UNIVERSITÀ DEGLI STUDI DI SALERNO

Department of Chemistry and Biology "A. Zambelli"

Ph.D. in Chemistry

XXXIV cycle

MOLECULAR BASES OF GLUTEN TOXICITY AND RELATIONSHIP WITH THE ENZYME TRANSGLUTAMINASE IN CELIAC DISEASE

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ACADEMIC YEAR 2020/2021

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ABSTRACT

Celiac disease (CD) is a life-long gluten-sensitive immune-mediated disorder that primarily affects the small intestine of genetically susceptible individuals worldwide. Type 2 transglutaminase (TG2) has two crucial roles in CD pathogenesis: as a deamidating enzyme, of crucial importance in enhancing gluten immunogenicity, and as a target autoantigen in the immune response. The presence of constitutive alterations in CD cells compared to non-CD ones has recently led to the definition of the so-called "celiac cellular phenotype", that may represent a predisposing condition to the damaging effects of gluten. TG2, and particularly anti-TG2 autoantibodies, also contributes to this phenotype. Indeed, anti-TG2 autoantibodies, by forming complexes with cell-surface TG2, specifically derange the uptake of the toxic α-gliadin peptide 31-43 by non-CD cells but not by CD ones. In this PhD thesis work, differences in TG2 subcellular distribution in CD and non-CD cells were investigated to determine how TG2 may be able to contribute to the different handling of p31-43 by the two groups of cells. Then, in the attempt to identify other constitutive differences regarding TG2 in CD and non-CD cells, it was investigated whether p31-43 differentially modulated TG2 expression and activity in fibroblasts from the two groups of subjects. The data obtained showed that TG2 was associated with the cell surface membrane, the early endosomal compartment and the autophagic compartment more in CD cells than in non-CD ones. The data also showed the p31-43 differently affected TG2 expression and activity in CD and non-CD cells, activating TG2 more in non-CD cells than in CD ones and inducing TG2 expression in CD cells but not in non-CD ones. These findings support the idea that TG2 localization inside CD cells contributes to defining the "celiac cellular phenotype", thus having an important but still undefined role in CD pathogenesis. Subsequently, it was investigated whether regulation of intracellular Ca^{2+} homeostasis regarding the endoplasmic reticulum (ER) was different in CD and non-CD cells. Relatedly, it was analyzed how CD and non-CD cells responded to stimulation with thapsigargin (THP), an ER-stress and autophagy inducer, also focusing the attention on TG2 modulation. The data obtained showed that, in CD cells, intracellular Ca^{2+} homeostasis was deregulated, thus causing both a strong Unfolding Protein Response (UPR) and an engulfed autophagy in response to stimulation with THP. Interestingly, p31-43 mobilized Ca^{2+} from intracellular stores more in CD cells than in non-CD ones; however, the p31-43-induced increase in Ca^{2+} only partially activated TG2. Overall, this PhD thesis work adds a small piece of knowledge about the complex interplay between gluten peptides and TG2 in the pathogenetic mechanisms of CD.

CHAPTER 1. INTRODUCTION

1.1 Celiac Disease

Coeliac disease or celiac disease (CD) is a life-long gluten-sensitive immune-mediated disorder that primarily affects the small intestine of genetically susceptible individuals worldwide [Caio et al. 2019]. Genetic and environmental factors have a critical role in CD. The main genetic risk factors are related to the presence of the Human Leukocyte Antigens (HLA) genes and, in particular, the HLA-DQ2 and HLA-DQ8 haplotypes. The main environmental risk factor is the gluten consumption, which can trigger immunological responses that result in intestinal inflammation and tissue damage in individuals genetically predisposed to CD. In CD, damage to the small-intestinal mucosa is induced by both adaptive and innate immune responses to ingested gluten [Koning et al. 2005]. Typical gluten-induced small-intestinal lesion includes various degrees of crypt cell hyperplasia and villous atrophy, as well as increased lymphocytic infiltration of the epithelium [Sollid et al. 2000] and increased density of various leukocytes in the lamina propria, with consequent global intestinal dysfunction. Elimination of gluten from the diet results in clinical improvement and the small-intestinal mucosa heals without scarring; however, the disease will relapse if gluten is reintroduced into the diet. The role of environmental causes other than gluten in the development of CD is far more obscure. Infant feeding habits as a risk factor for the disorder have been scrutinized for decades. It has been suggested that prolonged breast feeding, breast feeding during the introduction of dietary gluten and introducing gluten to infants between 4 and 6 months of age are associated with a reduced risk of CD [Akobeng et al. 2006; Silano et al. 2010]; however, according to recent evidence, no specific general recommendations concerning gluten introduction or breast feeding can be made with a view to lowering the risk [Vriezinga et al. 2014; Silano et al. 2016]. Also, early life infections [Mårild et al. 2015] and gastrointestinal infections, such as rotavirus infections [Stene et al. 2006], have been suggested to serve as additional triggers for CD development. In contrast, there is a possibility that clinical or subclinical infections by Epstein-Barr virus, cytomegalovirus and rubella might have a protective effect on the emergence of CD [Plot et al. 2009], a conception well in keeping with the finding that the prevalence of the disease is lower in areas with inferior prosperity and standard hygiene [Kondrashova et al. 2008].

1.1.1 Epidemiology

Previously thought to affect only (or predominantly) Northern and Western Europe people, CD is now recognized to be widely distributed worldwide. Epidemiological studies conducted in areas considered to be CD-free, such as South America, North Africa and Asia, have shown that most cases of CD were previously underdiagnosed [Lebwohl et al. 2021], probably due to the lack of typical gastrointestinal symptoms (i.e., chronic diarrhea and weight loss) and/or poor disease awareness [Caio et al. 2019]. Worldwide, indeed, CD "out of the intestine" is 15 times more frequent than CD "in the intestine", making the diagnosis extremely challenging. The prevalence (i.e., the number of affected persons, including subclinical cases, in a defined population at a certain point) of CD in the general population in different world areas is approximately 0.5-1%, with the exception of areas showing low frequency of CD-predisposing genes and low gluten consumption (e.g., sub-Saharan Africa and Japan) [Gujral et al. 2012; Ivarsson et al. 1999; Riestra et al. 2000; Volta et al. 2001; Mustalahti et al. 2010; Rubio-Tapia et al. 2012; Singh et al. 2016]. However, there is accumulating evidence that, beyond increased disease awareness, the incidence (i.e., the number of "new" diagnoses in the study population during a certain period) of CD has increased, which is affecting the prevalence of the disease [Lebwohl et al. 2021]. CD commonly appears in early childhood [Andrén Aronsson et al. 2019] but it can also develop in geriatric populations; such diagnoses may be due to a late discovery of longstanding CD or result from de novo loss of tolerance to gluten [Collin et al. 2018]. Also, the incidence of CD is higher in women than men, but this might be because men are more likely to remain undiagnosed; indeed, men are less likely to undergo duodenal biopsy examination during upper endoscopy for indications such as diarrhea and weight loss, which might contribute to underdiagnosis [Lebwohl et al. 2021; Gujral et al. 2012]. In addition, the prevalence of CD is higher in certain groups of the population, including first- and second-degree relatives of people with CD [Nellikkal et al. 2019] and patients with Down syndrome, type 1 diabetes, selective IgA deficiency, autoimmune thyroiditis, Turner syndrome, Williams syndrome and juvenile chronic arthritis [Fasano et al. 2003; Singh et al. 2015].

1.1.2 Clinical presentation

Clinical manifestations of CD vary markedly with the age of the patient, the duration and extent of disease and the presence of extra-intestinal pathologies. Intestinal symptoms commonly include abdominal pain, followed by diarrhea (that can be chronic or intermittent), failure to thrive, loss of appetite, abdominal distention and loss of weight [Vivas et al. 2008]. Although mainly detected in the pediatric population and children younger than 3 years, they can also occur in older children and adults [Reilly et al. 2011]. Extra-intestinal symptoms are common in both children and adults. They

include iron deficiency with or without anemia, reduced bone mineral density [Kamycheva et al. 2017], tooth enamel defects, aphthous stomatitis [Krzywicka et al. 2014] and hypertransaminasemia, which can be ascribed to food and bacterial antigen translocation reaching the liver due to increased intestinal permeability [Volta et al. 1998]. Clinical manifestations of CD may also include changes in reproductive function characterized by late menarche, amenorrhea, recurrent miscarriages, premature birth, early menopause and changes in the number and mobility of spermatozoa. In addition, a wide array of neurological symptoms, such as headache, paresthesia, neuroinflammation, anxiety, irritability and tendency to depression, can be detectable in CD patients.

1.1.3 Diagnosis

Currently, five criteria are useful for CD diagnosis: (1) presence of typical signs and symptoms (e.g., diarrhea and malabsorption), (2) detection of celiac-specific antibodies in serum, (3) HLA-DQ2/DQ8 positivity, (4) presence of intestinal damage (i.e., villous atrophy and minor lesions) and (5) clinical response to gluten-free diet (GFD) [Fousekis et al. 2020]. Mucosal changes detected by duodenal biopsy historically represent the gold standard for CD diagnosis though highly sensitive and specific serologic tests are available for diagnostic screening and work-up. Serum IgA antibodies to the autoantigen type 2 transglutaminase (TG2) are increased in CD patients on a gluten-containing diet [Dieterich et al. 1997] while they slowly disappear from the circulation when gluten is eliminated from the diet [Rostom et al. 2005]; for this reason, testing for anti-TG2 antibodies constitutes the first-line screening procedure in older children and adults [Rostom et al. 2005]. Other serologic tests evaluate serological levels of IgA anti-endomysial antibodies (EMA) and anti-deamidated gliadin peptide (DGP) antibodies [Mäki et al. 1995]. Patients with IgA deficiency can be tested with IgG anti-TG2, anti-EMA and/or anti-DGP serologic tests. Genetic testing for HLA susceptibility is also included in the work-up of CD and to determine risk in family members. However, despite the progress made in serology, at present, no antibody test available provides a sensitivity and specificity of 100% [Volta et al. 2010], therefore, the final diagnosis of CD rests on the demonstration of typical mucosal damage by histological examination of small-intestinal biopsies [Caio et al. 2012].

1.1.4 Treatment

At present, the only effective therapy available for CD individuals is a strict and life-long elimination of gluten from the diet. Indeed, maintenance of GFD leads to the gradual disappearance of intestinal and/or extra-intestinal symptoms, the negativity of serum celiac autoantibodies and the

regrowth of the intestinal villi [Caio et al. 2019]; however, intestinal damage recurs if gluten is reintroduced into the diet because immunologic intolerance to gluten does not go away. Conversely, poor diet compliance by patients and undiscovered disease are associated with increased mortality [Rubio-Tapia et al. 2009] and risk for intestinal lymphoma [Catassi et al. 2005]. Although a wide range of gluten-free foods are specifically manufactured for patients with CD, the consumption of some nutrients, particularly fibers, iron, calcium and folate, tends to be lower than normal in patients who adhere to GFD. Moreover, although considered safe and effective, life-long elimination of gluten from the diet has some disadvantages, including negative effects at a social level, psychological problems, fear of involuntary/inadvertent contamination with gluten, possible vitamin and mineral deficiencies, metabolic syndrome, an increased cardiovascular risk and often severe constipation [West et al. 2004; Hallert et al. 2002; Midhagen et al. 2003]. Also, in a minority of adult patients, CD does not respond to treatment with GFD. The most likely cause of non-responsiveness is continued gluten ingestion, which can be voluntary or inadvertent; other causes of non-responsiveness include other food intolerance diseases (e.g., milk, soya), pancreatic insufficiency, enteropathy-associated T-cell lymphoma, refractory sprue and ulcerative jejunitis [Fasano et al. 2001]. In addition, about 40% of CD patients are unsatisfied with their alimentary regimen and they would be keen to explore alternative treatments [Aziz et al. 2011]. For all these reasons, currently, there is an active area of research that focuses on the development of alternative treatments for CD. For example, attempts to render gluten non-toxic have been made. Unfortunately, this approach has been less appealing due to loss of baking characteristic, public refusal for genetically modified crops, contamination of genetically modified crops with gluten contained crops grown nearby and heterogeneous uncharacterised immunostimulatory epitopes in gluten and difference among patients response to epitopes and gluten levels [Donnelly et al. 2011]. Other approaches have involved the use of larazotide acetate, a synthetic hexapeptide that has been shown to be efficacy in gluten-related symptom control [Leffler et al. 2015], and ALV003, a mix of two gluten-specific proteases [Lähdeaho et al. 2014] that targets gluten and degrades it into small non-immunogenic fragments in the stomach before they pass into the duodenum; however, both strategies have been demonstrated to be effective in presence of only small amounts of gluten. Further potential treatments of CD currently under investigation are specific vaccines able to modulate gluten-specific T cells [Goel et al. 2017], or monoclonal antibodies with the capacity to block pro-inflammatory cytokines such as interleukin-15 (IL-15) and/or interferon (INF)-γ [Castillo et al. 2015].

1.2 Genetics

CD has a strong hereditary component. Epidemiological studies show that 7.5%-15% of first-degree relatives of individuals with CD are also affected by CD [Wessels et al. 2018, Singh et al. 2015], with concordance rates of 50%-80% in monozygotic twins and 10% in dizygotic twins [Nisticò et al. 2006; Kuja-Halkola et al. 2016]. CD occurs only in genetically predisposed individuals. Susceptibility to CD is strongly associated with the presence of particular haplotypes of the HLA system of class II known as HLA-DQ2 and HLA-DQ8. HLA genes are polymorphic genes located in a gene cluster called the Major Histocompatibility Complex (MHC) on chromosome 6p21.3; this region is known to contain hundreds of genes with immunological functions and to be responsible for the strongest association signals observed in most immune-mediated diseases [Dieli-Crimi et al. 2015]. Genome-wide association studies have identified more than 100 non-HLA-related genes associated with CD; however, the relevance of these additional genes in conferring genetic risk for CD is rather limited [Romanos et al. 2009; Hunt et al. 2008; Romanos et al. 2014]. More than 99% of people with CD are carriers of HLA-DQ2 and/or -DQ8 haplotypes [Hadithi et al. 2007]. The absence of both genes has a high negative predictive value and rules out the disease [Pietzak et al 2009]. However, only approximately 3% of DQ2- and/or DQ8-positive individuals develop CD [Megiorni et al. 2009]; therefore, the presence of HLA-DQ2 and -DQ8 haplotypes is necessary but not sufficient for the development of CD, suggesting that additional genetic and/or environmental factors are involved in the disease pathogenesis [Brown et al. 2019].

1.2.1 HLA-DQ2 and HLA-DQ8 in celiac disease pathogenesis

HLA-DQ2 and HLA-DQ8 genes encode for the MHC class II cell surface proteins normally expressed on the surface of the Antigen-Presenting cells (APCs) such as dendritic cells, macrophages and B cells; however, MHC II proteins can be conditionally expressed by all cell types, including enterocytes expressing HLA class II molecules. MHC II proteins are essential to activate the adaptive immune system. Indeed, they are responsible for the presentation of antigens (derived from self-proteins or from pathogens) by the APCs to appropriate T cells. This event leads to the activation of T cells. HLA-DQ2 and -DQ8 proteins play a crucial role in CD pathogenesis. Indeed, they bind with high affinity to gluten peptide epitopes and promote their presentation to CD4+ T lymphocytes, thus initiating inflammation [Jabri et al. 2006]. Gluten peptides immunogenicity is strongly enhanced after a post-translational modification catalyzed by TG2, a ubiquitous enzyme the expression of which increases in the inflamed CD intestinal mucosa [Esposito et al. 2003]. TG2 deamidation of specific glutamines of gluten peptides, indeed, increase

the gluten peptides affinity for HLA-DQ2/8 molecules, thus making gluten peptides presentation to T cells more efficient [Sollid et al. 2011]. HLA-DQ2 and -DQ8 molecules have a different set of gluten peptide epitopes that they are able to bind, based on the amino acid sequence present at their peptide-binding region. In particular, HLA-DQ2 is better suited than HLA-DQ8 to bind gluten peptide antigens; this may be the reason why HLA-DQ2 is a stronger susceptibility determinant for CD than HLA-DQ8 [Jabri et al. 2009].

1.3 Gluten

Gluten is a heterogeneous mixture of storage proteins found in wheat grains. Although, strictly speaking, the term "gluten" pertains only to wheat storage proteins, it is also used to refer to similar storage proteins found in any species of wheat, barley, rye and oat, as well as in any their derivatives (e.g., triticale and malt) and any other ancient wheat varieties (e.g., spelt and kamut). Gluten found in all of these grains has been identified as the component capable of triggering CD [Biesiekierski et al. 2017].

1.3.1 Structure and sources

Traditionally, wheat gluten proteins have been classified into two main fractions according to their solubility in aqueous alcohols: gliadins, the soluble fraction, and glutenins, the insoluble fraction. Gliadins are mainly monomeric proteins and, in turn, can be classified according to their different primary structures into the α/β-, γ- and ω-gliadins; usually, α/β- and γ-gliadins are in higher proportion than ω-gliadins. Glutenins are aggregated proteins linked by inter-chain disulphide bonds. Based on their primary structure, glutenins have been divided into high-molecular-weight (HMW) subunits and low-molecular-weight (LMW) subunits. Native glutenins are composed of a backbone formed by HMW subunit polymers and of LMW subunit polymers branched off from HMW subunits. Non-covalent bonds such as hydrogen bonds, ionic bonds and hydrophobic bonds are important for the aggregation of gliadins and glutenins and implicate structure and physical properties of gluten [Wieser et al. 2007]. At the level of their primary structure, the gliadin and glutenin proteins are considered prolamins, as they contain high amounts of glutamine (35%-38%) and proline (15%-20%) residues [Wieser et al. 2007]. The high percentage of proline residues makes gluten proteins resistant to degradation by gastric, pancreatic and intestinal digestive proteases in the gastrointestinal tract; thus, several long undigested gluten peptide fragments can

reach high concentration levels in the small-intestinal lumen [Hausch et al. 2002], where they can mediate adverse immune reactions in CD patients [Biesiekierski et al. 2017].

1.3.2 Gluten peptides

Some gluten peptides are toxic for CD patients while others are immunogenic. Toxic peptides (e.g., the α-gliadin peptide 31-43) exert direct damage to the intestinal mucosa of CD patients and can activate innate immune mechanisms [Maiuri et al. 2003]. Immunogenic peptides (e.g., the α-gliadin peptide 57-68) contain short repeated motifs (termed "epitopes") that are recognized by HLA-DQ2 and/or -DQ8 proteins on APCs and, after passing the intestinal epithelial border, trigger a dysregulated adaptive immune response at gut level dominated by CD4+ T lymphocytes of the T helper 1 (Th1) type. Damages to the intestinal mucosa of CD patients are the consequence of sustained mucosal inflammation due to both the T-cell response to immunogenic gluten peptides and the innate immune response to toxic gluten peptides. A variety of sequences from gliadin proteins, as well as from glutenin proteins, have been identified to be active in CD [Gianfrani et al. 2005]. Both gliadins and glutenins are toxic/immunogenic for CD patients; however, gliadins represent the main toxic/immunogenic component of gluten, and a very high number of gliadin peptides, mainly deriving from α- and γ-gliadins, have been reported to stimulate HLA-DQ2/8 restricted CD4+ T lymphocytes isolated from the small-intestinal mucosa of CD patients [Sollid et al. 2000].

1.3.3 Gluten peptides and the adaptive/innate immune response

A lot of T-cell stimulatory epitope-containing gluten peptides in native or deamidated form have been identified. Among these, the α-gliadin peptide 57-68 (p57-68) belonging to the undigested fragment named 33-mer (sequence from 57 to 89) is considered to be the most immunogenic sequence in CD; for this reason, p57-68 has been extensively studied as T-cell epitope model for studying the adaptive immune response in CD [Maiuri et al. 2003]. Immunogenic peptides such as p57-68 play a key role in the pathogenic mechanism of CD. Indeed, their presentation by HLA-DQ2 and/or -DQ8 molecules on APCs to CD4+ T lymphocytes induces a strong activation of the adaptive immune response in CD patients [Barone et al. 2014; Qiao et al. 2004] with the release of pro-inflammatory cytokines (mainly (INF)-γ). Such response initiates a cascade of biological effects leading to intestinal inflammation, enteropathy and villus atrophy, which can produce profound damage in the intestinal tissue, with severe negative consequences for absorption and barrier functions [Sollid et al. 2002]. The whole immune response to gluten is strongly enhanced by TG2-catalyzed post-translational deamidation of specific glutamine residues of gluten peptides. Indeed,

TG2-catalyzed deamidation introduces negative charges on gluten peptides at specific position that make gluten peptides recognition by HLA-DQ2 and/or -DQ8 molecules more efficient [Sollid et al. 2011]. Another important digestion-resistant α-gliadin peptide is 25-mer (sequence from 31 to 55) and, in particular, its shorter fragment 31-43/49 (p31-43). Non-immunogenic for T cells and not deamidated by TG2, p31-43 is considered to be the main peptide responsible for triggering a cascade of biological effects leading to both cell stress and innate immune response [Jabri et al. 2006], with IL-15 as a major mediator [Barone et al. 2014]. Cell structure alterations (altered cell shape and actin organization, increased permeability and altered vesicular trafficking [Clemente et al. 2003; Ménard et al. 2012; Nanayakkara et al. 2013], signaling/proliferation [Barone et al. 2007] and stress/innate immunity activation [Rivabene et al. 1999] are some of the biological non-T-cell mediated effects induced by p31-43 [Barone et al. 2014], which, in the presence of appropriate genetic susceptibility and environmental factors, may act together to drive CD. Indeed, although structural changes of the CD mucosa are considered a consequence of sustained mucosal inflammation due to the adaptive immune response, recent data have shown that p31-43 is able to induce proliferation of CD enterocytes: this process, IL-15 and epithelial growth factor (EGF) dependent, has profound upstream effects in inducing the crypt hyperplasia, which is characteristic of the remodeling of the CD mucosa [Barone et al. 2007]. Nevertheless, the mechanism by which p31-43 enters cells is not fully understood. No membrane receptor has been identified for this peptide [Paolella et al. 2018], therefore, it is unknown if and how it can be recognized by the sensors of the innate immune system. However, recent studies indicate that it may be internalized into early endosomes after direct interaction with the cell membrane [Barone et al. 2016]. In addition, there is increasing evidence that the initial innate immune response is necessary to trigger the local inflammation that drives adaptive immunity. Indeed, recent studies have shown that the IL-15 overexpression induced by p31-43 promotes an innate immune response that, in turn, promotes the CD4+ T lymphocytes adaptive immune response [Maiuri et al. 2003].

1.4 Type 2 Transglutaminase in celiac disease

TG2 (or cytosolic transglutaminase or liver transglutaminase) has two crucial roles in CD pathogenesis: as a deamidating enzyme, of crucial importance in enhancing gluten immunogenicity, and as a target autoantigen in the immune response.

1.4.1 General introduction to the transglutaminase protein family

Transglutaminases (TGs) are a family of structurally and functionally related proteins [Laszlo Lorand et al. 2003] that catalyze post-translational modifications of target proteins. Widely distributed in animals, plants and microorganisms, members of this family mainly catalyze the Ca^{2+}-dependent cross-linking reaction (transamidation) between various primary amines (most commonly the ε-amino group of protein- or peptide-bound lysine residues) and the γ-carboxamide group of protein- or peptide-bound glutamine residues [Laszlo Lorand et al. 2003]. In addition to the protein cross-linking, TGs catalyze post-translational modifications of proteins also via deamidation and amines incorporation. Members of this family also possess non-catalytic activities. By virtue of their both catalytic and non-catalytic activities, TGs serve as scaffolds, maintain membrane integrity, regulate cell adhesion and modulate signal transduction [Eckert et al. 2014]. In humans, nine distinct TGs isoenzymes have been identified at the genomic level; eight are catalytically active enzymes and one is inactive (i.e., the erythrocyte membrane protein band 4.2), which displays a scaffolding function in the erythrocyte membrane [Grenard et al. 2001]. Even though each type of TG has its own typical tissue distribution, individual enzymes are present in a number of different tissues and often in combination with other TGs.

1.4.2 Enzymatic and non-enzymatic functions of TG2

TG2 is the most widely distributed and extensively studied member of the human TGs. It is an 80-kDa protein ubiquitously expressed in almost all tissues and organs - for this reason it is also named "tissue" TG (tTG). Indeed, TG2 can be found both in the intracellular and the extracellular space of various types of tissues and it is present in many different organs, including the heart, the liver and the small intestine. Moreover, it is a multifunctional protein whose specific functions are strictly depending not only on its exact cellular localization (e.g., nucleus, cytosol, mitochondria, membrane inner face, cell surface, extracellular environment, etc.) and availability of its specific substrates [Esposito et al. 2005; Facchiano et al. 2009] but also on local concentrations of its activators/modulators (i.e., Ca^{2+}/guanine nucleotides/protein interactors), environmental redox conditions and, of course, which enzymatic or non-enzymatic function is eventually involved [Eckert et al 2014]. Like other members of the TGs family, the main and best-characterized catalytic activity of TG2 is the Ca^{2+}-dependent protein transamidation. More than one hundred of TG2 enzymatic substrates have been identified in a variety of cellular compartments, including cytosol, nucleus and mitochondria, as well as on the cell surface and in the extracellular matrix (ECM) [Laszlo Lorand et al. 2003]. Therefore, this enzymatic activity enables TG2 to generate an immense array of post-translational modifications in target proteins. The TG2 transamidation

activity consists of the Ca^{2+}-dependent formation of an intra- or inter-molecular N^{ε}-(γ-glutamyl)-lysine isopeptide bond between the γ-carboxamide group of a protein- or peptide-bound glutamine residue (glutamine donor) and the ε-amino group of a protein- or peptide-bound lysine residue (glutamine acceptor) [Laszlo Lorand et al. 2003]; both aminoacids can belong to the same intra- or extra-cellular protein or to different intra- or extra-cellular proteins [Laszlo Lorand et al. 2003] (Figure 1). Several polyamines can also act as glutamine acceptors and their covalent association with protein substrates of TG2 contributes to modulate functions and immunogenicity of these proteins [Lai et al. 2017] (Figure 1). The resulting cross-linked products in many cases have high molecular masses and are unusually resistant to proteolytic degradation and mechanical strain. Thus, they are of functional significance in tissues and processes in which these properties are important, for example extracellular matrix stabilization, apoptosis, blood clotting and wound healing [Laszlo Lorand et al. 2003]. The catalytic mechanism of the transamidation reaction involves the thiol group from a Cys residue in the active site of the enzyme, which attacks the γ-carboxamide group of the glutamine residue, releasing ammonia and forming a thioester intermediate. This enzyme-substrate intermediate then reacts with the primary amine group of the lysine residue or others polyamines, releasing the enzyme and giving the transamidated product [Folk et al. 1983]. Alternatively, in the absence of available amines and at a slightly acidic pH, the thioester intermediate can be hydrolysed, resulting in the conversion of the glutamine residue into the negatively charged glutamic acid, in a process termed deamidation [Stamnaes et al. 2008] (Figure 1).

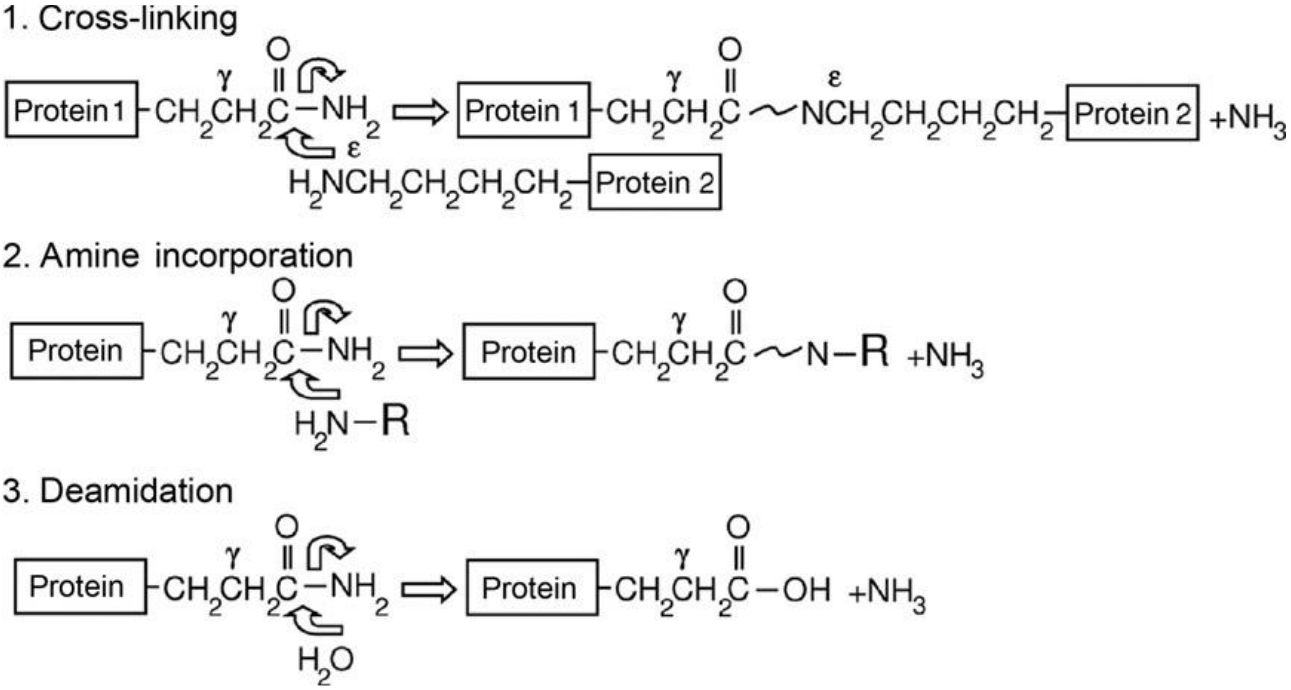

Figure 1. Main reactions catalyzed by TG2. (1) Protein cross-linking. (2) Protein aminylation. (3) Protein deamidation. [Nurminskaya et al. 2012]

In addition to the Ca^{2+}-dependent post-translational modification of proteins (i.e., transamidation and deamidation), TG2 also possesses several other Ca^{2+}-independent enzymatic activities. Indeed, the enzyme can also act as a GTPase, a protein disulphide isomerase (PDI) [Im et al. 1997; Hasegawa et al. 2003], a protein kinase [Mishra et al. 2004] and a isopeptidase in different cell compartments and biological contexts. Moreover, TG2 can also exert signaling/scaffolding/adapter functions independently of its enzymatic activity; indeed, it was found engaged in the formation of non-covalent complexes with multiple cellular proteins, both inside and outside the cell [Nurminskaya et al. 2012]. This signaling/scaffolding/adapter function of TG2 appears to regulate cell adhesion, ECM remodeling, survival, growth, migration and differentiation due to modulation of several signaling pathways [Belkin et al. 2011]. Once expressed, TG2 is primarily localized in the cytoplasm; here, the transamidating activity of TG2 is allosterically activated by Ca^{2+} and inhibited by guanosine triphosphate (GTP), guanosine diphosphate (GDP) and guanosine monophosphate (GMP). Because TG2 inside living cells is primarily GTP/GDP-bound and the Ca^{2+} level is physiologically low, the transamidating activity of the enzyme is inhibited and it functions mainly as a G-protein [Rauhavirta et al. 2013]. This may explain why overexpression of TG2 is not always associated with increased intracellular cross-linking activity. Nevertheless, despite low intracellular Ca^{2+} ions levels, multiple transamidation and cross-linking substrates of intracellular TG2 have been identified. This suggests that locally increased intracellular Ca^{2+} level and/or as yet uncharacterized interacting proteins may facilitate the induction of the transamidating activity of

TG2. Biochemical studies revealed that the transamidating and GTPase activities of TG2 are mutually exclusive. In essence, the protein can function as a G-protein or as a transamidation enzyme. Intracellular levels of Ca^{2+} and GTP allosterically regulate the transamidating and GTPase activities of TG2 in a reciprocal manner, by inducing an unusually large conformational change [Liu et al. 2002]: when Ca^{2+} level reaches a critical concentration, Ca^{2+} binding to TG2 causes the enzyme to shift to an 'open' (extended) conformation that unmask its active site, thus inhibiting the GTPase activity and activating the transamidating activity; conversely, when Ca^{2+} level returns to the normal intracellular concentration, the GTP binding to TG2 constrains the enzyme in a 'closed' (compact) conformation that shield its active site, thus turning off the transamidating activity and allowing the enzyme to function as GTPase [Liu et al. 2002]. Small amounts of TG2 are also present in the mitochondria and nucleus but not in the endoplasmic reticulum and Golgi [Laszlo Lorand et al. 2003]. In the nucleus, TG2 exerts regulatory functions on gene expression both by transamidating histones and some transcription factors and by acting non-enzymatically as a transcriptional co-regulator [Eckert et al. 2014]. In mitochondria, TG2 seems to act as a novel apoptotic BH3-only protein [Rodolfo et al. 2004] and also regulates mitochondrial physiology by acting as a protein disulphide-isomerase [Hasegawa et al. 2003]. TG2 is also present in association with the inner side of the plasmatic membrane [Laszlo Lorand et al. 2003], where it transduces signals in association with several receptors, by acting as a non-canonical GTP-hydrolyzing protein, as above described. Furthermore, despite lacking a secretory leader sequence, the enzyme can be transported to the cell surface and ECM by an as yet poorly known mechanism, possibly through an unconventional pathway involving recycling endosomes [Zemskov et al. 2011]. On cell surface, TG2 acts as a cross-linking stabilizing enzyme of the proteins of ECM, thus stabilizing the whole structure and modulating cell-matrix adhesion [Laszlo Lorand et al. 2003]; it also participates in the out-in signaling by virtue of its non-enzymatic functions [Eckert et al. 2014]. TG2 localized in the ECM is engaged in enzymatic and non-enzymatic scaffolding/adapter activities. It plays a significant role in cell adhesion, migration, and ECM organization and turnover, contributing to normal wound healing, tissue regeneration, inflammation, and fibrosis. On the whole, by means of its multiple activities, both enzymatic and non-enzymatic, TG2 is involved in several cellular processes, including cell proliferation and differentiation, cell survival and death, wound healing, cell adhesion and migration, extracellular matrix organization, etc. [Nurminskaya et al. 2012; Kanchan et al. 2015]. Thus, it is evident that anomalies in TG2 expression or in regulation of TG2 activities or in protein distribution could contribute to the development of pathological conditions. Several works have reported that TG2 is implicated in human pathologic conditions such as

degenerative disorders, certain types of cancer and inflammatory and autoimmune conditions, including CD [Iismaa et al. 2009].

1.4.3 Deamidation activity of TG2 in celiac disease

The deamidation activity of TG2 is shown to be linked to the pathological immune response towards gluten in CD [Molberg et al. 2001]. Gluten is an excellent substrate for TG2 as up to 36% of their glutamine residues are accessible to deamidation by the enzyme [Ciccocioppo et al. 2005]. TG2-induced deamidation of specific glutamines introduces negative charges in positions which enhances the binding affinity of gluten T-cell epitopes to the CD-predisposing HLA-DQ2 and -DQ8 molecules, thus promoting T-cell stimulation and cellular immune-responses; this makes gluten more immunogenic [Sollid et al. 2011]. It is well known that it has not been possible to derive a consensus sequence around the reactive glutamine residues; however, the spacing between the targeted glutamine and neighbouring proline residues in the sequence plays a dominating role in the specificity of TG2. Indeed, the enzyme preferentially targets residues within Gln-X-Pro sequences [Fleckenstein et al. 2002] but not residues within Gln-Pro or Gln-X-X-Pro sequences [Caputo et al. 2004]. The exact location where deamidation of immunogenic gluten peptides takes place is still unclear. Deamidation requires an adequate pH and a virtual total absence of amine substrates. Where such a microenvironment could be realized is still unclear. One obvious site would be the small intestine, where TG2 has been detected in the epithelial and endothelial cells and also abundantly in the basement membrane [Naiyer et al. 2008]. However, it has been demonstrated that, despite the presence of high levels of Ca^{2+}, under normal physiological conditions extracellular TG2 is enzymatically inactive in the resting state in the small-intestinal mucosa and it is only transiently activated after stress conditions (e.g., activation of the innate immune system) and changes in redox conditions [Siegel et al. 2008; Stamnaes et al. 2010]. Moreover, under physiological conditions, intracellular TG2 is also predominantly maintained in an inactive state due to a low Ca^{2+} concentration and inhibition by GTP/GDP. Nonetheless, a general increase in TG2 expression during inflammation in CD mucosa has been registered [Esposito et al. 2003] thus enhancing the probability that in situations of cell stress or trauma, and after disturbance or loss of Ca^{2+} homeostasis, TG2 may be activated and cause deamidation of gluten [Martucciello et al. 2018]. It has also been reported that p31-43 causes increased production of reactive oxygen species, which leads to TG2 overexpression and activation in intestinal epithelial cells [Liu et al. 2002].

1.4.4 Antibodies against TG2

TG2 is also the target of a strong autoimmune response in CD [Dieterich et al 1997]. In the process leading to the antibody response to TG2 in the small intestine of CD patients, gluten-specific $CD4^+$ T lymphocytes seems to play a central role while, to date, no TG2-specific $CD4^+$ (helper) T lymphocytes have been identified. As known, production of anti-TG2 autoantibodies can occur only in HLA-DQ2 or HLA-DQ8 individuals [Petterson et al. 1993; Mäki et al. 1995] and recedes when gluten is excluded from the diet [Basso et al. 2002]. This observation led Sollid et al. [Sollid et al. 1997] to formulate the so-called "hapten-carrier hypothesis" as a mechanism to explain how TG2-specific B cells get help from gluten-specific $CD4^+$ T cells to differentiate into IgA and IgG anti-TG2 plasma cells. According to this model, TG2 cross-links gluten peptides to itself, forming gluten-TG2 complexes that are recognized, internalized and processed by TG2-specific, normally silent, HLA-DQ2/8 positive B-cells [Sollid et al. 1997]. Subsequent exposure of processed gluten peptides on TG2-specific B-cells surface and their presentation by HLA-DQ2/8 molecules to gluten-specific $CD4^+$ T cells leads to activation of these latter (Figure 2). Once activated, gluten-specific $CD4^+$ T cells produce a pattern of pro-inflammatory cytokines dominated by (IFN)-γ and IL-21 [Nilsen et al. 1998; Fina et al. 2008], thereby creating an inflamed environment in the small-intestinal lamina propria and, on the other hand, in turn, activate TG2-specific B cells, which differentiate into plasma cells that secrete IgA and IgG antibodies against TG2 and deamidated gluten peptides [Sollid et al. 2017]. This model is compatible with the observation that the level of anti-TG2 antibodies gradually decreases when patients remove gluten from the diet [Hogen Esch et al. 2011]. Indeed, it means that the activity of TG2-specific B cells depends on persistent gluten presentation. Moreover, it could also explain how autoantibodies to some TG2 substrates (e.g., actin, desmin, calreticulin, collagen, etc.) have been found in CD [Shaoul et. al. 2007; Alaedini et al. 2008]. A revised model suggests that TG2 could form covalent cross-links between B-cell receptors, present on TG2-specific B-cell surface, and gluten peptides [Iversen et al. 2015]. Alternative models that explain the generation of anti-TG2 autoantibodies have been proposed, including the mechanism of molecular mimicry involving viral proteins [Dolcino et al. 2013] or the mechanism of production of neo-epitopes involving TG2 [Sollid et al. 2011].

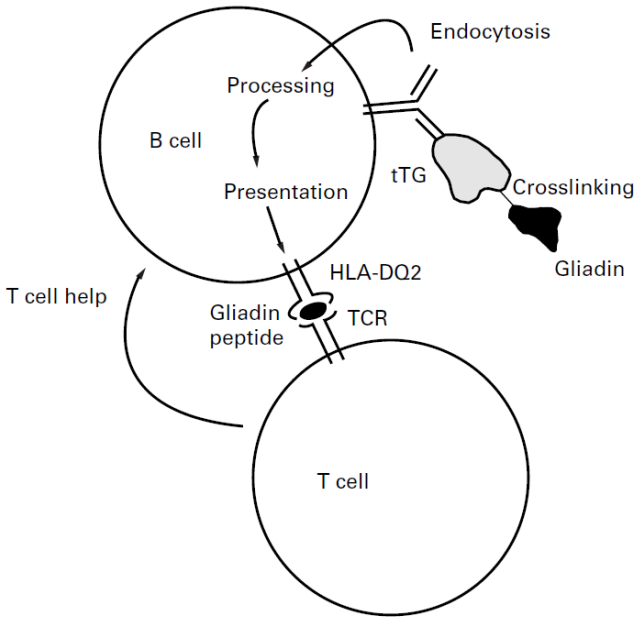

Figure 2. "Hapten-carrier" model. [Sollid et al. 1997]

1.5 Anti-TG2 autoantibodies in celiac disease

CD is considered an autoimmune disease because of the presence of autoantibodies to TG2 in the serum and small-intestinal mucosa of CD patients. The presence of anti-TG2 autoantibodies deposits in the CD small-intestinal mucosa in response to dietary gluten is a very early event of the disease and precedes the mucosal damage [Kaukinen et al. 2005]; from this site, anti-TG2 autoantibodies can successively spill over into the blood compartment when the mucosa is still intact [Kaukinen et al. 2005], thus reaching and accumulating in other organs. Even seronegative patients have mucosal anti-TG2 autoantibodies deposits when on a gluten-containing diet [Salmi et al. 2006]. For this reason, the presence of anti-TG2 antibodies, not only in the serum but also in the intestinal mucosa, represents a specific and sensible marker for clinical diagnosis of active CD. Several studies have shown that anti-TG2 autoantibodies, by interacting with TG2 present in the extracellular environment or on the cell surface, exert numerous biological effects that could actively contribute to the CD pathogenesis [Caputo et al. 2009]; however, it is still debated whether these autoantibodies actually play an active role in the onset and progression of the disease.

1.5.1 Biological roles of anti-TG2 autoantibodies

To date, there are no evidences indicating that anti-TG2 autoantibodies penetrate into cells, thus they have to exert their biological effects in the extracellular environment, where they can interact

with TG2 on cell-surface of TG2-expressing cell types or in ECM. Studies on the ability of anti-TG2 autoantibodies to modulate TG2 transamidating activity have given quite contradictory results. Indeed, they have been shown to either enhance [Király et al. 2006; Myrsky et al. 2009], inhibit [Dieterich et al. 2003; Byrne et al. 2010; Esposito et al. 2002] or have no effect on TG2 transamidating activity [Di Niro et al. 2012]. Perhaps, this could be due to different methodological approaches and different sources of antibodies (recombinant monoclonal or polyclonal, purified or not) used. Therefore, it is still unclear whether anti-TG2 autoantibodies can interfere in vivo with stabilization and repair functions of TG2 in the ECM and/or block those reactions involved in the breakdown of tolerance to gluten (i.e., deamidation of specific glutamines of gluten peptides and cross-linking of gluten peptides with self-proteins) [Martucciello et al. 2018]. Several studies have shown that anti-TG2 autoantibodies exert numerous biological effects on various cell types. The epithelium is one cell type where their effects have been extensively studied. Both monoclonal and CD patients serum anti-TG2 autoantibodies have been shown to inhibit the differentiation of T84 intestinal crypt epithelial cells [Halttunen et al. 1999]. In addition, anti-TG2 autoantibodies derived from serum of CD patients have been demonstrated to induce proliferation of intestinal epithelial cells [Barone et al. 2007] and also to increase their transepithelial permeability [Zanoni et al. 2006], thereby allowing gluten peptides to access the lamina propria and affecting epithelial cell biology [Lebreton et al. 2012]. Serum anti-TG2 autoantibodies from CD patients have also been shown to be capable of reducing the attachment of endothelial cells to the TG2-fibronectin matrix [Teesalu et al. 2012] and alter their mobility and dynamics [Kalliokoski et al. 2013], which might lead to the observed inhibition of in vitro, ex vivo and in vivo angiogenesis [Kalliokoski et al. 2013]. Vascular permeability seems also to be increased in the presence of CD patients serum anti-TG2 autoantibodies [Kalliokoski et al. 2013; Myrsky et al. 2009]. Whether the effects exerted by these antibodies on vascular biology are significant in the development of small-intestinal mucosal damage remains to be established; however, at least they could play a role in the development of the vascular abnormalities observed in CD. Anti-TG2 autoantibodies have also been reported to enhance lymphocyte adhesion, probably by up-regulating the expression of the adhesion molecule E-selectin, and to increase lymphocyte transendothelial migration [Myrsky et al. 2009]. As known, an increased cell proliferation, a reduced differentiation, an altered cell and tissue morphology, a compromised barrier function and a less well-organized vascular network all represent hallmarks of CD mucosal lesions. However, anti-TG2 autoantibodies did not significantly affect enterocyte apoptosis both in cell lines and in cultured intestinal biopsies, nor altered apoptosis rate of endothelial cells [Martucciello et al. 2018]. Other studies have demonstrated that, by interacting with the extracellular TG2, anti-TG2 autoantibodies act as signaling-like molecules and induce

extracellular signal-regulated kinase (ERK) phosphorylation and a rapid calcium mobilization from intracellular stores in Caco-2 cells (a model of human intestinal epithelial cells), thereby activating a signaling cascade involved in the activation of intracellular TG2 and potentially other Ca^{2+}-dependent enzymes [Caputo et al. 2013]; TG2 activation could cause gluten modification (e.g., gluten peptides deamidation) inside enterocytes and other specialized APCs. As signaling molecules, anti-TG2 autoantibodies have also been shown to induce actin rearrangement in several cell lines [Barone et al. 2007] and to trigger, in Caco-2 cells, a rapid change in intracellular phosphorylation patterns of several proteins acting in cellular processes such as cell cycle progression, stress response and apoptosis [Paolella et al. 2013]. The functional consequence of differential phosphorylation in CD mucosal environment remains to be established. However, these results, conducted using cell culture and focused on the effects of anti-TG2 autoantibodies on a sole cell type, cannot necessarily be translated to the level of the entire small intestine. Studies performed on mice immunized with TG2 have shown that although the animals developed an anti-TG2 antibody response, no morphological changes were evidenced in the small intestine [Freitag et al. 2004]. Similar results were obtained in another study where CD patient-derived single chain TG2-specific antibody fragments were expressed in mice using adeno-associated virus vectors [Di Niro et al. 2008]. In contrast to these two studies focused specifically on anti-TG2 autoantibodies, other studies have shown that the intraperitoneal injection of either CD patient serum or total immunoglobulin fractions caused an alteration in the small-intestinal mucosal morphology and an increased cellular infiltration in the lamina propria [Kalliokoski et al. 2017]. The general idea that emerges from all these studies, performed in in vitro and in vivo models, is that antibodies to TG2 could have an active role in CD pathogenesis as they are able to reproduce several features of CD intestinal mucosa, such as enhanced proliferation and reduced differentiation, altered permeability, cell architecture modification, etc. [Martucciello et al. 2018].

1.5.2 Anti-TG2 autoantibodies and gluten peptides

Another interesting aspect of anti-TG2 autoantibodies is related to their ability to influence the handling of gluten peptides at the level of the intestinal epithelium. Previous studies demonstrated that anti-TG2 autoantibodies enhanced the transepithelial passage of p57-68 and p31-43 across a monolayer of Caco-2 cells [Rauhavirta et al. 2011]. Authors also verified that the increase of cell-surface TG2 activity, in the presence of anti-TG2 autoantibodies, only in part was responsible for the enhanced peptide transport. Other studies have confirmed that anti-TG2 autoantibodies (both monoclonal and from CD patients serum) significantly reduced the uptake of the toxic p31-43 by Caco-2 cells [Caputo et al. 2010]; this effect was shown to be specific because anti-TG2

autoantibodies did not influence the uptake of the immunogenic p57-68 [Caputo et al. 2010]. As a consequence of interfering with p31-43 uptake by cells, anti-TG2 autoantibodies, at low concentration, also reduced the p31-43-induced increase of cell proliferation, thus exerting a relative protective role. Authors also demonstrated that neither TG2 nor another protein could act as receptor/carrier of peptide 31-43 [Paolella et al. 2018]. Thus, the molecular mechanism underlying the protective function of anti-TG2 autoantibodies is not related to a hypothetical receptorial role of TG2, and remains currently unclear. These findings suggest that anti-TG2 antibodies, by forming complexes with cell-surface TG2, to some degree protect cells from negative effects induced by p31-43. Thus, one can speculate that the humoral response to TG2, at the level of intestinal mucosa, could have the potentiality to protect cells, to some degree, from toxic effect of some gluten peptides. The hypothetical protective function exerted by anti-TG2 antibodies towards p31-43-induced negative effects was also observed in skin-derived fibroblasts (an in vitro CD-derived cell model) of healthy subjects but not in skin-derived fibroblasts of CD patients [Paolella et al. 2013]. The observation that CD fibroblasts handled p31-43 in a different manner compared to non-CD fibroblasts when anti-TG2 antibodies interact with TG2 on the cell surface led authors to suppose that in CD fibroblasts TG2 displays some different features compared to non-CD fibroblasts, such as a different pattern of surface interacting proteins or an altered trafficking (internalization, delivery to lysosomes, externalization) and, as a consequence, a different subcellular distribution [Paolella et al. 2017]. These results are in line with other observed differences between CD and non-CD fibroblasts, which are independent from gluten exposure and that have led to hypothesize the existence of a “celiac cellular phenotype”.

1.6 Celiac cellular phenotype

Several studies show that CD cells display constitutive alterations with respect to non-CD cells. These alterations are independent of the presence of gluten in the diet and, as shown by their presence in skin-derived fibroblasts, are also evident far from the main site of the inflammation. Altogether, these alterations define the so-called “celiac cellular phenotype”, that may represent a predisposing condition to the damaging effects of gluten. For example, a generally higher level of phosphorylated protein and an increased number of focal adhesions were observed in CD fibroblasts compared with non-CD ones [Nanayakkara et al. 2013]. A constitutive alteration involving early to late vesicular trafficking was also observed in CD enterocytes and fibroblasts,

with an increase of stress and inflammation markers, which could render CD cells more susceptible to the effects of gluten peptides [Lania et al. 2020].

In 2013, Nanayakkara et al. [Nanayakkara et al. 2013] showed that proliferation of crypts enterocytes was constitutively increased in CD patients on gluten-containing diet (including patients with atrophic mucosa and potential CD patients with normal mucosa), as well as in CD patients on GFD, with respect to non-CD healthy controls. Indeed, the increased proliferation of crypt enterocytes seen in CD patients was partially independent of the crypts hyperplasia (that does not occur in potential CD patients) and of the presence of gluten in the diet (as it was present in CD patients on GFD). The authors also found an increase in EGF mRNA and EGF-receptor (EGFR) protein in crypts enterocytes from CD patients compared with those from non-CD healthy controls. The increase was present also in CD patients on GFD without crypts hyperplasia, thus confirming that the EGF/EGFR system was enhanced in CD crypts enterocytes independently of the gluten intake and of the remodelling of the tissue. The authors also found that ERK1/2, the downstream effector of the EGFR signaling, was more phosphorylated in crypts enterocytes of both CD patients in the active phase of the disease (both CD patients with villous atrophy and potential CD patients) and CD patients in remission on GFD, compared with non-CD healthy controls. The increased proliferation of CD crypts enterocytes is dependent on EGFR signaling [Nanayakkara et al. 2013]. Indeed, upon EGF linking to the EGFR, a signaling cascade starts, that involves sequential phosphorylation of downstream effectors such as ERK1/2. Once activated, phosphorylated ERK1/2 can transfer to the nuclei, where it can start trans-activation of several genes that can induce cell proliferation and other biological effects [Ramos et al. 2008; Meloche et al. 2007]. The authors confirmed that the increased proliferation of CD crypts enterocytes was mediated by ERK1/2 activation. Indeed, they showed that blocking ERK1/2 phosphorylation with an ERK1/2-specific inhibitor resulted in a normalization of crypts enterocytes proliferation of CD atrophic mucosa, almost to the level of proliferation found in non-CD mucosa control. The same results were observed in skin-derived fibroblasts of CD patients on GFD, thus confirming that the EGFR/ERK pathway is constitutively altered in CD cells independently of the presence of gluten in the diet and of the main inflammation site. Altogether, these constitutive alterations may represent a predisposing condition to the damaging effects of gluten peptides in CD.

In another paper [Nanayakkara et al. 2013], the same authors described other constitutive differences in fibroblasts obtained from CD patients on GFD with respect to those obtained from non-CD healthy controls. These differences involved an altered cell shape and area, as well as the actin cytoskeleton. Indeed, CD fibroblasts showed shortened actin stress fibers and appeared less elongated with respect to non-CD ones; CD fibroblasts also showed a statistically significant

increase in cell area. The authors also found differences in the number of focal adhesions between the two groups of cells. Indeed, by analyzing the expression of Paxillin and Focal Adhesion Kinase (FAK), two focal adhesion markers, they found that both Paxillin- and FAK-positive focal adhesions were significantly increased in CD fibroblasts than in non-CD ones. Western blot analysis confirmed the increase of the Paxillin and FAK protein levels and of the phosphorylated forms of these proteins in CD fibroblasts with respect to non-CD ones, suggesting an over representation of these specialized cell adhesion sites in CD cells. Another difference they found involved an altered sub-cellular distribution of the LIM-containing lipoma-preferred partner (LPP) protein. The LPP gene is strongly associated with CD [Trynka et al. 2011] and its protein plays an important role in focal adhesion architecture and acts as a transcription factor in the nucleus. The authors found that the levels of LPP protein was significantly reduced in the nuclei of CD fibroblasts compared with non-CD ones. In contrast, a corresponding increase in LPP protein was observed in the cytosol, where the focal adhesion proteins are expected to be found. In other words, in CD cells, the sub-cellular distribution of LPP was increased in focal adhesions and reduced in the nucleus [Nanayakkara et al. 2013]. This different distribution of LPP protein in CD cells might have several biological effects. For example, the increased LPP localization at the focal adhesions could alter cellular adhesion, motility and shape, and the reduced amount of LPP in the nucleus could alter the transcriptional activity of this protein. This result is consistent with the hypothesis that an altered transcriptional activity of LPP might be involved in CD [Grunewald et al. 2009]. Interestingly, the authors also demonstrated that the treatment with the toxic peptide p31-43 induced the same “celiac cellular phenotype” in non-CD fibroblasts, suggesting a close association between these alterations and CD pathogenesis.

In a recent work, Paolella et al. [Paolella et al. 2017] demonstrated that, in presence of anti-TG2 antibodies, skin-derived fibroblasts from CD patients on GFD handled p31-43 in a different manner compared to fibroblasts from healthy controls. Indeed, the authors showed that pretreating cells with anti-TG2 antibodies before adding p31-43 significantly reduced the uptake of the toxic peptide in non-CD fibroblasts but not in CD ones. Furthermore, this effect was shown to be specific because anti-TG2 antibodies did not influence the uptake of the immunogenic peptide p57-68 by the two groups of cells. The authors suggested that anti-TG2 antibodies, by interacting with cell-surface TG2, somehow induce CD cells to behave differently than non-CD cells regarding the handling of gluten peptides that are responsible for innate immunity in CD. They also hypothesized that in CD fibroblasts, TG2 displays some different features compared to non-CD fibroblasts which could explain the ability of anti-TG2 antibodies to differentially alter the uptake of p31-43 by the two groups of cells. However, analyses of TG2 protein expression and enzymatic activity revealed that

this phenomenon was not related to different expression levels of TG2 or anomalous catalytic properties of this enzyme between fibroblasts from healthy and CD subjects, thus suggesting that other features related to TG2 may be responsible of this different behaviour, such as a different pattern of surface interacting proteins, or an altered trafficking (internalization, delivery to lysosomes, externalization), or a different subcellular distribution. However, these findings suggest that TG2 also may contribute to the "celiac cellular phenotype".

More recently, Lania et al. [Lania et al. 2020] showed that CD cells (both enterocytes and fibroblasts) also presented a constitutive alteration in the intracellular vesicular trafficking at the level of the early-recycling compartment, that could render these cells more sensitive to the effects of toxic gluten peptides. Indeed, the authors found that in CD cells, the numbers of early vesicles were increased and the EGF/EGFR trafficking was delayed at the level of the early endocytic vesicles with respect to non-CD cells. They also found that CD fibroblasts were more sensitive to the treatment with p31-43, and that this toxic peptide caused in CD fibroblasts a more prolonged delay in EGF/EGFR trafficking in the early endocytic and recycling compartments compared to fibroblasts from healthy control. This finding could explain why p31-43, which delays early to late vesicle trafficking, damages CD cells. The existence of a celiac cellular phenotype characterized by a constitutive alteration in intracellular vesicular trafficking could explain why CD cells were more sensitive to p31-43-induced inflammation (one of whose effects is to delay early and late vesicular trafficking) with respect to non-CD ones.

AIMS

In this PhD thesis work, differences in TG2 subcellular distribution in CD and non-CD fibroblasts were investigated to determine how TG2 may be able to contribute to the different handling of p31-by the two groups of cells. In the attempt to identify other constitutive differences regarding TG2 in CD and non-CD cells, it was investigated whether p31-43 differentially modulated TG2 expression and activity in fibroblasts from the two groups of subjects. Finally, it was also investigated whether regulation of intracellular Ca^{2+} homeostasis regarding the endoplasmic reticulum (ER) was different in CD and non-CD fibroblasts. Relatedly, it was analyzed how CD and non-CD cells responded to stimulation with thapsigargin (THP), an ER-stress and autophagy inducer, also focusing the attention on TG2 modulation.

CHAPTER 2. MATERIALS AND METHODS

2.1 Primary Fibroblasts Culture

All experiments described in this PhD thesis work were performed using fibroblasts obtained from skin biopsies of five CD patients on GFD (age range 17-43 years) and five HLA-DQ2-negative healthy controls (age range 25-30 years). CD patients were on GFD for at least 4 years and showed normal biopsies (Marsh T_0), anti-TG2 antibody serum levels ranging between 0 and 1.6 U/mL and negative anti-endomysium antibodies. Fibroblasts, obtained from Professor Maria Vittoria Barone (Department of Translational Medical Science - University of Naples Federico II), were cultured in Dulbecco's Modified Eagle's Medium (EuroClone, Milan, Italy) supplemented with 20% (*v/v*) Fetal Bovine Serum (Thermo Fisher Scientific, Rockford, Illinois), 1% (*v/v*) L-Glutamine (EuroClone) and 1% (*v/v*) Penicillin-Streptomycin (EuroClone). Cells were maintained at 37 °C in a 5% CO_2 and 95% air-humidified atmosphere. In all experiments, fibroblasts were used between the 4^{th} and 7^{th} passage.

2.2 Antibodies and peptides

The commercial monoclonal mouse anti-TG2 antibody, clone CUB 7402 (200 μg/mL), was from Thermo Fisher Scientific. Control antibodies were non-specific mouse IgGs (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA). Peptides p31-43 (sequence LGQQQPFPPQQPY) and p57-68 (sequence QLQPFPQPQLPY) were synthesized on the sequence of the α-gliadin (Inbios, Naples, Italy). Synthetic peptide p229-246, representing the C-terminal sequence of the α-gliadin, was used as irrelevant control peptide. Each peptide was solubilized into serum-free medium at 10 mg/mL and stored in small aliquots at -20 °C.

2.3 TG2 staining

2.3.1 Extracellular TG2

To visualize extracellular TG2 in CD and non-CD cells, fibroblasts were seeded on glass coverslips and cultured for 48 hours. Then, cells were incubated with 10 μg/mL of the primary mouse anti-

TG2 antibody CUB 7402 in 1% Bovine Serum Albumin (BSA, Sigma-Aldrich, Milan, Italy) in Phosphate Buffered Saline (PBS) consisting of 137 mM sodium chloride (NaCl), 10 mM disodium hydrogen phosphate (Na_2HPO_4), 1.76 mM potassium dihydrogen phosphate (KH_2PO_4) and 2 mM potassium chloride (KCl). After fixing for 10 minutes with 3% paraformaldehyde (PFA), coverslips were incubated with a secondary anti-mouse FITC-conjugated antibody (Invitrogen Srl, Milan, Italy) diluted 1:50 in PBS. Finally, coverslips were washed with PBS and mounted with Mowiol (Sigma-Aldrich). Images were acquired with a LSM 510 Zeiss microscope (Carl Zeiss MicroImaging Inc., Iena, Germany).

2.3.2 Intracellular TG2

To visualize intracellular TG2 in CD and non-CD cells, after fixing (3% PFA, 10 minutes) and permeabilizing (0.2% Triton X-100, 5 minutes), fibroblasts seeded on glass coverslips were incubated first with 1 μg/mL of CUB 7402 in 1% BSA in PBS and then with a secondary anti-mouse TRITC-conjugated antibody (Invitrogen Srl) diluted 1:200 in PBS. After washing with PBS, coverslips were mounted with Mowiol and stained cells were observed with an Axioplan 2 fluorescent microscope (Carl Zeiss MicroImaging Inc.). Images were acquired and processed with the KS300 software (Carl Zeiss MicroImaging Inc.).

2.4 TG2 expression: TG2 protein detection by western blot

2.4.1 Cell treatments and lysis

To analyze gliadin peptides-induced modifications in TG2 protein expression levels in CD and non-CD cells, fibroblasts were seeded in wells of a 6-well microplate and treated for 48 hours with 20 or 80 μg/mL of p31-43; in this experiment, p57-68 (20 μg/mL) was used as control peptide. To analyze THP (Invitrogen Srl)-induced modifications in TG2 protein expression levels in CD and non-CD cells, fibroblasts seeded in wells of a 6-well microplate were treated for 24 hours with different amounts of THP (0.01, 0.1 and 0.5 μM); in this experiment, dimethyl sulfoxide (DMSO, Sigma-Aldrich) was used as vehicle. At the end of treatments, after washing with PBS, cells were mechanically harvested in 70 μL of RIPA lysis buffer consisting of 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS, AppliChem, Darmstadt, Germany), 1 mM phenylmethylsulfonyl fluoride (PMSF, AppliChem), 1 mM sodium orthovanadate (Na_3OV_4, Sigma-Aldrich), 50 mM sodium fluoride (NAF, AppliChem) and 1% protease inhibitors cocktail (Sigma-Aldrich) and incubated for 1 hour on ice to promote cell lysis.

Finally, protein content of each sample was determined using the Bio-Rad reagent protein (Bio-Rad Laboratories, Milan, Italy), as described in Section 2.4.2, and 35 μg of proteins were used for western blot analysis, as described in Sections 2.4.3 and 2.4.4.

2.4.2 Bradford protein assay

Bradford protein assay is a simple, quick and fairly sensitive colorimetric assay for measuring the content of total proteins present in a sample. It is based on the shift in absorbance maximum of the Coomassie Brilliant Blue G-250 dye from 465 to 595 nm following its binding to denatured proteins present in solution [Bradford et al. 1976]. Absorbance at 595 nm varies linearly with the protein content: the greater is the amount of total proteins present in the sample, the greater the amount of Coomassie Brilliant Blue G-250 dye that binds to them.

To quantify proteins, for each sample, 1 μL of the total cell extract was added to 1 mL of Bio-Rad reagent protein (containing the Coomassie Brilliant Blue G-250 dye) diluted 1:5 in deionized water; then, absorbance was measured spectrophotometrically (VWRUV-3100PC) at 595 nm. For each sample, absorbance was measured in duplicate and the values obtained were averaged in order to obtain a more reliable measurement of the protein concentration. A standard curve was generated using solutions of known concentration (1, 2, 4, 8, and 10 μM) of the standard protein BSA. Each point of the curve was measured in duplicate and absorbances read at 595 nm were averaged.

2.4.3 SDS-PAGE

SDS-PAGE (Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis) is an electrophoretic technique that allows to separate proteins solely on the basis of their molecular weight. Proteins are amphoteric molecules, i.e. they have both positive and negative charges. So, to make proteins move in a single direction in a polyacrylamide gel, it is necessary to create a uniform negative charge on them. SDS is an anionic detergent that binds strongly to the protein backbone at a constant molar ratio (approximately one detergent molecule binds to two amino acids when SDS is present at 0.1%). When mixed with SDS, proteins acquire a net negative charge in proportion to their molecular size; thus, when a current is applied, all SDS-bound proteins migrate through the polyacrylamide gel toward the positively charged electrode according to their mass: proteins with less mass travel more quickly through the gel than those with greater mass because of the sieving effect of the gel matrix.

To denature proteins in cellular extracts, Laemmli sample buffer 4X consisting of 200 mM Tris-HCl pH 6.8, 20% glycerol, 8% SDS, 400 mM dithiothreitol (DTT, GE Healthcare, Milan, Italy) and 0.04% bromophenol blue was added to each sample in volume such that it was diluted to 1X. Then,

samples were boiled at 100 °C to promote protein denaturation. For SDS-PAGE, a two-phase polyacrylamide gel was prepared: the upper gel (or stacking gel), needed to concentrate proteins into one tight band before separating them, and the lower gel (or separating gel), that allows to separate proteins according to their molecular weight. The percentage of acrylamide in the stacking gel was 5%; the percentage of acrylamide in the separating gel depends on the molecular weight of the protein of interest: to detect TG2, a 10% acrylamide lower gel was prepared. 35 μg of total protein extracts of each sample were separated according to their molecular weight by 10% SDS-PAGE. Together with samples, a molecular weight marker (Thermo Fisher Scientific) was also loaded. The electrophoretic run was carried out at 100 V until proteins moved from the stacking to the separating gel; then, the voltage was increased to 120 V. Running Buffer 1X (25 mM Tris, 192 mM glycine and 0.1% SDS) was added to promote the passage of electrical current into the electrophoretic chamber. The electrophoretic run was stopped when the bromophenol blue escaped from the lower edge of the gel.

2.4.4 Western blot

Western blot (or western blotting or, more correctly, immunoblotting) is a widely used biochemical technique to identify with a specific antibody a specific protein within a complex mixture of proteins that has been fractionated in a polyacrylamide gel and immobilized into a membrane.

After separating by SDS-PAGE, proteins were electro-transferred for 1 hour at 120 V to a PVDF membrane (EMD Millipore Corporation, Billerica, Massachusetts, USA) previously activated in methanol for 1 minute. Transfer Buffer 1X (25 mM Tris, 192 mM glycine and 20% CH_3OH) was added to promote the passage of electrical current into the transfer chamber. After blocking nonspecific binding sites for 30 minutes with 5% non-fat dry milk (Santa Cruz Biotechnology Inc.) in Tris Buffered Saline (TBS) consisting of 50 mM Tris pH 7.5 and 150 mM NaCl, the membrane was washed with TBS containing 0.1% Tween-20 (T-TBS) to remove the blocking solution excess. TG2 protein was detected by incubating the membrane overnight at 4 °C with CUB 7402 diluted 1:1000 in T-TBS containing 0,1% non-fat dry milk. Then, the membrane was washed with T-TBS and incubated for 1 hour at room temperature with a secondary anti-mouse horseradish peroxidase-conjugated antibody diluted 1:10000 in T-TBS. After washing the membrane with T-TBS, immunocomplexes were revealed using a chemiluminescence detection kit (EMD Millipore Corporation, Burlington, Massachusetts, USA). The membrane was incubated for 5 minutes with ECL, containing luminol (i.e., the horseradish peroxidase substrate) and water: in the presence of water, luminol is oxidized by the enzyme, thus producing a chemiluminescent signal capable of impressing an autoradiographic slab (FujiFilm Corporation, Tokyo, Japan). The signal is more

intense the greater the amount of protein present in the loaded sample. Reagents were mixed in a 1:1 ratio, according to the manufacturer's instructions. The slab was developed and fixed with an automatic developer (Cawomat 2000 IR).

2.4.5 Slab acquisition and densitometry

Acquisition of slabs was carried out with a scanning densitometer GS-800 (Bio-Rad Laboratories); TG2 band analysis was carried out with the Quantity One software (Bio-Rad Laboratories). For each band, the Optical Density (OD) was calculated as the product of the band area and its density per unit area. ODs of the TG2 bands were normalized to those of the GAPDH.

2.5 TG2 expression: TG2 mRNA detection by real-time RT-PCR

2.5.1 Cell treatments and total RNA extraction

To analyze gliadin peptides-induced modifications in TG2 mRNA expression levels in CD and non-CD cells, fibroblasts were seeded in wells of a 6-well microplate and treated for 24 hours with different amounts of p-31-43 (20, 40 and 80 μg/mL). To extract total RNA, cells were lysed by incubating them for 15 minutes at room temperature with 500 μL of TRIzol-Reagent (Thermo Fisher Scientific). After adding 100 μL of chloroform and incubating for 3 minutes at room temperature, samples were centrifuged at 10000 rpm for 15 minutes at 4 °C and the aqueous phases, containing the total RNA extracted, were collected. Then, RNA was precipitated by adding 250 μL of isopropanol and, after incubating for 10 minutes at room temperature, samples were centrifuged at 10000 rpm for 10 minutes at 4 °C. Supernatants were discarded while pellets were washed with 75% ethanol. Samples were centrifuged at 7500 rpm for 5 minutes and ethanol was evaporated. Finally, 30 μL of RNAsi-free water was added to each sample to solubilize RNA and samples were heated at 55 °C for 10 minutes.

2.5.2 Total RNA quantization

For each sample, total RNA extracted was quantified by measuring absorbance at 260 nm of a 2 μL drop with the Nanodrop 2000. RNA purity was assessed by evaluating the ratio between absorbance read at 260 nm and absorbance read at 280 nm (range of accepted values 1.7-2.0), that allows to appreciate a possible contamination due to the presence of proteins, and the ratio between absorbance read at 260 nm and absorbance read at 230 nm (range of accepted values 2.0-2.2), that

allows to appreciate a possible contamination due to the presence of phenol, chloroform or guanidinium isothiocyanate (contained in TRIzol-Reagent).

2.5.3 RT-PCR

The first-strand cDNA synthesis reaction was performed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) and 1 μg of total RNA. For each sample, 2 μL of gDNA WipeOut Buffer and the volume of RNAsi-free water needed to reach the final volume of 14 μL were added to the volume corresponding to 1 μg of total RNA. After incubating samples at 42 °C for 2 minutes in a thermocycler (AppliedBiosystem PCR System 2720), 6 μL of Reverse Transcription Mix (consisting of 1 μL of Reverse Transcriptase, 4 μL of Quantiscript RT Buffer and 1 μL of RT Primer mix) have been added to each sample. Then, samples were first incubated at 42 °C for 25 minutes and then at 95 °C for 5 minutes. cDNAs obtained were used to amplify human TG2 transcripts as described in Section 2.5.4.

2.5.4 Real-time PCR

For amplification with real-time PCR, cDNAs were analysed in triplicate with the iQ™ SYBR Green Supermix (Bio-Rad Laboratories) and using the iQ™ 5 Multicolor Real Time PCR Detection System (Bio-Rad Laboratories). Primers used to amplify human TG2 transcripts were: 5'-TGCTGTGGAGGAGGGTGACT-3' (forward), 5'-ACCAGGCGTTGAAGAGCAAA-3' (reverse). Real-time PCR reactions were performed with 250 nM of each primer and 10 μL of SYBR Green Supermix, in a total volume of 20 μL. Real-time PCR program started with 3 minutes of incubation at 95 °C, followed by 40 cycles of 15 seconds at 95 °C, 15 seconds at 60 °C and 20 seconds at 72 °C. The concentration of TG2 mRNA was normalized to the concentration of the transcript for GAPDH. Primers used to amplify human GAPDH transcripts were: 5'-TTCAACAGCGACACCCACTG-3' (forward), 5'-CACCCTGTTGCTGTAGCCA-3' (reverse). Real-time PCR program started with 5 minutes of incubation at 94 °C, followed by cycles of 30 seconds at 94 °C, 45 seconds at 65 °C 120 seconds at 72 °C.

2.6 TG2 activity

To analyze gliadin peptides-induced modifications in TG2 activity in CD and non-CD cells, fibroblasts were seeded in plates of 60 mm in diameter and treated for 30 minutes with 10 μM ionomycin (Sigma-Aldrich) or different amounts of p31-43 (5, 10 and 20 μg/mL), p57-68 (20

μg/mL) and p229-246 (20 μg/mL) in the presence of 0.5 mM pentylamine-biotin (Thermo Fisher Scientific). To analyze THP-induced modifications in TG2 activity in CD and non-CD cells, fibroblasts seeded in plates of 60 mm in diameter were treated for 30 minutes with 10 μM ionomycin or different amounts of THP (0.01, 0.1, 0.5 and 1 μM) in the presence of 0.5 mM pentylamine-biotin. At the end of treatments, cells were washed with PBS and mechanically harvested in 70 μL of RIPA lysis buffer. After 30 minutes of incubation on ice, total cell extracts were centrifuged at 13,000×g for 10 minutes at 4 °C to remove cell debris. 25 μg of proteins were coated into the wells of a 96-well microplate. Then, wells were blocked for 3 hours with 10% BSA in Borate Buffered Saline (BBS) consisting of 80 mM NaCl, 100 mM H_3BO_3 and 20 mM $Na_2B_4O_7$. After washing with BBS and BBS containing 0.1% Tween-20 (T-BBS), wells were incubated for 1 hour with horseradish peroxidase-conjugated neutravidin (Thermo Fisher Scientific) diluted 1:3000 in 5% BSA in T-BBS. To reveal peroxidase activity, 100 μL of the horseradish peroxidase substrate 3,3',5,5'-tetramethylbenzidine (TMB, Sigma-Aldrich) were added to each well and, after stopping the reaction with 100 μL of H_2SO_4, absorbances were read at 450 nm.

2.7 TG2 subcellular distribution

2.7.1 Subcellular fractionation

To monitor TG2 distribution in cytosol and membranes of CD and non-CD cells, fibroblasts were seeded in wells of a 6-well microplate and cultured for 48 hours. Then, after washing with PBS, cells were mechanically harvested in 70 μL of a hypotonic lysis buffer consisting of 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM Na_3OV_4, 1 mM PMSF and 1% protease inhibitors cocktail. Cells were also disrupted by passing them several times through a 25 Ga needle using a 1 mL syringe. After 30 minutes of incubation on ice to promote cell lysis, 500 μL of total lysate were centrifuged at 800×g for 3 minutes at 4 °C to remove nuclei and unbroken cells. The obtained supernatant was centrifuged at 3000×g for 10 minutes at 4 °C to obtain a sheet membrane fraction. The supernatant was centrifuged again at 20,000×g for 2 hours at 4 °C to obtain the membrane fraction. The sheet fraction and the membrane fraction were each resuspended in 50 μL of Laemmli sample buffer. Finally, 50 μL of total lysate, of nuclei fraction, of cytosolic fraction and of both sheet and membrane fractions were loaded onto a 10% SDS-PAGE for western blot analyses. CUB 7402 (diluted 1:1.000 in 0.5% non-fat dry milk in TBS), anti-EGF-receptor (diluted 1:1.000), anti-GAPDH (diluted 1:2.000) and anti-lamin B antibodies (diluted 1:1.000) were used as markers for TG2, membrane, cytosolic and nuclear compartments, respectively.

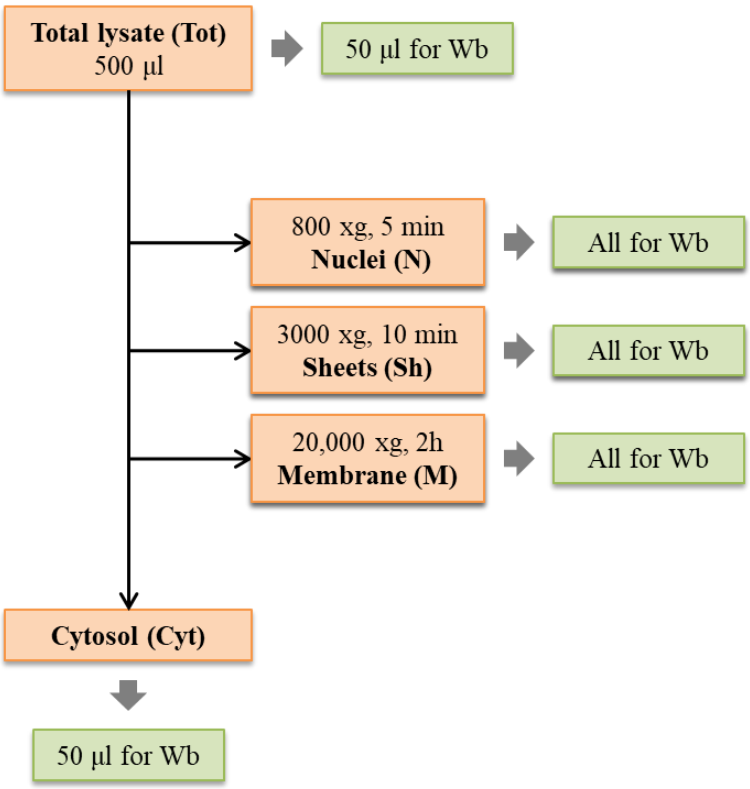

Figure 3. Scheme of the fractionating protocol.

2.7.2 Detection of membrane-surface TG2

To obtain a relative estimation of TG2 amount on the cell surface of CD and non-CD cells, fibroblasts were seeded at a density of 8000/cm^2 in wells of a 96-well microplate and cultured for 24 hours. Then, cells were incubated for 30 minutes at 4 °C with CUB 7402 diluted 1:50 in 5% BSA in PBS with Ca^{2+} and Mg^{2+} ions. After fixing for 5 minutes with 3% PFA, cells were incubated for 30 minutes with a secondary anti-mouse horseradish peroxidase-conjugated antibody diluted 1:100 in PBS. In parallel wells, cells were first fixed (3% PFA, 5 minutes) and permeabilized (0.2% Triton X-100, 5 minutes), then incubated with CUB 7402 diluted 1:100 and the secondary antibody, as described above. At the end of incubation, TMB was added to each well and, after 30 minutes, absorbances were read at 450 nm. Finally, the ratio between absorbances relative to extracellular TG2 (i.e., when CUB 7402 was added without fixation) and absorbances relative to intracellular TG2 (i.e., when CUB 7402 was added after fixation and permeabilization) was calculated. In this experiment, a non-specific mouse IgG (Santa Cruz Biotechnology Inc.) was used as the control of the unspecific staining. Furthermore, all wells were stained with the crystal violet dye (Sigma-Aldrich) to confirm that the same amount of cells was present in each well.

2.7.3 Confocal microscopy

Fibroblasts seeded on glass coverslips were fixed with 3% PFA for 10 minutes and permeabilized with 0.2% Triton X-100 for 5 minutes. Then, cells were incubated for 1 hour with primary antibodies diluted in 1% BSA in PBS as follows: mouse CUB 7402 diluted 1:150, rabbit anti-TG2 diluted 1:280 and mouse anti-transferrin receptor (TfR) diluted 1:500 (Thermo Fisher Scientific), mouse anti-lysosomal-associated membrane protein 2 (LAMP2) diluted 1:100 and goat anti-early endosome antigen 1 (EEA1) diluted 1:100 (Santa Cruz Biotechnology Inc.), rabbit anti-microtubule-associated protein 1A/1B light chain 3b (LC3) diluted 1:200 (Abcam, Cambridge, UK). Only to detect LAMP2, fixation and permeabilization were done with methanol (10 minutes) and acetone (1 minute), respectively. Then, cells were incubated for 1 hour with the following secondary antibodies diluted 1:100 in 1% BSA in PBS: anti-mouse TRITC-conjugated, anti-mouse Alexa-fluor 488-conjugated, anti-rabbit Alexa-fluor 488-conjugated, anti-rabbit Alexa-fluor-546-conjugated, anti-goat Alexa-fluor 488-conjugated (Thermo Fisher Scientific). Finally, coverslips were washed with PBS and mounted with Mowiol. Images were acquired with a Zeiss LSM 510 laser scanning microscope (Carl Zeiss MicroImaging Inc.). Magnification of the micrographs was the same for all the figures shown (63× objective). Colocalization analysis was performed with the AIS Zeiss software. Each value of the calculated colocalization index could range from 0 (no colocalization) to 1 (all pixels co-localize).

2.8 Intracellular Ca^{2+} concentration measurement

For microfluorimetric studies, fibroblasts were seeded on glass coverslips coated with 30 μg/mL poly-L-lysine (Sigma-Aldrich). Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured by single cell computer-assisted videoimaging, using the Ca^{2+} indicator Fura-2 acetoxymethyl ester (Fura-2AM), as described below. All microfluorimetric experiments were performed under the supervision Professor Agnese Secondo (Department of Neurosciences - University of Naples Federico II). Briefly, cells were loaded with 10 μM of Fura-2AM for 30 minutes at 37 °C in Normal Krebs (NK) solution consisting of 5.5 mM KCl, 160 mM NaCl, 1.2 mM magnesium chloride ($MgCl_2$), 1.5 mM $CaCl_2$, 10 mM glucose and 10 mM Hepes-NaOH, pH 7.4. Then, coverslips were placed into a perfusion chamber (Medical System, Co. Greenvale, New York, USA) mounted onto the stage of an inverted Zeiss Axiovert 200 microscope (Carl Zeiss MicroImaging, Inc.) equipped with a FLUAR 40× oil objective lens. The experiments were carried out with a digital imaging system consisting of a MicroMax 512BFT cooled CCD camera (Princeton Instruments, Trenton,

New Jersey, USA), a LAMBDA 10-2 filter wheeler (Sutter Instruments, Novato, California, USA) and a Meta-Morph/MetaFluor Imaging System software (Universal Imaging, West Chester, Pennsylvania, USA). After loading, cells were alternatively illuminated at wavelengths of 340 and 380 nm by a Xenon lamp. The emitted light was passed through a 512 nm barrier filter. Fura-2AM fluorescence intensity was measured every 30 seconds. Forty to sixty-five individual cells were selected and monitored simultaneously from each coverslip. All the results were presented as cytosolic Ca^{2+} concentrations. Assuming that the K_D for Fura-2AM was 224 nM [32, 33 di BHK cells transfected with NCX3 are more resistant to hypoxia followed by reoxygenation than those transfected with NCX1 and NCX2: Possible relationship with mitochondrial membrane potential], the equation of Grynkiewicz et al. [33 di BHK cells transfected with NCX3 are more resistant to hypoxia followed by reoxygenation than those transfected with NCX1 and NCX2: Possible relationship with mitochondrial membrane potential], whose parameters were determined for individual cells as described by Urbanczyk et al. [32 di BHK cells transfected with NCX3 are more resistant to hypoxia followed by reoxygenation than those transfected with NCX1 and NCX2: Possible relationship with mitochondrial membrane potential], was used for calibration [Grynkiewicz et al. 1985 di Anti-tissue transglutaminase antibodies activate intracellular tissue transglutaminase by modulating cytosolic Ca2+ homeostasis]. When p31-43 was used, it was added to the medium at the concentration of 20 μM.

2.9 GRP78 expression

2.9.1 GRP78 protein detection by western blot

To analyze THP-induced modifications in 78-kDa glucose-regulated protein (GRP78) protein expression levels in CD and non-CD cells, fibroblasts were seeded in wells of a 6-well microplate and treated for 24 hours with 0.1 or 0.5 μM of THP; in this experiment, DMSO was used as vehicle. Then, after washing with PBS, cells were mechanically harvested in 70 μL of RIPA lysis buffer and incubated for 1 hour on ice to promote cell lysis. Finally, 35 μg of proteins were loaded onto a 10% SDS-PAGE for western blot analyses, as described in Section 2.4.4. GRP78 protein was detected by incubating the membrane overnight at 4 °C with the primary mouse anti-GRP78 (Invitrogen Srl) diluted 1:1000 in T-TBS containing 0,1% non-fat dry milk.

2.9.2 GRP78 mRNA detection by real-time PCR

To analyze THP-induced modifications in GRP78 mRNA expression levels in CD and non-CD cells, fibroblasts were seeded in wells of a 6-well microplate and treated for 4 or 24 hours with different amounts of THP (0.01, 0.1 and 0.5 μM). Total RNA was extract and quantified as described in Sections 2.5.1 and 2.5.2, respectively. The first-strand cDNA synthesis reaction was performed using the QuantiTect Reverse Transcription Kit and 1 μg of total RNA, as described in Section 2.5.3. For amplification with real-time PCR, cDNAs were analysed in triplicate with the iQ™ SYBR Green Supermix and using the iQ™ 5 Multicolor Real Time PCR Detection System. Primers used to amplify human GRP78 transcripts were: 5'- TGATTCCAAGGAACACAGT-3' (forward), 5'-GTCAGATCAAATGTACCCA-3' (reverse). PCR reactions were performed with 250 nM of each primer and 10 μL of SYBR Green Supermix, in a total volume of 20 μL. Real-time PCR program started with 3 minutes of incubation at 95 °C, followed by 40 cycles of 15 seconds at 95 °C, 15 seconds at 60 °C and 20 seconds at 72 °C. The concentration of GRP78 mRNA was normalized to the concentration of the transcript for GAPDH, as described in Section 2.5.4.

2.10 XBP1 splicing

To investigate whether THP induced differences in X-Box Binding Protein 1 (XBP1) splicing between CD and non-CD cells, fibroblasts were seeded in wells of a 6-well microplate and treated for 4 hours with different amounts of THP (0.01, 0.1 and 0.5 μM). Total RNA was extract and quantified as described in Sections 2.5.1 and 2.5.2, respectively. The first-strand cDNA synthesis reaction was performed using the QuantiTect Reverse Transcription Kit and 1 μg of total RNA as described in Section 2.5.3. The obtained cDNAs were used to detect the unspliced and the spliced forms of XBP1 by PCR. Primers used to amplify human XBP1 transcripts (forward, 5'-CCTGGTTGCTGAAGAGGAGG-3'; reverse, 5'-CCATGGGGAGATGTTCTGGAG-3') were used at 500 nM each. PCR reactions were run on an Applied Biosystems® 2720 Thermal Cycler for 35 cycles with heating for 30 seconds at 94 °C, followed by annealing for 45 seconds at 58 °C and polymerization for 60 seconds at 72 °C. The mRNA concentration of unspliced and spliced forms of XBP1 were normalized to the concentration of the transcript for GAPDH, which was amplified with the same primers already used for real-time PCR. Finally, amplification products of XBP1 cDNAs were visualized on a 2.5% agarose gel stained with ethidium bromide.

2.11 MTT assay

The MTT assay is a colorimetric assay based on the ability of succinate dehydrogenase to reduce the water-soluble yellow dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into its insoluble purple formazan crystals, in the mitochondria of living cells. Formazan crystals are dissolved in DMSO and the resulting colored solution is quantified spectrophotometrically by measuring absorbance at 595 nm: the darker the solution, the greater the number of viable, metabolically active cells. Thus, because it depends on mitochondrial respiration, MTT assay indirectly serves to assess the cell viability, proliferation and cytotoxicity [Kumar et al. 2018].
To analyze the effect of THP on cell viability in CD and non-CD cells, fibroblasts were seeded in wells of a 96-well microplate and treated for 24 hours with different amounts of THP (0.01, 0.1, 0.5 and 1 μM). Then, after adding the MTT substrate (Sigma-Aldrich) to each well (final concentration 0.25 mg/mL), the plate was incubated for 90 minutes at 37 °C to allow formazan crystals to form. As positive control of the assay, 0.2% H_2O_2 was used for 30 minutes. Finally, crystals were dissolved in 100 μL of DMSO and absorbances were read at 595 nm. Cell viability was calculated by comparing absorbances registered in the wells where THP was added and absorbances registered in the wells where corresponding amounts of vehicle (DMSO) only were added. The percentage of cell viability was obtained by applying the following formula:

$$\% \text{ cell viability} = \frac{\text{OD tested compound}}{\text{OD DMSO}} \text{ x } 100$$

2.12 LC3 and p62 expression

To analyze THP-induced modifications in LC3 and p62/sequestosome 1 (SQSTM1) protein expression levels in CD and non-CD cells, fibroblasts were seeded in wells of a 6-well microplate and treated for 24 hours with different amounts of THP (0.01, 0.1 and 0.5 μM) and 4 hours with different amounts of THP (0.1 and 0.5 μM), respectively; in this experiment, DMSO was used as vehicle. Then, after washing with PBS, cells were mechanically harvested in 70 μL of RIPA lysis buffer and incubated for 1 hour on ice to promote cell lysis. 35 μg of proteins were loaded onto a 15% and 10% SDS-PAGE for western blot anti-LC3 and anti-p62 analyses, respectively. LC3 and p62 proteins were detected by incubating the membrane overnight at 4 °C with primary rabbit anti-LC3 (Invitrogen Srl) and mouse anti-p62 (Invitrogen Srl) antibodies, respectively, diluted 1:1000 in T-TBS containing 0,1% non-fat dry milk.

2.13 Statistics

Statistical analysis of the data obtained was performed where appropriate using Student's t-test. Differences were considered to be statistically significant at $p < 0.05$.

CHAPTER 3. RESULTS

3.1 Staining of extracellular and intracellular TG2 in CD and non-CD cells

To confirm that TG2 was indeed expressed in skin-derived fibroblasts obtained from CD patients and non-CD healthy controls used in the experiments described in this PhD thesis work, CD and non-CD cells were seeded on glass coverslips and, after incubation with the primary anti-TG2 antibody CUB 7402 and a secondary fluorescent molecule-conjugated antibody, observed with a fluorescent microscope (confocal or not). As already observed by Paolella et al. in dermal fibroblasts obtained from other CD and non-CD subjects [Paolella et al. 2017], fluorescent microscope images revealed that TG2 was present in all cell cultures tested (CD and non-CD), both in association with the cell membrane and intracellularly; images also revealed a similar distribution of intracellular and extracellular TG2 between the two groups of cells (CD and non-CD) (Figure 4). The results obtained thus confirmed that TG2 was actually expressed in CD and non-CD fibroblasts used in this PhD thesis work.

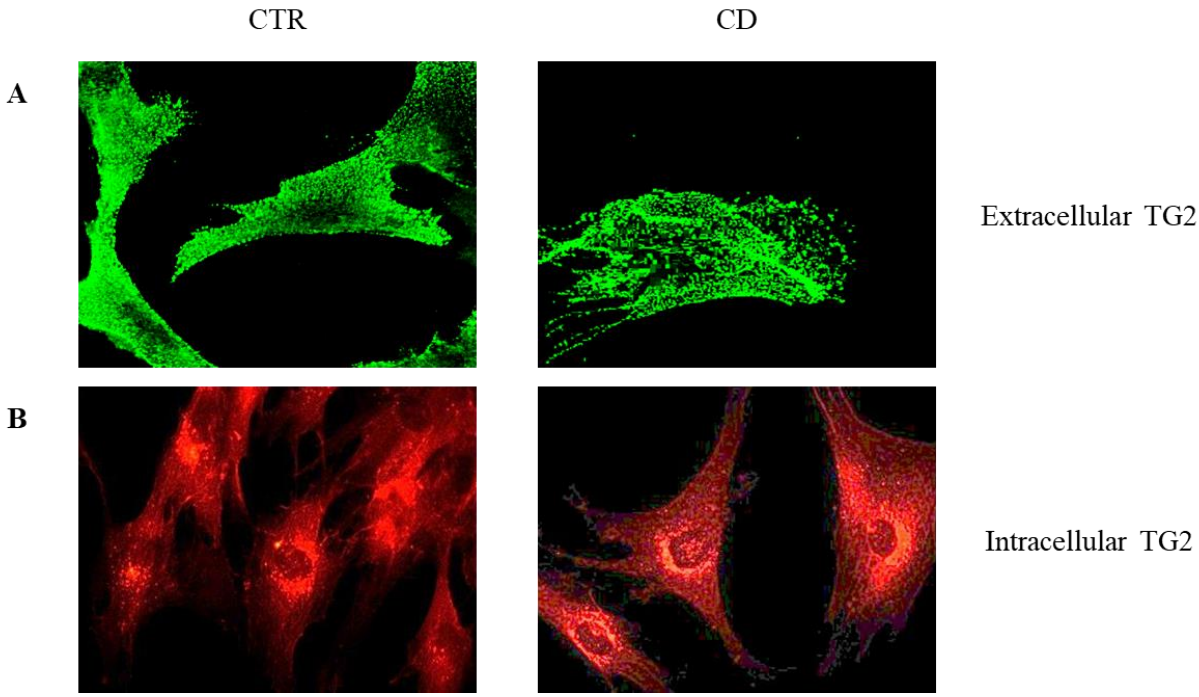

Figure 4. TG2 staining in skin-derived fibroblasts. (A) Representative confocal microscope images of TG2 distribution on cell surface of fibroblasts from one representative non-CD sample (left) and one representative CD sample (right). (B) Representative fluorescent microscope images of intracellular TG2 distribution into living fibroblasts from one representative non-CD sample (left) and one representative CD sample (right).

3.2 TG2 expression and activity in CD and non-CD cells

The experiments described in this paragraph were performed to confirm that basal expression and catalytic activity of TG2 do not differ significantly between CD and non-CD cells, as already shown by Paolella et al. in their 2017 paper cited above [Paolella et al. 2017]. Real-time PCR analysis of TG2 mRNA expression levels performed on skin-derived fibroblasts obtained from three CD patients and three non-CD healthy controls revealed an individual variability in basal TG2 mRNA levels in both CD and non-CD cell cultures (Figure 5A), with no significant difference between the two groups of cells. Western blot analysis of TG2 protein expression levels performed on skin-derived fibroblasts obtained from four CD patients and four non-CD healthy controls also revealed a wide individual variability in the basal expression levels of this protein in both CD and non-CD cell cultures (Figure 5B); however, no significant difference between these two groups of cells was observed (Figure 5C). The amount of TG2 protein was normalized to that of tubulin, a protein whose expression within the cell is constitutive.

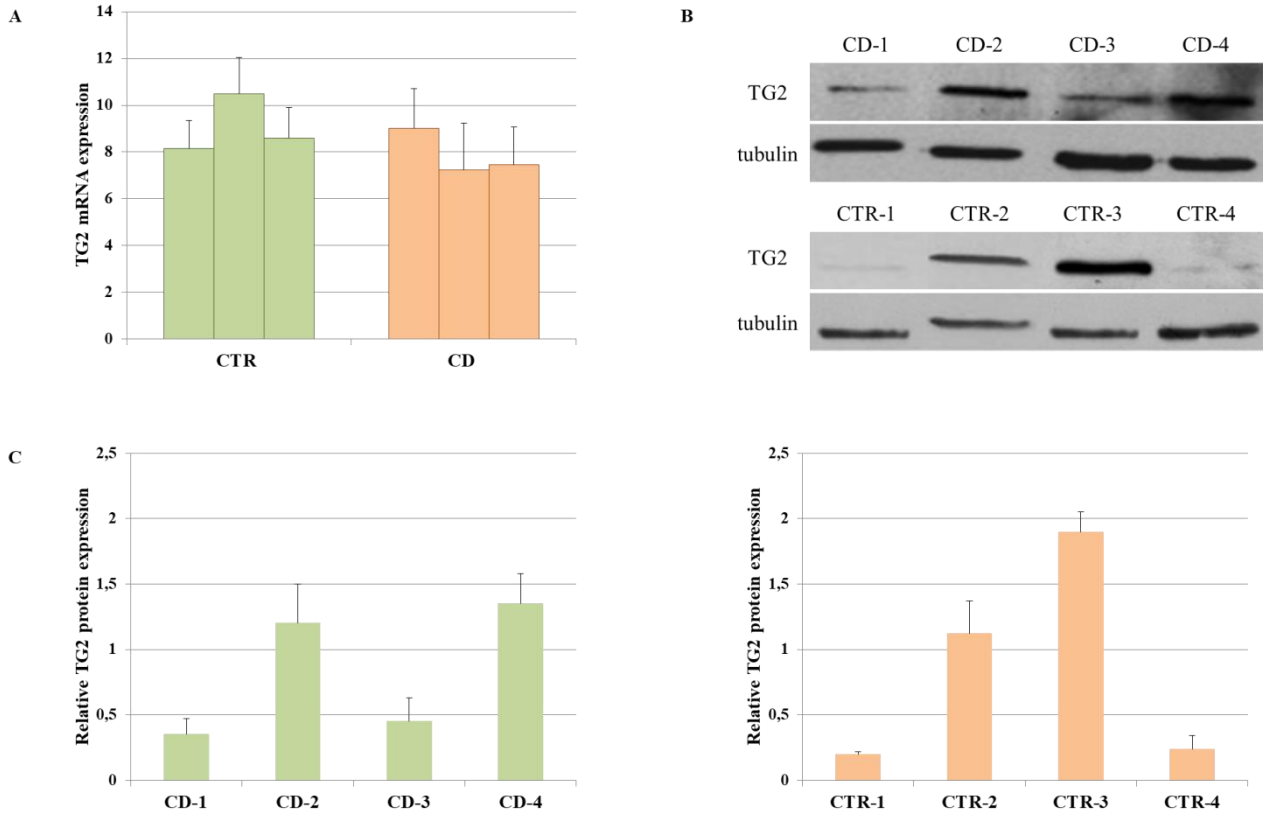

Figure 5. TG2 mRNA and protein expression in skin-derived fibroblasts. (A) TG2 mRNA levels measured in three non-CD samples and three CD samples. (B) Representative western blot anti-TG2 analysis performed on four non-CD samples and four CD samples. (C) Graphical representation of mean values (and standard deviations) of densitometric analysis of blots relative to four CD samples (left) and four non-CD samples (right). Tubulin was used as the internal reference.

The absence of significant differences in TG2 catalytic properties between CD and non-CD cells was confirmed by performing an *in situ* TG2 activity assay with the TG2 substrate pentylamine-biotin. Figure 6 shows representative images of the pentylamine-biotin incorporation, in the absence or in the presence of 10 μM of ionomycin, in one CD cell culture and one non-CD cell culture. Therefore, the data obtained confirmed what Paolella et al. reported in their paper [Paolella et al. 2017], i.e., CD and non-CD cells do not show significant differences in TG2 expression or catalytic activity that could explain the different handling of p31-43, in the presence of anti-TG2 antibodies, by the two groups of cells.

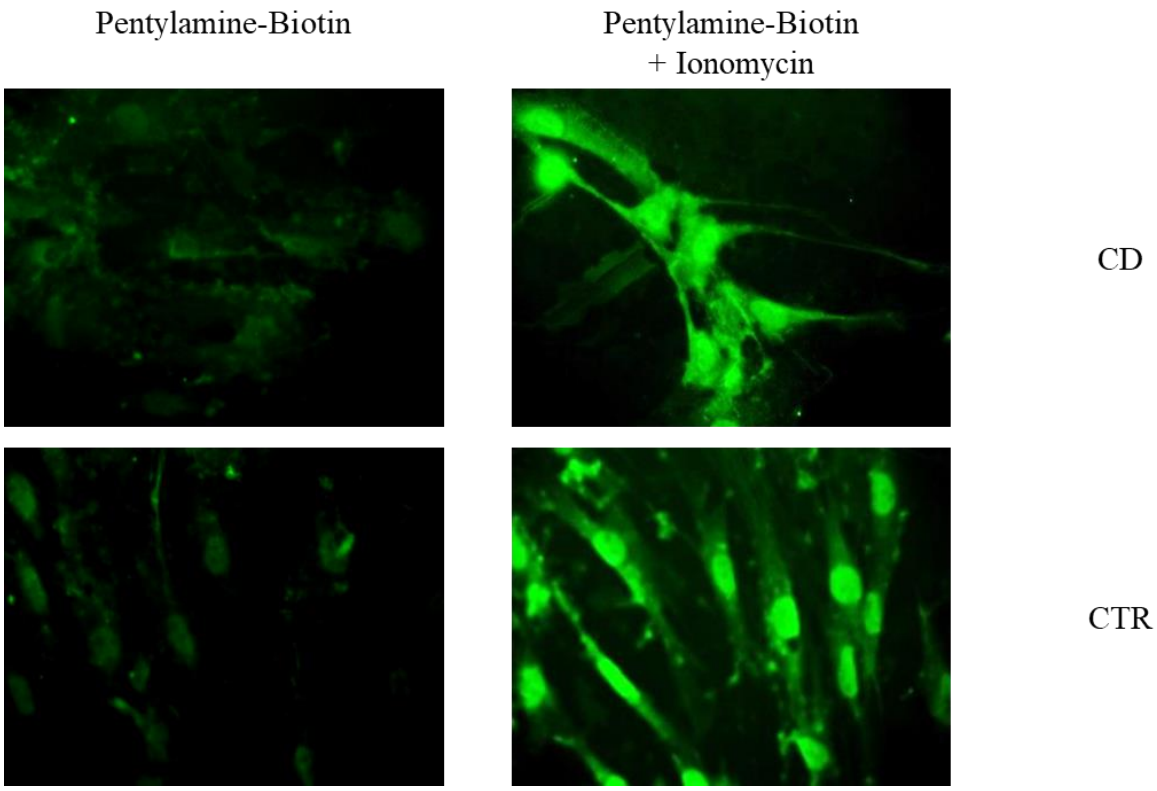

Figure 6. Representative images of *in situ* pentylamine-biotin incorporation in the absence (left) or in the presence (right) of ionomycin 10 μM, in one representative non-CD sample and one representative CD sample.

3.3 Differences in TG2 subcellular distribution between CD and non-CD cells

In the attempt to clarify whether the different uptake of p31-43 by CD and non-CD cells, in the presence of anti-TG2 antibodies [Paolella et al. 2017], could be due to a different TG2 localization into the cell, fractionating experiments were performed to investigate TG2 subcellular distribution in CD and non-CD fibroblasts. First, the efficacy of the fractionating protocol was verified on one representative sample of skin-derived fibroblasts obtained from a CD cell culture. The identity of

each fraction was verified by analyzing the expression of specific markers, i.e., EGFR for the membrane fractions (both sheets and membranes), GAPDH for the cytosol fraction and the lamin B for the nuclear fraction. As shown in Figure 7A, TG2 was present in each fraction; moreover, the membrane fractions (both sheets and membranes) were quite pure (however, they appear very little contaminated by the cytosol fraction), whereas the cytosolic fraction was pure and not contaminated by the membrane fractions. On the contrary, the nuclear fraction was contaminated by the membrane ones, thus it was not used for further analysis. Figure 7B shows a representative western blot anti-TG2 performed on 50 μL of total lysates and 50 μL of cytosolic and membrane fractions obtained from one representative CD cell culture and one representative non-CD cell culture. Western blot analysis of TG2 subcellular distribution in cytosol and membrane fractions performed on skin-derived fibroblasts obtained from four CD patients and four non-CD healthy controls showed that the amount of TG2 in the cytosolic fraction was slightly lower in CD cells than in non-CD ones, whereas the amount of TG2 associated with membrane sheets did not vary between the two groups of cells; on the contrary, the amount of TG2 associated with the membrane fraction was slightly, but significantly, more abundant in CD cells with respect to non-CD ones (Figure 7C). The data obtained thus showed a small but significantly higher association of TG2 to the membrane fractions in CD cells with respect to non-CD cells. However, this analysis did not define which membrane type TG2 was most associated with.

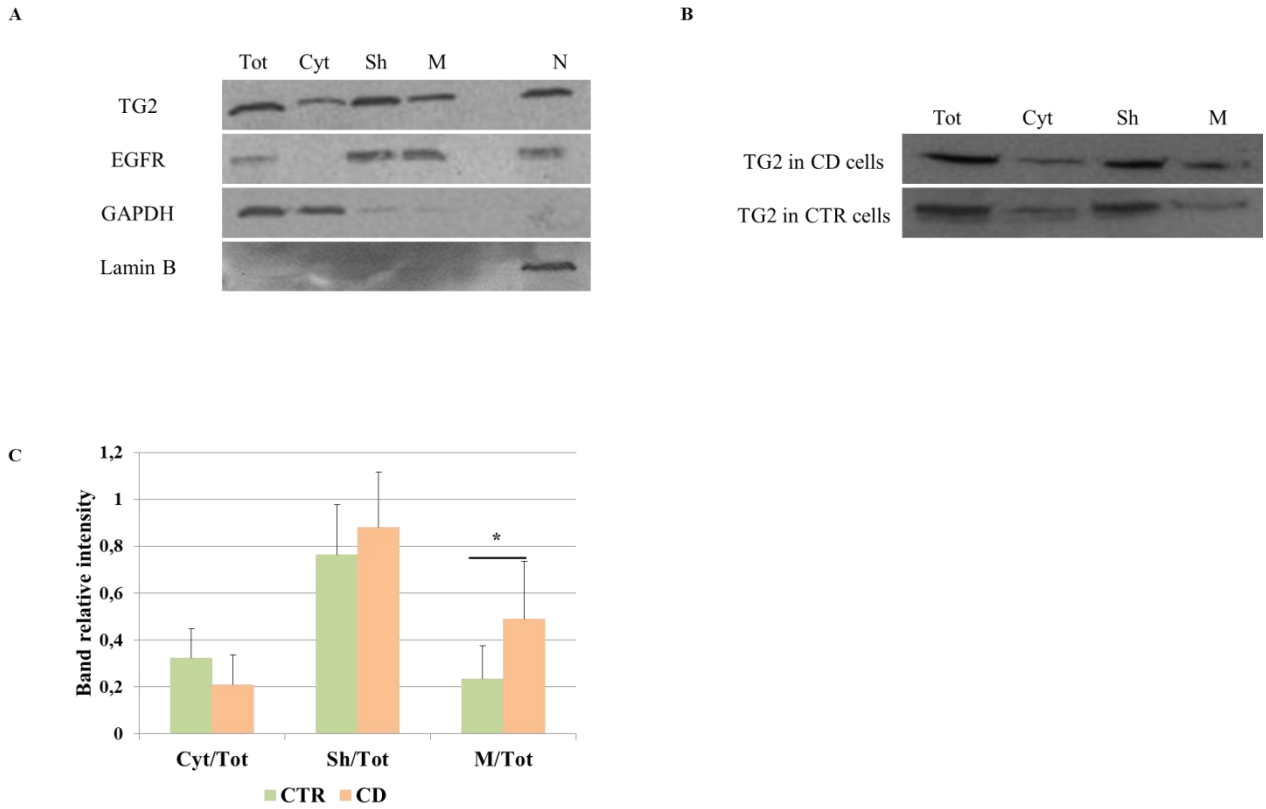

Figure 7. TG2 subcellular distribution in skin-derived fibroblasts. (A) Representative western blot analysis showing TG2 levels in subcellular fractions of fibroblasts from one representative CD sample (Tot, total lysate; Cyt, cytosol; Sh, sheets; M, membrane; N, nuclei). The identity of each fraction was verified by analyzing the expression of specific markers, i.e., the EGFR for membrane fractions (both sheets and membranes), GAPDH for the cytosol and lamin B for the nuclear fraction. (B) Representative western blot analysis showing TG2 levels in total lysates and cytosolic and membrane fractions of fibroblasts from one representative non-CD sample and one representative CD sample. (C) Graphical representation of mean values (and standard deviations) of densitometric analyses of relative TG2 levels into each fraction, expressed as the ratio between band intensity of the fraction and band intensity of the respective total lysate. Graph resumes western blots analysis data regarding experiments on four control and four CD samples. *$p < 0.05$.

3.4 Differences in the amount of extracellular membrane-bound TG2 between CD and non-CD cells

Based on the results described in the Section 3.3, a microplate immune assay was performed to evaluate the amount of TG2 associated with the extracellular membrane of cells from CD patients and non-CD healthy controls. Comparing one representative sample of skin-derived fibroblasts from a CD cell culture and one representative sample of skin-derived fibroblasts from a non-CD cell culture, it was found that absorbance relative to the extracellular TG2 was higher for the CD cell culture than the non-CD one (Figure 8A), whereas absorbance relative to the intracellular TG2 was slightly lower for the CD cell culture than the non-CD one (Figure 8B). In these experiments, a

non-specific mouse IgG was used as the control of the unspecific staining. Figure 8C shows a graphical representation of mean values (and standard deviations) of ratios between absorbance relative to extracellular TG2 ($TG2_{ex}$) and to intracellular TG2 ($TG2_{in}$), measured in corresponding wells, referred to analysis performed on skin-derived fibroblasts obtained from three CD patients and three non-CD healthy controls. The data obtained thus indicated that there was a slight, but significantly higher association of TG2 with the cell surface membrane in CD cells than in control ones.

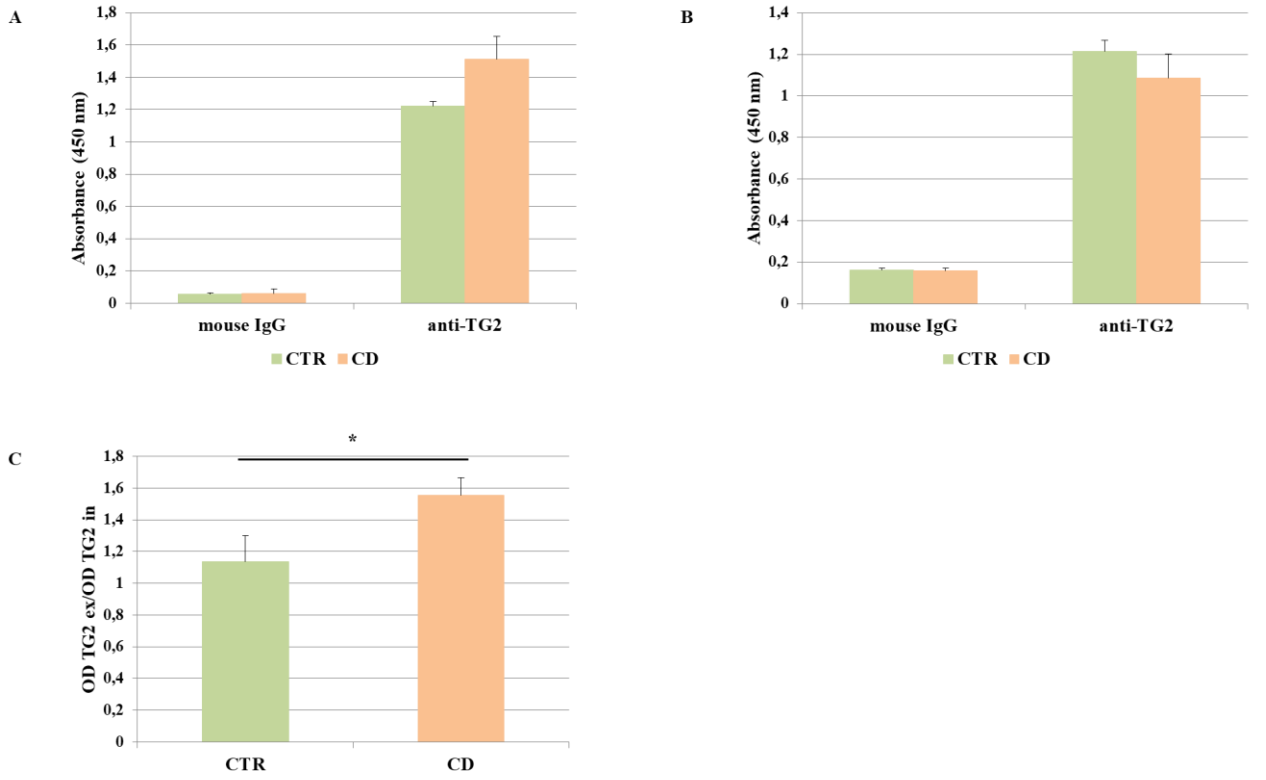

Figure 8. Relative amount of TG2 associated with the extracellular membrane surface in skin-derived fibroblasts. (A) Absorbances relative to detection of TG2 on cell surface of one representative non-CD sample and one representative CD sample. (B) Absorbances relative to detection of intracellular TG2 in one representative non-CD sample and one representative CD sample. In both (A) and (B) graphs, absorbances relative to non-specific mouse IgG, used as negative control, are also shown. Each determination was made in triplicate. (C) Graphical representation of mean values (and standard deviations) of ratios between absorbance relative to surface TG2 (OD $TG2_{ex}$) and to intracellular TG2 (OD $TG2_{in}$), measured in corresponding wells, referred to analysis performed on three non-CD samples and three CD samples. *$p < 0.05$.

3.5 Differences in intracellular colocalization of TG2 with vesicular markers between CD and non-CD cells

In the attempt to identify any other constitutive differences in TG2 distribution between CD and non-CD cells, TG2 colocalization with markers of different intracellular membrane compartments was investigated. Confocal immunofluorescence images revealed that TG2 colocalized with EEA1, a marker of the early endosomal compartment, in both groups of cells, but the colocalization between TG2 and EEA1 was higher in CD fibroblasts than in non-CD ones (Figure 9A). Images also revealed that TG2 colocalized with LAMP2 and TfR, markers of the late endosomal compartment and of the recycling vesicles, respectively, in both CD and non-CD cells, but without any significant difference between the two groups of cells (Figure 9B and 9C). Finally, the TG2 colocalization with LC3, a marker of the autophagic compartment, showed a higher colocalization between TG2 and LC3 in CD cells than in control ones (Figure 9D). Graphs resume colocalization data regarding experiments performed on skin-derived fibroblast obtained from four CD patients and four non-CD healthy controls.

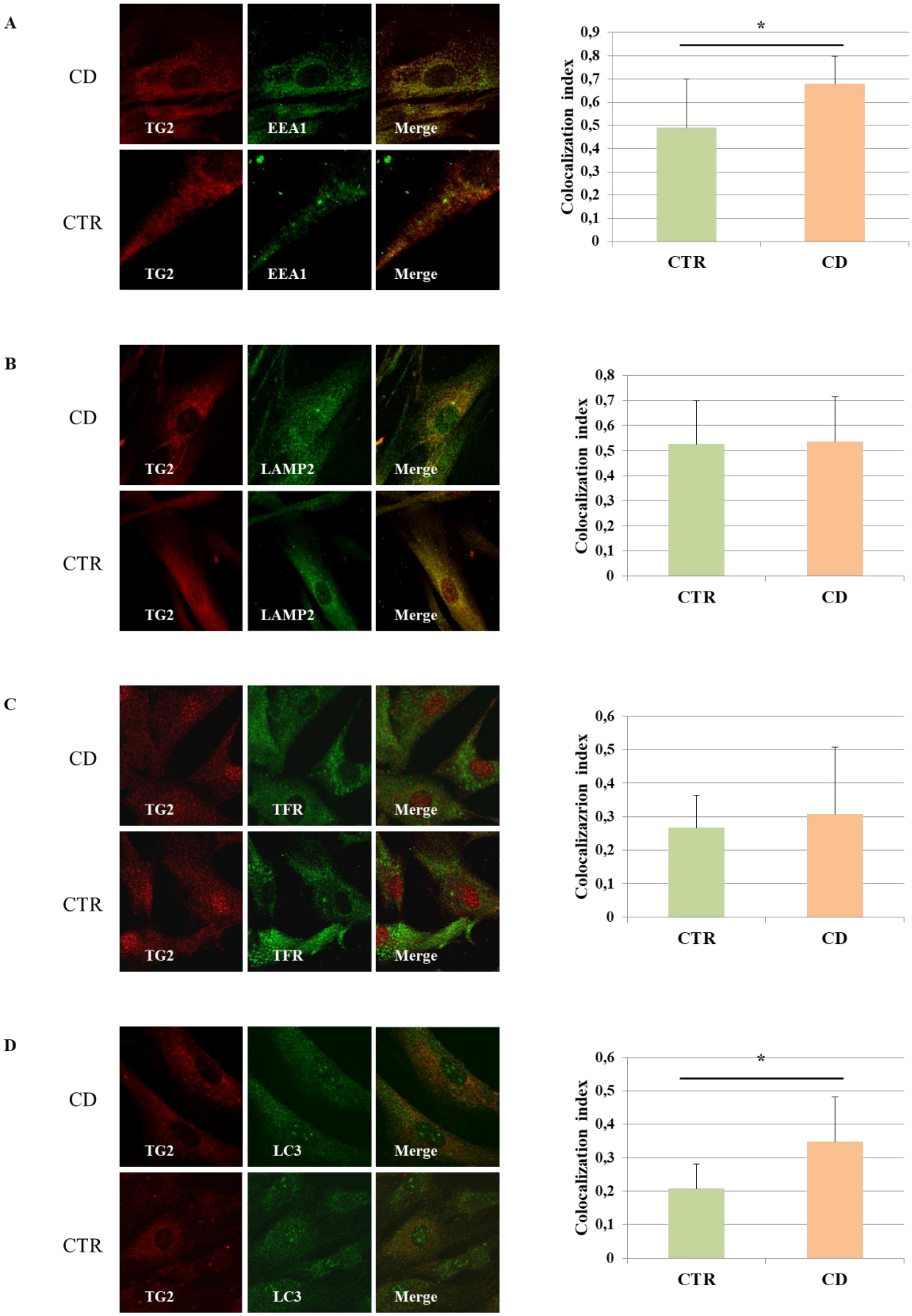

Figure 9. TG2 colocalization with endosomal markers in skin-derived fibroblasts. Representative confocal microscope images of fibroblasts from one representative non-CD sample and one representative CD sample stained with antibodies

against TG2 (red) and EEA1 (green) (A), LAMP2 (green) (B), transferrin receptor (TfR) (green) (C) and LC3 (green) (D); the merging of red and green fields is shown in yellow. Graphs resume colocalization data regarding experiments on four control and four CD samples. *$p < 0.05$.

3.6 Effects of p31-43 on TG2 expression and catalytic activity in CD and non-CD cells

To verify whether p31-43 was able to differently modulate intracellular TG2 enzymatic activity in skin-derived fibroblasts from CD patients compared with those from non-CD healthy controls, the *in situ* TG2 activity assay with the TG2 substrate pentylamine-biotin was performed again. Comparing the TG2 activity measured in one representative CD cell culture and one representative non-CD cell culture (both CD and non-CD subjects were chosen for their high expression level of TG2 protein), it was found that the treatment for 30 minutes with different amounts of p31-43 (5, 10 and 20 µg/mL) induced an increase in TG2 activity in both cell cultures; however, TG2 activation was clearly less pronounced in CD cells than in non-CD ones (Figure 10A). The experiment, performed on skin-derived fibroblasts obtained from three CD patients and three non-CD healthy controls, expressing similar levels of TG2, confirmed a dose-dependent increase of TG2 activity in the presence of increasing amounts of p31-43, but again showed a generally lower activation in CD cells than in control ones (Figure 10B); it was also observed a significant TG2 activation in the presence of 20 µg/mL of the immunogenic p57-68.

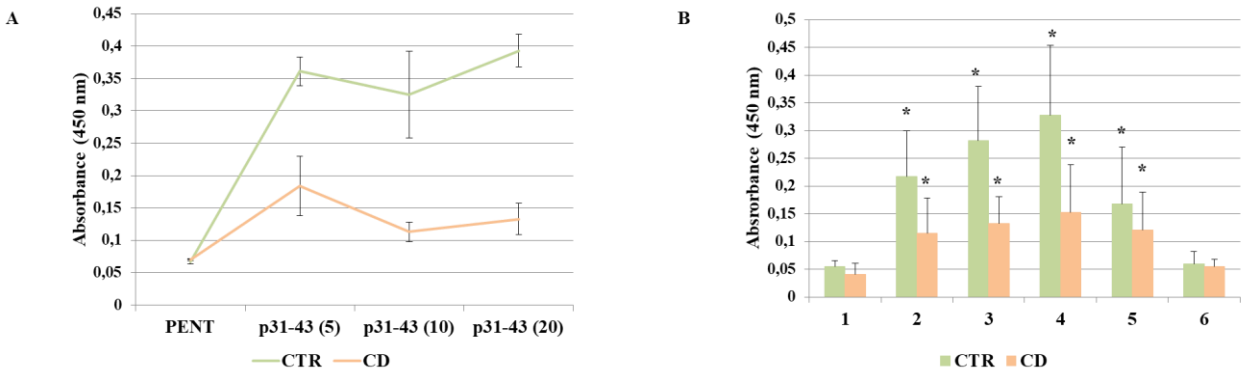

Figure 10. Effect of p31-43 on *in situ* TG2 activity in skin-derived fibroblasts. (A) Quantification of *in situ* TG2 activity induced by 5, 10 and 20 µg/mL of p31-43 by the microplate assay performed on 25 µg of cell lysates obtained from one representative non-CD sample and one representative CD sample. Basal *in situ* TG2 activity was obtained in the presence of pentylamine-biotin (PENT) only. Means and standard deviations of biological duplicates are reported. (B) Mean values (and standard deviations) relative to *in situ* TG2 activity measured in three non-CD samples and three CD samples treated with 5, 10 and 20 µg/mL of p31-43 and with 20 µg/mL of p57-68 (1, PENT; 2, p31-43 5 µg/mL; 3, p31-43 10 µg/mL; 4, p31-43 20 µg/mL; 5, p57-68 20 µg/mL; 6, pCTR 20 µg/mL). In these experiments, the α-gliadin

peptide 229-246 (pCTR) at 20 μg/mL was used as the irrelevant control peptide. *$p < 0.05$ versus respective pentylamine-biotin-treated cells.

The effect of p31-43 on TG2 protein expression was also analyzed. Skin-derived fibroblasts from CD and non-CD subjects were treated for 48 hours with 20 or 80 μg/mL of the toxic peptide; p57-68 at 20 μg/mL was used as the control peptide. Figure 11A shows a representative western blot anti-TG2 performed on skin-derived fibroblasts obtained from one representative CD patient and one representative non-CD healthy control. The amount of TG2 protein was normalized to that of the housekeeping gene GAPDH. Figure 11B shows densitometric analysis of blots relative to three CD cell cultures and three non-CD cell cultures. The results obtained showed that in non-CD cells, p31-43 did not induce TG2 protein expression after 48 hours of treatment, even at a high peptide concentration (i.e., 80 μg/mL), whereas, in CD cells, it was observed a significant increase of TG2 protein level already at 20 μg/mL; p57-68 only slightly induced TG2 protein expression in CD cells (Figure 11B). To verify whether the increase in TG2 protein expression levels was related to an increase in TG2 mRNA production, a real-time PCR analysis was performed. Skin-derived fibroblasts obtained from three CD patients and three non-CD healthy controls were treated for 24 hours with different amounts of p31-43 (20, 40 and 80 μg/mL). The results obtained showed that TG2 mRNA was over-expressed (about 2.5 fold), in CD cells only, in the presence of 80 μg/mL of p31-43 and not at lower concentrations (Figure 11C).

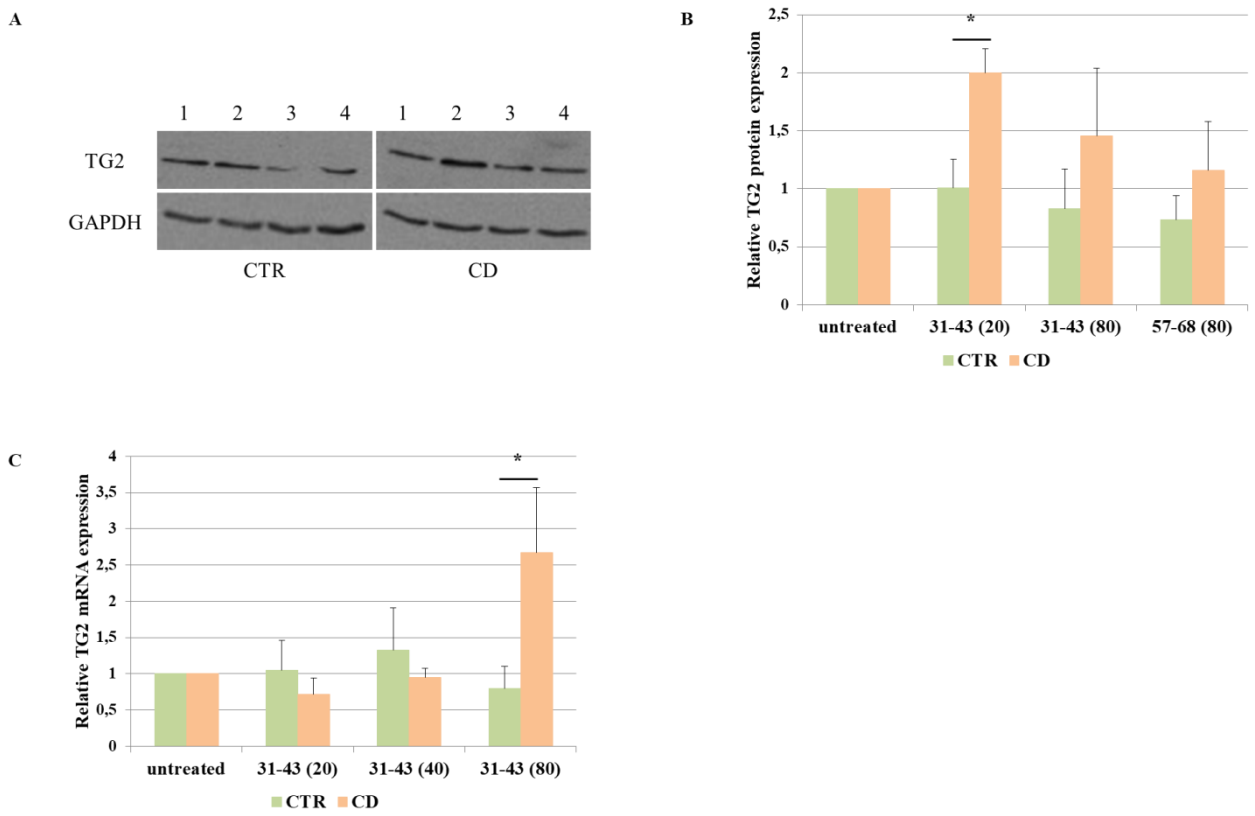

Figure 11. Effect of p31-43 on TG2 expression in skin-derived fibroblasts. (A) Representative western blot anti-TG2 analysis performed on fibroblasts from one representative non-CD sample and one representative CD sample after treatments of 48 hours with p31-43 (20 and 80 µg/mL) and p57-68 (20 µg/mL) (1, untreated; 2, p31-43 20 µg/mL; 3, p31-43 80 µg/mL; 4, p57-68 20 µg/mL). (B) Graphical representation of mean values (and standard deviations) of densitometric analysis of blots relative to three non-CD samples and three CD samples. GAPDH was used as the internal reference. (C) TG2 mRNA levels measured in three non-CD samples and three CD samples after treatments of 24 hours with 20, 40, and 80 µg/mL of p31-43. *$p < 0.05$ versus respective untreated samples.

3.7 Differences in intracellular Ca^{2+} homeostasis regulation between CD and non-CD cells

The lower activation of TG2 in CD fibroblasts with respect to non-CD ones after treatments with p31-43 could be related to a different regulation of intracellular Ca^{2+} homeostasis between these two groups of cells, particularly regarding the endoplasmic reticulum (ER), which is one of the targets of the Ca^{2+} mobilizing-activity of p31-43, as already demonstrated by Caputo et al. in Caco-2 cells [Caputo et al. 2012]. Therefore, to investigate whether regulation of intracellular Ca^{2+} homeostasis regarding the ER was different between CD and non-CD cells, single-cell Fura-2AM microfluorimetric studies were performed. In particular, it was measured the Ca^{2+} release from the ER in CD and non-CD cells by performing experiments in which THP, a molecule known to deplete Ca^{2+} store in the ER [Kitamura et al. 2011], was perfused in NK solution. As shown in

Figures 12A and 12B, basal intracellular Ca^{2+} level was lower in fibroblasts from CD patients than in those from non-CD healthy controls. Figure 12A also shows superimposed single-cell traces representative for the effect of THP on $[Ca^{2+}]i$ in CD and non-CD fibroblasts: the graph shows that perfusion with THP (whose starting time of addition is indicated, in the graph, by the arrow) induced a rapid rise in $[Ca^{2+}]_i$ in both CD and non-CD cells; however, THP-induced increase in $[Ca^{2+}]_i$ was higher in non-CD cells than in control ones. Importantly, THP reproduced the same effect on $[Ca^{2+}]i$ when perfused in a Ca^{2+}-free buffer, thus excluding the contribution of extracellular Ca^{2+}, flowing through plasma membrane, to THP-induced $[Ca^{2+}]_i$ increase (data not shown). By subtracting the value of the basal $[Ca^{2+}]_i$, measured before adding THP, from the value of $[Ca^{2+}]_i$, measured after adding THP, it was obtained the value of Ca^{2+} release from the ER (corresponding to the Ca^{2+} content in the ER) of CD and non-CD fibroblasts; this value, measured for several cells from each cell culture and expressed as percentage of variation, was reported in Figure 12C: the data obtained showed that the level of Ca^{2+} in the ER was lower in CD cells than in control ones. The same experiment, performed on further CD and non-CD cell cultures, gave similar results, as shown in Figure 12D. This suggested that CD cells had a constitutively lower content of Ca^{2+}, both in the ER and in cytosol, than non-CD ones and, more in general, Ca^{2+} homeostasis could be differently regulated between the two groups of cells. This could also explain why in CD fibroblasts it was found a lower activation of TG2 after treatments with p31-43 with respect to non-CD ones.

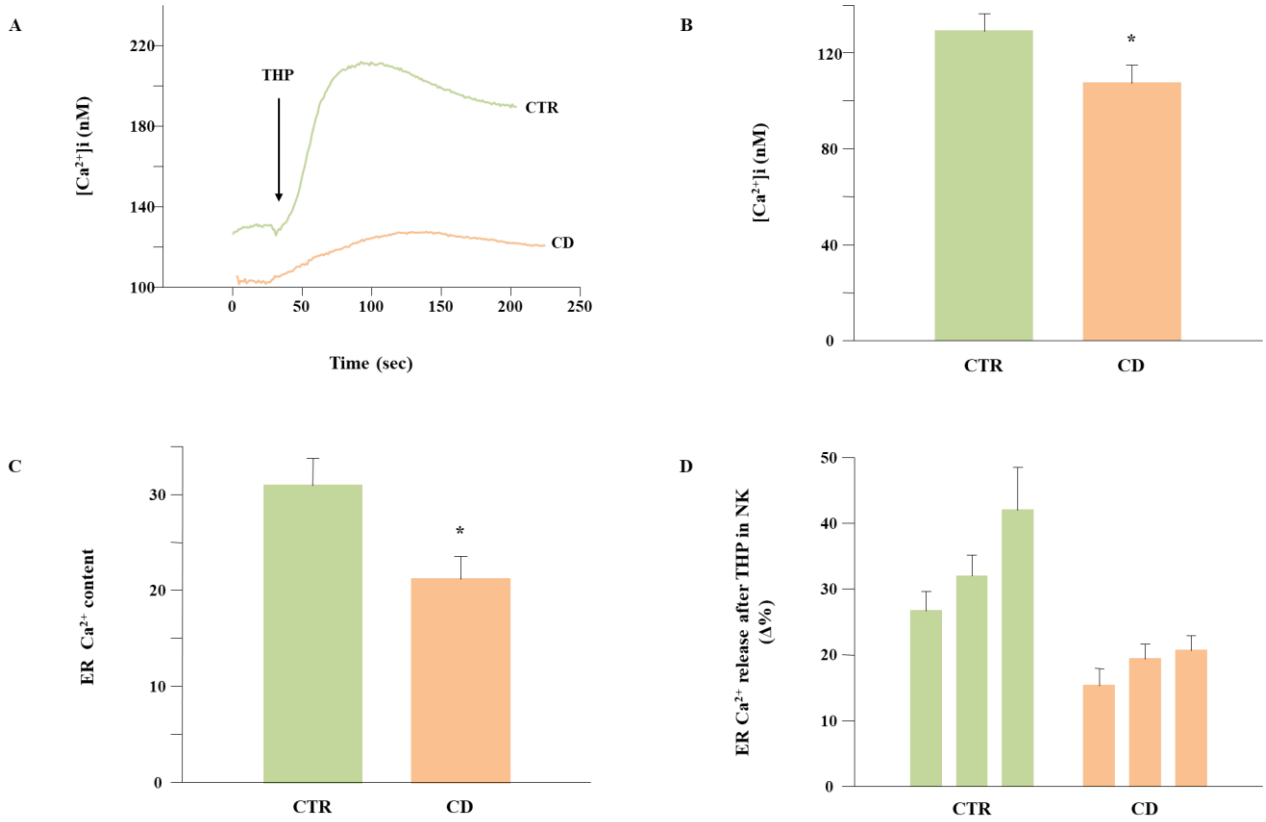

Figure 12. Ca^{2+} level measurement in skin-derived fibroblasts. (A) Superimposed single-cell traces representative for the effect of THP on $[Ca^{2+}]_i$ in one representative non-CD sample and one representative CD sample. Starting time of perfusion with THP is indicated by the arrow. (B) Quantification of basal $[Ca^{2+}]_i$ in non-CD and CD samples. (C) Quantification of the Ca^{2+} content in the ER of non-CD and CD samples. (D) Quantification of the Ca^{2+} release from ER after treatment with THP in three non-CD samples and three CD samples.

3.8 Effects of THP on TG2 expression and catalytic activity in CD and non-CD cells

In a 2012 paper, Caputo et al. demonstrated that, in Caco-2 cells, the THP-induced Ca^{2+} release from the ER was sufficient to activate the transamidating activity of the intracellular TG2 [Caputo et al. 2012]. To investigate whether THP was able to differently modulate intracellular TG2 catalytic activity between CD and non-CD cells, an *in situ* TG2 enzymatic assay by using the TG2 substrate pentylamine-biotin was performed. The data obtained comparing the TG2 activity measured in skin-derived fibroblasts obtained from three CD patients with that of skin-derived fibroblasts obtained from three non-CD healthy controls showed that the treatment of 30 minutes with different amounts of THP (0.01, 0.1, 0.5 and 1 μM) induced an increase in TG2 activity in non-CD cells only (Figure 13). Probably, in CD cells, the amount of Ca^{2+} released from the ER after stimulation with THP was not sufficient to activate the enzyme.

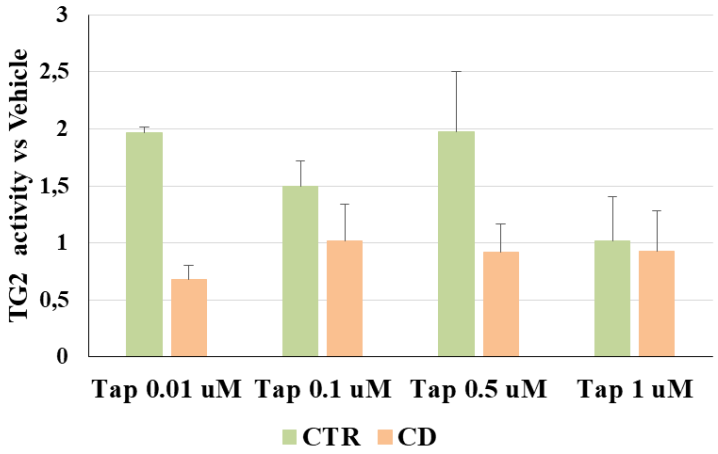

Figure 13. Effect of THP on TG2 activity in skin-derived fibroblasts.

The effect of THP on TG2 protein expression was also analyzed. Skin-derived fibroblasts from CD and non-CD subjects were treated for 24 hours with different amounts of THP (0.01, 0.1 and 0.5 µM). Western blot anti-TG2 analysis performed on skin-derived fibroblasts obtained from three CD patients and three non-CD healthy controls revealed that THP induced a reduction in TG2 protein expression levels in both groups of cells; however, this reduction was more pronounced in CD cells than in non-CD ones (Figure 14).

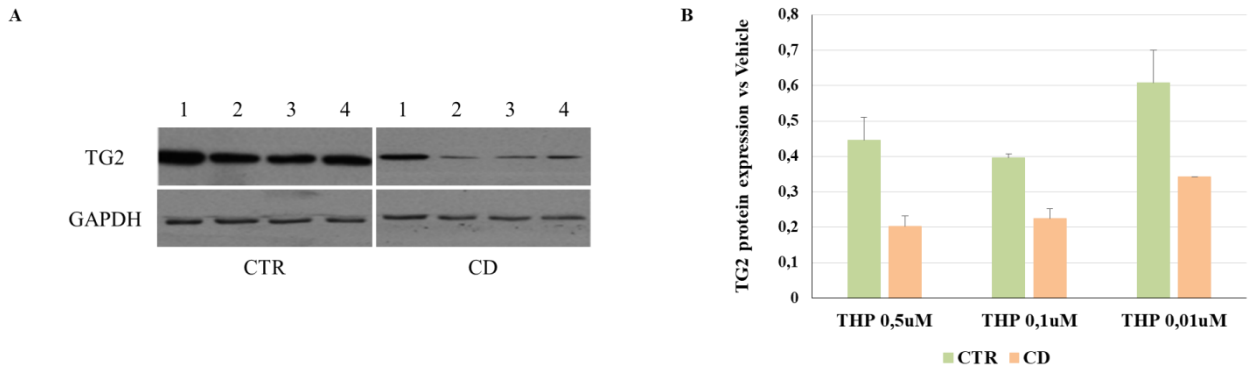

Figure 14. Effect of THP on TG2 protein expression in skin-derived fibroblasts. (A) Representative western blot anti-TG2 analysis performed on fibroblasts from one representative non-CD sample and one representative CD sample after treatments of 24 hours with THP 0.01, 0.1 and 0.5 µM (1, Vehicle; 2, THP 0,5 µM; 3, THP 0,1 µM; 4, THP 0,01 µM). (B) Graphical representation of mean values (and standard deviations) of densitometric analyses of blots relative to three non-CD samples and three CD samples. GAPDH was used as the internal reference.

3.9 Differences in p31-43-induced Ca^{2+} mobilization from intracellular deposits between CD and non-CD cells

Unlike THP, p31-43 activated TG2 also in CD fibroblasts (Section 3.6); this suggested that, in CD cells, other intracellular Ca^{2+} stores were involved in TG2 activation in the presence of the peptide. To verify this hypothesis, single-cell Fura-2AM microfluorimetric studies, where p31-43 was perfused in NK solution, were performed. Surprisingly, the resulted obtained comparing the increase in $[Ca^{2+}]_i$ in one CD cell culture and one non-CD cell culture showed that the Ca^{2+} release from intracellular deposits was higher in CD cells than in control ones (Figure 15). This confirmed that, in CD cells, other intracellular Ca^{2+} stores besides the ER were involved in Ca^{2+} release in the presence of p31-43. However, other cell cultures have to be tested to confirm this important finding.

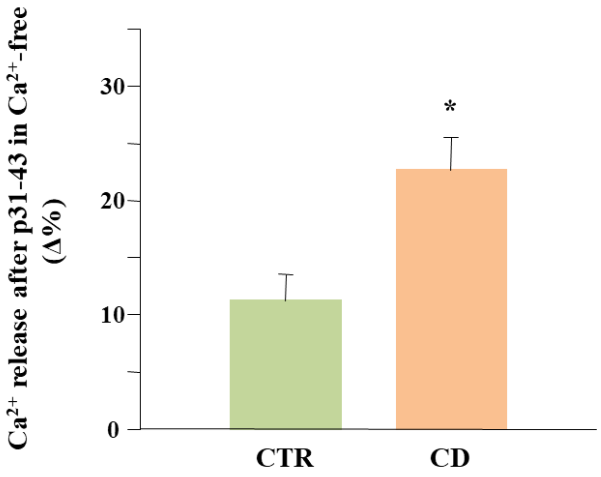

Figure 15. Quantification of the Ca^{2+} release from intracellular stores after treatment with p31-43 in four non-CD samples and three CD samples.

3.10 Differences in THP-induced ER stress response between CD and non-CD cells

THP is considered an ER stress-inducing agent. This molecule, in fact, inhibits SERCA, leading to a depletion of ER Ca^{2+} storage, which, in turn, decreases the activity of Ca^{2+}-dependent chaperones, thus leading to an increase in unfolded proteins and the corresponding induction of the Unfolded Protein Response (UPR) [Kitamura et al. 2011]. Based on differences found in regulation of intracellular Ca^{2+} homeostasis regarding ER, any differences in THP-induced ER stress response between CD and non-CD cells were investigated by analysing the expression of the well-known ER-stress biochemical marker GRP78, an ER-resident molecular chaperone belonging to the *heat*

shock family of proteins involved in correcting and clearing misfolded proteins in the ER [Gonzalez-Gronow et al. 2021], and the alternative splicing of XBP1, a key modulator of the UPR signaling [Wang et al. 2011].

3.10.1 Effects of THP on GRP78 protein and mRNA expression

GRP78 mRNA and protein expression levels were measured by real-time PCR and western blot analysis, respectively. Western blot anti-GRP78 analysis performed on skin-derived fibroblasts obtained from three CD patients and three non-CD healthy controls revealed that the basal expression level of the protein was significantly higher in non-CD cells than in CD ones (Figures 16A and 16B). Western blot anti-GRP78 analysis performed on skin-derived fibroblasts obtained from three CD patients and three non-CD healthy controls also revealed that treatment for 24 hours with THP at concentrations 0.1 and 0.5 μM caused a differential response between CD and non-CD cells: in non-CD cells, GRP78 protein expression levels increased after treatment with THP 0.1 μM and decreased after treatment with THP 0.5 μM; on the other hand, in CD cells, the increase in GRP78 protein expression levels was less evident at THP 0.1 μM but the response was clearly evident at THP 0.5 μM (Figures 16C and 16D).

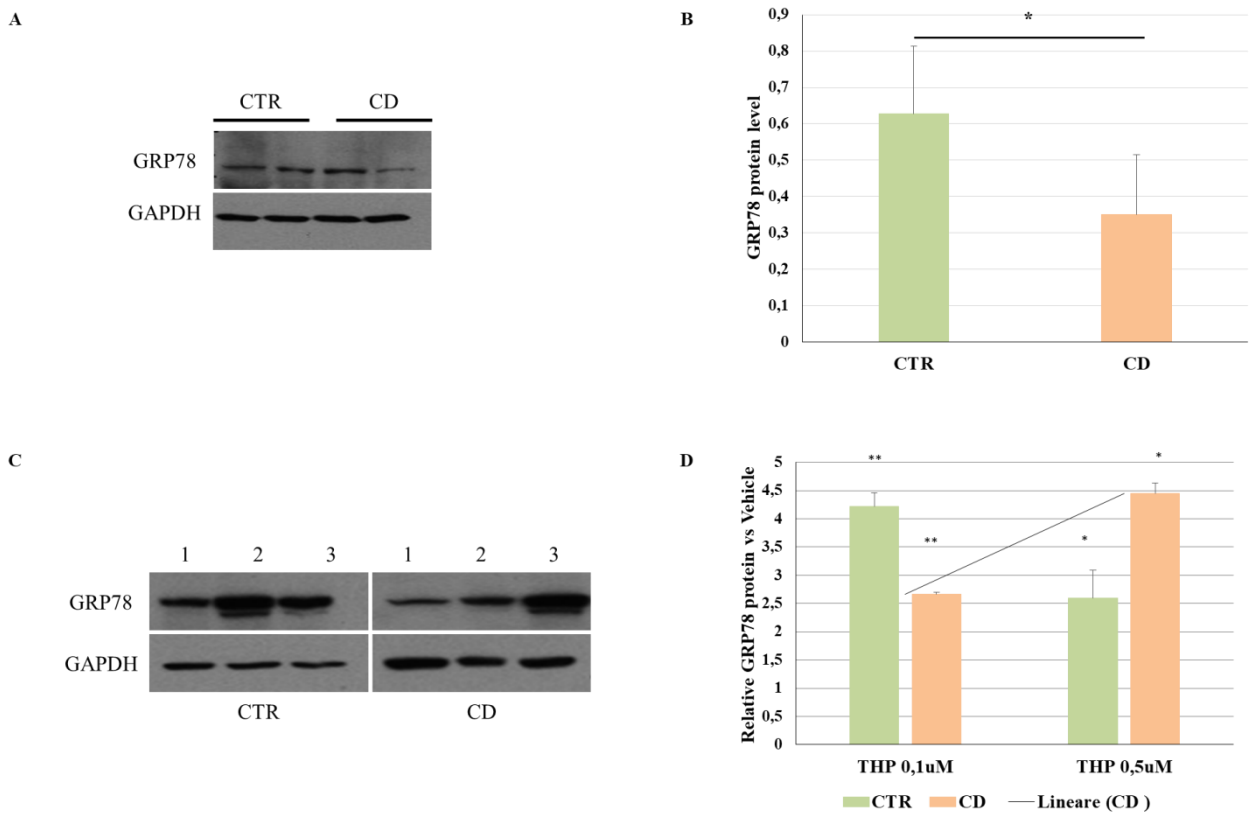

Figure 16. Effect of THP on GRP78 protein expression in skin-derived fibroblasts. (A) Representative western blot anti-GRP78 analysis performed on fibroblasts from two representative non-CD samples and two representative CD samples. (B) Graphical representation of mean values (and standard deviations) of densitometric analyses of blots relative to three non-CD samples and three CD samples. (C) Representative western blot anti-GRP78 analysis performed on fibroblasts from one representative non-CD sample and one representative CD sample after treatments of 24 hours with THP 0.1 and 0.5 μM (1, Vehicle; 2, THP 0,1 μM; 3, THP 0,5 μM). (D) Graphical representation of mean values (and standard deviations) of densitometric analyses of blots relative to three non-CD samples and three CD samples. GAPDH was used as the internal reference.

Real-time PCR analysis of GRP78 mRNA expression levels performed on skin-derived fibroblasts obtained from three CD patients and three non-CD healthy controls after 4 and 24 hours of treatment with different amounts of THP (0.01, 0.1 and 0.5 μM) revealed that GRP78 mRNA levels similarly increased in both groups of cells (Figure 17); however, after 4 hours of treatment with THP 0.01 μM, CD cells showed a higher increment in GRP78 mRNA levels than non-CD ones (Figure 17A).

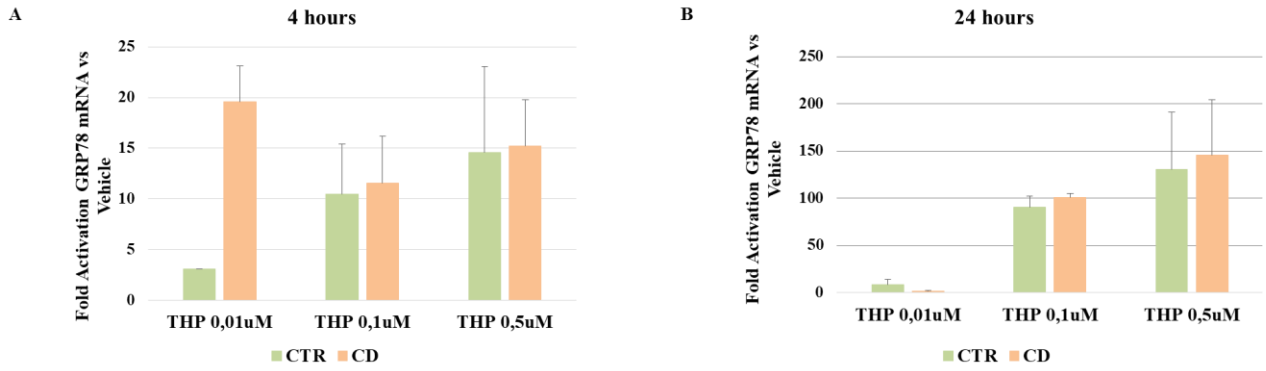

Figure 17. Effect of THP on GRP78 mRNA expression in skin-derived fibroblasts. GRP78 mRNA levels measured in three non-CD samples and three CD samples after 4 (A) and 24 (B) hours of treatment with THP 0.01, 0.1 and 0.5 µM.

3.10.2 Effects of THP on XBP1 splicing

XBP1 splicing was analysed by conventional PCR. Figure 18 shows the effect of treatments of 4 hours with different amounts of THP (0.01, 0.1 and 0.5 µM) on XBP1 splicing in CD and non-CD skin-derived fibroblasts. The data obtained showed that after treatment with THP 0.01 or 0.1 µM, the spliced form of XBP1 (sXBP1) similarly increased in both CD and non-CD cells; instead, after treatment with THP 0.5 µM, both CD and non-CD cells seemed to be in an adaptive stage with no further increase of sXBP1.

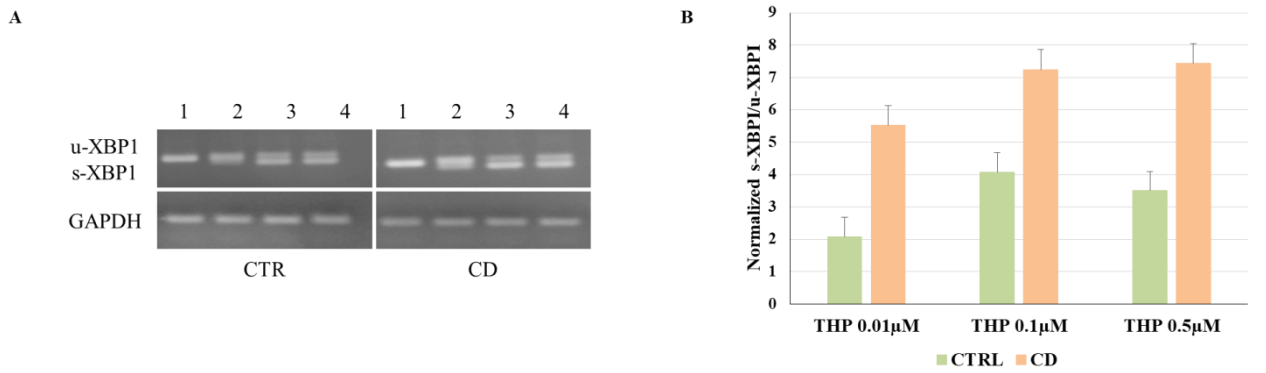

Figure 18. Effect of THP on XBP1 splicing in skin-derived fibroblasts. (A) XBP1 splicing in one representative non-CD sample and one representative CD sample after treatments of 4 hours with THP 0.01, 0.1 and 0.5 µM (1, Vehicle; 2, THP 0,01 µM; 3, THP 0,1 µM; 4, THP 0,5 µM). (B) Graphical representation of mean values (and standard deviations) of ratios between spliced (sXBP1) form and unspliced (uXBP1) form of XBP1, referred to analysis performed on three non-CD samples and three CD samples after treatment of 4 hours with THP 0.01, 0.1 and 0.5 µM.

3.11 Effects of THP on cell viability in CD and non-CD cells

Calcium is a fundamental regulator of cell signaling and function. By inhibiting SERCA, THP

disrupts Ca^{2+} homeostasis, thereby inducing a cytotoxic action that can causes cell death [Hiroi et al.2005]. To evaluate the effect of THP on cell viability in CD and non-CD cells, an MTT assay was performed. The results obtained showed that THP (tested for 24 hours at 0.01, 0.1, 0.5 and 1 μM) reduced cell viability in both groups of cells; however, CD cells appeared less sensitive to the cytotoxic effect of THP than non-CD ones (Figure 19): indeed, at the lowest concentration tested (0.01 μM) THP reduced viability of non-CD cells by approximately 20%, whereas CD cells were unaffected; only at the highest concentration tested (1 μM) THP reduced CD cells viability by 20% (in this condition, non-CD cells viability was reduced by approximately 40%.

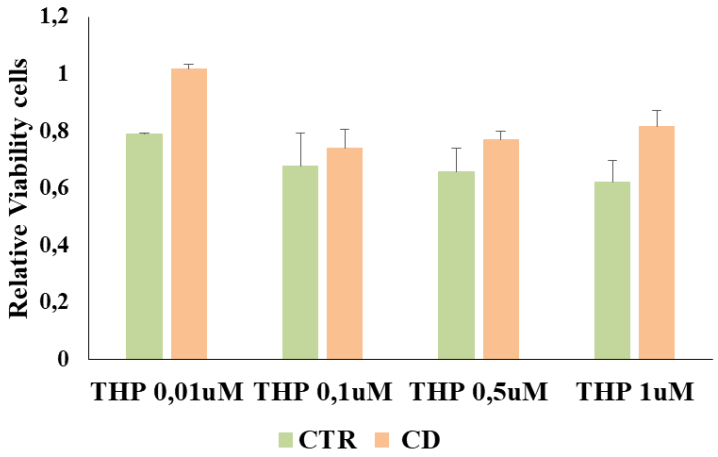

Figure 19. Cytotoxic effect of THP on skin-derived fibroblasts. Graphical representation of mean values (and standard deviations) of three independent MTT assays performed in triplicate on three non-CD samples and three CD samples.

3.12 Effects of THP on autophagy in CD and non-CD cells

Eukaryotic cells respond to the accumulation of unfolded proteins in the ER either by UPR, which results in the expression of chaperones and other proteins that act as folding catalysts, or by apoptosis, when the ER function cannot be restored. Accumulating data indicate that ER stress is also a potent trigger of autophagy, an important biological process whereby cells recycle their macromolecules and organelles. Depending on the context, autophagy counterbalances ER stress-induced ER expansion, enhances cell survival or commits the cell to non-apoptotic death [Høyer-Hansen et al. 2007; Yorimitsu et al. 2006]. Based on differences found in THP-induced ER stress response between CD and non-CD cells, any differences in THP-induced autophagy between the two groups of cells were investigated by analysing the expression of two important autophagy markers, LC3 (the accumulation of the LC3-II form of LC3 is indicative of autophagic

compartments formation) [Tanida et al. 2008] and p62 (the increase in p62 protein expression levels indicates the possibility of an insufficiency in autophagy) [Tanida et al. 2010]. Western blot anti-LC3 analysis performed on skin-derived fibroblasts obtained from three CD patients and three non-CD healthy controls revealed that the basal expression level of the LC3-II form of LC3 was significantly higher in CD cells than in non-CD ones (Figures 20A and 20B), whereas the basal expression level of p62 was similar in both groups of cells (Figures 20C and 20D).

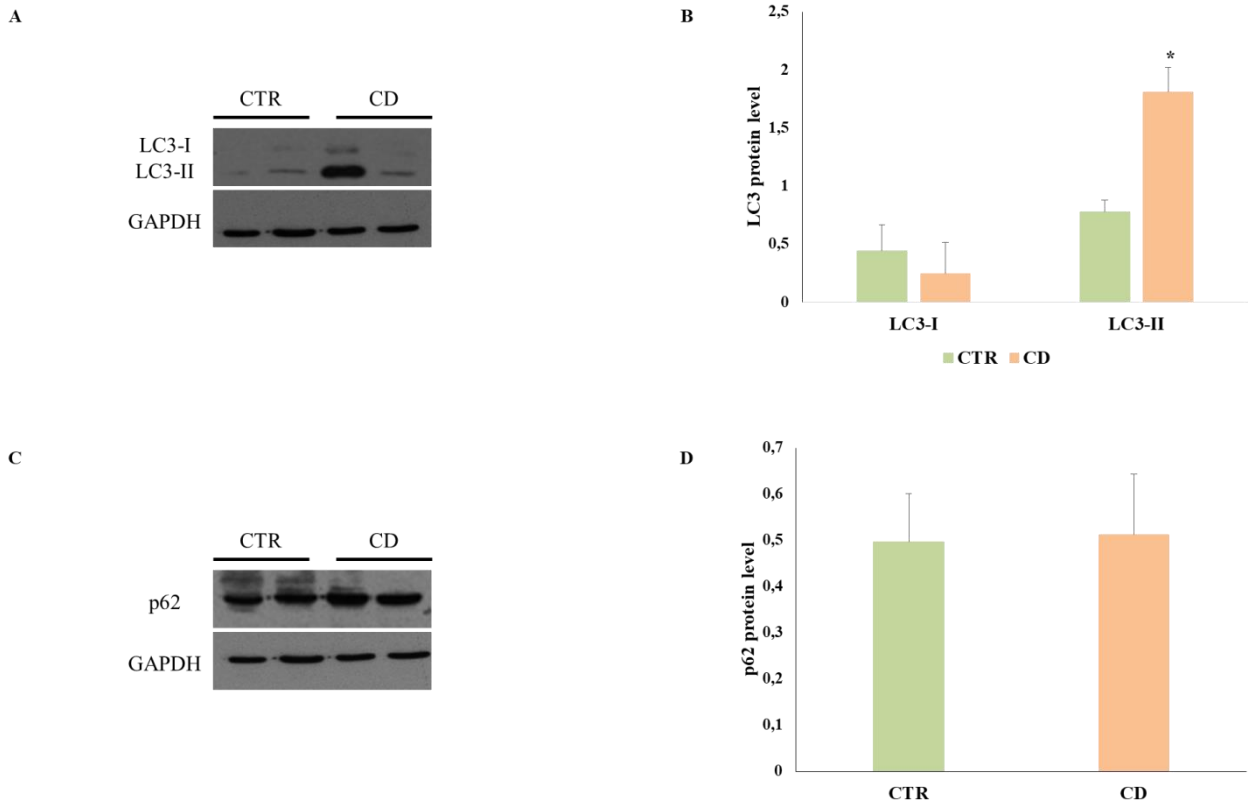

Figure 20. LC3 and p62 protein expression in skin-derived fibroblasts. (A) Representative western blot anti-LC3 analysis performed on fibroblasts from two representative non-CD samples and two representative CD samples. (B) Graphical representation of mean values (and standard deviations) of densitometric analyses of blots relative to three non-CD samples and three CD samples. (C) Representative western blot anti-p62 analysis performed on fibroblasts from two representative non-CD samples and two representative CD samples. (D) Graphical representation of mean values (and standard deviations) of densitometric analyses of blots relative to three non-CD samples and three CD samples. GAPDH was used as the internal reference.

Western blot anti-LC3 and anti-p62 analysis performed on skin-derived fibroblasts obtained from three CD patients and three non-CD healthy controls also revealed that treatment with THP caused a differential response between CD and non-CD cells: in non-CD cells, treatment with THP induced a dose-dependent increase in LC3-II and a dose-dependent decrease in p62; on the other hand, in

CD cells, treatment with THP induced a dose-dependent increase both in LC3-II and p62 (Figure 21).

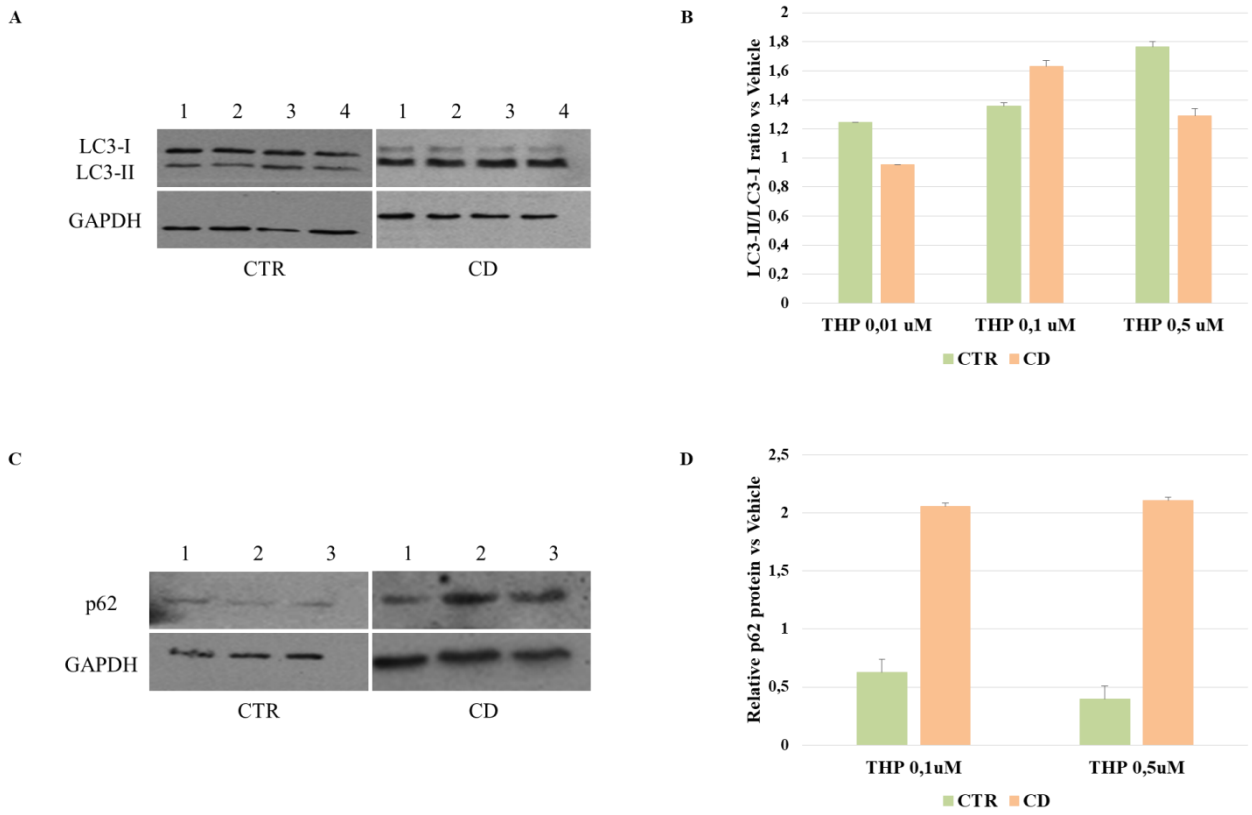

Figure 21. Effect of THP on LC3 and p62 protein expression in skin-derived fibroblasts. (A) Representative western blot anti-LC3 analysis performed on fibroblasts from one representative non-CD sample and one representative CD sample after treatments of 24 hours with THP 0.01, 0.1 and 0.5 µM (1, Vehicle; 2, THP 0.01 µM; 3, THP 0.1 µM; 4, THP 0.5 µM). (B) Graphical representation of mean values (and standard deviations) of densitometric analyses of blots relative to three non-CD samples and three CD samples. (C) Representative western blot anti-p62 analysis performed on fibroblasts from one representative non-CD sample and one representative CD sample after treatments of 4 hours with THP 0.1 and 0.5 µM (1, Vehicle; 2, THP 0.1 µM; 3, THP 0.5 µM). (D) Graphical representation of mean values (and standard deviations) of densitometric analyses of blots relative to three non-CD samples and three CD samples. GAPDH was used as the internal reference.

DISCUSSION

The presence of constitutive alterations in CD cells with respect to non-CD ones has recently led to the definition of the so-called "celiac cellular phenotype". For example, CD cells exhibit altered cell shape and actin organization, an increased number of focal adhesions and a generally higher level of total phosphorylated proteins than non-CD ones [Paolella et al. 2020]. TG2, and particularly anti-TG2 autoantibodies, also contributes to the "celiac cellular phenotype". Indeed, anti-TG2 autoantibodies, by forming complexes with cell-surface TG2, are able to reduce the uptake of the toxic α-gliadin peptide 31-43 by non-CD fibroblasts but not by CD ones [Paolella et al. 2017]. Previous studies have demonstrated that this different handling of p31-43 by CD and non-CD cells, in the presence of anti-TG2 autoantibodies, does not depend on differences in TG2 expression or catalytic activity [Paolella et al. 2017]. Also a differential TG2 subcellular distribution in CD and non-CD cells could explain the different behavior regarding the p31-43 uptake in the presence of anti-TG2 antibodies by the two groups of cells. Therefore, it was investigated whether CD and non-CD fibroblasts showed differences in TG2 localization in different cellular compartments. The results obtained showed that TG2 was associated with the membrane fraction (particularly the cell surface membrane), the early endosomal compartment and the autophagic compartment more in CD cells than in non-CD ones. Based on these results, it is possible to speculate that, in the intestinal CD mucosa environment, a slightly higher amount of TG2, potentially active, on the cell surface could promote gluten peptides cross-linking and deamidation, thereby enhancing the immune and auto-immune response. A higher association with the early endosomal compartment could indicate that, in CD cells, TG2 persists in early endosomes and, consequently, its targeting to lysosomes could be delayed; a higher amount of TG2 in the early endosomal compartment could also enhance gluten peptides deamidation by the enzyme due to the slightly acidic pH of this compartment. Lastly, a higher association with the autophagic compartment could be related to the complex role of TG2 in regulating autophagy, a process that could contribute, if deranged, to CD pathogenesis [Maiuri et al. 2019; Manai et al. 2018]. Overall, these data support the idea that TG2 localization inside CD cells contributes to defining the "celiac cellular phenotype"; however, the biological consequence of this contribution remains to be elucidated.

In the attempt to identify other differences regarding TG2 between CD and non-CD cells, it was also investigated whether p31-43 was able to differentially modulate TG2 expression and intracellular activity in CD and non-CD fibroblasts. The results obtained showed that prolonged stimulation (48 hours) with the peptide induced an increase in TG2 protein levels in CD cells only.

However, the increase in TG2 protein levels was not related to an increase in TG2 mRNA levels, suggesting that the increase in TG2 protein levels could be due, at least in part, to a modulation of TG2 turnover; the ability of p31-43 to delay endosomal trafficking [Nanayakkara et al. 2013] could also reduce the rate of TG2 endocytosis and consequent degradation of the protein into lysosomes. Stimulation with p31-43 also induced a less pronounced TG2 activation in CD fibroblast than in non-CD ones. Differences in the regulation of intracellular Ca^{2+} homeostasis regarding the ER, which is one of the targets of the Ca^{2+} mobilizing-activity of p31-43 [Caputo et al. 2012], could explain this different response to p31-43 in CD and non-CD cells; indeed, previous studies have demonstrated that TG2 does not possess different catalytic properties between CD and non-CD cells [Paolella et al. 2017]. The results obtained by analyzing the response to stimulation with THP, a molecule that selectively depletes ER, were in line with this hypothesis and showed that CD cells had a constitutively lower Ca^{2+} content, both in the ER and in cytosol, than non-CD ones. Ca^{2+} is an extremely versatile intracellular messenger that controls a wide range of cellular functions by regulating the activity of a vast number of target proteins [Newton et al. 2016]; therefore, in CD cells, alterations in intracellular Ca^{2+} levels could significantly influence the homeostasis of the whole cell.

THP was also used to discriminate different responses related to TG2 expression and intracellular activity between CD and non-CD cells. However, since THP is a known inducer of the ER-stress, this molecule could produce acute cytotoxic effects in cultured cells. Therefore, firstly, the effect of THP on cell viability in CD and non-CD fibroblasts was evaluated. The results obtained showed that CD cells appeared slightly less sensitive to the THP-induced cytotoxicity than non-CD ones. This might be due to the intrinsic higher proliferation rate of CD cells [Nanayakkara et al. 2013], which could make them more resistant to the presence of the toxic agent. In any case, the different response to THP by CD cells with respect to non-CD ones can be considered another aspect of the "celiac cellular phenotype". Stimulation with THP activated TG2 in non-CD fibroblasts only; probably, in CD cells, THP-induced Ca^{2+} release from the ER was not sufficient to activate the enzyme. However, as reported above, stimulation with p31-43 activated TG2 also in CD fibroblasts (although TG2 activation was less pronounced in CD cells than in non-CD ones), suggesting that, in CD cells, other Ca^{2+} stores besides the ER were involved in p31-43-induced TG2 activation. In this regard, in Caco-2 cells, Caputo at al. have demonstrated that p31-43 is able to mobilize Ca^{2+} from mitochondria too [Caputo et al. 2012]. The finding that stimulation with p31-43 induced a higher increase in $[Ca^{2+}]_i$ in CD fibroblasts than in non-CD ones supported this hypothesis. Stimulation with THP also reduced TG2 expression more in CD fibroblasts than in non-CD ones; however, the biological significance of this different response remains to be elucidated.

THP is able to disrupt ER homeostasis, thus causing a stress condition termed “ER stress”. When cells are subjected to ER stress, an adaptive mechanism referred to as UPR is triggered to allow them to restore ER homeostasis [Corazzari et al. 2017]. The response to THP-induced ER stress was investigated in CD and non-CD cells by analyzing two markers of ER stress, which are GRP78 expression and XBP1 alternative splicing. The results obtained showed, firstly, that basal GRP78 levels were significantly lower in CD cells than in non-CD ones. Secondly, stimulation with THP caused a differential response in CD and non-CD fibroblasts: in non-CD cells, GRP78 increased at the lowest THP concentration tested (0.1 μM) and decreased at the highest THP concentration tested (0.5 μM); in contrast, in CD cells, the increase in GRP78 was little evident at THP 0.1 μM but very marked at THP 0.5 μM. However, sXBP1 production was similar in both CD and non-CD cells. Overall, these data suggest that, in CD cells, the UPR was “less rapid” but stronger than in non-CD ones; moreover, unlike non-CD cells, CD cells appeared to fail to adapt to the THP-induced ER stress, probably because of the lower basal GRP78 levels. However, whether or not gluten peptides are able to induce a differential UPR in the two groups of cells has yet to be determined.

THP is also able to trigger autophagy, an adaptive stress response that contributes to restoring ER homeostasis [Lee et al. 2015]. To investigate how THP affects this process in CD and non-CD fibroblasts, the expression of LC3 and p62, two widely used autophagy-markers, was monitored. The results obtained showed, firstly, that basal LC3-II levels were significantly higher in CD cells than in non-CD ones. In non-CD cells, stimulation with THP induced a dose-dependent increase in LC3-II and a dose-dependent decrease in p62; this was indicative of a progressive autophagosome maturation, as expected. In CD cells, stimulation with THP induced a dose-dependent increase both in LC3-II (although the increase in LC3-II was not very pronounced) and p62, suggesting a delay or blockage of the autophagic flux. Høyer-Hansen et al. have demonstrated that the increase in $[Ca^{2+}]_i$ induced by Ca^{2+}-mobilizing agents is a potent inducer of autophagy [Høyer-Hansen et al. 2007]. Therefore, it is possible to speculate that, in CD cells, a lower THP-induced Ca^{2+} release from the ER could contribute to impair the autophagy flux in the presence of THP. However, p31-43 induced a higher increase in $[Ca^{2+}]_i$ in CD cells than in non-CD ones by mobilizing Ca^{2+} from other intracellular stores besides the ER; in any case, the amount of Ca^{2+} released could be insufficient to activate the autophagy flux. In this regard, Manai et al. have demonstrated that stimulation with gluten peptides leads to an impairment of the autophagy pathway in Caco-2 cells [Manai et al. 2019; Manai et al. 2018]. Therefore, it is possible to hypothesize that gluten peptides, and in particular p31-43, might exacerbate the “celiac cellular phenotype” with regard to the defective autophagy. Moreover, D’Eletto et al. have highlighted an important role for TG2-mediated post-

translational modifications in the autophagy pathway [D'Eletto et al. 2012]. Therefore, in CD cells, a non-activation of TG2 after stimulation with THP might could represent another factor that contribute to a negative modulation of autophagy. Finally, it is possible to hypothesize that, in CD cells, a lower p31-43-induced TG2 activation could be not sufficient for proper completion of the autophagic flux.

CONCLUSIONS

The data reported in this PhD thesis work show that TG2 is differently distributed between CD and non-CD cells; in particular, the protein is associated with the cell surface membrane and some intracellular vesicular compartments (early endosomal compartment and autophagic compartment) more in CD fibroblasts than in non-CD ones. Therefore, it was hypothesized that the different TG2 subcellular distribution between CD and non-CD cells is related, in a still unclear manner, to the different handling of p31-43, in the presence of anti-TG2 antibodies, by the two groups of cells. Moreover, p31-43 differently regulates TG2 expression and intracellular activity between CD and non-CD cells. Specifically, in CD cells, the peptide induces a lower TG2 activation than in non-CD cells; however, the low activation of the enzyme is partially restored by a p31-43-induced increase in its expression levels. The data also show that, in CD cells, intracellular Ca^{2+} homeostasis is deregulated, thus causing both a strong UPR and an engulfed autophagy in response to stimulation with THP. Interestingly, p31-43 mobilizes Ca^{2+} from intracellular stores more in CD cells than in non-CD ones; however, the p31-43-induced increase in Ca^{2+} only partially activates TG2. In conclusion, this PhD thesis work adds a small piece of knowledge about the complex interplay between gluten peptides and TG2 in the pathogenetic mechanisms of CD; however, further in-depth investigations are needed to better characterize the contribution of gluten and TG2 to the “celiac cellular phenotype”.

ABBREVIATIONS

(APCs) Antigen-Presenting Cells

(BBS) Borate Buffered Saline

(BSA) Bovine Serum Albumin

(CD) Celiac Disease

(cDNA) complementary DNA

(DGP) antibodies Deamidated Gliadin Peptide antibodies

(DMSO) Dimethyl Sulfoxide

(DTT) Dithiothreitol

(ECM) Extracellular Matrix

(EDTA) Ethylenediaminetetraacetic Acid

(EEA1) Early Endosome Antigen 1

(EGF) Epithelial Growth Factor

(EGFR) Epithelial Growth Factor- Receptor

(EMA) Endomysial Antibodies

(ER) Endoplasmic Reticulum

(ERK) Extracellular signal-Regulated Kinase

(FAK) Focal Adhesion Kinase

(FCCP) Carbonylcyanide-p-trifluoromethoxyphenylhydrazone

(FITC) Fluorescein-5-Isothiocyanate

(Fura-2AM) Fura-2 Acetoxymethyl Ester

(GAPDH) Glyceraldehyde-3-Phosphate Dehydrogenase

(GDP) Guanosine Diphosphate

(GFD) Gluten Free Diet

(GMP) Guanosine Monophosphate

(GRP78) 78-kDa Glucose-Regulated Protein

(GTP) Guanosine Triphosphate

(HLA) Human Leukocyte Antigens

(INF- γ) Interferon-γ

(IELs) Intraepithelial Lymphocytes

(IL-15) Interleukin-15

(LAMP2) Lysosomal-Associated Membrane Protein 2

(LC3) Microtubule-Associated Protein 1A/1B Light Chain 3B

(LPP) LIM-containing Lipoma-Preferred Partner

(MHC) Major Histocompatibility Complex

(MTT) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

(OD) Optical Density

(p31-43) peptide 31-43

(p57-68) peptide 57-68

(PBS) Phosphate Buffered Saline

(PCR) Polymerase Chain Reaction

(PFA) Paraformaldehyde

(PMSF) Phenylmethylsulfonyl Fluoride

(PVDF) Polyvinylidene Fluoride

(TRITC) 5/6-Tetramethyl-Rhodamine Isothiocyanate

(SDS) Sodium Dodecyl Sulfate

(SDS-PAGE) Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis

(SERCA) Sarcoendoplasmic Reticulum Calcium ATPase

(SQSTM1) p62/Sequestosome 1

(TfR) Transferrin Receptor

(TBS) Tris Buffered Saline

(T-BBS) Borate Buffered Saline-Tween

(T-TBS) Tris Buffered Saline-Tween

(TG2) Type 2 Transglutaminase

(THP) Thapsigargin

(TMB) 3,3',5,5'-Tetramethylbenzidine

(UPR) Unfolded Protein Response

(XBP1) X-Box Binding Protein 1

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