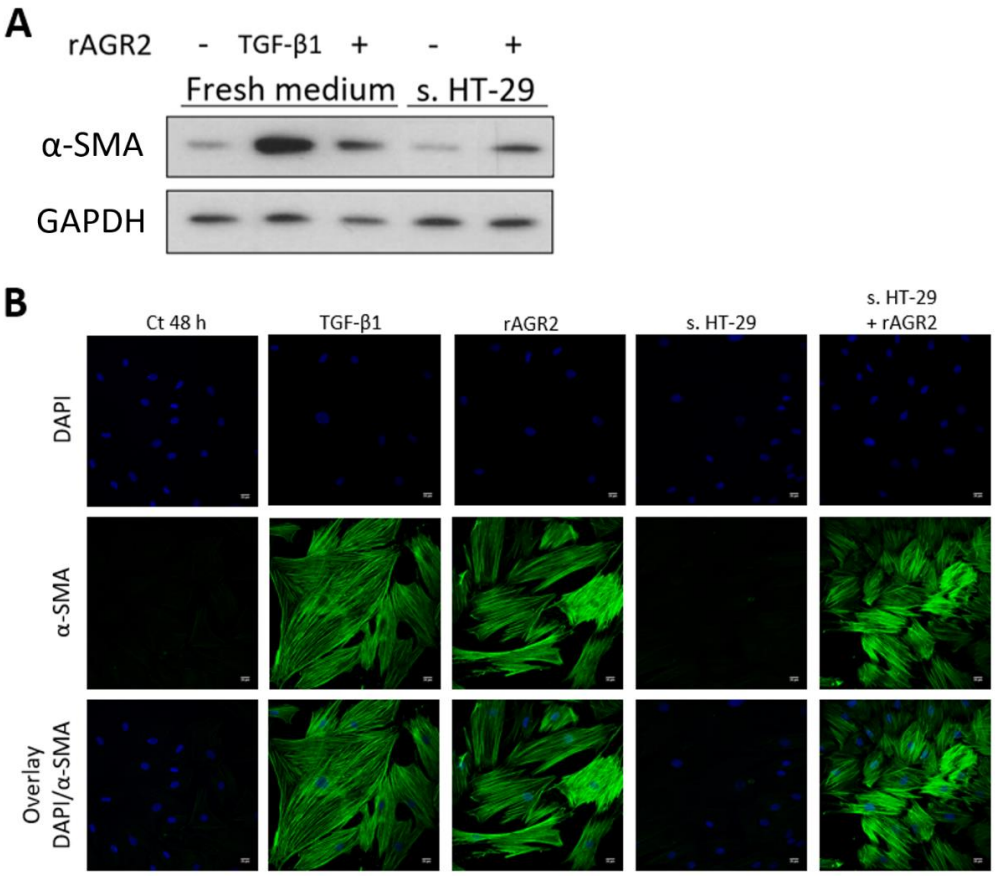



**Figure 40. Fibroblast to myofibroblast differentiation obtained with recombinant AGR2**
[A] WB and [B] IF analysis of α-SMA in CCD-18Co after application of recombinant AGR2 (rAGR2) in fresh media of CCD-18Co as well as in the supernatant of HT-29 cells, collected in control condition, and applied on CCD-18Co.

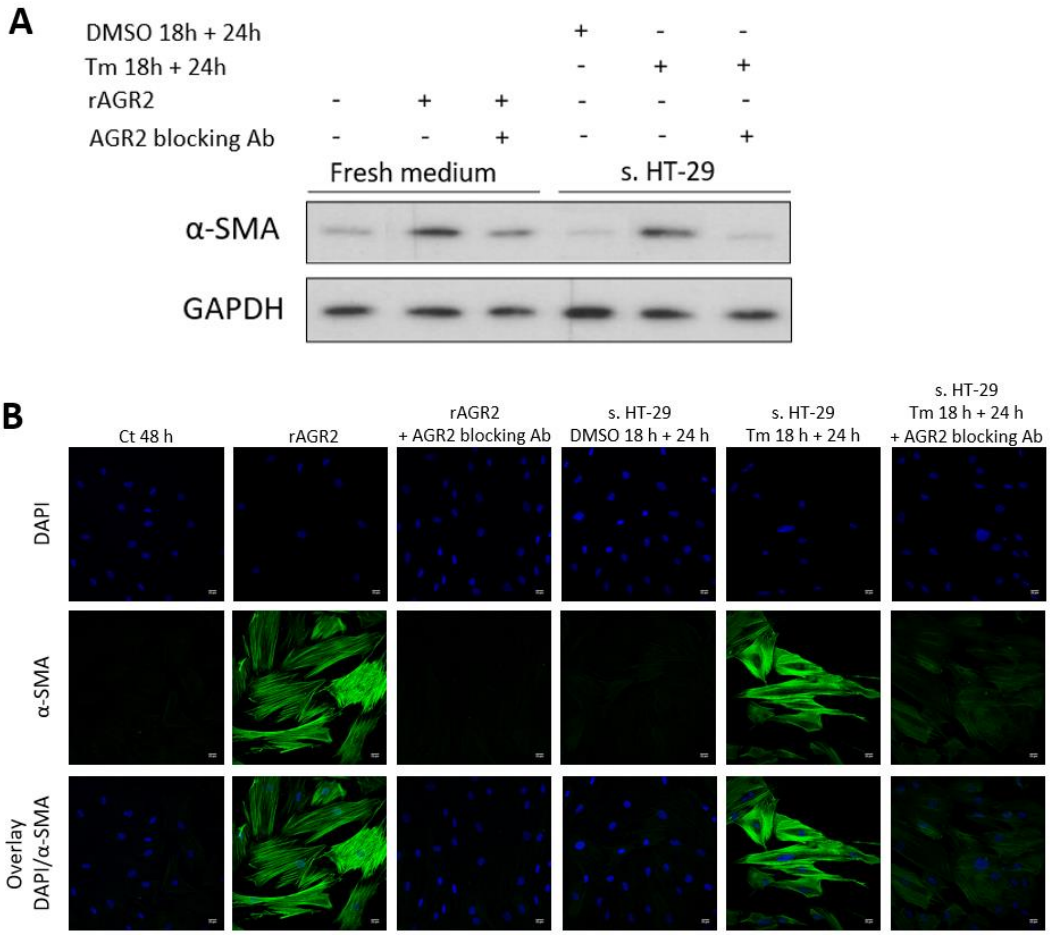



**Figure 41. Attenuation of the fibroblast to myofibroblast differentiation obtained with rAGR2 and HT-29 supernatant pre-conditioned by Tm, when an anti-AGR2 antibody is applied concomitantly**

[A] WB and [B] IF analysis of α-SMA in CCD-18Co after application of AGR2 blocking antibody (Ab) in the condition with rAGR2 or in the supernatant of HT-29 pre-conditioned by Tm.

### 3.4.7. Study of transforming growth factor β1 secretion in response to endoplasmic reticulum stress in intestinal epithelial cells

Then, we wanted to see if these IECs, under an induced ER stress, release TGF-β1, known to play a key role in the myofibroblastic differentiation. Using specific TGF-β1 ELISA, we compared the dosage of TGF-β1 (in its bioactive form and total TGF-β1) present in the supernatant of epithelial cells preconditioned by Tm. The bioactive TGF-β1 was not detected in the supernatant of HT-29 cells preconditioned by ER stress whereas it was in the control (DMSO) condition. Regarding the TGF-β1 total concentration, while myofibroblastic differentiation was mainly obtained with the supernatant collected 24 h after the media change (Figure 41), interestingly, no significant increase in TGF-β1 secretion was observed at this time (Figure 42).

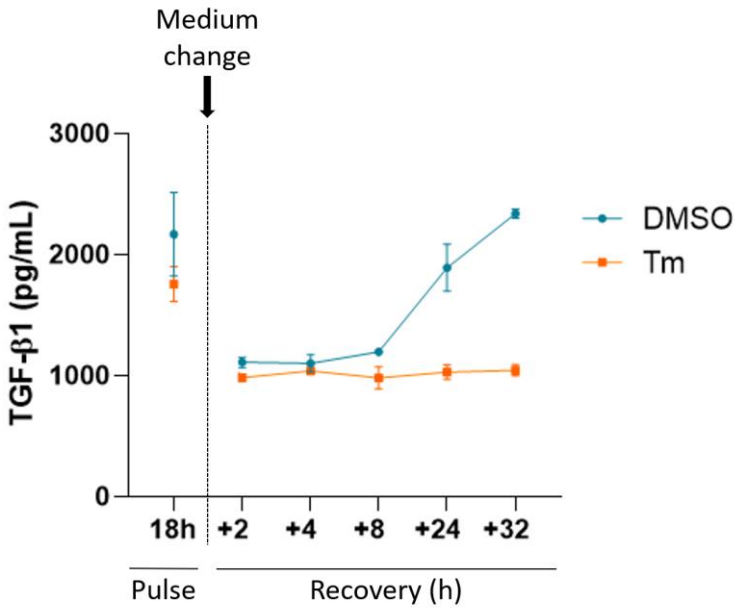



**Figure 42. Quantitative detection of total TGF-β1 (addition of active and latent TGF-β1) levels (in pg/mL) in supernatant of HT-29 cells pre-conditioned (in orange) or not (in blue) by Tm, measured using ELISA kit**

### 3.4.8. Epithelial-to-mesenchymal transition induced by endoplasmic reticulum stress in intestinal epithelial cells

To check whether the presence of an ER stress in IECs could contribute to fibrosis by inducing an EMT in these cells, we looked for modification of epithelial (CDH1) and mesenchymal (VIM, TGF-β1, FSP1, SNAI1) markers in IECs in response to and at distance from induced-ER stress (after medium change). While we did not observe a clear decrease in the epithelial E-cadherin marker, we did observe a significant increase in the mesenchymal markers studied in HT-29 pre-conditioned by Tm, especially 24 and 32 h after the media change (Figure 43).

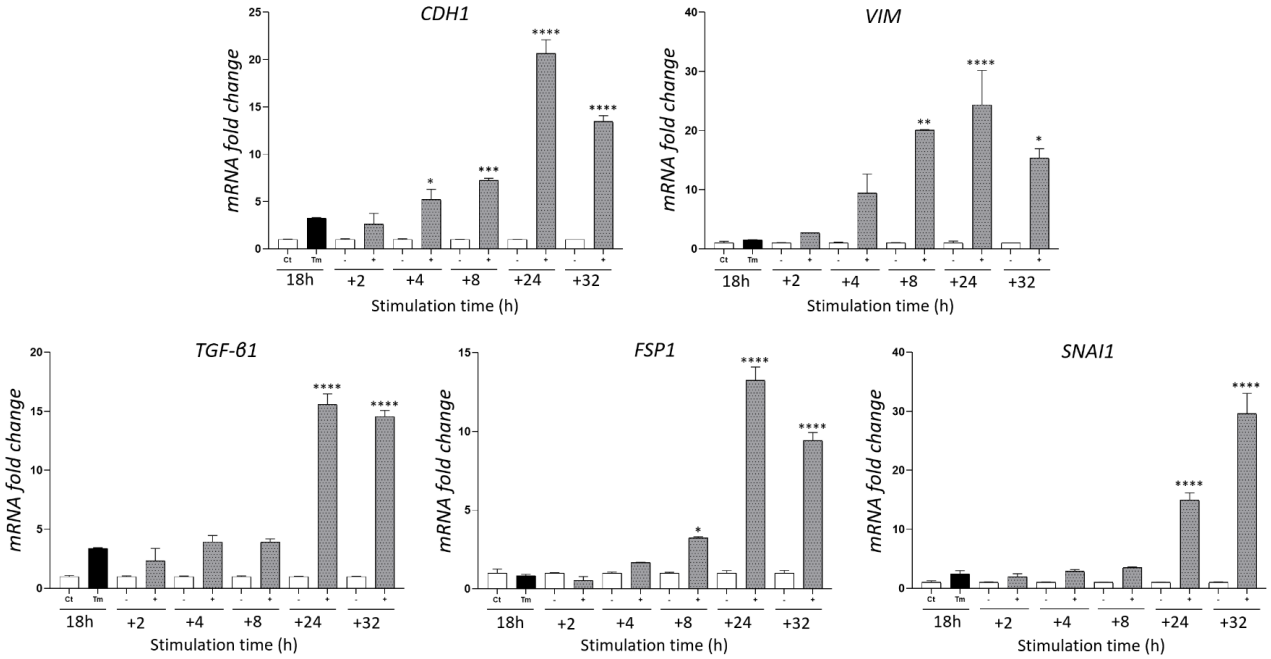



**Figure 43. Variation of EMT markers in HT-29 during cell recovery in fresh media after a 18 h transient treatment with Tm.** *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

### 3.4.9. Impact of transforming growth factor β1 or/and tumor necrosis factor α on epithelial endoplasmic reticulum stress

Finally, we looked at the impact of stimulation by TNF-α, TGF-β or both cytokines combined together, on markers of ER stress to see if these cytokines, usually present in CD, contribute to the induction of this epithelial ER stress. Compared to Tm, treatment with TNF-α and/or TGF-β, sequentially or combined together, were unable to show similar trends of ER stress markers (Figure 44).

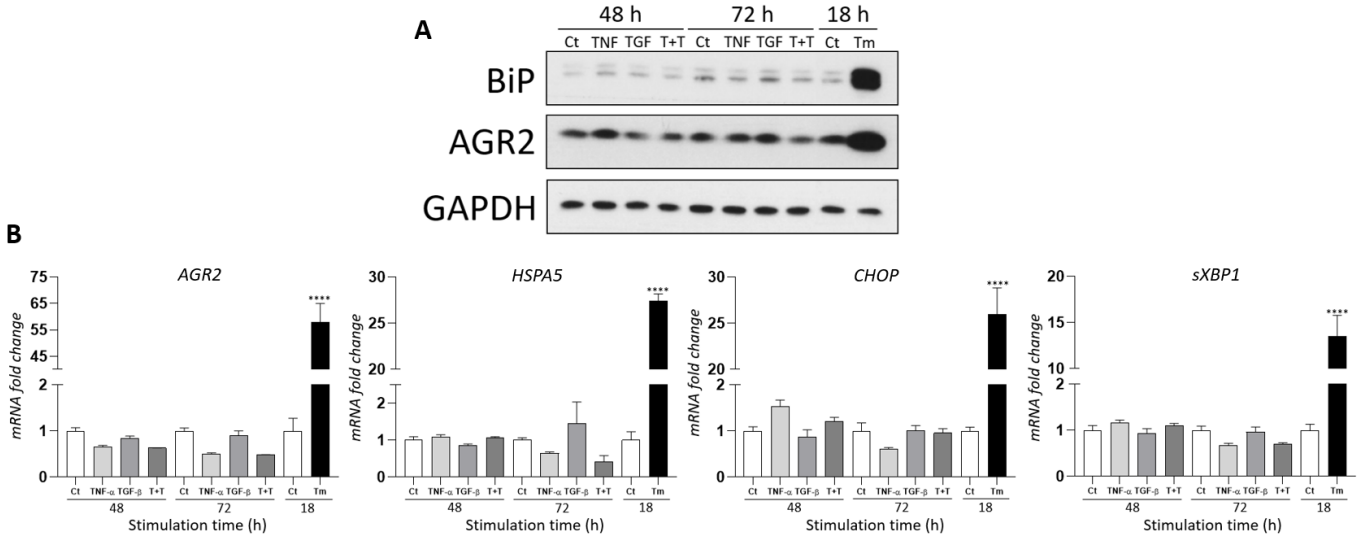



**Figure 44. Variation of ER stress under TNF-α, TGF-β1 and the combined cytokines stimulations on HT-29 compared to Tm treatment**

[A] WB analysis of AGR2 and BiP in HT-29 stimulated by TNF-α, TGF-β1, combined treatment during 48 and 72 h respectively or Tm during 18 h. Culture media or DMSO treatments were used as relevant time-control (Ct). [B] RT-qPCR expression fold change of AGR2, HSPA5, CHOP and sXBP1 in HT-29 stimulated by time-control Culture media or DMSO (Ct), TNF-α, TGF-β1 and both combined cytokines treatment during 48 and 72 h respectively or Tm during 18 h. *p<0.05, **p<0.01, ***p<0.001, ****p <0.0001

### 3.4.10. Alleviation of endoplasmic reticulum stress and protein disulfide isomerases secretion using tauroursodeoxycholic acid

Then, we evaluated the impact of concomitant treatment by TUDCA on Tm-induced ER stress, PDIs expression and their relative secretion. We assessed, in HT-29, the expression of AGR2, BiP and other PDIs in response to increasing doses of TUDCA, to determine the optimal dose to alleviate ER stress. As with 25 µmol, AGR2 expression was significantly decreased (Figure 45A), we looked at the impact of this dose of TUDCA on the secretion of PDIs 24 h after the medium change. Compared to the Tm-stimulated condition, AGR2 secretion, but also the PDIA6, were lower in the condition with Tm and TUDCA co-treatment (Figure 45B).

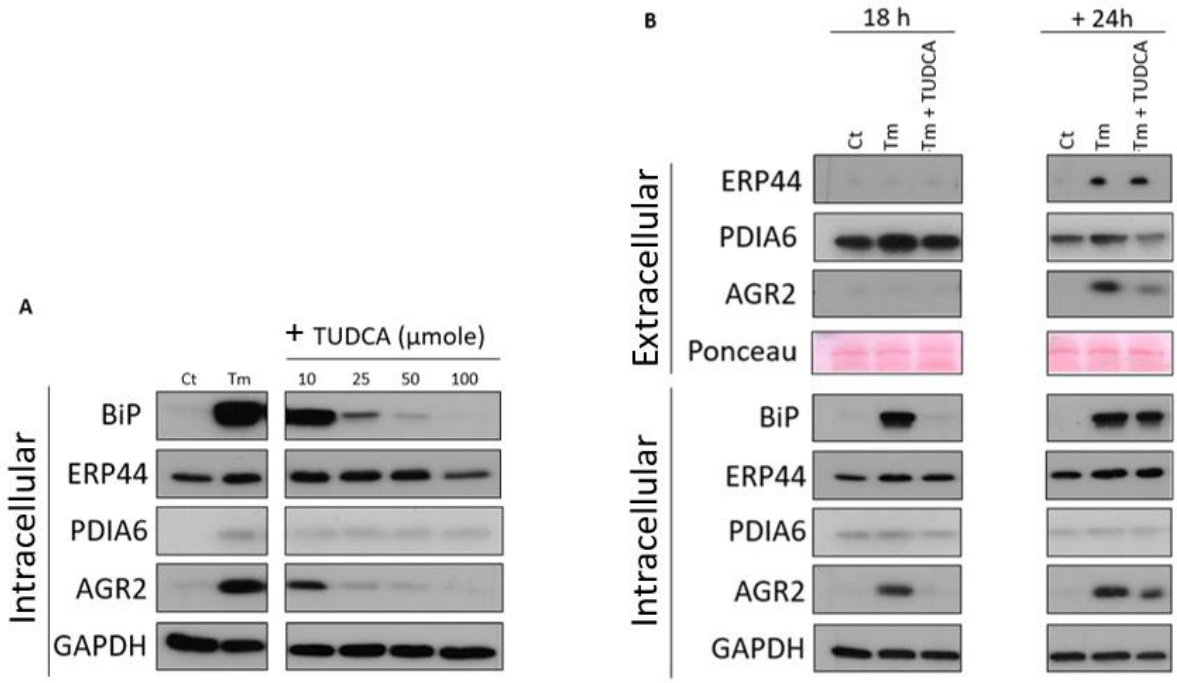



**Figure 45. Alleviation of ER stress and relative PDIs by TUDCA**
[A] WB analysis of AGR2, BiP, PDIA6 and ERP44 in HT-29 in control condition (Ct), stimulated by Tm, and with a concomitant co-treatment by Tm and increasing doses of TUDCA during 18 h. GAPDH was used as loading control. [B] WB analysis of the same proteins in intracellular protein extracts and in the supernatant of HT 29 cells, during a transient ER stress (18 h of Tm treatment) and after cell recovery (24h after Tm removal), with and without concomitant TUDCA treatment (25 µmol). GAPDH was used as loading control for the intracellular fraction of proteins and Ponceau red coloration gel was used for normalization of the extracellular fraction of proteins loading quantities.

## 3.5. DISCUSSION

### 3.5.1. Induction of an endoplasmic reticulum stress in the intestinal epithelial cell in response to tunicamycin treatment

We demonstrated by WB and RT-qPCR that in response to Tm stimulation there was an intracellular increase of AGR2, in HT-29 intestinal epithelial cells, as well as other ER stress markers such as BIP, encoded by the *HSPA5* gene, *CHOP* and *sXBP1*. These ER stress/UPR markers were chosen because they are classically used to study ER stress and UPR in the literature[401–403] and allow the study of all 3 UPR arms[99,101,102,106–108]. Indeed, BiP and CHOP depend essentially on the ER transmembrane sensors PERK and ATF6, whereas sXBP1 depends rather on IRE1α, as does AGR2[404]. sXBP1 may be promoted by ATF6 in a second step[108]. In response to induced ER stress, the *AGR2* peak expression (18h) appeared after those of the other studied ER stress markers[108]. *BiP* and *CHOP*, which depend on the PERK arm, which blocks mRNA translation, is the first initiated. sXBP1 is also upstream of AGR2, explaining why AGR2 is the last marker to appear[108], similarly to what is found in another study[405]. Compared to *BiP*, *sXBP1* and *CHOP*, the peak expression of AGR2 was obtained with higher doses of Tm. This is probably related to the fact that *BiP*, *CHOP* and *sXBP1* depend on several arms of the UPR, activated earlier, compared to AGR2 and therefore have an expression that can be amplified by the different UPR arms[108]. The dose of Tm that we used to induce ER stress was a high dose but similar to the one used in other models of ER stress induction in IECs[406–409]. We worked with this dose since our aim was mainly to study the role of the AGR2 protein and to see if we were able to induce an AGR2 secretion, the reason why we worked with an induction that was intended to be maximum (but with a stable housekeeping gene).

### 3.5.2. Pro-fibrotic paracrine role of extracellular anterior gradient protein 2 homolog

The myofibroblastic differentiation obtained with rAGR2 and its attenuation, by blocking AGR2 with anti-AGR2 antibody, suggest a specific paracrine pro-fibrotic role of the secreted AGR2. To the best of our knowledge, the interactions between eAGR2 and fibroblasts have never been studied in fibrosis, but are known to accelerate the cutaneous wound-healing process, where eAGR2 promotes the fibroblast recruitment and migration near the wounded area[410,411], and has been the subject of some publications in oncology[412–416]. Indeed, solid tumour malignant cells surround themselves by stromal cells such as fibroblasts, which they transform into cancer-associated fibroblasts (CAFs) to form a tumour microenvironment or TME (which results from complex interactions between tumour cells and the ECM) suitable

for its growth[417,418]. These malignant cells attract these stromal cells by secreting a range of growth factors, including fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF)[419], but also AGR2, which plays a key role in these TME by recruiting these fibroblasts surrounding tumour cells[415]. Tsuji et al. were the first to demonstrate, in 2015, that in gastric signet-ring cell carcinoma (SRCC), eAGR2 secreted by SRCC cells was incorporated by the surrounding gastric stomal normal fibroblasts, which are then activated and, in turn, coordinate the invasion of other normal fibroblasts promoting fibroblast-associated cancer invasion[412]. We could speculate that a very similar process takes place in fibrosis and more recent data indeed seem to support this hypothesis. The effects of AGR2 on fibroblast migration and organization are AGR2 concentration-gradient dependent and it was shown that fibroblasts only started migration when they received an optimal threshold concentration of AGR2[410,413,415], which seems to be effective only around the AGR2-secreting cells. This fibroblast chemotaxis by eAGR2 seems to be mediated by FGFR and VEGFR[413,415]. In addition to promoting the fibroblasts migration and elongation, eAGR2 also promote their proliferation (by stimulating RhoA expression which upregulate G1-S phase-regulatory protein cyclin D1)[415]. Finally, very recently, but after the publication of our manuscript, Merugu et al. found similar results to ours, showing that the presence of eAGR2 (secreted by lung cancer cell line) increases the α-SMA expression in neighbouring fibroblasts confirming their activation[416]. They report that this eAGR2 gets internalized into fibroblast cytoplasm by endocytosis, then binds to β-catenin and further co-translocates to the nucleus thereafter. Once in the fibroblast nucleus, this eAGR2 increase the expression, the stability, and the accumulation of β-catenin[416], which, interestingly, is known to promote pro-fibrotic genes[414]. However, to the best of our knowledge, our data are the first to suggest that eAGR2 could be a paracrine inducer of intestinal fibroblast to myofibroblast differentiation.

Beside its paracrine role on fibroblasts, eAGR2 can also contribute to endo-MT, by promoting the arrival of vascular endothelial cells through its action on VEGFR[415], as well as contribute to EMT (a point which will be discussed separately below)[352,420], both known to be implicated in intestinal fibrosis pathophysiology[145,203].

In addition to its action on cells potentially involved in fibrosis (fibroblasts, endothelial and adjacent epithelial cells), eAGR2 can also interact with the ECM protein[352,420], such as dystroglycan[421] (which binds to laminin, agrin and perlecan of the extracellular matrix[422]), or the protein C4.4A (LYPD3)[421,423]. This eAGR2 is also known to interact with core components

of the mTORC2 complex (RICTOR, RPS6, and P70S6K) and eAGR2 promotes phosphorylation of RICTOR, known to be involved in cell actin cytoskeleton organization in fibrosis[160].

Together, these data suggest that, beside its role in the fibroblasts to ECM-secreting myofibroblasts differentiation studied here, eAGR2 could contribute to intestinal fibrosis by a range of other mechanisms that deserve to be studied.

### 3.5.3. Other potential pro-fibrotic factors released by the epithelial cells under endoplasmic reticulum stress

As the supernatant of HT-29 cells, after transient ER stress, was able to induce a myofibroblastic differentiation, and as the addition of an anti-AGR2 to this supernatant does not completely reverse the induced myofibroblastic differentiation, the hypothesis that we support is that IECs under ER stress, release, in addition to eAGR2, a whole set of pro-fibrotic factors into their environment which could also exercise a paracrine pro-fibrotic action. To this end, one of the first things we wanted to test was whether these cells, under ER stress, release TGF-β1, the canonical pro-fibrotic factor. Indeed, TGF-β1 is known to be secreted by IECs[424] (in particular by HT-29 cells, and more abundantly when they are the target of pro-inflammatory factors)[425], and its intracellular expression is known to be promoted by the induction of ER stress in the cell[332]. The way by which ER stress induces TGF-β1 expression and activation has recently been demonstrated in subepithelial myofibroblasts of fibrostenotic CD patients[332] and we could speculate that a similar mechanism occurs in epithelial cells. The interaction between BiP and latent TGF-β1 increases its activation and XBP1 and ATF-6α (two ER stress sensors) act as transcription factors promoting *TGF-β1* gene expression. While our results confirmed that the induction of ER stress in IECs promotes the intracellular *TGF-β1* expression, the amount of TGF-β1, quantified by ELISA, in the supernatant of these ER stressed epithelial cells was lower than in the control condition. In contrast to what we observed, Carlisle RE et al. demonstrated that induction of an ER stress in human renal proximal tubule epithelial cells resulted in secretion of TGF-β1[426]. This secretion was obtained with thapsigargin (another ER stress inducer) but not with Tm. The authors underlined that the response to induced ER stress could depend on the inducer used, which could explain why no TGF-β1 secretion was observed in our model of Tm-induced ER stress[426]. Tm is known to induce ER stress in cells by inhibiting N-glycosylations. However, the TGF-β1 N-glycosylation is required for its

secretion[427–431]. It has indeed been shown that the inhibition of N-glycosylation by a treatment by Tm (in Chinese hamster ovary cells) or that mutation at one of the 3 N-glycosylation sites of β1-LAP (in human embryonic kidney cells) blocked the TGF-β1 secretion[427–431]. The secretion of TGF-β1 in response to induced ER stress deserves to be studied with other ER stress inducers. Therefore, in our model, the pro-fibrotic role of the HT-29 supernatant preconditioned by Tm treatment could be mediated by other paracrine factors.

Interestingly, some evidence suggests that other PDIs could be part of this set of pro-fibrotic factors[351]. In renal fibrosis indeed, in parallel with what we demonstrated in our proteomic and IHC studies for AGR2, a clear correlation has been demonstrated between ERP57 tissular expression level (another well-characterized member of the PDIs family) and the renal fibrosis degree in animal models and in patients' biopsies[351]. This ERP57 (also known as PDIA3) has also been shown to be secreted into the extracellular medium upon cytokine treatment and to exhibit a strong interaction with ECM proteins, especially fibronectin and collagen (COL1A1). ERP57 was supposed to prevent disulfide bond reductions and misfolding of these proteins, participating in ECM synthesis, accumulation, and stabilization, and thus promoting the renal fibrosis progression. Finally, already in the early stages of chronic kidney disease, Dihazi et al. demonstrated that ERP57 was excreted in the urine of patients, and its level correlated with the degree of renal fibrosis, suggesting that this PDI could be an early urinary marker for diagnosis of renal fibrosis, before the onset of visible kidney damage[351]. If ERP57 was not found to be significantly excreted in the supernatant of IEC preconditioned by ER stress (Figure 37), we highlighted, beside eAGR2, 2 other secreted PDIs, ERP44 and PDIA6, which were also significantly increased in fibrotic tissues in our proteomic study. The extracellular form of these proteins was, to our knowledge, not previously described in the literature and their role in intestinal fibrosis need to be further investigated.

### 3.5.4. Links between endoplasmic reticulum stress and epithelial-to-mesenchymal transition in intestinal epithelial cells

In addition to the release of pro-fibrotic factors into the environment, we wanted to see if epithelial ER-stress contributes to fibrosis by transforming the IECs into mesenchymal cells through the EMT process. The induction of EMT in epithelial cells in response to ER stress has been shown to play a key role in the development of fibrosis in several organs (and, for example, this has been demonstrated in alveolar epithelial cells in lung fibrosis[432,433], in renal tubular

epithelial cells in tubulointerstitial fibrosis[434,435], in mesothelial peritoneal cells in peritoneal fibrosis[436], as well as in human lens epithelial cells in cataract[437], …). Although evidence from the literature suggest that ER stress-induced EMT is neither cancer cells nor particular epithelial cell-type specific[438], this role of ER stress on EMT has been poorly studied in IECs to our knowledge. Shen et al. are the only ones to have demonstrated that gastric cancer cells can develop EMT in response to UPR in conditions of severe hypoxia[439]. Under reserve to what is observed for the E-cadherin (an epithelial marker thought to be decreased during EMT, which will be discussed later), we observed a transcript increase of 4 mesenchymal markers (*VIM*, *TGF-β1*, *FSP1* and *SNAI1*) 24 hours after a transient ER stress in the IEC, suggesting, indeed, a transformation towards the mesenchymal phenotype.

The transformation of the epithelial into mesenchymal cell in response to misfolded proteins appears to be initiated regardless of the underlying ER stress cause[438], is a sustained effect (cells may maintain partial mesenchymal cell characteristics after withdrawal of the ER stress inducer[438]) and is sometimes considered as an adaptative response that serves to protect cells against ER stress–induced apoptosis[433]. Several authors suggest that when the epithelial cell is subjected to ER stress, it has two possible cell fates: adaptation (by acquiring mesenchymal characteristics) or apoptosis (by activating a cell death program)[433,436]. The way it evolves towards one or the other fate depends on the induced-ER duration and intensity[433,436]. In mesothelial cells, it has been indeed shown that ER stress leads to EMT or apoptosis in a time and intensity dependent way[436]. While mild ER stress might induce a reversible EMT (by a transient CHOP expression), the prolonged ER stress (48 h) might induce apoptosis (by a persistent upregulation of CHOP)[436]. In the gut, where a series of dietary and microbial agents can induce an ER stress, transient or prolonged, the epithelial cell can therefore evolve towards either EMT or apoptosis, but whether it evolves towards one or the other, both contributing to fibrosis[330].

The induction of this mesenchymal transformation in response to ER stress can be mediated by the increased TGF-β signalling in epithelial cells (as we observed in our epithelial cell line in response to ER stress), known to be the more powerful EMT driver[440–442], but not the only one. Interestingly, the AGR2 protein, mainly in its extracellular form[352,443], can also contribute to this epithelial to mesenchymal transformation and represents another pathway by which this eAGR2 could contribute to intestinal fibrosis. Fessart et al. as well as Li et al. demonstrated, in lung and breast cancer, respectively, that eAGR2 could lead to a disruption of the apico-basal polarity of epithelial cells but also of the cell-cell adhesion and promotes transformation of

these epithelial cells into a fibroblast-like phenotype[352,443] (which are EMT features)[444]. In the Fessart et al. study, the addition of eAGR2 to human bronchial epithelial cell organoids significantly modified EMT transcripts (such as *VIM*, *ZEB1*, *MMP3*, *MMP9*, *MAP1B* and *Wnt5b*) in epithelial cells[352]. In malignancies, this mesenchymal transformation confers a more invasive phenotype of these cancer cells and is notably involved in the metastasis formation process. The mechanism proposed by Li et al. to explain this eAGR2-induced EMT is that eAGR2 enhances insulin like-growth factor 1 (IGF-1) signalling, known to induce EMT[443] (IGF-1 which, intriguingly, is also a factor involved in the fibroblasts to myofibroblasts differentiation[445]).

While AGR2 seems to have a pro-EMT role in its extracellular form, this protein appears rather to be associated with the preservation of the epithelial phenotype in its intracellular form[446–450]. Several studies have shown that iAGR2 expression was positively correlated with the expression of epithelial markers such as E-cadherin and was associated to the epithelial phenotype of the cell[446,447]. In contrast, the decrease in iAGR2 expression was linked to the presence of classical mesenchymal features including the loss of E-cadherin, the induction of N-cadherin, as well as the acquisition of a mesenchymal-like phenotype[446,447]. The presence of interactions between iAGR2 and the EMT process was confirmed in *in vitro* models[446,447]. On one hand, treatment of epithelial cells by a powerful EMT inducer (TGF-β), resulted in a decrease in iAGR2 expression (through the canonical TGF-β/Smad signalling pathway (SMAD4)[451] and a Smad-independent pathway[447]) and the neutralisation of this TGF-β signalling allowed to restore this iAGR2 expression[447]. On the other hand, iAGR2 was able to prevent the occurrence of an EMT by inhibiting ZEB1 and SNAI2 (2 EMT-inducing transcription factors) and by inhibiting p38 MAPK signalling, which is frequently involved in the triggering of EMT[447–450]. To sum up, these data underscore that the role of AGR2 in EMT depends on its localization, but the mechanism regulating the intracellular and extracellular AGR2 forms and their impact on the epithelial phenotype deserves to be further investigated[447]. The opposite actions on EMT of a same protein, depending on its intracellular or extracellular localisation, has already been reported and described for other proteins, such as HSP70 (or heat shock protein 70, another chaperone protein) or calcium-binding proteins S100A8 and S100A9[447,452–454]. In our model, we observed, in parallel, 24h after Tm removal (and thus, induced ER stress), an intracellular increase of AGR2 (presumed to preserve the epithelial phenotype) and eAGR2 (presumed to induce EMT). It would be surprising, but not impossible, if the cell generated intracellular AGR2 at the same time, responsible for maintaining the

epithelial phenotype and extracellular AGR2 promoting mesenchymal transformation. We could speculate, but it would need to be studied, that the intracellular AGR2 form showing a role in the preservation of the epithelial phenotype would be the dimeric form. Whereas the intracellular peak found in our model 24 hours after ER stress (but concomitant with the increase in other ER stress factors), could correspond to a monomeric form, such as the secreted one[374], whose role on the epithelial barrier deserves to be studied.

A point worth discussing is the persistence of the epithelial marker E-cadherin, which we would expect to see decrease during EMT process. Other authors studying EMT in IBD have already reported the persistence of this epithelial marker, especially in intestinal fibrosis[321,323]. Indeed, Flier et al. reported E-cadherin+ αSMA+ double positive cells in biopsies from IBD patients (in CD and UC)[321] and Scharl et al. found similar findings in CD fibrotic tissues with EMT features[323]. While they offer no explanation for these findings, several explanations seem possible in the context of our cellular model to explain the persistence of this epithelial marker in parallel with the induction of mesenchymal markers. Firstly, it is possible that these epithelial cells, in response to ER stress, put themselves in a state of partial EMT, also known as "transitional state", where the 2 types of markers co-exist[450]. The existence of this hybrid epithelial/mesenchymal phenotype has already been described in many developmental, wound healing, and cancer processes[455–457]. Then, as EMT is a reversible phenomenon, which can be followed by mesenchymal to epithelial transition (MET), the increase in these 2 types of markers could also reflect the plasticity of these cells to induce or reverse the EMT process[444]. It is also possible that there are more specific interactions between E-cadherin and AGR2 in CD[446,447]. Finally, this may also be related to the fact that we worked on a cancerous epithelial cells line, in which, this concomitant increase in both epithelial and mesenchymal markers has already been demonstrated and sometimes described as an "epithelial paradox" (such as breast cancer cells) and is associated with the more aggressive phenotype of the cell[455–458].

### 3.5.5 Links between intestinal epithelial endoplasmic reticulum stress and mucosal cytokines and inflammatory mediators

While it is widely accepted that ER stress triggers the pro-inflammatory cascade[98], the role of pro-inflammatory and other mucosal cytokines on ER stress, and the way these modulate it, has been poorly studied[459]. We tested this hypothesis in our work by stimulating IECs by a pro-inflammatory cytokine (TNF-α), by a pro-fibrotic cytokine (TGF-β), as well as a by

combination of the 2 cytokines, to see if they were able to induce an ER stress. Our results showed that stimulation of HT-29 cells by TNF-α, TGF-β or both cytokines do not induce a significant increase of the ER stress markers, compared to Tm treatment.

The lack of ER stress in response to TNF-α treatment in our model is probably related to the doses and timing of stimulation tested and deserves to be further studied. It has already been demonstrated, in IECs, that TNF-α is able to induce an ER stress, but with generally shorter stimulation times (1-2h)[460]. TNF-α promoted ER stress in particular by inducing a BiP relocation from the ER lumen into the cytoplasmic compartment, thus limiting the amount of chaperone proteins in the ER. On the other hand, TNF-α selectively blocked the recruitment of the ER transmembrane ATF-6 protein (an UPR-arm)[460]. The induction of an ER stress in response to TNF-α treatment has also been confirmed in other cell types than IECs but generally at higher doses than those we used[461–463]. Furthermore, the cocktail of TNF-α and IFN-γ cytokines is frequently used on IECs to induce ER stress[461,464]. Finally, IL-6 is another pro-inflammatory cytokine that is an important regulator of ER stress-UPR. It is known to induce ER stress as well as apoptosis in IECs (by promoting expression of CHOP, GADD34, caspase-12) in a similar way that other pro-inflammatory cytokines[464–466]. Regarding the TGF-β pro-fibrotic cytokine, it is known to induce ER stress in fibroblasts[467] and podocytes[468] but its role on intestinal epithelial ER stress has been poorly studied to our knowledge, this cytokine being rather used as an EMT inducer as we mentioned.

### 3.5.6. Modulation of intestinal epithelial endoplasmic reticulum stress by a luminal agent, the tauroursodeoxycholic acid

In addition to these cytokines, ER stress in IECs can also be modulated by luminal agents. Among those potential luminal modulating agents are bile acids, whose main role is to emulsify large fat droplets into micro-droplets to facilitate their intestinal absorption. These molecules, derived from cholesterol, are synthetised by the liver as primary bile acids, including cholic acid (CA) and chenodeoxycholic acid (CDCA). These are then conjugated with glycine and taurine in the liver, to form glyco-conjugated (glycocholic acid or GCA and glycochenodeoxycholic acid or GCDCA) and tauro-conjugated forms (taurocholic acid or TCA and taurochenodeoxycholic acid or TDCA), which, after storage in the gallbladder, are discharged into the intestine via the bile. Once in the intestinal lumen, these bile acids undergo a series of modifications by luminal bacteria (including deconjugation, dehydroxylation,

oxidation, and epimerization[469]) to become so-called secondary bile acids, including ursodesoxycholic acid or UDCA. If a small proportion (<5%) of this intestinal bile acids pool is excreted in the faeces, most of them are reabsorbed in the terminal intestine and returns to the liver via the portal system (a process known as the enterohepatic cycle)[470]. In the liver, they can be re-conjugated with glycine and taurine, to become, in this latter case, the tauroursodeoxycholic acid or TUDCA. The reabsorption of these bile acids in the terminal intestine occurs either easily by crossing the membrane for hydrophobic bile acids (such as UDCA) or via a sodium-dependent bile acid transporter for hydrophilic bile acids (such as TUDCA)[471]. Bile acids not reabsorbed in the terminal intestine can be deconjugated by colonic bacterial flora, can be passively absorbed by colonocytes and can also cross ER membranes[470].

This endogenous bile acid, only found in trace amounts in the human bile but naturally present at the intestinal epithelium contact[471], has been more widely studied and is known to alleviate experimental intestinal inflammation. This compound acts by (1) down-regulating inflammatory cytokines (such as TNF-α, IFN-γ, IL-1β, MPO, CXCL2)[472–474]; (2) having anti-apoptotic effect[473,475], preventing activation of the caspase-3 key proapoptotic factor[473]; (3) reducing colitogenic dysbiosis[476] (through the normalization of the colitis-associated increased ratio of *Firmicutes* to *Bacteroidetes* as well as the prevention of the loss of species known to be decreased in IBD including *Clostridium cluster XIVa* and *Akkermansia muciniphila*[477,478]); (4) improving the intestinal barrier function (by increasing the levels of tight junction molecules[479] and the mucin production[473]) and (5) alleviating ER stress in IECs[471,472].

If it has been shown that this secondary bile acid could inhibit the genes expression of the ER stress as well as of the 3 arms of UPR[471,472], it is mainly through its chaperone properties that TUDCA promotes ER homeostasis, it masked parts of denatured proteins, as well as underglycosylated proteins and thereby prohibits their aggregation[471,480]. TUDCA is composed of taurine and UDCA. If taurine (also known for its chaperone properties[481]) fails to inhibit the Tm-induced ER stress, both UDCA and TUDCA are able to inhibit it[471], but at concentrations ten times lower for the UDCA, which is substantially more lipophilic and can thereby seamlessly go through biological membranes (and does not require a transporter, unlike the TUDCA)[471]. However, although UDCA appears to be more potent in inhibiting ER stress, Van den Bossche et al. showed that both UDCA and TUDCA decrease the severity of DSS-induced colitis with similar effectiveness[476]. Interestingly, pro-inflammatory mediators (partially produced in response to ER stress[98,115,116,128,129]) negatively regulate the expression of the

sodium-dependent bile acid transporter[482,483], reducing the intracellular uptake of these secondary bile salts[470] (whose concentration is already known to be decreased in the stools of IBD patients due to their dysbiosis[484]), and prevent them to exercise their immunomodulatory functions[485]. Proinflammatory cytokines therefore induce a vicious cycle leading to the perpetuation of the intestinal inflammation[485].

While the role of TUDCA in intestinal inflammation is well known[472–476,479], its potential role in intestinal fibrosis is much less so. Only Cao et al. have demonstrated (using a trichrome staining), on their DSS-induced colitis mice model, that fibrosis in the large intestine was significantly reduced by the feeding of TUDCA, which alleviated ER stress as well as apoptosis in colonic epithelium[472]. The potential anti-fibrotic effect of this chemical chaperone has been better studied in fibrosis of other organs, such as heart[486,487], kidney[488,489], liver[490–492] and lung[493–495]. In this latter, Tong et al.[495] showed that a TUDCA pre-treatment could prevent bleomycin-induced lung fibrosis, by countering ER stress its evoke, allowing to inhibit pulmonary TGF-β/Smad2/3-mediated EMT of alveolar epithelial cells and alleviates collagen deposition as well as alveolar septal destruction, a mechanism which could be extrapolated to intestinal fibrosis. Our results showed that a treatment with TUDCA (administered concomitantly to an ER stress inducer) attenuates the induced ER stress (and hypothetically the associated EMT) as well as the secretion of extracellular AGR2 (which we have shown to have a potential pro-fibrotic role) and may, therefore, have an impact on IBD-associated intestinal fibrosis.

The chemical chaperone TUDCA is a Food and Drug Administration-approved drug for the treatment of primary biliary cirrhosis[496] and is in clinical trial for the treatment of a number of conditions associated with protein misfolding in the ER, including ulcerative colitis[497], cystic fibrosis[498], progressive multiple sclerosis[499], amyotrophic lateral sclerosis[500], type 2 diabetes[501], Alzheimer Disease[502] as well as senile and transthyretin cardiac amyloidosis[503] (https://clinicaltrials.gov/ct2/results/details?cond=TUDCA)[472]. The above-mentioned beneficial effect of TUDCA and UDCA on experimental intestinal inflammation[472–476,479], its preventive effects on IBD-related colorectal carcinogenesis[504–506] together with some emerging data on improvement of intestinal fibrosis[472], suggest that these chemical chaperones could be a safe, readily available and potential oral delivery adjuvant treatment option for IBD[472].

### 3.6. LIMITATIONS

Our work presents several limitations. First of all, our experiments were performed on a cancerous cells line, the HT-29 cells, derived from human colon adenocarcinoma[507]. While our observations related to AGR2 were mainly confirmed in the ileum (in our pilot proteomic study and our IHC study), we therefore worked on an epithelial cell line which, although they mimic some characteristics of small intestine IECs, are colonic cells[508]. These cells are considered as a pluripotent intestinal cell line (but with characteristics of mature intestinal cells) which can differentiate into enterocyte-like cells or into goblet cells, depending on the growing conditions[509]. We worked in conditions that allowed them to remain undifferentiated, this does not allow us to attribute our observations to a particular cellular type (goblet cells, enterocytes). Furthermore the AGR2 protein is known to be overexpressed by cancer cells[510,511] (a fact that we confirmed by evaluating the basal expression of this protein in our different cell lines) but this is a situation quite close to what can be found in CD patients who also show an overexpression of this protein in their IECs[381,382]. Despite these limitations, this human colon carcinoma cell line has some advantages including a fairly stable phenotype over the course of cell passages but also the ability to secrete factors, including pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), growth factors (platelet-derived growth factor AA, TGF-α and TGF-β) as well as chemokines and immune-modulatory cytokines, which are similar to the cytokine secretion profile observed in vivo[512]. HT-29s are generally considered as a valuable tool to study molecular mechanisms of intestinal cell differentiation, and are considered as relevant to human in vivo situation[508]. The results of this work deserve to be reproduced on other IECs lines or, even better, on explants or organoids. Among other limitations, we must also point out the limited number of experimental conditions for testing the impact of cytokines (doses, timeframe etc.) on epithelial cells, explaining the discrepancies with the literature. In a similar way than what we reported for the HT-29, the fibroblasts we used (CCD-18Co) were not fibroblasts from CD patients with fibrostenotic disease, which probably have a different phenotype. Furthermore, the application of HT-29 supernatant preconditioned with Tm and rAGR2 on CCD-18Co resulted in a myofibroblastic differentiation markers increase at the protein level, but not at transcript level, suggesting a pro-fibrotic post-transcriptional action of these.

## 3.7. CONCLUSIONS AND PERSPECTIVES

While this work provides some answers on the role of ER stress and AGR2 protein in intestinal fibrosis (Figure 46), several issues remain to be clarified to see if it could be used as a potential therapeutic target. Indeed, our results suggest, for the first time, that the presence of an ER stress in IECs may play a role in intestinal fibrosis through the secretion of AGR2 and its paracrine action on intestinal fibroblasts. However, if we demonstrated that extracellular AGR2 could activate these intestinal fibroblasts, its involvement in their recruitment, migration and proliferation remains to be further investigated.

While we expected to have TGF-β1 secretion in response to induced ER stress, the amount of TGF-β1 found in the control condition was higher than in the stimulated condition. This is probably related to the type of ER stress inducer used (Tm which inhibits N-glycosylations necessary for the TGF- β1 secretion)[427–431]. The secretion of this prototypical pro-fibrosis factor in response to other ER stress inducers (such as thapsigargin[513,514]) would deserve to be studied. However, we have highlighted the secretion of 2 other PDIs, namely PDIA6 and ERP44, whose expression was also increased in tissues with a fibrotic component (in our proteomic study). The pro-fibrotic role of these PDIs should be studied by the application of recombinant proteins (as we did for AGR2), as well as by the application of PDIA6 or ERP44 blocking antibodies in the supernatant of HT-29 cells pre-conditioned by Tm. In addition to these PDIs, epithelial ER stress probably leads to the release of a whole serie of other pro-fibrosing factors into the cell environment. To this end, to compare the secretome of these cells in control condition and in induced-ER stress condition (either by mass spectrometry-based proteomics or by proximity extension assay or PEA, using a panel targeting immune response or inflammation, in absence of panel targeting fibrosis) could allow to better characterise the paracrine pro-fibrosing role of this epithelial ER stress and to identify other factors which could be potential therapeutic targets.

We then showed that the accumulation of misfolded proteins in these IECs could also contribute to fibrosis by promoting the evolution to a mesenchymal phenotype, source of ECM-producing myofibroblasts. However, these links between ER stress/UPR, AGR2 and EMT are poorly studied in intestinal fibrosis and would require further explorations. As there is a small discrepancy with E-cadherin, further investigations may be useful to confirm this ER stress-induced EMT in IECs. We could look at the modulation of other epithelial markers (such

as claudin-1 or zonula occludens-1) in response to Tm as well as at cell morphology changes (e.g. by phase contrast imaging, immunofluorescence). To study under which conditions this epithelial ER stress leads the cell to EMT or apoptosis process would also be interesting, as the literature reveals that the fate of the cell depends on the intensity and duration of the induced-ER stress. The role of TGF-β1 and AGR2 (in its intracellular and extracellular forms) in this mesenchymal transformation would also deserve to be further studied.

Given the potential role of the AGR2 in this intestinal fibrosis highlighted by this work, it is interesting to ask whether this protein could be a therapeutic target. As many tumour cells (including breast, lung, ovarian, oesophageal, prostate, pancreatic, gastric, and colorectal cells[510,511]) overexpress AGR2 and as this aberrant expression was reportedly associated with poor clinical outcomes[368], the use of AGR2 monoclonal antibody has already been considered in oncology[515,516]. As this protein is associated with a range of functions[368], assessing the impact of its inhibition on the organism is not straightforward[516]. Recently, an AGR2 monoclonal antibody has been tested in lung cancer in mice and has proven to be effective on the lung cancer growth and the metastasis, without exerting any toxic effects[517]. However, unlike cancer, in the context of intestinal fibrosis, complications are essentially local with the formation of strictures. In view of the local action of eAGR2 on intestinal fibroblasts (but also the recruitment, migration, proliferation and activation reported in the worldwide literature), but also its interaction with growth factors VEGF and FGF, the possible role in the EMT induction, as well as the contacts of the secreted fraction of this protein with the ECM, the local inhibition of AGR2, by an intralesional injection, associated or not to an EBD, could be an attractive therapeutic proposal, further limiting the risk of side-effects, but would not then allow its systemic more preventive action on the whole inflammatory lesions.

Beside AGR2, ER stress probably contributes to intestinal fibrosis by other mechanisms that we have mentioned, such as the secretion of other PDIs, the release of other pro-fibrosing factors, the induction of EMT as well as by inducing cell apoptosis. It would therefore be more interesting to modulate this epithelial ER stress rather than to selectively inhibit AGR2. Indeed, in the intestinal epithelium, this ER stress is probably modulated by a whole series of factors that may be endogenous molecules including cytokines (modulators particularly relevant in the context of CD, characterized by a particular cytokine climate), but also by luminal agents (such as dietary and microbial agents). In this context, we searched, in the literature, the different modulators of intestinal epithelial ER stress already identified to date (Table 9), and which

could be tested on our cellular model. Regarding the modulation of epithelial ER stress by cytokines, it would be interesting to test other doses and timing of stimulation by TNF-α/TGF-β, to see how these cytokines, usually present in CD, impact the ER stress/UPR processes and modulates this (attenuates or amplifies it) once it is present.

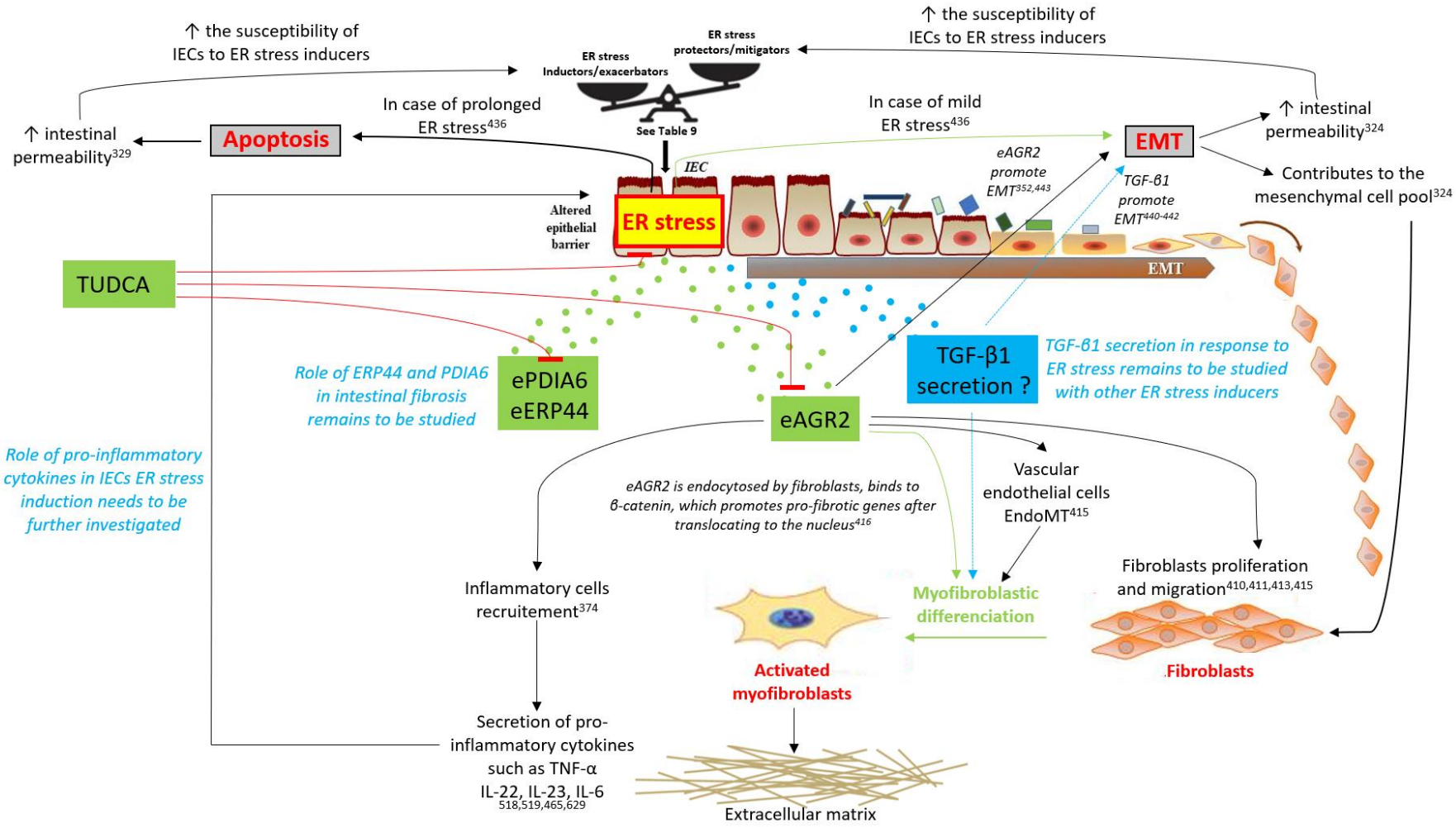
The impact of other cytokines also involved in the pathophysiology of CD could also be tested such as IL-10, IL-22, IL-23, IL-17A[460,518–524]. IL-10 and IL-22, two cytokines playing a key role in the maintenance of mucosal homeostasis have been shown to mitigate ER stress in IECs[525] (but with a more controversial role for IL-22[518]). IL-10 is highly relevant in IBD as GWAS have linked common IL-10 polymorphisms to IBD (and rarer IL-10 and IL-10R genes polymorphisms with early onset IBD[526–528]) and as IL-10 knockout mice are well established genetic model of spontaneous enterocolitis (mimicking IBD)[529,530], accompanied by ER stress[460]. In the intestinal microenvironment, IL-10 (produced by Treg cells, T effector cells, macrophages, dendritic cells and IECs[520]) prevents cytokines production and chemokines expression[531], regulates different innate and adaptative immune cells, thus preventing the development of an inappropriate immune response[525]. In addition to all these functions, IL-10 also avoids inflammation by suppressing ER stress (by blocking ATF6 translocation to the nucleus[460]), but also by facilitating MUC2 production in goblet cells (by up-regulating genes involved in MUC2 folding such as AGR2[364], its transport from the ER, and its *O*-glycosylation and secretion), thus maintaining the mucus production as well as the mucosal barrier function[520], and its impact on intestinal fibrosis would need to be studied for this purpose. IL-22 is a member of the IL-10 family cytokines, produced by Th17 and γδ T cells[532], natural killer T cells, innate lymphoid cells, macrophage and neutrophiles[533], which mainly targets epithelial cells[534], and contributes to protection against pathogens at mucosal surfaces[535] and to epithelial repair (by inducing required genes for epithelial stem cells migration and proliferation)[536–538]. IBD patients presented an elevated level of IL-22 in their mucosal tissues[537] and the role of this cytokine, secreted in response to IL-23 stimulation[535], on ER stress and in IBD pathogenesis is particularly interesting in view of the advent of new IL-23-targetting molecules. If prevailing view is that IL-22 could have a protective role by decreasing ER stress[522,524,539], correcting MUC2 misfolding and alleviating autophagy[522], some preclinical models of IBD indicate that IL-22 may actually contribute to disease[540–542] and very promising results obtained with anti-IL-23 in early phase clinical studies in IBD[543,544], together with the fact that IL-22 serum levels predict response to these anti-IL23 treatment[544] challenge this belief[518]. If it seems, indeed, that IL-22 plays a protective role in acute inflammation context (in the short-term insults, with

abrupt tissue injuries and where primary insult is epithelial disruption[545,546]), IL-22 could rather be a potentiator of ER stress (where it acts in conjunction with IL-17A) in situations of chronic inflammation[518]. Interestingly, consistent with these data, Powell et al. investigated the colonic transcriptome (thanks to colonic biopsies performed at week 8 and week 44) of a subset of patients from the UNITI trial program (ustekinumab, an anti-IL-12/23 p40 monoclonal antibody versus placebo) and demonstrated that patients treated with ustekinumab had a significant reduction of ER stress markers transcripts expression at week 44 compared to placebo[518]. Regarding these data, and the highlighting of epithelial ER stress as a predominant pathway in fibrosis (especially in our pilot study but also via the myofibroblastic differentiation obtained after application, on CCD-18Co, of supernatant of epithelial cells preconditioned by Tm), the impact of ustekinumab and anti-IL-23 on intestinal fibrosis deserves to be studied.

Finally, as the IECs are the first to encounter a whole series of environmental toxins and infectious agents, studying the impact of these agents on epithelial ER stress, and consequently on intestinal fibrosis, could be particularly relevant. In particular, several elements of the Western diet such as high-fat diet[524,547–549], palmitic acid[550–552], mycotoxin[553–557] or maltodextrin[558–560], have demonstrated an exacerbating effect on ER stress. For example, maltodextrin (MDX) is a powder rich in starch added by manufacturers to many foods to improve their flavour, texture (thickener) or shelf life. It can be found in many industrial/ultra-processed products including sauces, soups, sweets, dairy products as well as energy drinks, and it has been shown that a prolonged MDX-enriched diet induce low-grade intestinal inflammation[559]. MDX-induced ER stress causes indeed an alteration of the mucus barrier, promoting thus epithelial intestinal invasion by pathogenes[559] (whose impact could also be tested), which, in turn, can lead to an accumulation of misfolded proteins in the ER and to a vicious circle. Finally, a range of strategies exists to reduce this intestinal epithelial ER stress such as the consumption of a diet rich in glutamine[561–564] and polyphenols[513,565–573], the use of curcumin[574–576] (which has already demonstrated an impact on intestinal fibrosis by reducing EMT[577]), engaging a physical activity[578,579] but also the use of therapeutic agents such as TUDCA[471,475,580], IL-10[460,520,521], anti-IL-22[518], anti-IL-23 therapies[518,519] as well as mesenchymal stem cells[581], which should be the subject of future investigations.

| Intestinal ER stress | Protectors/mitigators | Inductors/exacerbators |
|---|---|---|
| ***Dietary agents*** | Amino acids such as glutamine[561–564] and glycine[582]<br>Natural polyphenols[513,565–573]<br>Omega-3 fatty acids and omega-6 fatty acids[551,583,584]<br>Vitamin C[585], Vitamin E[586]<br>Selenium[587]<br>Curcumin[574–576]<br>Allicin (garlic)[588]<br>Vinegar[589]<br>Koumiss[590]<br>Chitosan oligosaccharide[591]<br>Chinese herbs (berberin[464,592], bitter melon[407], catalpol[593], lycium barbarum[594], crataegi Fructus[595])<br>*Lachnum* polysaccharide[433] | L-cysteine[596]<br>Maltodextrin[558–560]<br>High-fat diet[524,547–549] and frying fat[597]<br>Dietary saturated fat (tetranoic acid[598])<br>Long-chain fatty acids such as palmitic acid[550–552], oleic acid[599] and elaidic acid[600]<br>Mycotoxin[553–557]<br>Gliadin[601]<br>Nonylphenol[602]<br>Epibrassinolide (vegetal hormone)[603]<br>Cucurbitacin E[604]<br>Wheat Bran[605]<br>Alcohol[606]<br>Thickener and emulsifier[607]<br>Acrolein (cigarette component)[608]<br>Azo dye[609]<br>Colostrum oxytocin[610] |
| ***Microbial agents*** | Escherichia coli O157:H7[611]<br>Schistosoma japonicum[612] | Escherichia Coli[613,614]<br>Lipopolysaccharides (LPSs)[615,616]<br>Citrobacter rodentium[582,617]<br>Rotavirus[618]<br>Helicobacter[619]<br>Shiga toxins[620]<br>Cryptosporidium parvum[621]<br>Clostridium difficile toxin B[622]<br>Lentivirus[623]<br>Trichinella spiralis[624]<br>Gastroenteritis virus N protein[625]<br>Porcine epidemic diarrhea virus E[626] |
| ***Endogenous molecules*** | IL-22 (in acute inflammation)[522–524]<br>IL-10[460,520,521]<br>Bile acids[627]<br>Hormones (progesterone, estrogen[628], oxytocin[610]) | IL-22 (in chronic inflammation)[518]<br>IL-23[518,519]<br>IL-17A[518]<br>IL-6[465]<br>TNF-α[629] |
| ***Chemical molecules/therapeutic agents*** | Tauroursodeoxycholic acid (TUDCA)[471,475,580]<br>4-phenylbutyric acid (4-PBA)[580,630–632]<br>Salubrinal[632,633]<br>Probiotics[601,617,634]<br>Naltrexone[635]<br>Hydrogen-rich saline (HRS)[636,637]<br>Roxithromycin (antibiotic)[638]<br>Glucocorticoids[639]<br>Ocreotide[640]<br>Fexofenadine (anti-H1)[641]<br>Mesenchymal stem cells[581] | Tunicamycin[471,518,520,628,635,639,642–646]<br>Brefeldin A[513]<br>Dithiothreitol (DTT)[599]<br>Thapsigargin[513,514]<br>NSAIDs ( nonsteroidal anti-inflammatory drug)[647–649]<br>Probiotics (Lactobacillus paracasei[632], Lactobacillus plantarum[650])<br>Iron sulfate[651]<br>Vitamin D3[652]<br>HIV ( human immunodeficiency virus) protease inhibitors[653,654]<br>EGFR ( epidermal growth factor receptor) inhibitor[655,656]<br>Trametinib (Mitogen-activated protein kinase enzymes inhibitor) and Pictilisib (PI3K inhibitor)[657] |
| ***Others*** | Physical activity (through irisin, a factor secreted by myocytes in response to exercise)[578,579] | Ischemia-reperfusion[658]<br>Acidosis[659]<br>Heat[660–664]<br>Shake[661]<br>Ionizing radiation[580]<br>Maternal separation[633,665] |

**Table 9. Modulators of intestinal epithelial ER stress**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↑ the susceptibility of IECs to ER stress inducers
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ER stress
EMT
TUDCA
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eERP44
TGF-β1 secretion ?
TGF-β1 secretion in response to ER stress remains to be studied with other ER stress inducers
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Inflammatory cells recruitement374
Myofibroblastic differenciation
Activated myofibroblasts
Fibroblasts
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Extracellular matrix

**Figure 46. Summary hypothesis of the role of ER stress and AGR2 in intestinal fibrosis**

To our knowledge, we are the first to demonstrate a role of the epithelial ER stress in intestinal fibrosis. This figure summarizes a hypothesis of the role of epithelial ER stress and the AGR2 protein in intestinal fibrosis. What has been demonstrated in this thesis is in green and is repositioned in the data of worldwide literature (in black). Hypotheses to be tested have also been implemented (in blue).

The surface intestinal epithelium is subjected to a range of ER stress inducers and attenuators (including bacterial luminal agents or dietary fragments). Exposure of the cells to (an) ER stress inducer(s) (or to an imbalance between ER stress inducers and attenuators, as referenced in Table 9) results in ER stress in the IECs. We have shown that in response to induced ER stress in the IECs, the latter releases eAGR2, but also 2 other PDIs such as PDIA6 and ERP44. While the role of PDIA6 and ERP44 in intestinal fibrosis needs to be further investigated, we demonstrated that eAGR2 could induce the transformation of fibroblasts into activated myofibroblasts, secreting the ECM that accumulates in intestinal fibrosis. In addition, eAGR2 could contribute to fibrosis by: (1) promoting the recruitment of inflammatory cells[374], which secrete pro-inflammatory cytokines such as TNF-α or IL-6, which could induce ER stress in IECs (although this would need to be studied in more details in the IECs), thus contributing to a vicious cycle[465,629]; (2) promoting the proliferation and migration of fibroblasts[410,411,413,415]; (3) promoting the recruitment of vascular endothelial cells for Endo-MT[415]; (4) promoting the EMT[352,443]. In response to this ER stress, the cell could also secrete TGF-β1 (but this needs to be checked with another ER stress inducer) which could contribute to fibrosis by promoting the myofibroblastic differentiation and EMT[440–442]. The epithelial cell may also, in response to induced ER stress, evolve into a mesenchymal transformation (in the case of mild ER stress[436]) contributing to the pool of mesenchymal cells[324], able to become activated myofibroblasts, but also promoting an increase of intestinal barrier permeability[324], making the cells more susceptible to ER stress inducers and may contribute to a vicious cycle. In the event of persistent ER stress, the IEC may undergo apoptosis[436], also promoting increased intestinal permeability and contact between IECs and ER stress inducers[329].

Finally, we demonstrated that TUDCA, an endogenous bile acid, naturally present at the intestinal epithelium contact, was able to inhibit ER stress induced in IECs when applied concomitantly with the inducer and inhibited, in addition to intracellular PDIs, the secretion of AGR2 and PDIA6.

# EVALUATION OF SAFETY AND EFFICACY OF LOCAL MESENCHYMAL STEM CELLS INJECTION IN CROHN'S DISEASE STRICTURE

# 4. EVALUATION OF SAFETY AND EFFICACY OF LOCAL MESENCHYMAL STEM CELLS INJECTION IN CROHN'S DISEASE STRICTURE

## 4.1. INTRODUCTION

### 4.1.1. Stem cells and cell therapy

Stem cells are at the origin of every human being and have 2 main characteristics: the ability of self-renewing and the ability to differentiate into multiple cell lineages through successive cell divisions. They can be classified as totipotent, pluripotent, multipotent and unipotent according to their ability to differentiate. The embryonic stem cells resulting from the first divisions of the fertilized oocyte, until the 4$^{th}$ day (morula stage), are **totipotent** and can differentiate into all the cell types of the organism (both embryonic and extraembryonic, including placental cells). They are the only ones that allow the complete development of an individual[666,667]. The daughter cells of these embryonic stem cells, derived from the inner mass of the embryo (at the blastocyst stage), from day 5 to 7, are termed as **pluripotent** and began to differentiate from the trophectoderm cells (that form the placenta)[666–668]. They are the source of the three germ lines cells (endodermal, mesodermal and ectodermal layers), giving rise to more than 200 cell types representative of every body's tissues[666–668]. Then, the stem cells become **multipotent**, and are characterised by the ability to differentiate into all cell types within one particular lineage, including hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs)[669–671]. Finally, **unipotent** stem cells are so involved in a process of differentiation that they are only able to provide one cell type but are able of self-renewing (which distinguishes them from precursor cells) to ensure the proper functioning of organs by replacing dead cells[672].

The cell therapy is the use of these stem cells, by injection or transplantation, to repair a destroyed tissue, restore a deficient function or modulate a pathophysiological process[673]. The stem cells used in cell therapy can come from the patient himself (**autologous stem cells** that will be perfectly tolerated by the patient from an immune point of view) or from someone else than the patient (**allogeneic stem cells** that may trigger immune tolerance problems)[674]. In this case, the donor's cells can be recognised by the recipient's immune system as foreign elements and be eliminated, leading to graft rejection[674]. The use of embryonic totipotent and pluripotent stem cells (by opposition to adult stem cells), being associated to obvious ethical and legal issues, it is mainly **induced pluripotent stem cells** (iPSCs) or **multipotent stem cells** (such as HSCs or MSCs) that are used in cell therapy[675,676]. **iPSCs** are adult somatic cells reprogrammed into pluripotent cells by genetic engineering, using different transcription factors (e.g. OCT4 or

Octamer binding transcription factor-4, Sox2 or (Sex determining region Y)-box 2, Klf4 or Kruppel Like Factor-4 and c-Myc)[677], which possess unique potential to differentiate into any cell of the human body, and have a wide range of applications, especially in regenerative medicine[678]. **HSCs** are at the origin of all blood cells and can be harvested from bone marrow (BM), cord blood or peripheral blood (after stimulation with granulocyte-colony stimulating factor or G-CSF allowing HSCs to migrate from BM into the circulating blood)[679]. They have been used to treat haematological malignancies through a procedure called haematopoietic stem cell transplantation (HSCT), where these stem cells reconstitute a stock of healthy blood cells in the patient, after having destroyed his own diseased cells by chemotherapy[680]. Beside HSCs, the other multipotent cells used in cell therapy are **MSCs**, which can be easily isolated from bone marrow (BM-derived MSCs)[311] or adipose tissue (adipose-tissue-derived MSCs)[681], but which can also be found in various other tissues such as peripheral blood[682,683], placenta[684,685], umbilical cord[686], cord blood[687] and connective tissues of most organs[311,688,689]. These MSCs have emerged as a novel significant approach to improve inflammation in a number of inflammatory diseases, due to their cell differentiation and immunomodulatory properties[673].

### 4.1.2. Mesenchymal stem cells properties

MSCs have 3 main characteristics: (1) they have a fibroblast-like morphology and are plastic adherent in standard culture conditions, (2) they express cell surface markers such as CD13, CD29, CD44, CD54, CD73, CD90, CD105, CD166, Stro-1 and MHC-I and lack the expression of haematopoietic markers such as CD11b, CD14, CD19, CD31, CD34, CD45, CD79A, and human leucocyte antigen (HLA)-DR surface molecules and (3) they keep the capacity to *in vitro* differentiate into osteoblasts, chondroblasts and adipocytes[311,690–693].

Whatever their origin or their route of administration[311,694–699], these MSCs have the ability to selectively migrate to the injured intestinal wall (lamina propria, submucosa and muscular layers), in response to growth factors (VEGF, PDGF)[311,700,701], thanks to interactions between chemokines and various C-C chemokine receptors[702] and their expression of integrins and adhesion molecules (such as intercellular adhesion molecule or ICAM and vascular cell adhesion protein or VCAM)[311,697].

Once at the inflammation site, MSCs can differentiate into different types of mesenchymal cells and engraft in tissue[311,703–705], where they can promote tissue repair[311,706–711], in particular epithelium regeneration[712–714]. The tissue repair properties of MSCs are not only related to their

capacity to differentiate but also to their ability to prevent apoptosis and to stimulate survival as well as proliferation of un-injured parenchymal cells[715–718].

Beside these regenerative properties, MSCs have immunomodulatory properties contributing to alleviate the on-going inflammation[719]. Firstly, they inhibit the T cells proliferation (whether the lymphocytes are CD4+ or CD8+, naïve or memory, regardless of their major histocompatibility complex or functional state)[311,720–724]. The mechanism by which these MSCs inhibit T cells proliferation is not yet clarified[311,722,725], but could be through apoptosis[725] or cell cycle blockade in G0/G1 phase[311,726]. Secondly, MSCs inhibit proliferation and function of Th1 and Th17 cells (leading to lower level of Th1 and Th17 pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-6, IL-12, IL-17, IL-23 and higher levels of the IL-10 anti-inflammatory cytokine[311,697–699,727]) and increase the recruitment of Treg from both naïve and memory T cells[311,728,729]. MSCs also act on other cell types including antigen presenting cells (inhibition of their maturation and migration, impairment of their antigen-presenting and modulation of their secreted cytokines)[311,730–733], B-cells (inhibition of their activation, proliferation as well as chemotaxis)[311,734–737] and natural killer cells (inhibition of their proliferation and secretion of pro-inflammatory cytokines)[311,738–741]. Finally, MSCs secrete a range of anti-inflammatory molecules, such as IL-10, HGF[742,743], prostaglandin $E_2$ (PGE2), indoleamine 2,3-dioxygenase (IDO), nitric oxide (NO), HLA-G as well as extracellular vesicles (EVs) including exosomes[311,738,744–747].

As these MSCs produce anti-inflammatory factors leading to local immunosuppression[311,738,744–747] and weakly express HLA markers[748,749], immune responses against the graft are limited and no previous exogenous immunosuppressive treatment is therefore necessary when using allogeneic mesenchymal stem cells[750].

### 4.1.3. Mesenchymal stem cells therapy in Crohn's disease

Clinical trials regarding MSCs in CD have focused on fistulising and luminal diseases[311,751].

***MSCs in perianal fistulising CD***

The use of MSCs to treat CD-associated fistulas shows encouraging results, whatever the origin of these MSCs (autologous versus allogeneic, derived from bone marrow versus adipose tissues), the MSCs dose and the injection process[311]. In a recent meta-analysis[751], CD patients with fistulising disease treated by a stem cells injection had a higher healing rate of fistula than

patients in placebo group (61.75% vs 40.46%, OR 2.21, 95% CI 1.19 to 4.11, $p < 0.05$)[751]. Regarding the source of these stem cells, the healing rate of CD fistula seemed numerically higher with darvadstrocel (Cx601, Alofisel, Takeda which are expanded human adult allogeneic MSCs from adipose tissue[743]) (61.02%) than with adipose tissue-derived stem cells from homemade cultures (51.43%, $p < 0.05$) but no firm conclusion can be drawn without a head-to-head trial[751]. The stem cells injection at the dose of $3 \times 10^7$ stem cells/mL was associated with the highest healing rate of CD fistulas (71.0%) compared to other doses group of stem cells, ranging from $1 \times 10^7$ cells/mL to $9 \times 10^7$ cells/mL (RR 1.3, 95% CI 0.76 to 2.22)[751]. Regarding the type of fistula, perianal and trans-sphincteric fistulas responded better to MSCs than rectovaginal fistulas (77.95% and 76.41% vs. 27.18% respectively, $p < 0.01$)[751]. Intriguingly, Crohn's disease activity index (CDAI) and Perianal Disease Activity Index (PDAI) scores were transiently increased 1 month after stem cells injection (up to 90% of stem cells are vanished and destroyed by subjects' immune clearance themselves), and were reduced to a level below the baseline at 3 months (some authors suggest that this is because the remaining stem cells (about 8%) were differentiated into functional epithelial cells or stromal cells)[751,752 751,752]. Furthermore, the incidence rate of treatment-related adverse events in the stem cell group was significantly lower than in the placebo group (RR 0.58, 95% CI 0.30 to 1.14)[751], confirming their great safety and tolerability profile[311,753].

***MSCs in luminal CD***

Autologous BM-MSCs intravenous injection has only been evaluated in two small trials[754,755], with inconclusive results[311]. In contrast, allogenic MSCs intravenous injection[756–758] was associated to promising results with around 60% of clinical response and 40% of clinical remission, but MSCs have not yet been shown to be superior to placebo in this context[311].

## 4.2. RATIONALE AND OBJECTIVES

Although clinical studies have demonstrated the safety and variable efficacy of IV MSCs administration to treat luminal CD[756–758] and local MSCs administration to treat perianal CD fistulas[751], and that MSCs had anti-fibrotic properties reported in fibrosis of several organs[759], the impact of MSCs on CD stricture has never been studied to our knowledge. Our aim was to perform an exploratory study on the use of local MSCs injection to treat stricturing CD lesions.

Beyond the feasibility study, our purposes were to:

- Assess the safety of local MSCs injection in CD stricture

- Evaluate the efficacy of local MSCs injection on the healing of these lesions

## 4.3. MATERIAL AND METHODS

### 4.3.1. Patients

We included patients diagnosed with CD at least 6 months previously, aged 18 years or older, and having a symptomatic non-passable CD stricture, less than 5 cm in length (according to magnetic resonance enterography (MRE) measure), endoscopically accessible and refractory to conventional or biologic therapies (azathioprine, 6-mercaptopurine, methotrexate, anti-tumor necrosis factor, vedolizumab, ustekinumab). Patients were considered symptomatic if they had at least 4 episodes of obstructive pain (minimal or moderate) in the last 8 weeks before inclusion, with or without nausea, vomiting, food restriction and intestinal obstruction. Patients could have one or more strictures (the presence of several strictures was not an exclusion criterion). Exclusion criteria were intestinal obstruction or indication for immediate surgery, intra-abdominal fistula or abscess, pregnancy or planning pregnancy within one-year, terminal organ failure, human immunodeficiency virus positivity, uncontrolled infection, and a history of malignancy within the past 5 years. The study was approved by the ethics committee of Liège University and written informed consent was obtained from all patients.

### 4.3.2. Mesenchymal stem cell preparation and injection

MSC donors were healthy adult volunteers, unrelated to the recipient, and eligible for allogeneic hematopoietic cell donation. No human leukocyte antigen (HLA) matching was required. Bone marrow collection and MSC cultures were carried out at the Laboratory of Cell and Gene Therapy (LTCG) at the CHU of Liège, as previously described[308,760]. Freshly P3-harvested cells were washed and resuspended at 3.75 x $10^6$ cells/mL in a 75% NaCl 0.9%/ 25% HAS 20% solution and conditioned in 2 mL syringes at room temperature. The MSC solution was locally injected into the stricture during ileocolonoscopy performed by a gastroenterologist with an expertise in IBD and therapeutic endoscopy. A total of $3X10^7$ MSCs were equally injected in the 4 quadrants in the submucosa of the strictured bowel wall. The stem cells were injected alone and were not combined with EBD.

### 4.3.3. Study design and endpoints

Adverse events, clinical scores (CDAI[761], Short Health Scale or SHS[762] and CDOS[239]) as well as biomarkers (CRP and fecal calprotectin) were evaluated at each follow-up visit (at week 4,

12, 24, 36 and 48). The Crohn Disease Obstructive Score, is a 0-6 scale assessing the intensity of obstructive symptoms taking into account duration and intensity of obstructive pain, associated signs such as nausea and vomiting, dietary restriction and hospitalisation[239]. Endoscopy and MRE were performed at baseline, week 12 and 48. Biopsies of the stricture lesion and of the surrounding healthy tissue have been performed at weeks 0, 12 and 48. The main judgement criterion for efficacy was the complete or partial resolution of the stricture, assessed during ileocolonoscopy, at 12 weeks. Complete resolution was defined by the ability to pass an adult ileocolonoscope through the stricture and partial resolution was defined by an increase of the diameter of the stricture, as measured in comparison to the size of an open biopsy forceps (7 mm). Second efficacy criteria included complete or partial resolution of the stricture at 48 weeks as well as the evolution of clinical scores and MRE images (stricture length, bowel wall thickness, pre-stricture dilatation, intramural T2 hyperintensity, three-layered target sign, homogeneous mural enhancement, late enhancement and presence of a comb sign) between weeks 0, 12 and 48. These images were reviewed by a single radiologist blinded for the clinical and endoscopic outcomes (PM). All concomitant medications including conventional therapies, immunomodulators or biologics were allowed during the study but had to remain stable till evaluation for primary endpoint at week 12.

### 4.3.4. Statistical analysis

Demographic and disease-specific data are given descriptively or tabulated. Results are presented as medians and quartiles for continuous parameters or as frequency tables for qualitative parameters. Statistical tests were performed by using GraphPad Prism, version 8.1.2 for Windows (GraphPad Software, San Diego, Calif).

## 4.4. RESULTS

### 4.4.1. Patient and stricture characteristics

A total of 11 MSC injections were performed in the stricture of 10 patients (6 females; median age 45 years, range 38-65 years; 70% were smoker) between May 2018 and July 2020; one patient was injected twice in the same anastomotic stricture at 26 months interval. The strictures' (3 de novo and 7 anastomoses) location was as followed: 7 at an ileo-colonic or ileo-rectal anastomosis, 1 colonic and 2 of the terminal ileum. The median stricture length was 23 mm (range, 6.3–150 mm; three patients were protocol violations after central reading by PM with a length of 70 for two and 150 mm for one) and 5 patients (50%) presented abnormal mucosa (inflammation/ulceration) on endoscopy. A previous EBD was performed in 6 patients. Patients and strictures' characteristics are detailed in Table 10 (see Table 11 for details by patient).

| Parameter | n=10 (%) |
|---|---|
| Female gender | 6 (60.0) |
| Age at inclusion (years, median [IQR]) | 45 (42.5-49) |
| Disease duration (years, median [IQR]) | 21 (14.5-25.5) |
| Smoking habits | |
| Never | 1 (10.0) |
| Past smoker | 3 (30.0) |
| Active smoker | 6 (60.0) |
| Medication during follow-up[a] | |
| None | 2 (20.0) |
| Budesonide | 3 (30.0) |
| Methylprednisolone | 1 (10.0) |
| Immunomodulators | 4 (40.0) |
| Anti-TNF therapy | 4 (40.0) |
| Ustekinumab | 4 (40.0) |
| Previous EBD | 6 (60.0) |
| One | 3 (30.0) |
| Two | 2 (20.0) |
| Seven | 1 (10.0) |
| Previous luminal surgery | |
| Yes | 7 (70.0) |
| No | 3 (30.0) |
| CDOS (median [IQR]) | 3 (2-4) |
| Disease activity at inclusion | |
| CDAI (median [IQR])[b] | 204 (127-271.5) |
| CRP (mg/L, median [IQR])[b] | 4.75 (1.8-7.7) |
| Faecal calprotectin (µg/g, median [IQR])[b] | 67 (32-195) |

**Table 10.** Patients' characteristics.

CDAI, Crohn Disease Activity Index; CDOS, Crohn Disease Obstructive Score; CRP, C-Reactive Protein; EBD, Endoscopic Balloon Dilatation; IQR, interquartile range; TNF, Tumor Necrosis Factor.

[a]Some patients are under combination therapy

[b]Data not available for all subjects

| Patient | Age (y) | Sex | Smoking habits | CD Duration (y) | Concomitant drug therapy | Naive versus anastomotic stricture | Previous EBD | Stricture diameter (mm) | Inflammation on endoscopy | Stricture length (mm) | Maximum bowel wall thickness (mm) | Pre-stricture dilatation (mm) | Intra-mural T2 hyper-intensity | Three-layered target sign | Homogeneous mural enhancement | Late enhancement | Comb sign |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 (1st inj.) | 45 | F | Yes | 28 | USTE, MTX | A (ileo-rectal) | 6 | 8 | No | 10 | 7,4 | 67 | No | No | Yes | Yes | No |
| 1 (2nd inj.) | 47 | | Yes | 30 | USTE, CS | | 8 | 2 | No | 6.3 | 7,8 | 70 | Patient evaluated by abdominal CT scan | | | | |
| 2 | 42 | M | Yes | 23 | BUD | A (ileo-colic) | 0 | 10 | Yes | 49 | 9 | 27.5 | Yes | Yes | Yes | No | Yes |
| 3 | 37 | M | Former | 21 | IFX | A (ileo-colic) | 0 | 10 | No | 70 | 20 | 33 | No | No | Yes | Yes | No |
| 4 | 65 | F | Former | 41 | ADA, BUD | A (ileo-colic) | 2 | 4 | Yes | 22 | 10,1 | 29 | Yes | No | Yes | No | No |
| 5 | 65 | F | Former | 15 | ADA, AZA, BUD | A (ileo-colic) | 1 | 6 | No | 19 | 8 | 23 | Yes | No | Yes | Yes | No |
| 6 | 49 | F | Yes | 20 | None | N (left colon) | 0 | 8 | Yes | 23 | 6 | 70 | No | No | Yes | Yes | No |
| 7 | 43 | M | Yes | 12 | USTE, MTX | N (ileo-caecal valve) | 1 | 10 | No | 70 | 12,5 | 17.5 | Yes | Yes | Yes | No | No |
| 8 | 38 | F | Yes | 14 | Modulen | A (ileo-colic) | 0 | 6 | Yes | 150 | 10 | 28 | No | Yes | Yes | Yes | Yes |
| 9 | 49 | M | Yes | 22 | USTE | A (ileo-colic) | 1 | 4 | Yes | 32 | 8 | 25 | No | No | Yes | Yes | Yes |
| 10 | 43 | F | Former | 2 | ADA, MTX | N (ileo-caecal valve) | 2 | 8 | No | 16 | 10 | 22 | No | No | Yes | Yes | No |

**Table 11. Patient demographics and stricture details**

A, Anastomotic stricture; ADA, adalimumab; AZA, azathioprine, BUD, budesonide; CS, corticosteroids; EBD, endoscopic balloon dilatation; IFX, infliximab; inj, injection; MTX, methotrexate; NA, not applicable; N, naive stricture; USTE, ustekinumab.

### 4.4.2. Safety

MSC injection was safe, and no immediate side effect was identified. No patient developed abscess or fistula in the follow up. One patient had actually a small intestinal fistula at baseline (not diagnosed by the routine imaging report but disclosed by the central reading by PM). A total of 5 hospitalizations were reported: one for a disease flare-up and 4 for occlusion (1 secondary to a different stricture than the injected one, at week 37) leading to a surgical resection for one patient (at week 22) and to an EBD, twice, for the patient who underwent 2 procedures (at week 39 for the first and week 2 for the second one). One patient developed a basocellular carcinoma (week 24) which was completely resected.

### 4.4.3. Efficacy

At 12 weeks, 5 patients presented a complete (2 patients) or partial (3 patients) resolution of the stricture. The stricture diameter could not be measured in one patient due to poor preparation. Out of the 11 MSC injections, 7 strictures were re-evaluated at week 48 (1 operated, 1 dilated twice, and 1 lost to follow-up) and 4 patients had a complete resolution (Figure 47). Figures 48 shows the evolution of stricture diameter, CDOS and pre-stricture dilatation between weeks 0, 12 and 48. There was no statistically significant change of these parameters as well as of other clinical scores, biomarkers and the stricture characteristics analysed by MRE (Supplemental Table 7), except the disappearance of T2 submucosal oedema in two endoscopic responders.

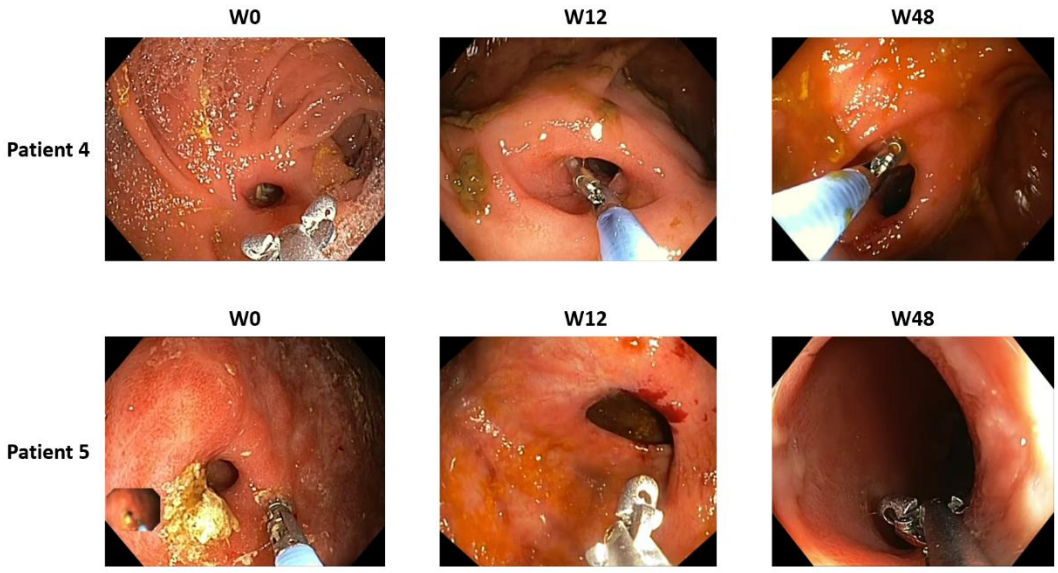


**Figure 47. Evolution of the stricture diameter in patients 4 and 5 at weeks 0, 12 and 48**

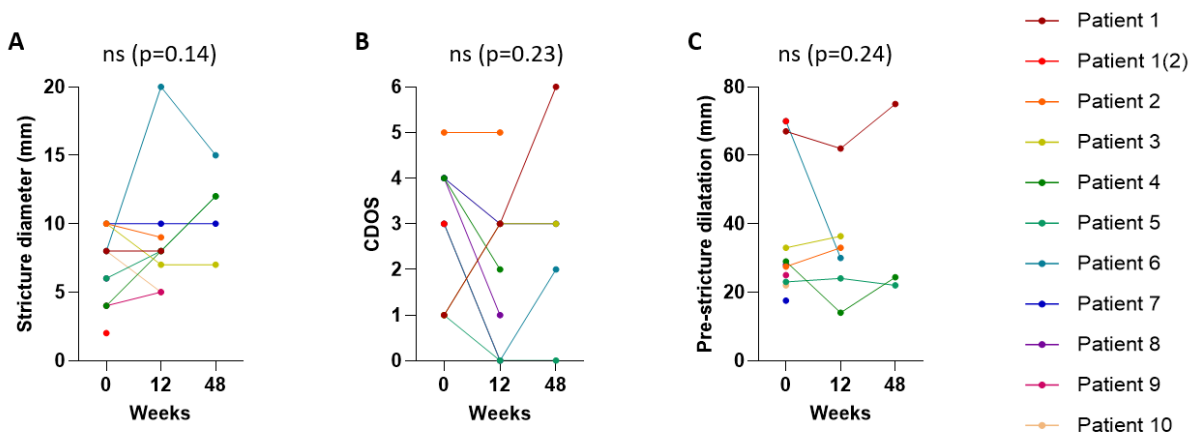


**Figure 48. Evolution of the stricture diameter (mm) (A), Crohn's Disease Obstructive Score (CDOS) (B) and pre-stricture dilatation (mm) (C) between weeks 0, 12 and 48**
ns: not significant.

The Table 12 compares the survival free of the need for re-intervention (EBD or surgery) after EBD and after MSC injection for patients who had previously received EBD. Patients 4, 7 (who were 2 responders) and patient 10 had longer survival without need of re-intervention after MSCs injection than after EBD. The outcome of the patient who was injected twice deserves special consideration. She was injected a first time on 24/05/2018 and had to be dilated 39 weeks later (22/02/2019), for sub-occlusive symptoms while, surprisingly, the stricture showed an increased diameter (since the stricture was passable by the gastroscope). After this EBD, the patient developed again sub-occlusive symptoms and required a new EBD 6 weeks later (04/04/2019) and then, 12 weeks later (26/06/2019). As the patient remained 39 weeks without sub-occlusion after the MSCs injection while recurred after 6 and 12 weeks after the EBD, we attempted a second injection. The patient had to undergo a new dilatation 2 weeks after the 2$^{nd}$ injection, but the stricture diameter re-increased again (from 2 to 4 mm). A submucosal oedema was probably caused by the injection of MSCs.

| Patient's number | Survival free of the need for re-intervention (EBD or surgery) after EBD | Survival free of the need for re-intervention (EBD or surgery) after MSCs injection |
|---|---|---|
| 1 | 26 months, 6 months, 10 months, 26 months, 1 month, 1 month<br>After the 2nd injection: 1,5 months and 3 months | EBD after 10 months and 2 weeks |
| 4 | 11 months and 19 months | No re-intervention to date (38 months after MSCs injection) |
| 5 | 42 months | No re-intervention to date (38 months after MSCs injection) |
| 7 | 2 months | Surgery after 24 months |
| 9 | 21 months | Surgery after 14 months |
| 10 | 1 month and 14 months | No re-intervention to date (18 months after MSCs injection) |

**Table 12. Comparison of survival free of the need for re-intervention (EBD or surgery) after EBD and MSCs injection, for patients who had previously benefited from an EBD**
EBD, endoscopic balloon dilatation; MSCs, mesenchymal stem cells

## 4.5. DISCUSSION

Whereas clinical studies have demonstrated the safety and variable efficacy of IV MSC administration to treat luminal CD[308] and local MSC administration to treat perianal CD fistulas[309–311], the present study is the first, to our knowledge, to evaluate MSC injection in CD strictures. Similarly to intralesional injection of steroids[257,263,763] or infliximab[764,765], local MSC injection was well tolerated on the short term and no complication such as intestinal perforation, abscess or new intra-abdominal fistula was reported. Several occlusions were reported in the follow-up probably related to the insufficient efficacy of the MSC injection in those cases.

The efficacy of MSC injection in the strictures remains difficult to interpret in this study in absence of a control group. We can't exclude that the diameter increase observed in some patients was related to the medical CD concomitant treatment[766] rather than the MSCs. However, if we evaluate the need for re-intervention (EBD or surgery) within 24 to 48 weeks, generally considered as an appropriate endpoint according to experts' consensus[169], only 2 patients (20%) needed further endoscopic or surgical intervention. This result is in fact quite close to what has been observed with EBD[258,767] and seems encouraging for a local treatment of symptomatic non-passable CD stricture. In the assessment of effectiveness, it should also be taken into account that 2 non-responders were part of the 3 patients who had stricture longer than 5 cm. These strictures had been judged shorter by the routine imaging report but were re-evaluated longer at the central reading by PM, using standard definition of Crohn's disease (CD) stricture[169]. As EBD implies elongation and rupture of fibrous tissue rich in collagen and could result in further tissue damage by inflammation, oedema, additional fibrosis and scarring, a combined treatment by MSCs might be beneficial and could prevent re-scarring[262].

Indeed, besides their known anti-inflammatory properties, MSCs have been shown to have an anti-fibrotic effect in several organs[302] by acting through different pathways including release of anti-fibrotic molecules (such as hepatocyte growth factor[303] and TNF-stimulated gene 6[304]), and by inhibiting ECM remodeling[768] and TGF-β activation[302,769], known to play a key role in intestinal fibrosis. In animal models, it has been shown that MSC culture supernatant can prevent luminal stricture development after ESD in the esophagus[770] and rectum[771] of pigs by inhibiting inflammatory cell infiltration, myofibroblast activation, fiber accumulation, and hypertrophy of the muscularis propria. Recently, the role of MSCs as prophylactic or therapeutic treatment on CD-associated intestinal fibrosis was investigated in a TNBS-induced

mouse model[772]. While prophylactic treatment with MSCs allowed to inhibit the expression of fibrotic proteins as well as the accumulation of fibrotic tissue, the use of MSCs as therapeutic treatment reversed the established intestinal fibrosis. It was shown that MSCs down-regulated the secretion of fibrogenic factors (such as IL-1beta, IL-6 and IL-13) and up-regulated anti-fibrogenic factors (such as IL-10), reduced the epithelial-to-mesenchymal transition process (which is a contributing source of fibroblasts in intestinal fibrosis[321]) and inhibited the expression of TGF-beta/Smad signalling pathway (the most important signalling pathway associated with CD-intestinal fibrosis[136]), which makes it a promising adjuvant treatment for CD stricture.

## 4.6. LIMITATIONS

This study has several limitations. First, the small sample size does not allow to conduct a meaningful statistical analysis to identify predictive factors of response. Second, the lack of control group and the fact that patients received concomitant systemic biologic therapy does not allow to really evaluate the beneficial role of MSCs.

## 4.7. CONCLUSIONS AND PERSPECTIVES

In conclusion, local MSC injection in non-passable CD strictures was well tolerated over the short term although several occlusions occurred in the follow-up indicating insufficient therapeutic effect in those cases. Combining the effects of MSCs with the proven effect of EBD could possibly improve the outcome of CD stricture. For this purpose, a comparative study evaluating the effects of EBD associated with local MSCs injection versus EBD alone (control group) deserves to be carried out.

Several questions regarding the use of MSCs to treat CD stricture remain to be addressed. First, we need more information about the dose of MSCs to be injected[311]. If the few studies that have looked at this issue showed a higher healing rate in CD fistula with $3x10^7$ cells/mL[309,311,743,773], different doses would be worth investigating in the treatment of CD stricture. Secondly, it would be interesting to see which of the 2 major sources of MSCs (bone-marrow versus adipose-tissue) offer the better therapeutic approach. Although each has been separately studied in the treatment of CD fistulas, the 2 sources have never been directly compared to each other[743]. Then, we need additional information to be able to choose an optimal healthy donor, as the proliferation and viability of MSCs may vary from donor to another[743,774]. We need to know more about the behaviour of these MSCs once injected into the stricture lesion and with that the optimal frequency of administration[743]. Beyond this, the ex vivo modulation of these MSCs before therapeutic use could be promising and will be discussed in the general perspectives.

# GENERAL PERSPECTIVES

## 5. GENERAL PERSPECTIVES

To date, there is no medication to prevent or reverse intestinal fibrosis and to find an effective anti-fibrotic agent in CD is a global challenge. The epithelium, which is at the interface between the host and the "outside" lumen, and which is the first to encounter many diet toxins and bacterial agents, probably plays a key role. The results of our research showed that epithelial ER stress was probably an important process in the generation of CD stricture. This ER stress could contribute to fibrosis through a variety of mechanisms that we mentioned earlier such as the PDIs secretion (including AGR2), possibly other paracrine pro-fibrosis factors secretion (which remain to be further investigated) but also by inducing EMT in these epithelial cells, contributing to epithelial barrier disruption. Interestingly, MSCs, for which we have shown the feasibility of local injection in CD stricture, have a known series of actions on the epithelium[311,697,712,714,775–777]. As a next step to the work carried out on these two cell types, investigate further the links between epithelial cells, mesenchymal stem cells and intestinal fibrosis (particularly the way by which MSCs may modulate epithelial abnormalities found in intestinal fibrosis) could be an interesting research prospect for the future.

*What is known about the role of MSCs on the intestinal epithelial cells?*

Several studies have shown that MSCs exert their role on target cells through differentiation but mainly by the secretion of a range of extracellular vesicles (EVs) and soluble factors[778,779]. Indeed, once transplanted, these MSCs go to the lesion site by homing[780] and then deliver these EVs and soluble factors by fusion with the cells or by the formation of tunnelling nanotubes[778,781]. These EVs form heterogenous population of small vesicles (30 nm to 5 µm) which can be divided into exosomes (30-150 nm), microvesicles or MVs (100-1000 nm) and apoptotic bodies (1000-5000)[782] and which transfer bioactive molecules (including nucleic acids such as mRNA, microRNA, siRNA and DNA but also proteins, lipids and other bioactive molecules)[782,783] involved in intercellular communication. Most of the effects of MSCs on epithelial cells depend on their paracrine action[305,784] and in order to be able, in the future, to counteract the epithelial abnormalities found in intestinal fibrosis, by MSCs, we looked at the existing data on the subject.

Regarding the impact of MSCs on **intestinal epithelial ER stress and PDIs**, it was shown that the systemic administration of umbilical cord mesenchymal stem cells (UCMSCs) in a DSS-induced colitis experimental mouse model (in which ER stress plays a crucial role) led to a

significant reduction in the BiP and PDIs expression that was comparable with controls[581]. The use of UCMSCs allowed to reduce the degree of collagen deposition in both the mucosa and the submucosa compared to DSS + phosphate-buffered saline mice (which therefore did not benefit from UCMSCs)[581]. However, this study did not allow to establish a link between the attenuation of collagen deposition and the attenuation of ER stress. The mechanism by which MSCs attenuated the intestinal epithelial ER stress has not been studied[581]. In a bleomycin induced-murine model of lung fibrosis, the systemic administration of MSCs attenuated ER stress of alveolar epithelial cells via the PERK-Nrf2 pathway[785]. In addition, in other conditions than fibrotic disease, MSCs have been shown to have an impact on the ER stress attenuation of several cell types and through various mechanisms such as the release of EVs on the ER stress in cardiac cells[786] or in human corneal endothelial cells[787], the release of exosomes (containing miR-21) on the ER stress in pancreatic beta cells[788] but also through the secretion of specific factors such as tumor necrosis factor α-induced gene/protein 6 or TSG-6 (which attenuated ER stress in pancreatic acinar cells[789]) or stannioncalcin-1 or STC-1 (which attenuated ER stress in alveolar epithelial cells[790])[788]. The use of STC-1 derived from MSCs in the case of endothelial lipotoxicity induced by palmitic acid, allowed, in addition to alleviating the ER stress, to reduce endo-MT[791].

Regarding the impact of MSCs on **intestinal EMT**, Lian et al. demonstrated in a mouse model of intestinal fibrosis (induced by 7 weeks of TNBS infusions) that prophylactic MSCs treatment (intraperitoneally injected 24 h before each TNBS infusion) inhibited EMT and therapeutic MSCs treatment (administered every day for 7 days after TNBS infusions) reversed EMT but they did not investigate how[772]. Another group (Yang et al.) demonstrated that a microRNA derived from MSCs (miR-200b), transferred into MVs, were able to play an anti-fibrosis role by inhibiting EMT, via an action on *ZEB1* and *ZEB2* (2 EMT-orchestrating transcription factors)[305]. Indeed, they transfected BM-MSCs with a lentivirus to overexpress mi-R200b and then harvested the MVs that they used as an anti-fibrosis treatment. In vitro, the application of these miR-200b-containing microvesicles (miR-200b-MVs) to intestinal epithelial cells (IEC-6), increased the level of miR-200b in these cells and inhibited TGF-β1 mediated EMT[305]. In vivo, the application of miR-200b-MVs in a TNBS-induced intestinal fibrosis in rats, increased the level of miR-200b in colon, prevented EMT and improved the TNBS-induced colon fibrosis histologically[305]. In addition, this inhibition of EMT by these miR-200b has also been demonstrated in other organs (such as lung fibrosis[792,793] and tubulointerstitial fibrosis[794]).

Besides this role on ER stress and EMT, MSCs are known to **prevent and repair intestinal epithelial damages**[311,697,712,714,775–777]. MSCs alleviate epithelial damage by preventing the depletion of IECs and mucin-producing goblet cells in TNBS-induced colitis[697] or DSS-induced colitis[776]. MSCs also promoted the repair of epithelial damages by promoting proliferation of IECs[712] (notably by the miR-200b fraction of these MSCs[775]), by promoting differentiation of intestinal stem cells[712], by decreasing paracellular permeability of the epithelial barrier by restoring claudin-2, claudin-12 and claudin-15 expression in DSS-induced colitis in rats[311,714], by enhancing the formation of MUC 5AC in DSS (known to reduce MUC 5AC expression) treated mice (through EVs from MSCs conditioned with a cytokine cocktail)[777].

*Proposed future directions*

As we performed, in our phase I/II pilot study, biopsies (in stricture lesions and in the surrounding healthy tissue) before the MSCs injection, as well as at weeks 12 and 48, one of the first things we could do, is to study the surface epithelium proteome changes induced by MSCs. We could compare these changes in responders versus non-responders, to see if these MSCs had an impact on the epithelium, especially on epithelial ER stress, PDIs expression (including AGR2) and EMT process.

Secondly, we would like to modify these MSCs, by ex vivo strategies, to improve their specific effects on the epithelial abnormalities observed in intestinal fibrosis (and thus improve their attenuating effects on ER stress, PDIs expression and EMT) but also to improve their anti-fibrotic effects. As mentioned earlier, MSCs mainly exert their effects through a paracrine action[777,795–797], especially via the secretion of EVs[798–800]. It has been shown that these mesenchymal stem cell-derived extracellular vesicles (MSCs-EVs) had the immunomodulatory and regenerative capacities as well as therapeutic activities of their parent cells on their own[777,801,802]. Recently, several studies even indicated that the effects of MSCs-EVs were probably superior to their parents cells, with a better safety profile and can be stored without losing their function[777,778,802,803]. These data led the researchers to use these EVs as therapeutic tools, after changing their composition (by induced genetic changes in MSCs) in order to enhance specific effects on target cells[804–807].

Depending on the preventive effect (targeting, for example, the epithelial abnormalities present in fibrosis) or the therapeutic effect (targeting rather the ECM degradation, for example) that

we want to give to these MSCs-EVs in fibrosis, different modifications of the composition of these EVs could be studied and induced. Different methods are available to genetically modify these MSCs such as retroviral transduction, lentiviral-based micro-RNA, or inductible lentiviral[808]. To improve epithelial anomalies found in intestinal fibrosis, the induction of TSG-6 secretion[789], the transfer of miR-200b (to attenuate EMT)[305,792–794] or miR-21 (to attenuate ER stress)[788] from genetically modified MSCs (through their EVs) on epithelial cells should be studied. To improve the anti-fibrotic effects of these MSCs-EVs, several genetic modifications have been tested in fibrosis of other organs and could be studied in intestinal fibrosis (see Figure 49 for example)[778]. In addition, IL-10 mRNA engineered MSCs[809] or the overexpression of HGF in MSCs[306] could improve the anti-fibrotic action of MSCs-EVs. Finally, the induced modifications could also target the viability and proliferation of epithelial cells or promote the immunomodulatory properties of MSCs[311,810–815].

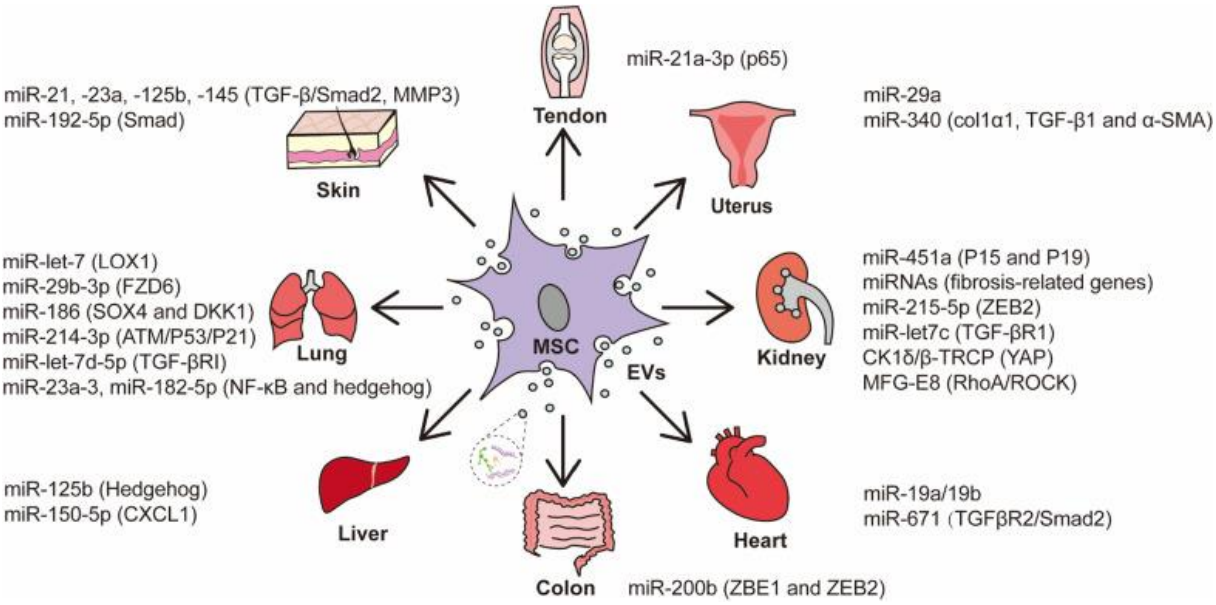


**Figure 49. MSC-EVs can exert an anti-fibrosis effect through all kinds of mechanisms**[305,778,816–838]

TGF-β1 (transforming growth factor β1)/Smad pathway[818–820,823,837,839,840], TGF-βR1 (transforming growth factor β receptor 1)[836,840], Hedgehog[816,817,841], α-SMA[823,824,842], ZEB2[305,827], Dickkopf-related protein 1 (DKK1)[843–845] are relatively well-known targets of miR. ATM, ataxia telangiectasia mutated; CXCL1, C-X-C motif chemokine ligand 10; DKK1, Dickkopf-1; EVs, extracellular vesicles; FZD6, frizzled 6; LOX1, lectin-like oxidized low-density lipoprotein receptor-1; MFG-E8, milk fat globule-epidermal growth factor-factor 8; MMP3, matrix metallopeptidase 3; MSCs, mesenchymal stem cells; SOX4, SRY-related HMG box transcription factor 4; YAP, Yes-associated protein

After genetic modifications of these MSCs, one of the first things to do will be to check that these cells retain the MSCs characteristics such as spindle-shaped aspect, but also that they

preserve cell surface marker profile or surfactome (such as a positivity for classical mesenchymal markers and the absence of hematopoietic markers) by flow cytometric analysis[681]. Then, it will be necessary to compare the over-expression of the different genes that we wanted to overexpress in modified-EVs compared to a control condition or null-EVs (by RT-qPCR for example).

Next, we could test the impact of these modified EVs on an epithelial cell line model. The application of these modified MVs and null-MVs on control IECs, IECs with induced ER-stress and IECs with induced-EMT could allow us to verify the impact of these modified-EVs on epithelial changes observed in intestinal fibrosis. First, it will be necessary to confirm the integration of these modified-EVs in target cells (compared to cells having received null-EVs) by assessing the expression of upregulated mi-R, for example, by PCR or fluorescence microscopy. Then, we could evaluate the expression of ER stress markers, PDIs and EMT markers (by WB, RT-qPCR or IF) in IECs treated with modified versus null-EVs. The impact of these modified-EVS on epithelial cells proliferation and apoptosis could also be assessed. Finally, as we are also familiar with the model of fibroblast to myofibroblast differentiation, the effect of these modified EVs on this myofibroblastic differentiation model could also be tested[818].

Finally, the impact of the application of these modified-EVs versus null-EVs on animal models or organoids with induced fibrosis could be studied. After checking that the EVs were well incorporated into the target cells, both the macroscopic changes (such as the evolution of a visible strictures with proximal dilatation), body weight, colon length, colon weight, but also microscopic changes should be assessed. Light-field microscopic analysis of the evolution of fibrosis under PBS (as control condition), modified EVs-group and null-EVs group should be performed using hematoxylin and eosin (H&E) as well as Masson's trichrome-stained colon sections tissues[305]. Finally, epithelial changes and ER stress, PDIs and EMT markers could be assessed by the same techniques as mentioned above.

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# 6. REFERENCES


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# APPENDICES

## 7. APPENDICES

**Appendice 1.** Potential Role of Epithelial Endoplasmic Reticulum Stress and Anterior Gradient Protein 2 Homolog in Crohn's Disease Fibrosis
**Appendice 2.** Supplemental methods of Potential Role of Epithelial Endoplasmic Reticulum Stress and Anterior Gradient Protein 2 Homolog in Crohn's Disease Fibrosis
**Appendice 3.** Mesenchymal Stem Cell Injection in Crohn's Disease Strictures: a phase I-II clinical study