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BIOCHEMICAL MECHANISMS UNDERLYING THE CELIAC CELLULAR PHENOTYPE

Ph.D. Tutor:

Professor Ivana Caputo

Discussant:

Professor Anna Marabotti

Ph.D. Coordinator:

Professor Alessandra Lattanzi

Ph.D. Candidate:

Antonio

Montefusco

Matr.

8860400011

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*“A te, tre anni da ora.
Da te, tre anni fa.”*

ABSTRACT

Celiac disease (CD) is an enteropathy characterised by an immune response to dietary gluten. Emerging evidence highlights the existence of a constitutive cellular phenotype that persists independently of gluten exposure. This thesis provides a comprehensive biochemical and molecular characterisation of this phenotype using, as study models, primary fibroblasts derived from skin and intestinal biopsies of CD patients, compared with matched healthy controls.

Skin fibroblasts from CD patients displayed reduced sensitivity to the ER stress-inducer thapsigargin, prolonged unfolded protein response (UPR), and impaired autophagic flux, linked to altered calcium homeostasis and decreased activation of type 2 transglutaminase (TG2). Similar defects were observed in intestinal fibroblasts, indicating that these alterations may be systemic and not tissue restricted.

Omics approaches further highlighted shared molecular signatures in both models. Proteomics identified consistent differential expression of proteins linked to extracellular matrix organisation, exosomes, vesicular trafficking, cytoskeletal remodelling, Ca^{2+} homeostasis, UPR signalling in CD cells. Interestingly, among proteins downregulated in both cell systems, we also found TG2, thus underlining the central role of this enzyme in the celiac phenotype. In addition, several proteins appeared differentially phosphorylated in skin fibroblasts. On the other hand, a metabolomic analysis conducted on intestinal fibroblasts revealed distinct energy metabolism defects, including altered levels of glucose, glutamine, and dihydroxyacetone, supporting impaired glycolysis and amino acid metabolism. Finally, transcriptomic profiling of CD intestinal fibroblasts treated with the clinically relevant irreversible TG2 inhibitor ZED1227 could provide new insights into TG2-dependent transcriptional networks, with differential expression analysis currently underway.

Altogether, these findings uncover a multi-layered cellular phenotype in CD across distinct tissue-derived fibroblast populations, comprising altered stress responses, autophagy, Ca^{2+} homeostasis, vesicular trafficking, and metabolic pathways. In conclusion, this thesis contributes to increasing knowledge about the celiac cellular phenotype and may pave the way for the research of new disease biomarkers and therapeutic targets.

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Abbreviations

BSA – Bovine serum albumin;

CD – Celiac disease;

CTR – Control subjects;

DMSO - Dimethyl sulfoxide;

DGP - anti-deamidated gliadin peptide;

ER - endoplasmic reticulum;

HRP - horseradish peroxidase

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

PBS - Phosphate Buffered Saline;

PLS-DA - Partial Least-Squares Discriminant Analysis;

SDS-PAGE - Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis

SNP - Single Nucleotide Polymorphism;

TG2 – Transglutaminase 2;

THP – thapsigargin;

TPI - triose phosphate isomerase;

UPR – Unfolded protein response;

4D-DIA - Four-Dimensional Data-Independent Acquisition;

1. Introduction

1.1 Celiac disease

Celiac disease (hereafter referred to as CD) originated in ancient times and was described by a Greek physician as a condition of malabsorption and diarrhea in infants. For its first more accurate description, we had to wait until the year 1888, when Samuel Gee described chronic indigestion in children and suggested that the cure was to be found in diet.¹ Today, after years of progress in research in this area, we can define CD as a chronic immune-based enteropathy caused by an adverse reaction to gluten, a protein found within wheat, barley, and rye.^{2,3} With a more in-depth description of the condition, we can also state that CD presents a clinical picture characterized by abdominal pain, bloating, chronic diarrhea, weight loss, and alterations in the structure and morphology of the intestine.^{4,5} Most of these symptoms can also be traced back to a deficiency of vitamins, minerals, and electrolytes due to malabsorption caused by villous atrophy due to gluten sensitivity.⁶ Furthermore, there is a spectrum of extra-intestinal manifestations related to CD, including dermatitis herpetiformis⁶, headache⁷, fatigue⁷, gluten ataxia⁸, and epilepsy.⁹

1.2 Epidemiology

The epidemiological aspect of CD is exciting. Although the current incidence rates of the disease in the world range from 0.7% to 2.9%¹⁰, this value appears to be the result of various factors and therefore must be analyzed carefully. First, it is important to underline that the incidence of the pathology has undergone a significant increase in recent decades.¹¹ This is due to a series of factors such as a more efficient screening network, more sensitive serological tests (further information in the diagnosis chapter), and reduced tolerance to gluten in the population. Furthermore, it must be considered that this range of incidence varies considerably throughout the world. Initially, it was believed that this pathology was limited to the caucasian population. Erroneously, this data does not consider the evolutionary history of man, his habits, and his

migratory flows. Furthermore, this consideration arises from the fact that in the European population, the gastro-intestinal symptoms typical of CD are much more common, while in the rest of the world extra-intestinal manifestations are fifteen times more frequent, thus complicating the correct diagnosis. Indeed, *Gujral et al.* brilliantly illustrated how the prevalence and incidence of CD follow and has always followed wheat consumption around the world. Factors such as the modification of eating habits, the transition to a sedentary lifestyle, and the improvement of environmental conditions have led humans to increase the consumption of wheat over the years, with a consequent increase in exposure to gluten.¹² CD is present in the population with a higher prevalence in women than men¹³; this is because many cases remain undiagnosed, often because men have a lower propensity for endoscopy. In addition to gender differences, the incidence of CD appears to be different in some groups of the population, such as in the case of first-degree relatives, where the incidence appears to be 10-20% higher.¹⁴ Furthermore, the prevalence of CD in identical twins has been found as high as 75%-80%.^{15,16} Lastly, the prevalence of CD affects other groups of the population with a greater frequency, such as patients with type 1 diabetes, Turner syndrome, Down syndrome, and autoimmune thyroiditis.¹⁷

1.3 Pathophysiology of CD

The pathogenesis of CD is a complex, multifactorial process primarily involving genetic, immune, and environmental factors. The physio-pathological manifestations arise in genetically predisposed subjects following the ingestion of gluten, which is the main triggering factor. Compared to a few decades ago, today many studies have been conducted on all the possible actors involved in the pathology. The genetic background is certainly a predominant aspect as the presence of HLA DQ2/DQ8 *loci* is strongly associated with the development of the pathology (this aspect will be explored in depth in the next paragraph). On the other hand, the most important exogenous factor is gluten, which determines the loss of tolerance towards itself in predisposed subjects, thus triggering the pathology. Although these are the two most well-known actors, to date a wide range of works in the literature show that the situation is even more complex. New evidence suggests that intestinal microbiota may play a role in CD, potentially influencing the immune response and modulating the disease onset. Dysbiosis (an

imbalance of gut microbes) has been observed in CD patients. T-cells are considered a key component in the pathogenesis of CD, as they seem to play a role in innate immune mechanisms. They act as initiators of the inflammatory response after gluten peptides are presented to them.^{12,18–20}

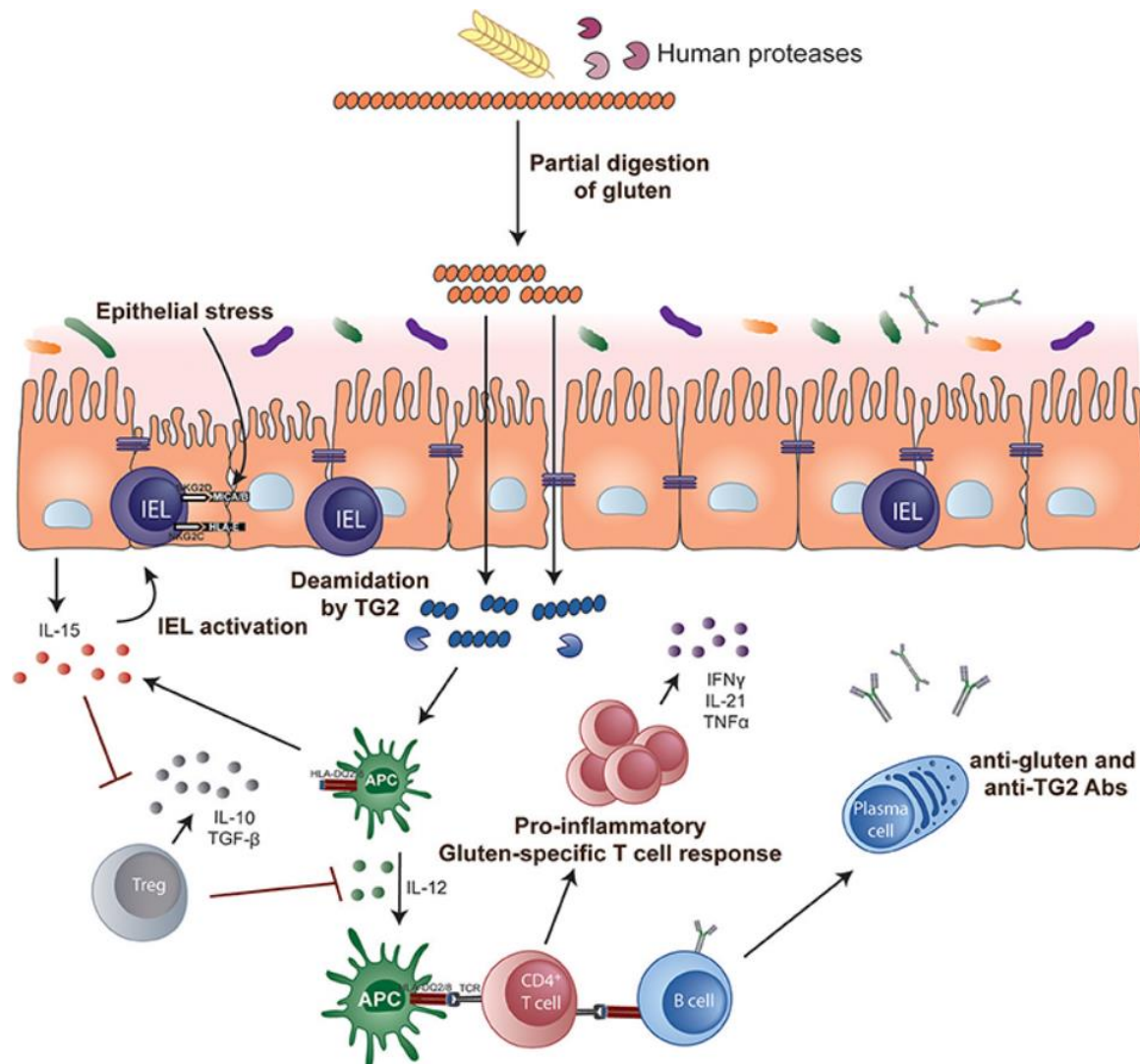


Figure 1. Key processes in the pathogenesis of celiac disease, which include several interconnected steps. Gluten peptides containing T-cell epitopes evade degradation in the gastrointestinal tract. TG2 facilitates the deamidation of these gluten peptides, enhancing their ability to bind efficiently to disease-associated HLA-DQ molecules on antigen-presenting cells. This leads to the recruitment of immune system cells with a subsequent release of cytokines. These, such as IFN-γ and IL-21, contribute to intestinal damage, promote the activation of intraepithelial lymphocytes (IELs), and stimulate B-cell responses. Image obtained from the work of *Tye-Din et al. 2018*²¹.

1.3.1 Genetics

As already discussed in paragraph 1.1.1, CD boasts a strong hereditary component, with a prevalence percentage between 10% and 20% among first-degree relatives and between 75% and 80% among homozygous twins.^{13–15} These data depend on a strong genetic component that is present in CD. Although the genetic component is very important in determining the outcomes of CD, the presence of specific genes represents a necessary but not sufficient condition for the development of the pathology, as many of the involved genes appear to be widely spread in the population. The main genes involved concern haplotypes (the combination of allelic variants along a chromosome is defined as a haplotype) of the MHC class II system, in particular the HLA haplotypes DQ2 and DQ8, present on chromosome 6p21. Between 40 and 50% of the genetic variance in CD is attributable to the MHC *locus*.²⁰ Several studies with genome-wide approaches have demonstrated the existence and correlation of other non-HLA genes with CD (Table 1).

Table 1. Some of the polymorphisms that refer to non-HLA genes and that correlate with increased susceptibility to CD.

Gene Name	Single Nucleotide Polymorphism (SNP)
MYO9B	rs1545620, rs1457092, rs2305767 ²²
PTPN22	rs1310182 ²³
SH2B3	rs3184504 ²⁴
IL2/IL21	rs6822844 and rs6840978 ²⁴
TLR7/TLR8	rs5979785 ²⁵
IL12A-AS1	rs17810546 ²⁶

Even if HLA-DQ2/DQ8 genes are present in the population at a percentage of 40%, the incidence of the pathology is much lower than this value. About 3% of people with these genes have the disease. In the population with CD, approximately 99% have one of the two genes, with a predominance of roughly 95% of DQ2.^{27–29} HLA genes, which are responsible for producing surface molecules involved in the immune system. Specifically, these molecules may be present on the surface of enterocytes, which are important in the context of CD, although they are typically specific to antigen-presenting cells (APCs). HLA molecules on the surface of APCs present antigens to T lymphocytes, whether self or non-self, to stimulate the immune

response. Susceptibility to CD is increased by a post-translational modification by the TG2 enzyme, which deamidates Gln residues to Glu residues, thus enhancing the immunogenicity of gluten-derived peptides. Indeed, these Glu-containing-peptides acquire negative charges, allowing them to be recognized more efficiently by MHC molecules in subjects presenting the HLA - DQ /DQ8 alleles. This is due to a better fitting of the molecules in the MHC pocket.^{30,31}

Figure 2 schematizes what has just been said.

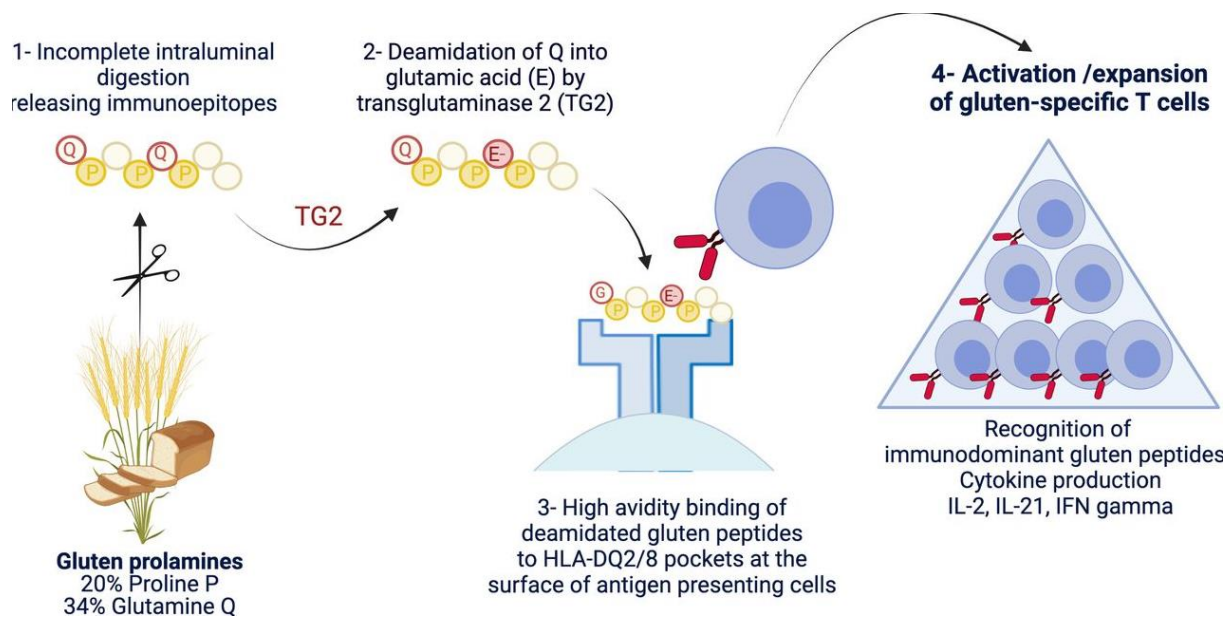


Figure 2. Gluten fate inside the gut. **1)** Incomplete digestion of gluten peptides due to their chemical composition, with a high content of proline and glutamine; **2)** Deamidation of a glutamine residue to a glutamic acid, with an increase of the negative charge of peptides; **3)** Higher affinity of deamidated peptides to the HLA-DQ2/8 pockets; **4)** Activation and expansion of gluten-specific T cells.³²

1.3.2 Gluten

The term gluten refers to a protein complex that is found in numerous cereals, the main ones being wheat, barley, and rye. Thanks to their qualities such as elasticity and cohesion, gluten-containing cereals are widely consumed. Wheat is responsible for around 20% of global calorie consumption, demonstrating the great importance it has in the world population.³³ In a clinical context, gastrointestinal breakdown of the gluten complex leads to the production of peptides that can trigger CD.³⁴ Indeed, as already discussed in the previous paragraphs, the genetic

component alone is not sufficient for the trigger of the pathology, but other factors, such as the presence of gluten peptides in the gut, are required for the development of the pathophysiological characteristics. Subjects suffering from CD should not exceed the daily dose of 10-50 mg. This information allows us to outline a delicate picture for CD as follows: gluten is widely present in our lives, and, since the gluten-free diet is the only effective treatment nowadays, it is difficult to avoid possible contamination with these proteins. Indeed, although many cereals do not contain gluten, they are often processed or transported together with products that contain it, thus causing cross-contamination. Last important aspect, many authorities set the limit for gluten-free products at a value no higher than 20 ppm; otherwise, the possibility of triggering CD in genetically predisposed subjects is highly probable.⁵⁴

1.3.2.1 Structure

The storage proteins that make up gluten are composed, for the most part, of prolamins. This denomination considers two characteristics: a different solubility in mixtures of alcohol and water and their high content of prolines and glutamines. More in detail, these prolamins can in turn be divided based on their solubility as either alcohol-soluble monomeric prolamins or alcohol-insoluble polymeric glutenins. As for monomeric prolamins, they can be called Gliadins, and there are three types, α/β , γ -, and ω -types based on their electrophoretic mobility, although their structure is very similar.³⁵ In the context of CD, α -gliadins contain the most immunogenic sequences. Gliadin proteins have analogues among different cereals, both functionally and structurally. The nomenclature varies depending on the specific cereal: secalins in rye, hordeins in barley, and avenins in oats.³¹ The other fraction contains molecules called Glutenins. They are high-molecular-weight proteins that also contain proline-rich regions, and they are more complex in structure. They contribute to gluten's viscoelastic properties when mixed with water. The primary structure of gluten peptides, characterized by a high content of proline and glutamine residues, contributes to their resistance to digestion in the human gastrointestinal tract. The percentages of these amino acids within these molecules are as follows: glutamines (35%-38%) and proline (15%-20%) of the residues.³⁶ The presence of proline (which causes bending in the peptide chain) makes gluten peptides highly resistant to digestion by human proteases, such as pepsin and trypsin. Thus, several long undigested gluten

peptide fragments can reach high concentration levels in the small intestine, where they can mediate adverse immune reactions in susceptible subjects.³⁴

1.3.2.2 Gluten peptides and the immune system

The gluten peptides play a pivotal role in the pathogenesis of CD. As just described, due to their amino acid structure rich in proline and glutamine, gluten peptides are very resistant to digestion/fragmentation at the level of the gastrointestinal system. Gluten fragments, particularly of α -gliadin, appear to be active toward the immune system. Some of them are able to activate the adaptive immune system, while others appear to be more active in processes involving the innate immune system.^{37,38} One highly immunogenic peptide, derived from α 2-gliadin, is a 33-mer sequence (residues 57 to 89), in which the active sequence is the peptide p57-68. This peptide is a substrate for TG2, which deamidates specific glutamine residues to glutamic acid, increasing its binding affinity to HLA-DQ2 or HLA-DQ8 molecules. The resulting peptide-HLA complex is recognized by gluten-specific CD4⁺ T cells in the lamina propria of the small intestine, leading to their activation. Activated T cells produce pro-inflammatory cytokines, such as interferon-gamma (IFN- γ), which contribute to mucosal inflammation and villous atrophy characteristic of CD.^{39,40} Effects on the adaptive immune system are not the only ones triggered by gluten peptides. The α -gliadin 25-mer peptide (sequence 31 to 55) appears to be very important for its effects on the innate immune system. In more detail, its shorter fragment, 31-43 (p31-43), is responsible for a wide spectrum of biological effects in subjects affected by CD.⁴¹ Several studies have been conducted on this peptide. To date, no membrane receptor for this peptide has been identified, and the process by which the peptide enters cells is not fully known. Recent studies propose its internalization through early endosomes following a direct interaction with the membrane.⁴² Furthermore, we do not have a clear idea of how the immune system recognizes it and then determines its biological effects. Regarding the latter, the following have been described: activation of innate immunity and cellular stress, with IL-15 as the main mediator⁴⁰, changes in cellular structure, shape alterations, actin organization, altered vesicular trafficking, and increased permeability.⁴³⁻⁴⁵ Furthermore, there is an activation of the inflammasome, an activation of TG2, and an increase in the proliferation of intestinal crypts (IL-15 dependent mechanism), a key mechanism in the remodeling of the intestinal mucosa observed in CD.⁴⁶

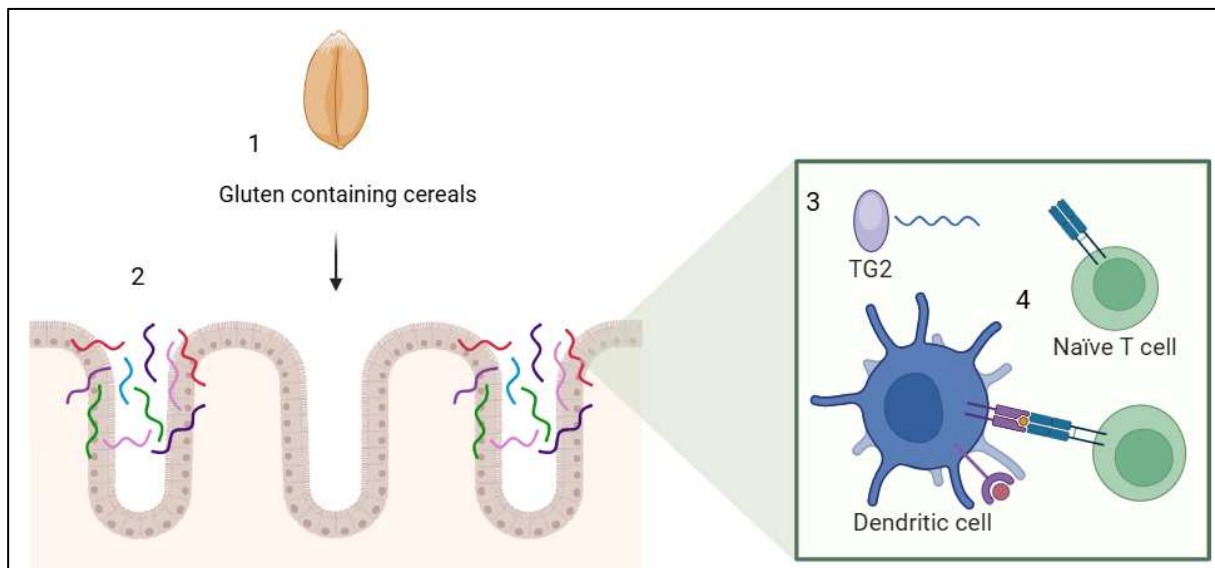


Figure 3. Fate of gluten in the CD. **1)** Gluten present in some cereals is ingested and reaches the gut lumen. **2)** At the level of the gastrointestinal system, many gluten proteins resist the action of the enzymes present there and arrive in situ as partially digested fragments. **3)** At the intestinal level, some peptides can be modified by TG2, enhancing their immunogenic action. **4)** When antigen-presenting cells that have internalized peptide fragments present them to cells of the immune system, a broad spectrum of biological effects can occur, triggering both innate and adaptive immunity. Image created with BioRender Software.

1.3.3 Transglutaminases

Transglutaminases are multifunctional and ubiquitous enzymes, present both in bacteria and in plant or animal organisms, including mammals. Its functions range from a multitude of biological effects important for the body normal physiology. At the industrial level, these enzymes are used in transformation processes as they modify qualities such as elasticity, thermal stability of proteins, viscosity, and many others.^{47,48} Furthermore, when there is an alteration in the activity or expression of members of this family of proteins, pathological conditions may develop in humans. Among them, we find various type of cancer, fibrosis, skin diseases, diabetes, blood disorders, neurodegenerative disease, and CD. A correct understanding of the mechanisms underlying the functioning of these enzymes can also lead to a greater understanding of the pathologies associated with them.

1.3.3.1 Family of TGase

Although transglutaminases have been found in different types of organisms, in this thesis, the focus is on TG present in mammals. The transglutaminase family comprises several isoenzymes with distinct tissue distributions and functions. These enzymes play pivotal roles in various biological processes, including blood coagulation, skin barrier formation, wound healing, G protein function, and extracellular matrix stabilization. The following table (**Table 2**) lists the 8 mammalian members of the TGase family, indicating some of the salient information regarding them.

Table 2. TGase family members. Key information of protein members belonging to the TGase family. The informations were obtained from the work of *Griffin, M. et. al. 2002*⁴⁹.

Identified forms of Tgase	Synonyms	Residues (molecular mass in kDa)	Gene	Gene map locus	Prevalent function
Factor XIII A	Catalytic A subunit of Factor XIII found associated with B subunit in plasma as A2B2 heterotetramer. Fibrin stabilizing factor	732 (83)	F13A1	6p24-35	Blood clotting and wound healing
Type 1 Tgase	Keratinocyte Tgase	814 (90)	TGM1	14q11.2	Cell envelope formation in the differentiation of keratinocytes
Type 2 Tgase	Tissue Tgase	686 (80)	TGM2	20q11-12	Cell death and cell differentiation, matrix stabilization, adhesion protein
Type 3 Tgase	Epidermal Tgase	692 (77)	TGM3	20q11-12	Cell envelope formation during terminal differentiation of keratinocytes
Type 4 Tgase	Prostate Tgase	683 (77)	TGM4	3q21-22	Reproductive function involving semen coagulation particularly in rodents
Type 5 Tgase	Tgase X	719 (81)	TGM5	15q15.2	Epidermal differentiation
Type 6 Tgase	Tgase Y		TGM6	20q11 15	Not characterized
Type 7 Tgase	Tgase Z	710 (80)	TGM7	15q15.2	Not characterized

Among these members, TG2 stands out for its ubiquitous expression and its multifunctional nature, characteristics that make this isoform an interesting protein to study. TG2 is unique in its ability to exhibit multiple enzymatic activities beyond its primary transamidating function. It can act, depending on its localization and physiological conditions (for example, the concentration of Ca^{2+} inside the cell), as a G protein in transmembrane signalling, as a protein disulfide isomerase, and as a protein kinase. Furthermore, another important function not

involving enzymatic functions is that of a mediator of adhesion to the cell surface, distinguishing it from other members of the TGase family.⁴⁹

TG2 has been found in the nucleus, cytosol, mitochondria, membrane inner face, cell surface, extracellular environment, etc.^{50,51} Furthermore, the presence and local concentration of its regulators influence what its activity will be (i.e., Ca^{2+} /guanine nucleotides/protein interactors). TG2 protein displays two distinct conformations: a closed conformation, in which the enzyme is catalytically inactive, and an open conformation, in which the enzyme mediates different reactions, thanks to a catalytic triad including a Cys residue in the active site. Finally, the presence or absence of its substrates determines its specific functions. As regards the substrates, numerous have already been described, with others listed as potential substrates, as TG2 is implicated in multiple biological pathways. For a greater understanding of the best-characterized substrates, the work of *Facchiano et al.*⁵² it is certainly a good guide.

1.3.3.2 Transglutaminase in CD

In the context of CD, the transglutaminase implicated is TG2, also named Tissue transglutaminase, a name due to its ubiquitous presence in almost all organs and tissues. TG2 enzymatic reactions seem to have a key role with respect to the pathogenesis of the disease. The main reactions are reported in **Figure 4**. TG2 transamidation (also called protein cross-linking) 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). This reaction plays a key role because cross-linked products can have high molecular masses, a feature that could make them more resistant to proteolytic degradation. This reaction is fundamental in many physiological processes, such as blood clotting formation (by FXIIIa), wound healing, and stabilization of the extracellular matrix⁵³. Transamidation consists of a ping-pong mechanism in which the γ -carboxamide group forms a thiol ester with the Cys residue of the enzyme active site; here, with the release of ammonia, there is the transfer of the acyl intermediate to a residue that contains a nucleophilic amine. Usually, this reaction occurs between different proteins or parts of the same protein (therefore intra- or inter-protein). When the acceptors of the acyl intermediate are missing, the

enzyme can use other low-mass primary amines (e.g., spermidine). In this case, we therefore speak of polyamination. The concept and mechanism of action are the same as the cross-linking activity; the difference strictly concerns the acceptor substrate of the reaction intermediate⁵⁴.

Finally, the last enzymatic reaction that turns out to be very important in CD is deamidation of specific glutamine residues. The rich presence of glutamines in gluten peptides makes these peptides excellent candidates for TG2 reactions. In an environment such as that of the gastrointestinal tract, with a slightly acidic pH and the low abundance of primary amine acceptors, TG2 catalyzes the deamidation of glutamine residues into glutamate. Therefore, the peptides acquire an increasing number of negative charges, which allows for an increased fitting with the HLA-DQ groove of antigen-presenting cells. Thus, an increase in the immunogenicity of the peptides themselves is obtained, leading to the activation of an adaptive immune response to gluten⁵⁵.

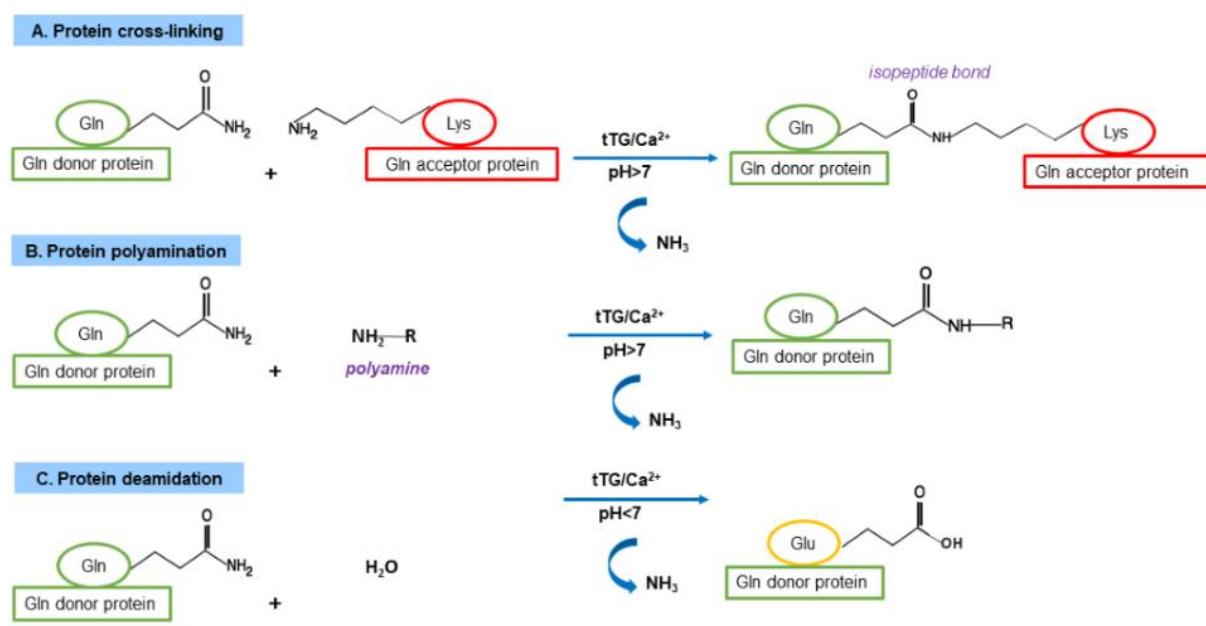


Figure 4. Main enzymatic reactions of TG2. **A)** Protein cross-linking reaction; **B)** Protein polyamination reaction; **C)** Protein deamidation reaction.⁵⁴

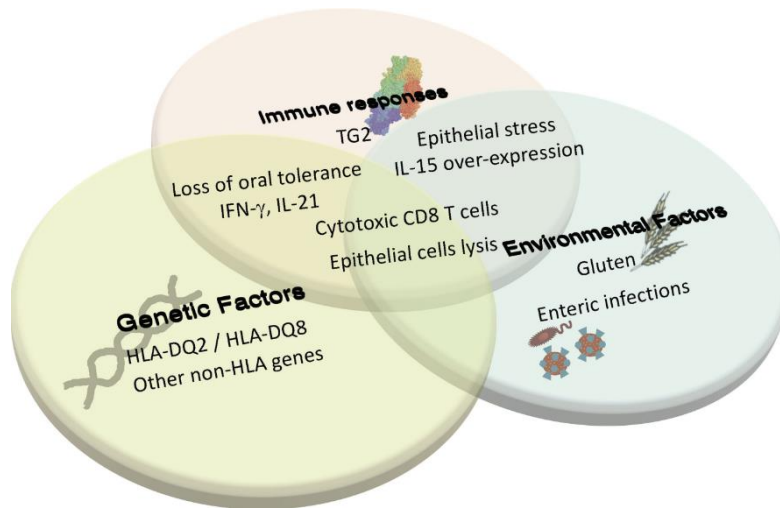


Figure 5. Interplay between the main actors who determine the pathological aspects of celiac disease. Genetic susceptibility (e.g., HLA-DQ2/DQ8) enables efficient presentation of gluten-derived peptides to the immune system. TG2 modifies gluten peptides by deamidation, increasing their affinity for these HLA molecules and amplifying T-cell activation. This triad drives loss of tolerance, autoantibody formation against TG2, and the characteristic intestinal pathology of celiac disease. Other secondary concurrent factors may contribute to the formation of pathological features. Image obtained from the work of *Voisine et al. 2021*.⁷⁷

1.4 Clinical Aspects of CD

Clinical manifestations and diagnosis are strictly associated in the case of CD and require particular attention.

1.4.1 Clinical manifestations

The clinical manifestations of this condition vary due to its multivariate nature⁵⁶ and can be classified into two types: intestinal and extra-intestinal. The first appears to be more commonly diagnosed in the pediatric population and children under 3 years of age⁵⁷, while in the second one, it seems that the symptoms are present with the same frequency in all groups of the population. Precisely because of this dual nature, the diagnosis of the pathology has remained difficult for many years, leading to an underestimation of the incidence in the global population.

Abdominal pain remains the best-established clinical manifestation, a finding consistently linked to gluten ingestion.⁵⁸ Other symptoms that the pathology commonly presents are malabsorption syndrome, failure to thrive, and diarrhea, which can be chronic or intermittent, directly related to the malabsorption of nutrients.⁵⁹ Although these are some of the most encountered symptoms in CD, many others are less common and far from the primary site of the pathology, represented by the intestine, a factor that adds greater difficulty in the correct diagnosis and the drafting of univocal criteria for the diagnosis of the pathology. Extra-intestinal symptoms are common in both children and adults. The vast spectrum of extra-intestinal symptoms outlines a complex picture of the pathology. Some of them are discussed in the following lines, showing the multifaceted aspect of CD. Some may be associated with malabsorption, such as iron deficiency with or without anemia, reduced bone mineral density⁶⁰, tooth enamel defects, aphthous stomatitis⁶¹, and hypertransaminasemia, which can be ascribed to food and bacterial antigen translocation reaching the liver due to increased intestinal permeability.⁶² In addition, alterations 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. Finally, a wide array of neurological symptoms, such as headache, paresthesia, neuroinflammation, anxiety, irritability, and a tendency to depression, can be detectable in CD patients. According to the 2011 Oslo definition, there exists, in addition to the intestinal and extra-intestinal ones, a form of CD defined as subclinical, where patients are below the threshold for clinical detection without sufficient signs or symptoms to trigger CD testing in routine practice.⁶³

1.4.2 Diagnosis

As already described in the previous paragraph, due to the multifaceted nature of CD it is difficult to establish univocal criteria useful for diagnosis. The gold standard for the diagnosis of this condition is represented by the evaluation of intestinal morphology in search of mucosal damage (i.e., villous atrophy) via duodenal biopsy⁶⁴; the Marsh classification (grades 0–3) is commonly used to assess the degree of damage to the small intestine⁶⁵.

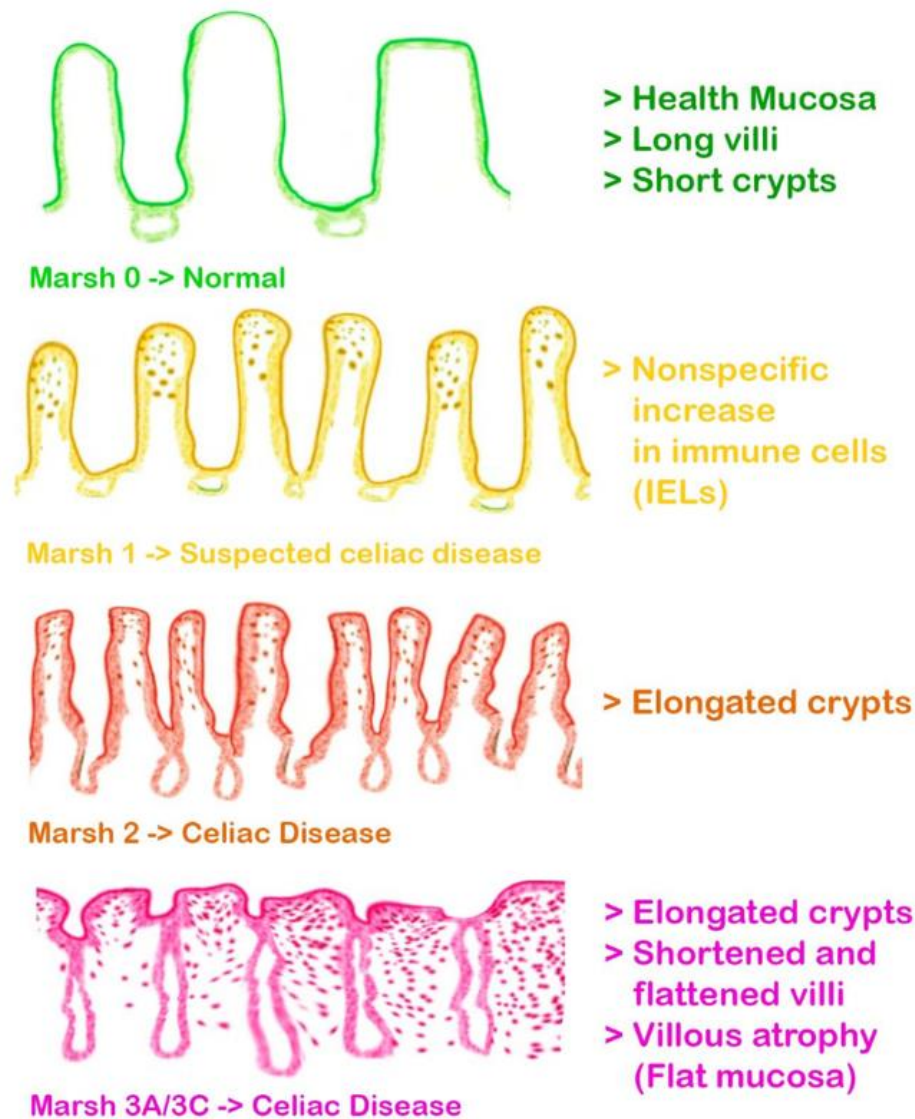


Figure 6. Different stages of intestinal mucosal lesions according to the Marsh scale.

Image obtained from the work of *Inchingolo et al. 2024*⁶⁶.

There are heated debates on this topic as the test appears to be invasive and many subjects could be discouraged from continuing towards the diagnosis, frightened by the analysis itself. Thus, the incidence of CD is probably underestimated. According to the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), in the presence of a serum anti-TG2 IgA value > 10 times greater than the limit, a biopsy may not be required for diagnosis, but this guideline is not then implemented in the Central Europe Guideline.⁶⁶ Finally, the fundamental criteria that can be used to diagnose CD are clinical presentation, therefore the evaluation of the symptoms described in this same paragraph, and the serologic tests, because the first step in diagnosing CD often involves serologic testing for antibodies anti-TG2 IgA, which is typically the first line test. Anti-endomysial antibodies (EMA) are more specific but

less sensitive than anti-TG2 IgA; anti-deamidated gliadin peptide (DGP) antibodies are particularly useful in children and may serve as an adjunct to other serologic tests. Genetic tests (which will be explored in more detail in a later section) are also available for HLA-DQ2 and HLA-DQ8 genotypes: most individuals with CD carry the HLA-DQ2 or HLA-DQ8 genetic variants, but these are also present in a significant portion of the general population. Negative HLA testing (absence of DQ2/DQ8) effectively excludes the diagnosis of CD. Lastly, a positive response to a gluten-free diet (improvement of symptoms and normalization of serology) supports the diagnosis of CD. However, diagnosis should not be based solely on diet response in the absence of confirmatory tests^{63,67,68}.

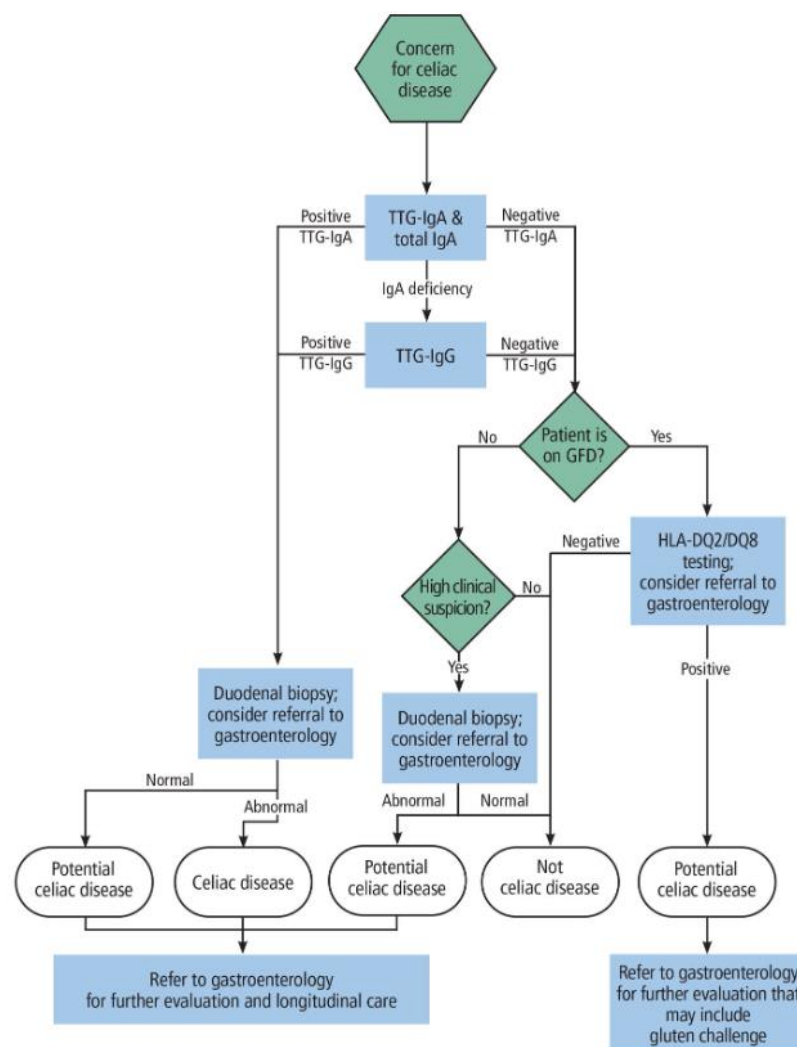


Figure 7. Diagnostic strategy for suspected celiac disease. Image obtained from the work of *Nasser et al. 2023*⁶⁹.

There are some differences in the testing process between pediatric and adult patients. Specifically, these differences focus on the possibility of avoiding an invasive biopsy in pediatric patients. This can be avoided if IgA levels against TG2 are greater than 10 times the normal range, there is a positive EMA test, and a positive genetic test for HLA-DQ2/8, according to the ESPHGAN guidelines⁶⁶ (as previously mentioned).

1.5 Treatments

In the pathological context of CD, two possible approaches to a cure can be considered: following a specific diet or taking medication. In the first case, there is lower adherence due to the greater difficulty of the population in adhering to a specific regimen (42 - 91% of adherence declared by patients⁶⁹), an aspect that makes the treatment less effective. In the second case, however, participation is generally higher. This premise is important in the clinical picture of CD since, to date, the only clear-cut remedy that allows for complete recovery from intestinal and extra-intestinal symptoms and disappearance of serological signs is a strict adherence to a diet free of gluten (hereinafter referred to as GFD). In addition, an important aspect that makes this approach even more difficult is the possibility of gluten contamination, which appears to be very common, and which affects the complete recovery of a healthy condition for patients. Nowadays, *Machado*⁷⁰ *et al.* illustrated the information about some Pathophysiology-Driven Strategies to Treat CDs. Some research is underway for the development of an oral therapy based on enzymes that allow the degradation of gluten in such a way as to break the molecule before it triggers its immunogenic action in the gastrointestinal tract, a strategy that would also benefit subjects who encounter gluten involuntarily.⁷¹ In this direction, TAK-062, an oral enzyme capable of breaking down gluten very efficiently, appears to be an effective strategy, although its clinical efficacy remains to be proven.⁷² An innovative approach is to be found in the immunomodulation of the immune response to gluten. This approach aims at inhibition through different methods, such as the use of monoclonal antibodies, to suppress the immune response that is triggered in the intestine. Some examples are anti-CD3 monoclonal antibodies to prevent T-cell activation and anti-IL-15 therapies.⁷³ The enzyme TG2 plays a crucial role in CD as it modifies gluten peptides, thus triggering the immune response. Its inhibition could lead to the reduction of immune complexes that activate a series of pathological processes in

the disease.⁷⁴ Several molecules have been synthesized for this task, among which one of the most important is certainly the compound ZED1227, which appears to be a good candidate for the treatment of CD. In early testing, the compound, a TG2 inhibitor, appears to reduce intestinal mucosal damage from gluten peptides.⁷⁵ A further innovative aspect is to be found in vaccination. The goal is to desensitize and increase tolerance to gluten peptides. In Phase I clinical trial, the Nexvax2® vaccine, which consists of three gluten peptides from wheat, barley, and rye, was administered to make gluten-specific CD4 T cells unresponsive to subsequent exposure to the peptides of gluten.⁶⁶ In addition, the use of nanoparticles to achieve gluten tolerance represents one of the most innovative approaches currently available. There are several phase II studies of some compounds encapsulated in nanoparticles for the treatment of the pathology.⁷⁶ An *in vivo* study on mice demonstrated how the use of these nanoparticles induced a reduction in inflammation markers and enteropathy, all thanks to an increase in gluten tolerance with the help of PLGA-based particles.⁷⁷

1.7 Cellular and molecular insights into CD

Several experimental findings show that cells from CD patients appear to exhibit a characteristic phenotype, independent of the site of inflammation and gluten exposure. Although a classic biochemical approach allows the characterization of this cell type to define its phenotypic consequences, omics technologies have emerged as powerful tools to interrogate the genetic, transcriptomic, proteomic, and metabolic landscape of CD. Integrating cellular phenotype data with omics-based approaches enables a systems-level understanding of how molecular alterations translate into functional dysfunction.

1.7.1 Celiac cellular phenotype

The concept of celiac cellular phenotype is quite recent. This denomination refers to several studies that have highlighted constitutive differences present in the cells of celiac subjects compared to those of non-celiac subjects. These alterations could in some way represent a

predisposition to the negative effects that gluten has on individuals. Furthermore, the differences appear to be constitutive since they have been highlighted in the absence of gluten in the diet of individuals, a condition that should cancel out any alteration, at least histological and health-related, of CD. In addition, they have also been found in sites other than the main inflammation site, which is the intestine, particularly in skin fibroblasts. The differences that have been described concern various biological aspects, both morphological and functional, summarised in the tables below (**Tables 3 and 4**).

Table 3. Morphological differences reported in CD cells to define the celiac cellular phenotype.

Cell Type	Morphological Difference	Details	Reference
Skin Fibroblasts	Increased number of focal adhesions	Higher expression of Paxillin and FAK proteins in CD fibroblasts	<i>Nanayakkara et al., 2013</i> ⁷⁸
Skin Fibroblasts	Altered cell shape and area	CD fibroblasts less elongated, increased cell area, and shortened actin stress fibers	<i>Nanayakkara et al., 2013</i> ⁷⁸
Skin Fibroblasts	Altered subcellular distribution of LPP protein	LPP reduced in the nucleus and increased in cytosol of CD fibroblasts	<i>Nanayakkara et al., 2013</i> ⁷⁸
Enterocytes	Constitutive increase in crypts enterocyte proliferation	Observed even in CD patients on a gluten-free diet (GFD) and in potential CD	<i>Nanayakkara et al., 2013</i> ⁴⁴
Enterocytes and Fibroblasts	Increased number of early endosomes	Related to altered EGF/EGFR trafficking	<i>Lania et al., 2019</i> ²

Table 4. Functional differences reported in CD cells to define the celiac Cellular Phenotype.

Cell Type	Functional Difference	Details	Reference
Skin Fibroblasts	Altered vesicular trafficking	Early to late vesicle trafficking is altered in CD fibroblasts	<i>Lania et al., 2019</i> ²
Skin Fibroblasts	Different handling of toxic gluten peptide p31-43	Anti-TG2 antibodies reduced p31-43 uptake in non-CD fibroblasts but not in CD fibroblasts	<i>Paolella et al., 2017</i> ⁷⁹
Enterocytes	Increased EGFR signaling	Enhanced EGF mRNA and EGFR protein levels, independent of gluten intake	<i>Nanayakkara et al., 2013</i> ⁴⁴
Enterocytes	Increased ERK1/2 phosphorylation	Mediates increased proliferation of crypt enterocytes	<i>Nanayakkara et al., 2013</i> ⁴⁴
Skin Fibroblasts	Increased sensitivity to p31-43 induced trafficking delay	p31-43 causes more prolonged EGF/EGFR trafficking delay in CD fibroblasts	<i>Lania et al., 2019</i> ²
Intestinal Epithelial Cells	Increased expression of IL-15	Promotes survival and activation of intraepithelial lymphocytes	<i>Jabri et al., 2006</i> ⁴¹
Enterocytes	Overexpression of stress markers (e.g., HSP70, GRP78)	Constitutive activation of cellular stress response pathways	<i>Maiuri et al., 2003</i> ³⁸
Skin Fibroblasts	Impaired autophagy	Reduced capacity to eliminate damaged proteins and organelles	<i>Lania et al., 2019</i> ²

Starting from the morphological point of view, several constitutive characteristics have been highlighted. *Nanayakkara et al.*, in 2013⁷⁸, highlighted an increase in the number of focal adhesions in fibroblasts obtained from patient biopsies. The increase in these is due to alterations in the proteins Paxillin and Focal Adhesion Kinase (FAK), two markers used for focal adhesions. In the study, the two markers appear to have increased expression in celiac fibroblasts. Moreover, the phosphorylated forms of these proteins, which appear to be important

in the physiology of focal adhesions⁸⁰, also appear to be more present in celiac fibroblasts. In the same work, the authors also show differences regarding the shape and the cellular area, in the same cellular system. In fact, celiac fibroblasts showed shorter stress fibers. Therefore, the alteration of the cells concerns both the shape and some cytoskeletal components. The authors also highlighted in the same article an altered subcellular distribution of the LIM-containing lipoma-preferred partner (LPP). This protein has a dual nature: it has a key role in the architecture of focal adhesions, and it acts as a transcription factor. In particular, the authors highlighted a decrease in the levels of this marker in the nucleus, therefore with reduced activity as a transcription factor, while they highlighted the increase of the protein in the cytosol, a compartment in which the protein fulfills its function as a component of focal adhesions. In this way, as previously mentioned, a modification in the shape and motility of these cells is outlined. Finally, treatment of these cells with peptide 31-43, one of the cytotoxic peptides derived from gluten, induced cellular alterations in non-celiac cells, which rendered them more similar to the cells of celiac subjects; a celiac cellular phenotype was therefore "induced" in normal cells, thus suggesting an association between these alterations and the pathogenesis of CD.

Regarding functional differences, *Lania et al.*² highlighted how, in the fibroblasts of celiac subjects, there is an alteration of vesicular trafficking, in particular from the early compartment to the late compartment. *Nanayakkara et al.*⁴⁴ showed how in cells from CD subjects, there was a constitutive increase in the proliferation of the enterocyte crypts compared to non-celiac subjects. The authors highlighted an increase in EGF mRNA and an increase in the protein quantities of the EGF-receptor (EGFR) in the enterocyte crypts of celiac subjects compared to control subjects (hereafter called CTR). The signaling cascade in response to the EGF/GFR system, mediated by ERK1/2, was also evaluated. Indeed, the authors demonstrated how, in CD cells, there were higher values of phosphorylated ERK1/2. Following these phosphorylations, these effectors can be translocated to the nucleus, where they can modulate the gene expression of genes of the proliferative response.⁸¹ To confirm these data, the authors used an ERK1/2 inhibitor to block its phosphorylation, thus obtaining a normalization of the proliferation of the crypts in subjects affected by CD (both atrophic and in remission by adhering to a gluten free diet), almost at the same level as that observed in the CTR subjects. The same event was also observed in the cellular system of skin fibroblasts, thus not only confirming this initial hypothesis, but also giving a further contribution to the definition of constitutive differences, which are therefore not limited to a single site and are also present independently of gluten intake.

Finally, another interesting aspect has been discussed by *Paolella et. al.*⁷⁹ In their work, the authors showed how anti-TG2 antibodies induced differences in the uptake of the gliadin peptide p31-43, known as one of the major activators of innate immunity in CD. Pre-treatment of skin fibroblasts with these antibodies showed a reduction in the uptake of the peptide in non-celiac cells, but not in CD fibroblasts; in addition, this behavior was not observed when a different peptide was added, namely the immunogenic peptide p57-68. The explanation for this phenomenon is still uncertain. The authors suggested that the antibodies, interacting with the surface TG2, somehow induced a different behavior towards the peptide in the two groups of subjects. The same authors demonstrate how there is a different sub-cellular distribution of the enzyme in the same cells, with a fraction bound to membranes greater in celiacs than in controls (CTRs). This, in some way, could determine changes in the phenomenon of uptake of the peptide related to anti-TG2 antibodies. In conclusion, it is possible that the TG2 enzyme also contributes to the existence of the celiac cellular phenotype.

1.7.2 Omics approaches applied to CD

An innovative method for studying the celiac cellular phenotype and, more generally, CD, is represented by omics techniques. This approach requires a deep knowledge of the system being analyzed but has a great advantage, that of obtaining unbiased insight into biological processes. To date, few studies have focused on omics approaches for CD. Furthermore, most studies have been conducted on intestinal tissue, with particular attention paid to enterocytes. The two main approaches, which are also concerned with the first to be used, are transcriptomics and proteomics. In the first case, greater depth is obtained, and up to about 10,000 genes are analyzed simultaneously. Instead, for proteomics, there is a bias due to the presence of poorly expressed proteins (such as some cytokines)⁸². Furthermore, the study of the metabolome could provide further information on subjects affected by CD and CTR, to provide a further integration to the omics data already present in the literature. This multi-omics approach represents a very important resource as it allows the analysis of different levels of cellular complexity. It starts with genomics, passes through transcriptomics and proteomics, and finally concludes with metabolomics. In this way, a global vision of what happens inside the samples is obtained.

1.7.2.1 Genomics and CD

Genomics is the study of the entire genome of an organism. This approach can obtain a lot of information about the genes implicated in a disease. In particular, for CD, the study of the genome has allowed us to define a hereditary component of the disease. As already mentioned in paragraph 1.3.1, HLA genes are important predisposing genes for the disease. However, these genes contribute approximately 40% to the disease susceptibility²⁷. Nearly all individuals diagnosed with CD possess the HLA-DQ2.5 heterodimer²⁰, whereas only a small fraction carry alternative, lower-risk variants such as HLA-DQ8, HLA-DQ2.2, or HLA-DQ7.5. In addition, genomic studies are essential for the search for other non-HLA genes that may be somehow related to the development of the disease. Initially, chromosomal regions were identified that were related to the genetic predisposition to CD. These are three and are regions: 5q31-q33 (CELIACS 2), 2q33 (CELIAC 3), and 19p13.1 (CELIAC 4)²⁷. To date, some specific *loci* have been identified through genomic studies (**Table 1**).

1.7.2.2 Transcriptomics and CD

With transcriptomics, we study a snapshot of the cell transcripts. Therefore, through these techniques, it is possible to analyze all the transcripts present in a biological system at a given time, considering both the transcripts that could be translated into proteins and non-coding RNA, with other important functions. These analyses are important because, since mRNA is a transient form of information from a cell under a specific condition, it allows us to obtain key information on the physiology of what we observe⁸³. The use of these techniques in the study of CD could provide important information both regarding the molecular mechanisms underlying the pathology and for the search for possible markers of the pathology. The two main methods that are used are RNA-Sequencing (RNA-Seq) and Microarray. Depending on the type of sample, the quantity of starting RNA, and the characteristics that you want to evaluate, one may be preferred over the other. Regarding the differences that have been observed to date in cells or biopsies of patients affected by CD compared to CTRs, these analyses have highlighted changes in gene expression that highlight alterations in immune responses, epithelial integrity, and metabolic functions. Among the most relevant markers, it

has been reported an increased expression of inflammatory markers or those involved with innate immunity has been reported, an important feature in CD. *Loberman-Nachum et al.*⁸⁴ reported increases in the expression of markers, including interferon response, alpha-beta, and gamma-delta T-cells. The authors specified that many of them can somehow overlap with similar markers of other pathologies, as in the case of Crohn's disease. But, as described in the same work, some genes related to cell proliferation or nuclear division, such as CCNB1 or Ki67, appeared to be specific for CD and, therefore, to be promising candidates to represent specific markers.

1.7.2.3 Proteomics and CD

Proteomics allows for the large-scale study of proteins and their functions. Several approaches can be used, such as the former gold standard represented by two-dimensional electrophoresis. In the context of CD, the proteomic approach has been used to analyze the protein composition of intestinal tissues and fluids. These studies have identified differential expression of proteins involved in immune responses, epithelial structure, and metabolism. To date, in general, few studies have focused on cells other than intestinal enterocytes. However, as already abundantly highlighted, the study of different cells could provide not only important information on the celiac cell phenotype but could also add relevant information on the behavior of the CD microenvironment, since primary cultures of intestinal fibroblasts from patients affected by CD were used in our studies. A recent study by *Na Li et al.*⁸⁵ used proteomics through a Four-Dimensional Data-Independent Acquisition (hereafter called 4D-DIA) for the analysis of plasma and intestinal biopsy samples in the search for non-invasive markers for the diagnosis of CD. The results highlighted three possible markers, FGL2, TXNDC5, and CHGA. For the first two, significant increases in protein amounts were observed in the tissues of subjects affected by CD compared to the CTR group. In addition, they highlighted four possible candidates through exploratory analysis of patient serum for markers associated with pathological grading (villous atrophy). These four are FABP, CPOX, BHMT, and PPP2CB. Although the authors suggested that further analyses were needed, this evidence showed that the proteomic approach can not only provide information to understand the basis of a pathology, but also for the search for new non-invasive markers.

1.7.2.4 Metabolomics and CD

On a scale of biological complexity, metabolites represent the lowest level. Indeed, humans contain approximately 20,000-25,000 genes,⁸⁶ but these, due to the increased complexity and number of biological reactions, allow for the production of a greater quantity of metabolites. According to the Human Metabolome Database (HMDB), one of the most comprehensive resources, it lists approximately 250,000 metabolites, including both accurately described and predicted ones. Metabolomics studies enable us to capture a sample phenotype, its expression at a given time. This phenomenon, however, is difficult to observe with other omics techniques, such as genomics and transcriptomics, because in these cases, we observe a "potential" phenotype, where other phenomena come into play before defining the "actual" phenotype (for example, gene transcript maturation). Metabolomics, on the other hand, analyzes a "snapshot" of the sample and is therefore highly sensitive to changes. Furthermore, this allows for an important tool for the detection of biomarkers for a specific condition in a minimally invasive manner, for example, using body fluids.^{87,88} Two approaches are typically used: untargeted metabolomics, for studying the total metabolites in a sample, useful when analyzing unexpected variations, or targeted metabolomics, in which a single metabolite and its relative concentrations are investigated. Mass spectrometry and NMR are used for this purpose.⁸⁷ In the context of CD, there are still few studies, due to the relatively young age of metabolomics and the complexity of the disease itself. In 2009, *Bertini et al.*⁸⁹ provided one of the first pieces of evidence of the use of metabolomics for CD. Using an NMR-based approach, the research team highlighted, using fluids such as urine and serum, alterations in energy production, amino acid catabolism, and gut microbe–host cometabolites in subjects with CD compared to CTRs. *Upadhyay et al.*⁹⁰ later confirmed a disruption of the TCA cycle and amino acid metabolism. From the perspective of investigating possible biomarkers, *Girdhar et al.*⁹¹ highlighted how in children who were followed up to 5 years of age, metabolomic signals from "potential" subjects can predict the full-blown disease. Analyzing plasma, the authors explained how taurodeoxycholic acid (TDCA) levels were ~2-fold higher. Furthermore, in the same study, this metabolite was administered to mice, and mild inflammation, along with villous atrophy, was observed, thus linking this metabolite to a potential biomarker for diagnosing the disease.

2. Aims

The celiac cellular phenotype is the set of constitutive differences present in cells from individuals with CD compared to cells from healthy CTRs. This thesis aims to delve deeper into this topic with a biochemical approach. Specifically, the study was structured in two main parts closely related to the two cellular models that will be used: primary fibroblasts from skin biopsies of celiac patients on a gluten-free diet and CTRs in the first case, and primary fibroblasts from intestinal biopsies of celiac patients with acute disease and CTRs in the second case. These primary cells were provided by the University of Naples Federico II. The first part of the research focuses on the study of the skin cellular system, with an initial section dedicated to the analysis of cellular responses following treatments with the stress agent thapsigargin (THP), followed by analyses of the basal proteome and phosphoproteome. For the second cellular system, the approach will mainly focus on omics techniques, starting with the analysis of the basal proteome, followed by the study of the metabolome. Finally, after obtaining a molecular basal characterization of the above-mentioned systems, the transcriptome was investigated in celiac intestinal fibroblasts following stimulation with an inhibitor of TG2, a key enzyme in the celiac pathogenesis, in order to define how TG2 modulation may affect the expression pattern in celiac cells.

3. Materials and Methods

3.1 Cell cultures

Primary skin and intestinal fibroblasts were obtained from the University of Naples Federico II. Skin fibroblasts were derived from five CD patients on a gluten-free diet (aged 17–43 years) and five HLA-DQ2/8-negative healthy subjects (aged 25–30). CD patients were on a gluten-free diet for at least 4 years and showed normal (Marsh T0) biopsies, negative serology, and negativity for anti-endomysium antibodies. None of the CD patients were affected by dermatitis herpetiformis. Intestinal fibroblasts were obtained from duodenal biopsies from five CD patients with a Marsh T3c histology (mean age of 10 years), with a positive serology. Four CTR were also obtained (mean age of 13 years), with a negative serology. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, supplemented with L-Glutamine) with 20% v/v of Fetal bovine serum (FBS) and 1% v/v of Pen/Strep (Gibco, Invitrogen SRL, Milan, Italy). For the intestinal fibroblasts, FBS was previously heat-treated to inactivate the complement component in serum at 56°C for 20 min. All experiments were performed on cells at passages between 4 and 7.

3.2 MTT assay

The MTT assay is a colorimetric method that evaluates cell viability by utilizing the activity of succinate dehydrogenase in living cells. This enzyme reduces the water-soluble yellow dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), into insoluble purple formazan crystals within the mitochondria. Afterward, the formazan crystals are dissolved in dimethyl sulfoxide (DMSO, Carlo Erba, Milan, Italy), producing a colored solution measured spectrophotometrically at an absorbance of 595 nm. A higher absorbance indicates a higher number of metabolically active and viable cells. Given that the assay relies on mitochondrial respiration, it indirectly measures cell viability, proliferation, and cytotoxicity.⁹² To assess the impact of THP on cell viability in both CD and CTR dermal fibroblasts, cells were seeded in a

96-well microplate and exposed to various concentrations of THP and different times. Following the treatment, MTT substrate from Sigma-Aldrich was added to each well at a final concentration of 0.25 mg/mL, and the plate was incubated at 37°C for 90 min to facilitate the formation of formazan crystals. A positive control using 0.2% H₂O₂ was included and administered for 30 min. Subsequently, the crystals were dissolved in 100 µL of DMSO, and absorbances were measured at 595 nm. Cell viability was determined by comparing the absorbance values from THP-treated cells to those from control cells containing only corresponding amounts of the vehicle (DMSO). For the intestinal fibroblasts, treatments were performed with ZED1227 (D.B.A. S.r.l., Milan, Italy), in a range of concentrations from 300 nM to 50 µM for 24 h. The MTT assays were performed both on intestinal and dermal fibroblasts.

3.3 Proteins quantification and detection

3.3.1 Cell treatment and lysis

To investigate the biochemical differences in protein levels in both CD and non-CD cells, dermal fibroblasts were cultured in 6-well microplates and exposed to different compounds, THP and Bafilomycin A1 (Invitrogen Srl) at concentrations ranging from 0.01 to 1 µM for 24 or 48 h, and 0.1 µM for 4 h, respectively. For the intestinal fibroblasts, stimulation was performed with ZED1227 (D.B.A. S.r.l., Italy), in a range of concentrations from 300 nM to 50 µM for 24 h. DMSO was used as the vehicle for all compounds. After treatments, cells were washed with phosphate buffered saline (hereafter called PBS) and mechanically harvested into 70 µL of RIPA lysis buffer. This buffer consisted of 20 mM Tris-HCl (pH 7.5) (D.B.A. S.r.l.), 150 mM NaCl (D.B.A. S.r.l.), 10% glycerol (Carlo Erba), 1% Triton X-100 (D.B.A. S.r.l.), 0.1% sodium dodecyl sulphate (SDS, AppliChem, Darmstadt, Germany), 1 mM phenylmethylsulfonyl fluoride (PMSF, AppliChem), 1 mM sodium orthovanadate (Na₃VO₄, Sigma-Aldrich, Missouri, United States), 50 mM sodium fluoride (NaF, AppliChem), and 1% protease inhibitor cocktail (Sigma-Aldrich). The samples were then incubated on ice for one hour to facilitate cell lysis. The protein content of each sample was subsequently measured

using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Milan, Italy) as detailed in Section 3.3.2. A range from 30 to 50 μg of extracted proteins from each sample was then subjected to Western blot analysis following the protocols outlined in Sections 3.3.3 and 3.3.4. The lysis protocol was the same for both cellular systems.

3.3.2 Bradford Assay

The Bradford protein assay is a straightforward, rapid, and relatively sensitive colorimetric method used to estimate the total protein content in a sample. This technique relies on the shift in the absorbance maximum of Coomassie Brilliant Blue G-250 dye from 465 nm to 595 nm upon binding to denatured proteins in solution, as described by Bradford and colleagues in 1976⁹³. The absorbance at 595 nm correlates linearly with protein concentration: higher protein levels increase the amount of dye bound, resulting in greater absorbance. For protein quantification, 1 μL of total cell extract from each sample was combined (in duplicate) with 1 mL of Bio-Rad protein reagent (containing Coomassie Brilliant Blue G-250 dye), diluted at a 1:5 ratio in deionized water. The absorbance was then measured at 595 nm using a VWR-UV-3100PC spectrophotometer. To enhance the reliability of results, absorbances for each sample were measured in duplicate and averaged. A standard curve was employed using bovine serum albumin (BSA) solutions of known concentrations (1, 2, 4, 8, and 10 μM). Averaged absorbances at 595 nm for each sample were calculated to ensure accurate determination of protein concentration in the samples.

3.3.3 SDS-PAGE

Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) is an electrophoretic method used to separate proteins based solely on their molecular weight. Proteins are amphoteric by nature, having both positive and negative charges. To enable proteins to migrate uniformly in a single direction within a polyacrylamide gel, it is essential to impart a consistent negative charge to them. SDS, an anionic detergent, binds tightly to the

protein backbone at a relatively fixed molar ratio (approximately one detergent molecule per two amino acids when SDS is at 0.1%). This interaction results in proteins acquiring a uniform net negative charge proportional to their molecular size. As a voltage is applied, the SDS-bound proteins migrate through the gel matrix toward the positively charged electrode, with smaller proteins moving faster, due to the sieving effect of the gel. To denature proteins from cellular extracts, Laemmli sample buffer (4X), containing 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 samples and diluted to a final concentration of 1X. Samples were then boiled at 100°C to facilitate complete protein denaturation. A two-phase polyacrylamide gel was prepared for SDS-PAGE: an upper stacking gel to condense proteins into a tight band and a lower separating gel for molecular weight-based separation. The stacking gel contained 5% acrylamide, while the percentage of acrylamide in the separating gel varied based on the target protein size; for TG2 detection, a 10% separating gel was used. A total of 35 µg of protein extract from each sample was resolved using 10% SDS-PAGE alongside a molecular weight marker (Invitrogen S.r.l.). Electrophoresis was conducted at 100 V until protein bands migrated from the stacking to the separating gel, after which the voltage was increased to 120 V. A 1X running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS) was used to fill the electrophoretic chamber. The run was stopped once the bromophenol blue reached the gel's lower boundary.

3.3.3.1 Pro-Q™ Diamond Phosphoprotein Gel Stain

For the dermal fibroblasts, after the phosphoprotein enrichment (described in section 3.6) and the SDS-PAGE, the gels were stained with the Pro-Q™ Diamond Phosphoprotein Gel Stain (Invitrogen, S.r.l.) to stain selectively the phosphoprotein. First, the gel was fixed with a solution made with 50% methanol and 10% acetic acid for 30 min (for two times). Then, three wash steps were performed with ultrapure water. A total amount of 60 mL of the gel stain was used for each gel and left for 60-90 min. To reduce the background signal and the nonspecific signal, a destaining solution was prepared with 20% acetonitrile and 50 mM of sodium acetate at pH 4. Three steps with this solution were made, 30m each. Finally, after three washes with ultrapure water, the signals were acquired with the iBright image system (Invitrogen, S.r.l.).

3.3.4 Western Blot

Western blotting is a highly utilized biochemical approach for identifying specific proteins within complex mixtures that have been resolved via SDS-PAGE and immobilized on a membrane. Following separation by SDS-PAGE, proteins were transferred onto a pre-activated PVDF membrane (EMD Millipore Corporation, Billerica, Massachusetts, USA) in methanol for one minute. The electrotransfer was performed at 120 V for one hour using a transfer buffer (1X: 25 mM Tris, 192 mM glycine, and 20% methanol) with the Trans-Blot Turbo Transfer System (BioRad Laboratories). Non-specific binding sites on the membrane were blocked by incubating it in Tris-buffered saline (TBS) with 5% non-fat dry milk (Santa Cruz Biotechnology Inc., Dallas, United States) for 30 min. Excess blocking solution was removed through TBS-Tween-20 washes (TBS with 0.1% Tween-20). Protein detection involved overnight incubation at 4°C with different antibodies, all diluted 1:1000 in TBS-Tween-20 containing 0.1% non-fat dry milk. The antibodies used were purchased from different companies. Antibodies obtained from Thermo Fischer Scientific (Massachusetts, United States): CUB 7402 antibody, diluted 1:1000; GRP78 antibody, diluted 1:1000; LC3 antibody, diluted 1:1000; p62 antibody, diluted 1:1000. Antibodies purchased from Aurogene (Aurogene S.R.L., Rome, Italy): gamma-synuclein (SNCG) antibody, diluted 1:1000; involucrin (IVL) antibody, diluted 1:1000; Mitofusin-1 (MFN-1) antibody diluted 1:1000; Intercellular Adhesion Molecule 1 (ICAM-1) antibody, diluted 1:1000; Glycoprotein 9 (CD9) antibody, diluted 1:1000; Glycoprotein 44 (CD44) antibody, diluted 1:1000. Antibodies purchased by Santa Cruz (Santa Cruz Biotechnology Inc.): glyceraldehyde 3-phosphate dehydrogenase (GAPDH), diluted 1:1000. The membrane was subsequently washed and incubated at room temperature for one hour with a secondary anti-mouse horseradish peroxidase (HRP)-conjugated antibody diluted 1:10000 in TBS-Tween-20. After additional washes, immunocomplexes were visualized using a chemiluminescence detection kit (EMD Millipore Corporation). The membrane was treated with an enhanced chemiluminescence reagent (ECL) containing luminol (the substrate for HRP) and water. Luminol emits a chemiluminescent signal, upon oxidation by HRP in the presence of water, which was captured using the iBright image system (Invitrogen, S.r.l.). Signal intensity directly correlated with the protein quantity in the sample. Reagents were mixed in equal proportions according to the manufacturer's guidelines.

3.3.5 Signal acquisition

Acquisition of western blot images was carried out with the iBright image system; protein band analysis was carried out with the iBright Imaging Systems 5.4.0 (Thermo Fisher Scientific). 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 protein bands were normalized to those of a housekeeping protein.

3.4 TG2 activity assay

TG2 activity was assessed through an *in vitro* and *in situ* assay following established protocols.^{94,95} Regarding the *in vitro* assay, we followed the steps that have already been published by researchers of our group⁹⁴. For the *in situ* assay, we used cell cultures, and a minimum of two independent experiments, each conducted in triplicate, were performed. In summary, dermal fibroblasts were exposed to THP at concentrations of 0.01, 0.1, and 0.5 μM for 30 min at 37°C, in the presence of the TG2 substrate pentylamine-biotin (0.5 mM; Thermo Fisher Scientific, Monza, Italy). Intestinal fibroblasts were exposed to ZED1227 at concentrations of 300 nM and 10 μM for 30 min and with THP for 1 h. In addition, CACO-2 cells were also used to assess the capability of ZED1227 to inhibit the TG2 activity, using the same chemicals and concentrations used for the dermal and intestinal fibroblasts. Post-treatment, cells were lysed using RIPA buffer (see paragraph 3.3.1 for the exact composition). The lysates (25 μg of protein) were subsequently coated onto 96-well microplate wells and left overnight (coating step). Following this step, wells were treated with a blocking solution (10% BSA in borate-buffered saline) before incubation with HRP-conjugated streptavidin (1:3000; Thermo Fisher Scientific). The enzymatic activity of peroxidase was detected using 3,3',5,5'-tetramethylbenzidine as the substrate. Absorbance readings at 450 nm provided an estimate of intracellular TG2 activity.

3.5 Confocal microscopy for LC3 protein

Fibroblasts grown on glass coverslips underwent fixation using 3% paraformaldehyde (PFA) for 10 min, followed by a 5- min permeabilization with 0.2% Triton X-100. Subsequently, the cells were incubated for 1 hour with primary antibodies prepared in 1% BSA dissolved in PBS. The rabbit anti-LC3 antibody was used at 1:100, then cells were incubated for 1 h with an anti-rabbit tetramethylrhodamine-conjugated antibody (1:100, in 0.1% BSA) (Thermo Fisher Scientific). Post-incubation, the coverslips were thoroughly washed with PBS and mounted using Mowiol. Imaging was acquired using a Zeiss LSM 510 laser scanning microscope from Carl Zeiss MicroImaging Inc., employing a magnification of 63× across all figures presented.

3.6 Mass spectrometry samples preparation

To perform mass spectrometry analysis to analyze the basal proteomic and phosphoproteomic patterns of the primary fibroblasts, cells were harvested and collected in two different ways. For the proteomics, cells were grown in a 60 mm dish (Corning, New York, United States) until they reached 80% confluence and then were lysed in a buffer (for the composition see section 3.3.1). Protein extracts, both for intestinal and dermal fibroblasts, were pulled (25 µg each, for a total of 100 µg) to reduce interpersonal differences between different subjects. Then, the mixtures were separated by 12% SDS-PAGE. The gels were then sectioned into 10 pieces, and each sample underwent a trypsin in gel digestion procedure. For the phosphoproteome, the Pierce™ Phosphoprotein Enrichment Kit (Invitrogen S.r.l.) was used, according to the manufacturer's instructions, to collect the samples, before performing the steps already described for the proteome analysis.

3.7 Mass spectrometry

The analyses involving the proteome and phosphoproteome were performed in collaboration with the Department of Medicine, Surgery, and Dentistry of the University of Salerno. Briefly, the resulting peptide mixtures were analyzed in positive ion full scan and dependent scan mass spectrometry mode on a Q-Exactive Orbitrap mass spectrometer coupled with a nanoUltimate UHPLC system (Thermo Fisher Scientific). Peptide separation was performed on a capillary EASY-Spray PepMap column (0.075 mm × 50 mm, 2 µm, Thermo Fisher Scientific) using aqueous 0.1% formic acid (A) and CH₃CN containing 0.1% formic acid (B) as mobile phases in 45 min and a 300 nL/min flow rate. Mass spectra were acquired over an m/z range from 375 to 1500.

3.8 Bioinformatics analyses

The mass spectrometry analysis yielded a list of up- and down-regulated proteins based on their abundance ratios. For the significant ones (p -value < 0,05), unique peptides >2, abundance ratio > 1.8 and <0.5), bioinformatics analysis was performed to group all those proteins to highlight the pathways that were differentially regulated between CD cells and CTRs. The analysis was not excessively stringent for some proteins. All proteins with a significant p -value change were considered, as the collective action was assessed, seeking to identify disrupted pathways rather than individual proteins. First, STRING (a database that contains predicted and known protein-protein interactions (<https://string-db.org/>, accessed on 15/03/2024)⁹⁶ was employed to evaluate potential interactions between our pool of proteins. Moreover, the Gene Ontology terms referred to our proteins were analyzed using ShinyGO software (v0.741, South Dakota University, accessed on 18/04/2024), a powerful tool that was used to make an efficient enrichment analysis, supplied with an efficient plot visualization due to its R-based structure.⁹⁷ Furthermore, processing was performed to allow the functional organization of proteins according to the GO terms. Additionally, for each GO term, all the genes related to each term were extracted (using ShinyGO software).

One of the proteins identified by phosphoproteome analysis (cDNA fly58187) seems to be extremely similar to TG2. Using the flj58187 cDNA obtained from phosphoproteomic analyses, its origin was assessed using the UniProt database (accessed 08-12-2025, code B4DIT7). Once the amino acid sequence was obtained, a comparative alignment was performed using the same

software to assess the similarities to TG2, due to the importance of this protein in the CD pathology. Similarities to the TG2 protein were identified (UniProt accession code P21980). Subsequently, the flj58187 cDNA sequence was modelled based on a template of TG2 using Phyre2 software⁹⁸. The software used the TG2 protein as a template, with a coverage of 96% and a confidence of 100% (template used to model the sequence: c1kv3F), confirming the presence of differences regarding the levels of TG2 phosphorylation in the CD.

3.9 Metabolome analysis

Metabolomic analyses on intestinal fibroblasts were performed in collaboration with Theoreo S.r.l. (Pontecagnano, Italy). Briefly, an untargeted metabolomics analysis was carried out using gas chromatography - mass spectrometry (GC–MS) methodology. Intestinal fibroblasts were plated until they reached 80% of confluence. Cells were washed twice with cold PBS to stop metabolism and then harvested. After, 5×10^5 cells for each sample were collected for pellet metabolome analysis. Moreover, the supernatants of these cell cultures were collected to analyze their metabolome composition. The samples were immediately put at -80°C to correctly preserve the integrity of the metabolome until the subsequent analysis. To summarise, the processes of metabolome extraction, purification, and derivatization were conducted with the MetaboPrep GC kit (Theoreo, S.r.l.) according to the manufacturer's instructions. 2-isopropyl malic acid served as the internal standard. The instrumental analysis was performed on a GC–MS system comprising the GC-2010 Plus gas chromatograph and QP2010SE mass spectrometer from Shimadzu Corp., Kyoto, Japan.

3.10 RNA extraction and transcriptome analysis

Intestinal fibroblasts from CD patients were used for transcriptomic analyses. Briefly, cells were plated and grown to 80% confluence. Four CD cultures were treated with the inhibitor ZED1227 as described in section 3.3.1. Cells were then lysed, and RNA was extracted using

the reagents from the miRNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A total of 1×10^6 cells for each sample were used for lysis and extraction. After obtaining the samples, they were quantified using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific) and their quality was also assessed by visualize them on an ethidium bromide-stained agarose gel. Then, samples were prepared at a final concentration of 200 ng/ μ L for 30 μ L total and stored at -80°C until shipment to BMR Genomics S.r.l. (Padua, Italy), for further sample quality control and transcriptomic analysis.

3.11 Statistical analysis

The statistical analysis and graphing were performed using GraphPad Prism 9.5.1 (San Diego, CA). The mean and standard error of the experiment were determined, and their significance was assessed using a *t*-Student test. A *p*-value of less than 0.05 was considered the threshold for statistical significance in the evaluation of means and standard errors. The differences in the Western blot images generally showed statistical differences between the samples and their respective vehicle controls. Where specifically indicated, the difference between CD and CTR was depicted.

4 Results

4.1 Skin Fibroblasts

4.1.1 THP effects on primary skin fibroblasts

4.1.1.1 How THP affects cell Viability

THP was used as a stressor agent to characterize constitutive differences between celiac and CTR cells; therefore, its effects on cell viability were first assessed. After 24 h of exposure to THP, a reduction in cell viability was observed in both CD and CTR cells. However, the CTR cultures demonstrated greater sensitivity compared to the celiac samples, as illustrated in **Figure 8**. Notably, even at the lowest concentration tested (0.01 μ M), THP decreased the viability of CTR cells by approximately 20%, while CD fibroblasts remained unaffected. By 48 and 72 h, both CTR and CD cells exhibited similar responses to THP exposure.

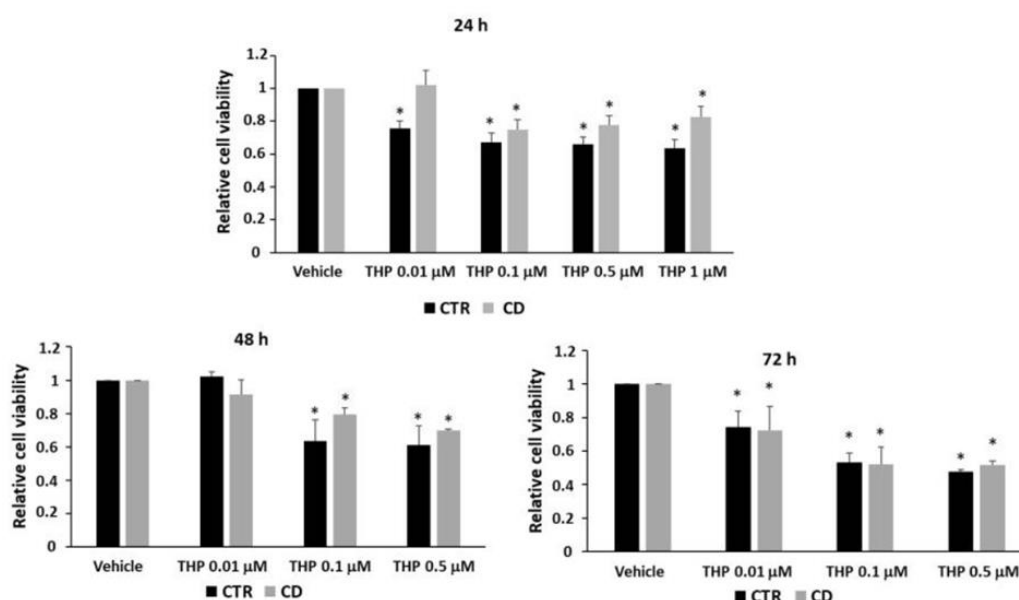


Figure 8. Effects of THP on cell viability at different treatment times for a range of concentrations from 0.01 to 1 μ M (24 h) and 0.01 to 0.5 μ M (48 and 72 h). Basal viability was assessed in the presence of the vehicle, in this case DMSO (0.05%). In all cases, the vehicle did not reduce viability by more than 10-15%. Data are reported as

means $\pm$ standard error (SE) from two independent experiments, each performed in triplicate, on three CTR and three CD cultures. * $p < 0.05$ *versus* (vs.) the respective vehicle.

4.1.1.2 Effects of THP on GRP78 expression

Previous studies conducted by our research group indicated that Ca^{2+} homeostasis in celiac dermal fibroblasts was altered⁹⁹. In particular, the basal intracellular $[\text{Ca}^{2+}]$ was significantly lower in fibroblasts of CD subjects than in CTRs. Moreover, the amount of Ca^{2+} ions released from the endoplasmic reticulum (ER) after THP treatments was significantly less in CD cells than in CTR ones⁹⁹. The evidence of differences in Ca^{2+} content and homeostasis in cells from celiac patients has led to the hypothesis of involvement of the UPR of the ER. To assess the triggering of the UPR in THP-treated cells, the expression of the 78 kDa glucose-regulated protein (GRP78) marker was analyzed. Western blot analyses revealed that basal GRP78 protein expression was lower in celiac subjects compared to CTRs (**Figure 9a, b**). Furthermore, following a treatment with THP for 24 h, the behavior of the two groups of subjects differed (**Figure 9c, d**). For CTRs, an increase in protein expression was evident even at lower doses (0.1 μM). In celiac subjects, however, the response to the same concentration appeared to be milder, with a marked increase at higher concentrations (0.5 μM). These data are in line with the previous evaluation performed by the research group regarding the splicing of X-Box Binding Protein 1 (XBP1), another UPR marker⁹⁹. Employing conventional semi-quantitative PCR, a marked increase in XBP1 splicing in both cell groups following treatments with THP for 4 h was registered. However, in CTR cells, a pronounced and comparable splicing response was observed at both 0.1 and 0.5 μM concentrations of THP, suggesting that, following an initial rapid reaction, the cells may adapt to the induced stress. In contrast, in CD cells, splicing was already prominent at 0.1 μM THP and became considerably more pronounced at 0.5 μM , indicating a heightened or progressive response under these conditions⁹⁹.

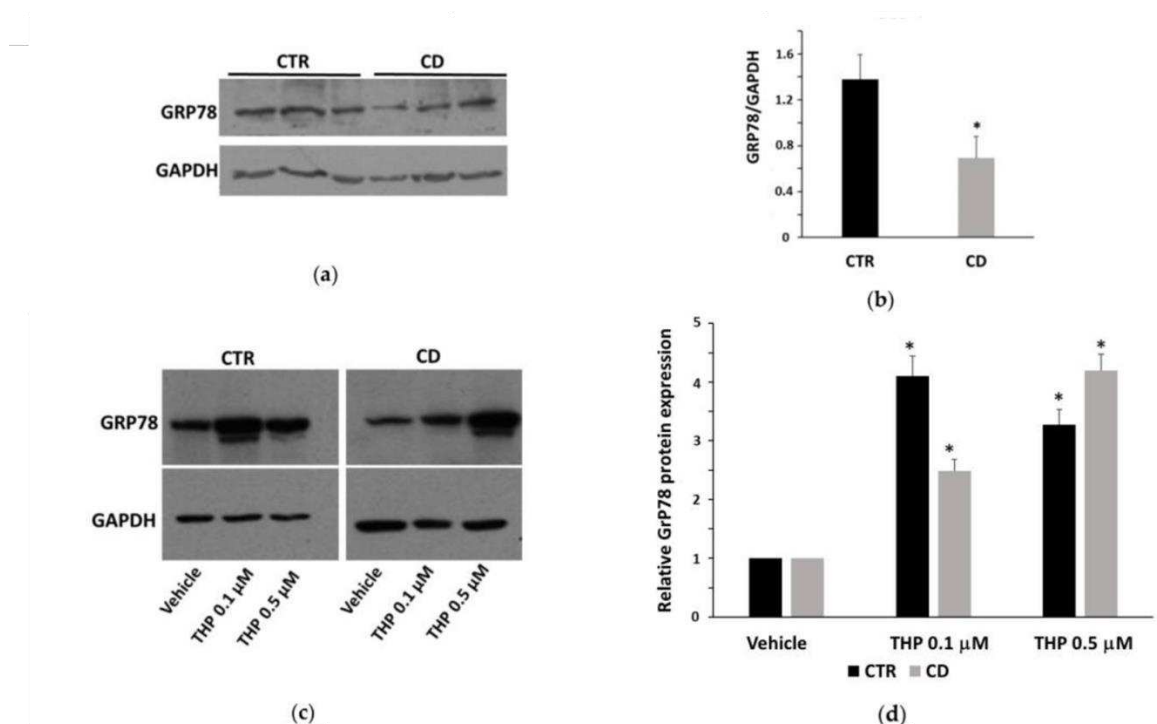


Figure 9. Effect of THP on GRP78 expression. (a) Representative Western blot of basal GRP78 levels in three CD and three CTR samples. (b) Densitometric analysis of GRP78 expression. Levels are normalized to those of GAPDH in five CD and five CTR cell cultures. * $p < 0.05$ vs. controls (Student's t -test for unpaired data) (c) Representative Western blot of GRP78 levels following 24 h exposure to THP in a celiac cell culture and a CTR culture. (d) Densitometric analysis of GRP78 levels following 24 h stimulation with THP in three CD cultures and three CTR cultures. Protein levels are normalized to GAPDH expression and expressed as variation with respect to the relative vehicle-treated sample. Data are reported as mean $\pm$ SE. * $p < 0.05$ vs. the respective vehicle.

4.1.1.3 Effects of THP on TG2 expression and catalytic activity

As previously mentioned, differences have been observed by our group in intracellular Ca^{2+} content and calcium release from the ER. Consequently, this phenomenon could affect the transamidase activity of the TG2 enzyme. To test this hypothesis, we performed an *in situ* TG assay using the substrate biotin-pentylamine in cells treated for 30 min with THP at different concentrations. As shown in **Figure 10**, a dose-dependent increase in enzyme activity was

observed in CTRs; in contrast, no increase in activity was detected in CD cells at any of the concentrations tested.

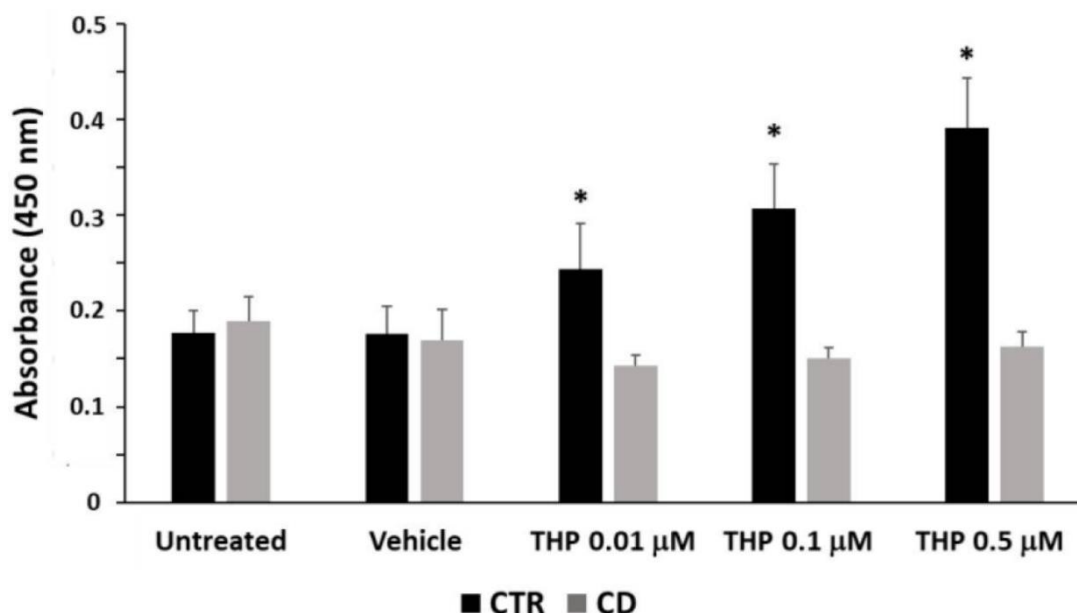


Figure 10. Intracellular TG2 enzymatic activity expressed as absorbance at 450 nm in skin-derived fibroblasts from three CTR and three CD subjects. Data are reported as mean $\pm$ SE. * $p < 0.05$ vs. respective untreated samples.

Also, we investigated whether THP could affect TG2 protein level. TG2 expression was analyzed following 24 h of treatment with THP. Western blot results demonstrated that THP led to a reduction in TG2 protein levels in both CD and non-CD cells, with the decrease being slightly more pronounced in CD samples (**Figure 11a, b**). Interestingly, after 4 h of treatment, Western blot analysis revealed that the TG2 levels declined only in CTR cells (**Figure 11c**). Furthermore, the TG2-specific inhibitor Z-DON (40 μ M) was able to counteract the THP-induced reduction of TG2 in CTR cells but did not show any significant effect in CD cells (**Figure 11c**).

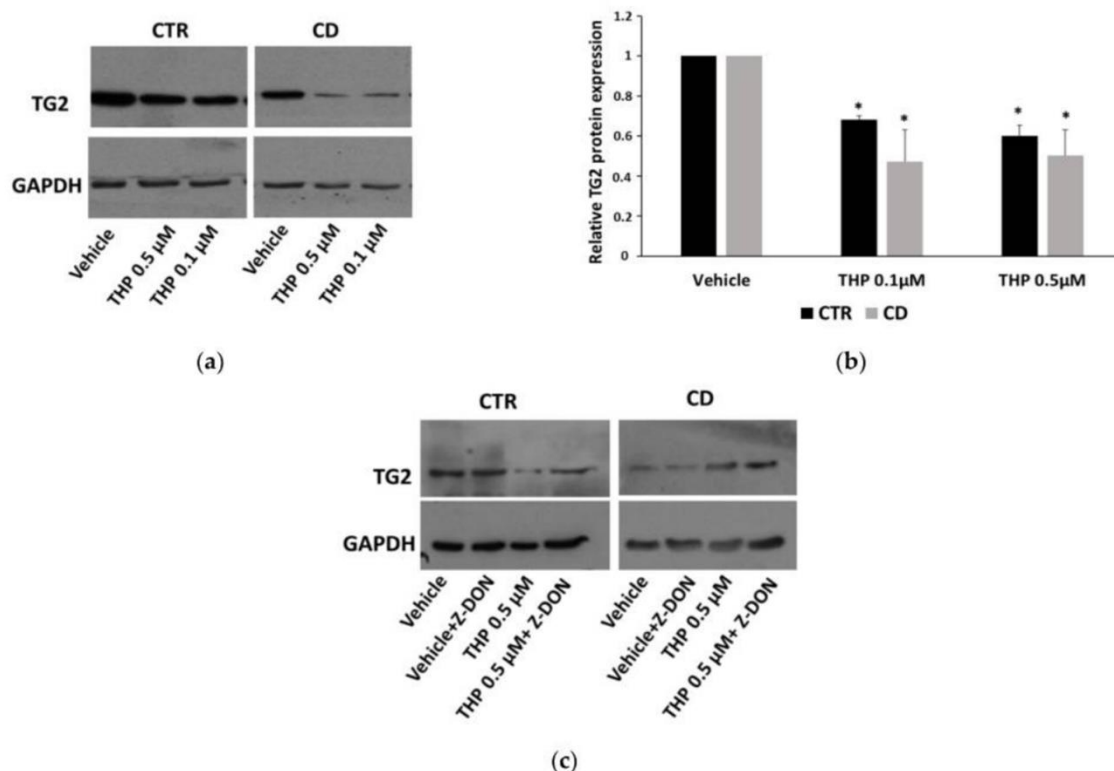


Figure 11. TG2 expression in the presence of THP. (a) Representative Western blot anti-TG2 on samples from one CTR and one CD culture treated with THP for 24 h. (b) Densitometric analyzes relative to Western blot anti-TG2 on samples from three CTR and three CD cultures treated with THP for 24 h. (c) Anti-TG2 Western blot on samples from one CTR and one CD culture treated with THP for 4 h and with Z-DON. Data are reported as mean $\pm$ SE. * $p < 0.05$ vs. respective vehicle.

4.1.1.4 Effects of THP on Autophagy

Alterations observed so far in the UPR by the ER suggest the involvement of autophagy, as ER stress is a potent trigger of autophagy.¹⁰⁰ In situations of severe stress, where misfolded proteins are abundant, a mechanism aimed at recycling their components, called autophagy, can be triggered. The proper functioning of this biochemical process plays a key role in the cell, allowing the cell to relieve stress induced by the ER, or it can be a signal to the cell to initiate apoptosis.¹⁰⁰ Furthermore, TG2 appears to play a key role in the maturation of autophagic vesicles, so alterations in the enzyme could imply alterations in autophagy.^{101–103} Since THP strongly triggers the UPR, differences in autophagic flux following the use of this substance were evaluated in CTR and CD cells. Two key markers of autophagic flux were analyzed: LC3, whose accumulation of its LC3-II isoform indicates an increase in autophagic vesicles, and p62, whose increased protein levels may indicate an insufficient autophagic flux. First, Western blot

analyses on three CD cultures and three CTR cultures showed that, in CD subjects, basal expression of LC3-II and p62 was markedly increased compared to CTRs (**Figure 12**). Moreover, confocal images of basal LC3 showed a more marked presence of large perinuclear vesicles in CD samples than in CTR ones (**Figure 12**).

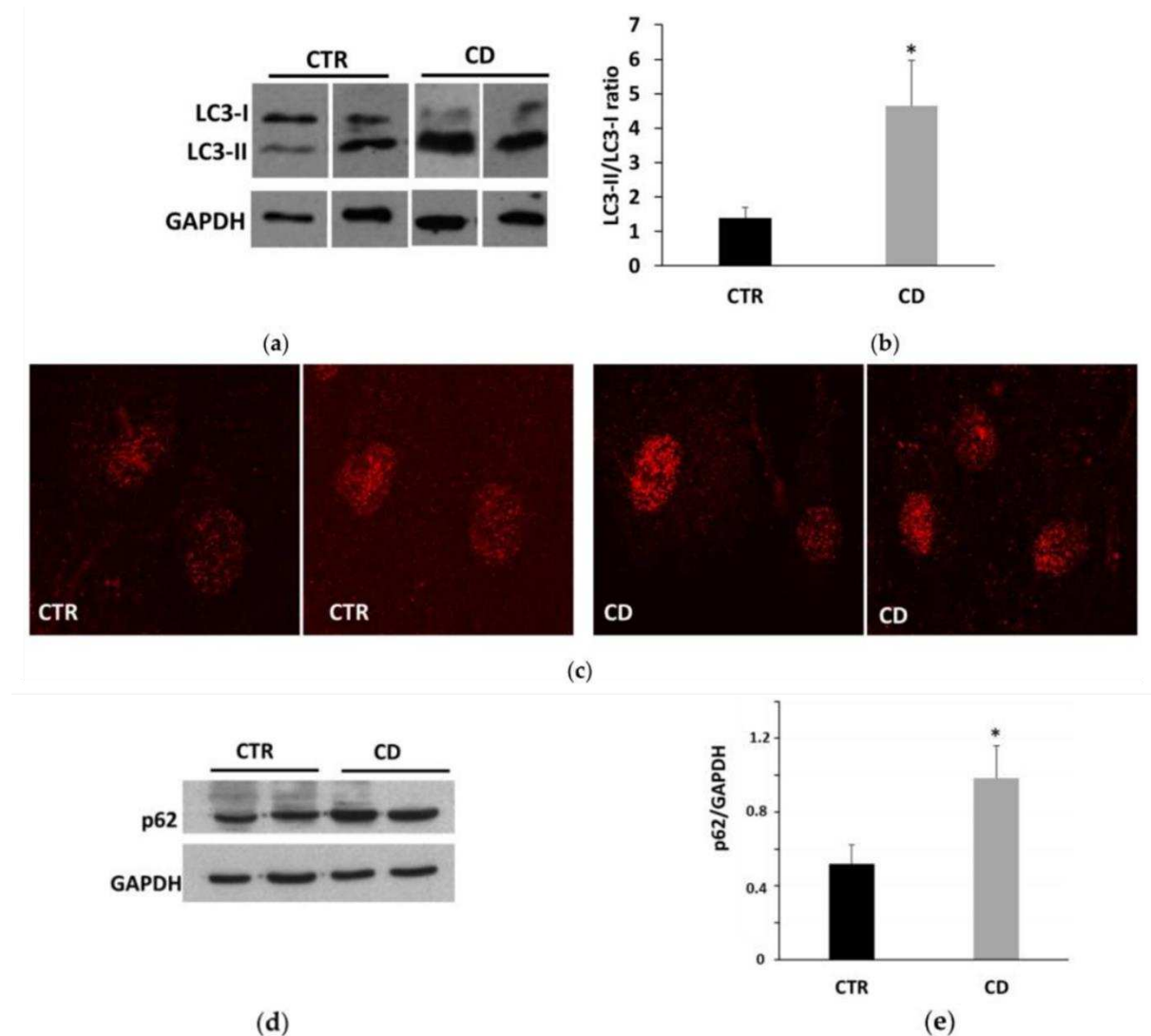


Figure 12. Basal expression of autophagy markers. **(a)** Representative Western blot anti-LC3 showing basal levels of LC3-I and LC3-II on samples from two CTR and two CD cultures. **(c)** Representative confocal images of LC3 fluorescence staining in cells from two CTR cultures and two CD ones (63× magnification). **(d)** Representative Western blot of the basal level of p62 in samples from two CTR and two CD cultures. **(b, e)** Densitometric analysis of the relationship between LC3-II and LC3-I forms and of p62 basal levels, normalized with respect to GAPDH expression, for both obtained in samples from four CTR and four CD cultures. Data are reported as mean $\pm$ SE. * $p < 0.05$ vs. CTRs (Student's t-test for unpaired data).

Afterwards, the possible differential response of CD and CTR subjects following treatment with THP was evaluated by Western blot analysis with anti-LC3 and anti-p62 antibodies. In CTR cells, 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 in LC3-II, whereas it was not possible to appreciate a reduction of p62 (**Figure 13**).

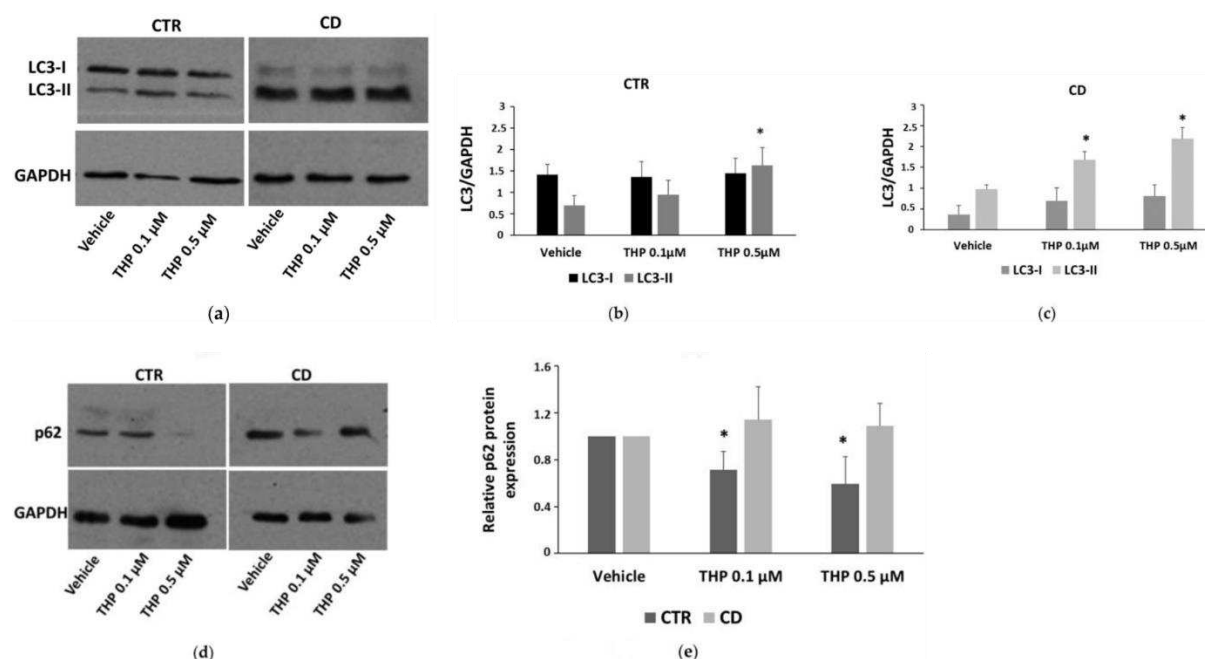


Figure 13. Analysis of the effect of THP on LC3 and p62 protein expression in skin-derived fibroblasts. **(a)** Representative Western blot anti-LC3 performed on fibroblasts from one CTR sample and one CD sample after treatments of 24 h with THP 0.1 and 0.5 μ M. **(b, c)** Densitometric analysis of blots related to three CTR samples and three CD samples. Protein levels of LC3-I and LC3-II are normalized with respect to GAPDH expression. **(d)** Representative Western blot anti-p62 performed on fibroblasts from one CTR sample and one CD sample after treatments of 24 h with THP 0.1 and 0.5 μ M. **(e)** Densitometric analyses of blots relating to three CTR samples and three CD samples. GAPDH was used as the internal reference. Data are reported as mean $\pm$ SE. * $p < 0.05$ vs. the respective vehicle.

These findings collectively suggest that autophagic flux may be impaired in CD cells. To further confirm this hypothesis, we examined the effects of starvation, a known inducer of autophagy, and bafilomycin A1, an inhibitor of autophagic flux. In CTR fibroblasts, starvation induced a moderate but significant increase in the LC3-II isoform and led to a noticeable accumulation of LC3-positive autophagosomes (**Figure 14a–c**). Bafilomycin A1 treatment alone resulted in a

marked elevation of the LC3-II isoform, and this effect was more pronounced (accompanied by a reduction in LC3-I levels) when cells were previously exposed to starvation (Figure 14a, b). These observations are consistent with an enhanced autophagic flux in CTR cells. In contrast, CD cells exhibited a diminished response to starvation, as indicated by lack of increase of LC3-II isoform detected on Western blot analysis (Figure 14a, b) and of LC3-positive vesicles (Figure 14c). While treatment with bafilomycin A1 alone caused significant accumulation of LC3-II, the combined effect of bafilomycin A1 with starvation was less pronounced compared to CTR samples (Figure 14a, b). Finally, preliminary results (Figure 14d) showed that the effect of bafilomycin A1 was also evident on the accumulation of the p62 marker. P62 accumulation was less marked in CTRs, consistent with what was observed for LC3. Taken together, these data suggest that autophagic activity in CD cells may be partially compromised.

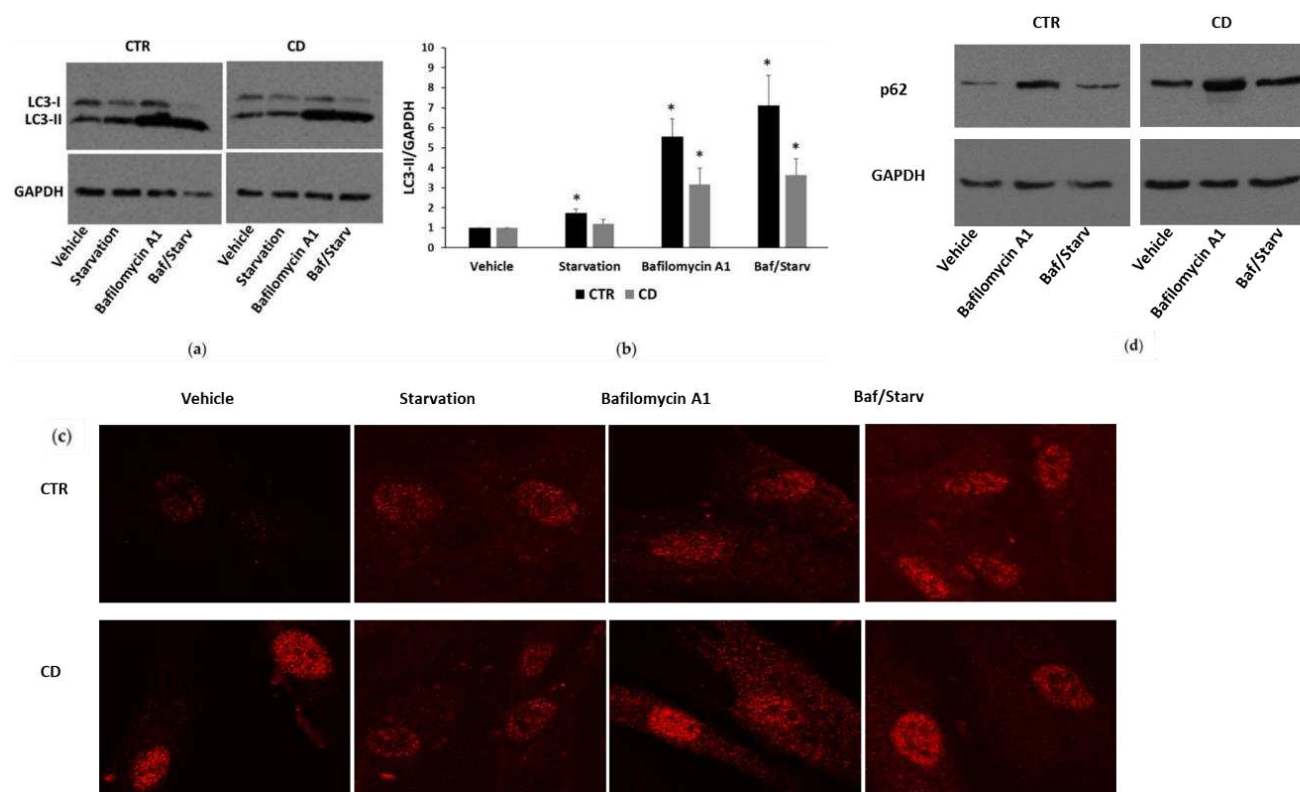


Figure 14. LC3 and p62 levels in the presence of starvation medium and/or of bafilomycin A1. (a) Representative Western blot anti-LC3 on samples from one CTR and one CD culture, treated with bafilomycin A1 0.1 μ M for 4 h; where indicated, cells were previously starved for 2 h. (b) Densitometric analyses of LC3-II levels relative to samples from three CTR and three CD cultures treated as in (a). Protein levels are normalized with respect to GAPDH expression and reported as variations with respect to the relative vehicle-treated sample. Data are reported

as mean $\pm$ SE. * $p < 0.05$ vs. the respective vehicle. (c) Confocal immunofluorescence images of fibroblasts from CTR and CD subjects stained with anti-LC3 antibodies (magnification 63 $\times$). (d) Representative Western blot anti-p62 on samples from one CTR and one CD culture, treated with bafilomycin A1 0.1 μ M for 4 h; where indicated, cells were previously starved for 2 h.

4.1.2 Omics analyses on skin fibroblasts

The use of omics techniques allows us to analyze biological complexity and molecular interactions, going beyond single components for a comprehensive view. For both the proteome and phosphoproteome, the analysis was conducted on a pool of four CD and four CTR samples derived from primary skin fibroblasts. Samples were obtained by pooling 25 μ g of each sample to minimize interpersonal differences.

4.1.3 Proteome analysis on skin fibroblasts

As previously discussed, the proteomic approach not only allows for a greater understanding of the biological system under examination, especially in the case of a disease compared to a CTR, but can also provide indications of potential new biomarkers that are less invasive than traditional methods, such as biopsy, as the gold standard for CD. Four CTR and four CD samples were analyzed by mass spectrometry using a bottom-up approach. To minimize interpersonal differences and increase statistical efficiency, the samples were pulled before analysis, using 25 μ g of protein for each patient. This analysis identified **3340** proteins. Of these, according to a p -value threshold of <0.05 , unique peptides >2 and an abundance ratio of >1.8 , **79** proteins were found to be more highly expressed in CD subjects than in CTRs (reported in **Supplementary Table 1**). In contrast, using the same parameters and an abundance ratio <0.5 , **99** proteins were found to be less expressed in CD subjects than in CTRs (reported in **Supplementary Table 2**).

A Gene Ontology (GO) enrichment analysis was then performed on these proteins. GO term enrichment involves a statistical approach to assess whether a specific group of genes shows a significant overrepresentation of GO terms. This analysis highlighted an enrichment of

biological functions, cellular components, or pathways within the gene set, beyond what would occur randomly. In this way, it was possible to observe functions or biological processes that were more representative of up- and down-expressed proteins in CD. Also, the ShinyGO software was used with a False Discovery Rate (FDR) <0.05 , showing 10 pathways (**Figure 15**). The analysis was performed for the three classes of Ontologies: Biological Processes (**Figure 15a, d**), Molecular Functions (**Figure 15b, e**), and Cellular Components (**Figure 15c, f**). From a biological and functional perspective, the most important nodes appear to include proteins related to the proteasome complex, the extracellular exosome and vesicles, and proteins involved in catabolic processes.

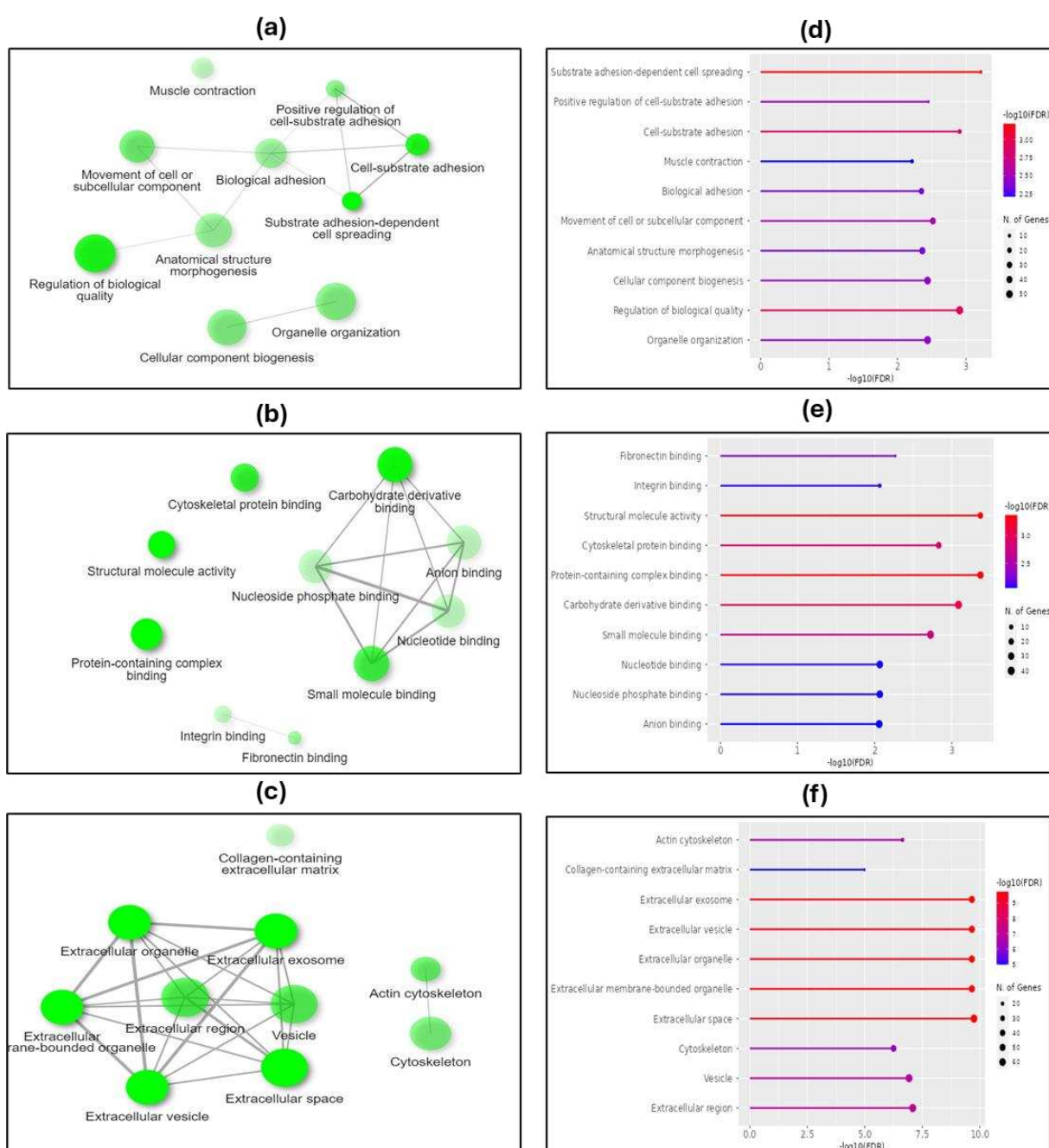


Figure 15. GO enrichment analysis. Enrichment of up- and down-expressed proteins in CD skin fibroblasts compared to CTR. Clustering and the most representative terms in the protein population, with a minimum of 20 genes per term, of the different GO classes: (a, d) biological processes, (b, e) molecular functions, (c, f) cellular components.

From the enrichment analysis, the various GO terms were extracted and organized by entry to obtain a functional clustering. The terms were taken from GO AmiGO, a software that collects the functional information and annotations of genes. This allowed us to visualize which genes, and therefore which proteins, were most represented in the GO terms. Furthermore, it allowed us to identify which terms may be most involved in the differences between CD and CTR, so that we can direct our investigations in that direction. These results are reported in **Tables 5, 6, and 7**, which represent biological process, molecular function, and cellular component, respectively.

Table 5. Proteome Enrichment analysis of skin fibroblasts. Enrichment of GO terms by biological process. Ontology source <http://amigo.geneontology.org/amigo/term>.

Gene Ontology ID	Enrichment FDR	Fold Enrichment	Gene Ontology Term	Number of Genes	Gene Found Associated with the GO term
GO:0034446	6.1E-04	10.7	Substrate adhesion-dependent cell spreading	9	FBLN1 NRP1 FN1 GBP1 LAMC1 DOCK5 AXL FGB PARVB
GO:0010811	3.5E-03	8.5	Positive regulation of cell-substrate adhesion	8	MAP4K4 FBLN1 CCDC80 NRP1 CDK6 FN1 DOCK5 FGB
GO:0031589	1.2E-03	5	Cell-substrate adhesion	14	MAP4K4 FBLN1 ITGB5 CCDC80 NRP1 CDK6 FN1 GBP1 CCN2 LAMC1 DOCK5 AXL FGB PARVB
GO:0006936	6.1E-03	4.5	Muscle contraction	12	MYLK ITGB5 MYL6 ACTA2 CRYAB CERT1 CCN2 CALD1 DOCK5 TPM4 DES TPM2
GO:0022610	4.5E-03	2.4	Biological adhesion	29	CD99 CD9 MAP4K4 FBLN1 ITGB5 CCDC80 NRP1 CDK6 ENG CD81 FN1 GBP1 CCN2 LOXL2 LAMC1 TMEM47 DOCK5 IL18 PLPP3 COL6A3 IGFBP7 AXL FGB LRRC15 YES1 PAWR PARVB YTHDF2 TGM2
GO:0006928	3.0E-03	2.2	Movement of cell or subcellular component	37	CD99 CD9 GPC1 MYLK MAP4K4 MYDGF FBLN1 EPHA6 ITGB5 TF MYO15A MYL6 NRP1 RPS19 CDK6 ENG PPP1R9B CD81 FN1 CCN2 LOXL2 LAMC1 DAB2IP DOCK5 ADAMTS1 APPL1 COPG2 PLPP3 CTHRC1 TPM4 AXL SLC3A2 CLIC4 LRRC15 DES YES1 SLIT3 TPM2
GO:0009653	4.3E-03	2	Anatomical structure morphogenesis	42	CD9 GPC1 MYLK MAP4K4 MYDGF FBLN1 EPHA6 ITGB5 MYO15A NRP1 PPP1R16B ENG ACTA2 CRYAB CD81 CERT1 FN1 GBP1 CCN2 CALD1 PTGIS SPART LOXL2 LAMC1 DAB2IP FGD4 DOCK5 IL18 ADAMTS1 LEMD2 CTHRC1 AXL CLIC4 FGB RAPH1 CDC42EP4 ALDH1A3 SLIT3 PARVB YTHDF2 TGM2 HMGNI
GO:0044085	3.6E-03	1.8	Cellular component biogenesis	50	ZNF207 CD9 UTP18 GPC1 MYLK MAP4K4 ITGB5 NRP1 PPP1R16B COTL1 RPS19 MPP6 UBE2S NUP88 PPP1R9B CRYAB TIMMDC1 FN1 CCN2 CALD1 RPL23 CLIP1 DKC1 NOL11 LAMC1 YME1L1 DAB2IP FGD4 SETDB1 CHMP7 CCT8 NDUFV3 LEMD2 FGB UBE2C PAWR RABEP2 CDC42EP4

					SF3A3 ALDH1A3 H1-5 ERC2 H2AX PARVB YTHDF2 LCMT1 NUP62 TUBB TUBB8 UTP4
GO:006500 8	1.2E-03	1.8	Regulation of biological quality	58	ZNF207 CD9 CPS1 ISOC2 MAP4K4 CYBRD1 FBLN1 AFM TF COMT NRP1 COTL1 RPS19 CDK6 ENG ACTA2 UBE2S CCDC47 CRYAB CD81 CERT1 FN1 KMO CCN2 RPL23 GNAI1 NXNL2 DKC1 SPART LAMC1 NMT1 DAB2IP FGD4 NPC1 DOCK5 IL18 CCT8 PLPP3 CTSB CKB ENDOG AXL COG7 CLIC4 MFN1 FGB SNCG UBE2C YES1 CDC42EP4 ALDH1A3 H1-5 ERC2 PARVB YTHDF2 TGM2 TAF15 CALB2
GO:000699 6	3.6E-03	1.8	Organelle organization	55	ZNF207 ITGB5 TF MYO15A NRP1 COTL1 RPS19 UBE2S CCDC47 PPP1R9B CRYAB CERT1 TIMMDC1 CCN2 CALD1 GNAI1 CLIP1 DKC1 SPART LOXL2 NMT1 YME1L1 FGD4 CARM1 SETDB1 CHMP7 ARFGAP2 N6AMT1 CCT8 CHCHD6 NDUFV3 LEMD2 TPM4 COG7 CLIC4 TOR1AIP2 MFN1 UBE2C DES PAWR RABEP2 CDC42EP4 H1-5 RAD51D H2AX PARVB TPM2 YTHDF2 TOP1 HMGNI1 LCMT1 NUP62 TUBB TUBB8 UHRF1

Table 6. Proteome Enrichment analysis in skin fibroblasts. Enrichment of GO terms by molecular function.
Ontology source <http://amigo.geneontology.org/amigo/term>.

Gene Ontology ID	Enrichment FDR	Fold Enrichment	Gene Ontology Term	Number of Genes	Gene Found Associated with the GO term
GO:0001968	5.4E-03	18.6	Fibronectin binding	4	FBLN1 CCDC80 CCN2 LRRC15
GO:0005178	8.6E-03	6.2	Integrin binding	7	CD9 FBLN1 ITGB5 CD81 FN1 CCN2 PLPP3
GO:0005198	4.3E-04	3.4	Structural molecule activity	20	FBLN1 MYL6 RPS19 NUP88 CRYAB FN1 RPL23 LAMC1 COPG2 COL6A3 IGFBP7 CTHRC1 TPM4 FGB DES ERC2 TPM2 NUP62 TUBB TUBB8
GO:0008092	1.5E-03	2.8	Cytoskeletal protein binding	22	ZNF207 MYLK MAP4K4 MYO15A COTL1 PPP1R9B CRYAB GBP1 CALD1 CLIP1 FGD4 MYH15 APPL1 TPM4 AXL HSPB7 DES PAWR RAD51D PARVB TPM2 NUP62
GO:0044877	4.3E-04	2.7	Protein- containing complex binding	29	CD9 CPS1 FBLN1 ITGB5 MYO15A COTL1 ENG UBE2S CCDC47 PPP1R9B CRYAB CD81 FN1 GBP1 CCN2 GNAI1 DAB2IP MYH15 APPL1 COLEC12 PLPP3 CTSB TPM4 LRRC15 H1-5 TPM2 HMGNI1 NUP62 UHRF1
GO:0097367	8.2E-04	2.1	Carbohydrate derivative binding	39	ZNF207 CPS1 MYLK MAP4K4 EPHA6 MYO15A CCDC80 NRP1 CDK6 ENG ACTA2 UBE2S HARS2 FN1 GBP1 CCN2 GNAI1 CARS2 YME1L1 NME7 MYH15 ADAMTS1 CCT8 AGAP1 CTSB CKB TK2 AXL GLB1 MFN1 UBE2C YES1 SLIT3 UBE2G2 RAD51D TOP1 TGM2 TUBB TUBB8
GO:0036094	1.9E-03	2	Small molecule binding	40	CPS1 MYLK MAP4K4 AFM EPHA6 MYO15A CDK6 ENG ACTA2 UBE2S CD81 HARS2 SPR KMO GBP1 GNAI1 CARS2 YME1L1 NPC1 NME7 MYH15 PLOD2 CCT8 AGAP1 COLEC12 MOCS2 CKB TK2 AXL MFN1 UBE2C YES1 ALDH1A3 UBE2G2 RAD51D TOP1 TGM2 TUBB TUBB8 UHRF1
GO:0000166	8.6E-03	1.9	Nucleotide binding	34	CPS1 MYLK MAP4K4 EPHA6 MYO15A CDK6 ACTA2 UBE2S HARS2 SPR KMO GBP1 GNAI1 CARS2 YME1L1 NME7 MYH15 CCT8 AGAP1 MOCS2 CKB TK2 AXL MFN1 UBE2C YES1 ALDH1A3 UBE2G2 RAD51D TOP1 TGM2 TUBB TUBB8 UHRF1
GO:1901265	8.6E-03	1.9	Nucleoside phosphate binding	34	CPS1 MYLK MAP4K4 EPHA6 MYO15A CDK6 ACTA2 UBE2S HARS2 SPR KMO GBP1 GNAI1 CARS2 YME1L1 NME7 MYH15 CCT8 AGAP1 MOCS2 CKB TK2 AXL MFN1 UBE2C YES1 ALDH1A3 UBE2G2 RAD51D TOP1 TGM2 TUBB TUBB8 UHRF1
GO:0043168	8.7E-03	1.9	Anion binding	36	MGST1 CPS1 MYLK MTHFD2 MAP4K4 AFM EPHA6 MYO15A CDK6 ACTA2 UBE2S HARS2 CERT1 KMO GBP1 GNAI1 CARS2 YME1L1 NME7 MYH15 PLOD2 CCT8 AGAP1 CKB TK2 AXL MFN1 UBE2C YES1 ALDH1A3 UBE2G2 RAD51D TOP1 TGM2 TUBB TUBB8

Table 7. Proteome Enrichment analysis in skin fibroblasts. Enrichment of GO terms by cellular component. Ontology source <http://amigo.geneontology.org/amigo/term>.

Gene Ontology ID	Enrichment FDR	Fold Enrichment	Gene Ontology Term	Number of Genes	Gene Found Associated with the GO term
GO:0015629	2.3E-07	5	Actin cytoskeleton	20	MYLK MYO15A MYL6 COTL1 ACTA2 PPP1R9B CRYAB GBP1 CALD1 MYH15 AFAP1L1 TPM4 AXL CLIC4 HSPB7 YES1 PAWR CDC42EP4 PARVB TPM2
GO:0062023	1.0E-05	4.8	Collagen-containing extracellular matrix	16	GPC1 FBLN1 CCDC80 FN1 CCN2 LOXL2 LAMC1 ADAMTS1 COL6A3 IGFBP7 CTSB CTHRC1 SERPINB12 FGB LRRC15 TGM2
GO:0070062	2.2E-10	2.9	Extracellular exosome	50	CD9 MON2 GPC1 PLPP1 CYBRD1 FBLN1 AFM ITGB5 TF MYO15A MYL6 COMT COTL1 RPS19 MPP6 ACTA2 CRYAB CD81 FN1 SPR RPL23 GNAI1 SLC44A2 LAMC1 NPC1 CNKSR2 PLOD2 CCT8 APPL1 IVL COL6A3 IGFBP7 CTSB CKB TPM4 AXL SLC3A2 CLIC4 GLB1 FGB LRRC15 SNCG DES YES1 ALDH1A3 H2AX TGM2 TUBB TUBB8 CANX
GO:1903561	2.2E-10	2.9	Extracellular vesicle	50	CD9 MON2 GPC1 PLPP1 CYBRD1 FBLN1 AFM ITGB5 TF MYO15A MYL6 COMT COTL1 RPS19 MPP6 ACTA2 CRYAB CD81 FN1 SPR RPL23 GNAI1 SLC44A2 LAMC1 NPC1 CNKSR2 PLOD2 CCT8 APPL1 IVL COL6A3 IGFBP7 CTSB CKB TPM4 AXL SLC3A2 CLIC4 GLB1 FGB LRRC15 SNCG DES YES1 ALDH1A3 H2AX TGM2 TUBB TUBB8 CANX
GO:0043230	2.2E-10	2.9	Extracellular organelle	50	CD9 MON2 GPC1 PLPP1 CYBRD1 FBLN1 AFM ITGB5 TF MYO15A MYL6 COMT COTL1 RPS19 MPP6 ACTA2 CRYAB CD81 FN1 SPR RPL23 GNAI1 SLC44A2 LAMC1 NPC1 CNKSR2 PLOD2 CCT8 APPL1 IVL COL6A3 IGFBP7 CTSB CKB TPM4 AXL SLC3A2 CLIC4 GLB1 FGB LRRC15 SNCG DES YES1 ALDH1A3 H2AX TGM2 TUBB TUBB8 CANX
GO:0065010	2.2E-10	2.9	Extracellular membrane-bounded organelle	50	CD9 MON2 GPC1 PLPP1 CYBRD1 FBLN1 AFM ITGB5 TF MYO15A MYL6 COMT COTL1 RPS19 MPP6 ACTA2 CRYAB CD81 FN1 SPR RPL23 GNAI1 SLC44A2 LAMC1 NPC1 CNKSR2 PLOD2 CCT8 APPL1 IVL COL6A3 IGFBP7 CTSB CKB TPM4 AXL SLC3A2 CLIC4 GLB1 FGB LRRC15 SNCG DES YES1 ALDH1A3 H2AX TGM2 TUBB TUBB8 CANX
GO:0005615	1.8E-10	2.5	Extracellular space	65	CD9 MON2 GPC1 MTHFD2 PLPP1 CYBRD1 MYDGF FBLN1 AFM ITGB5 TF MYO15A MYL6 COMT NRP1 COTL1 RPS19 MPP6 ENG ACTA2 CRYAB CD81 FN1 SPR KMO CCN2 PTGIS RPL23 GNAI1 SLC44A2 LOXL2 LAMC1 NPC1 CNKSR2 IL18 PLOD2 CCT8 APPL1 COLEC12 IVL COL6A3 IGFBP7 CTSB CTHRC1 CKB SERPINB12 TPM4 AXL SLC3A2 CLIC4 GLB1 FGB LRRC15 SNCG DES YES1 DIPK2A ALDH1A3 SLIT3 H2AX TGM2 C1QTNF5 TUBB TUBB8 CANX
GO:0005856	5.6E-07	2.4	Cytoskeleton	45	ZNF207 PLEKHH1 MYLK MYO15A MYL6 NRP1 COTL1 CDK6 ACTA2 PPP1R9B CRYAB GBP1 CALD1 GNAI1 CLIP1 FGD4 NME7 MYH15 CCT8 TBC1D31 AFAP1L1 SYNC PEA15 IVL TPM4 AXL CLIC4 RAPH1 SNCG HSPB7 DES YES1 PAWR RABEP2 CDC42EP4 RAD51D ERC2 H2AX PARVB TPM2 YTHDF2 NUP62 TUBB CEP95 TUBB8
GO:0031982	1.2E-07	2	Vesicle	66	MGST1 CD9 GOPC MON2 GPC1 PLPP1 CYBRD1 FBLN1 AFM ITGB5 TF MYO15A MYL6 COMT NRP1 COTL1 RPS19 MPP6 ACTA2 CRYAB CD81 FN1 SPR GBP1 PIGT RPL23 GNAI1 SLC44A2 CLIP1 LAMC1 DAB2IP NPC1 EBP CHMP7 CNKSR2 PLOD2 ADAMTS1 CCT8 APPL1 COLEC12 COPG2 IVL COL6A3 IGFBP7 CTSB CKB SERPINB12 TPM4 AXL SLC3A2 CLIC4 GLB1 FGB LRRC15 SNCG DES YES1 RABEP2 CDC42EP4 DIPK2A ALDH1A3 H2AX TGM2 TUBB TUBB8 CANX
GO:0005576	8.2E-08	2	Extracellular region	69	CD9 MON2 GPC1 MTHFD2 PLPP1 CYBRD1 MYDGF FBLN1 AFM ITGB5 TF MYO15A CCDC80 MYL6 COMT NRP1 COTL1 RPS19 MPP6 ENG ACTA2 CRYAB CD81 FN1 SPR KMO GBP1 CCN2 PTGIS RPL23 GNAI1 SLC44A2 LOXL2 LAMC1 NPC1 CNKSR2 IL18 PLOD2 ADAMTS1 CCT8 APPL1 COLEC12 IVL COL6A3 IGFBP7 CTSB CTHRC1 CKB SERPINB12 TPM4 AXL SLC3A2 CLIC4 GLB1 FGB LRRC15 SNCG DES YES1 DIPK2A OLFML1 ALDH1A3 SLIT3 H2AX TGM2 C1QTNF5 TUBB TUBB8 CANX

To validate these data, four proteins were selected for Western blot analysis, two of which were down-expressed in CD (MFN-1 and TG2) and two of which were up-expressed in CD (IVL and gamma synuclein SNCG). The results are shown in **Figure 16**. In summary, the data appeared to confirm what was recorded in the mass spectrometry analysis, although there was some inter-sample variability.

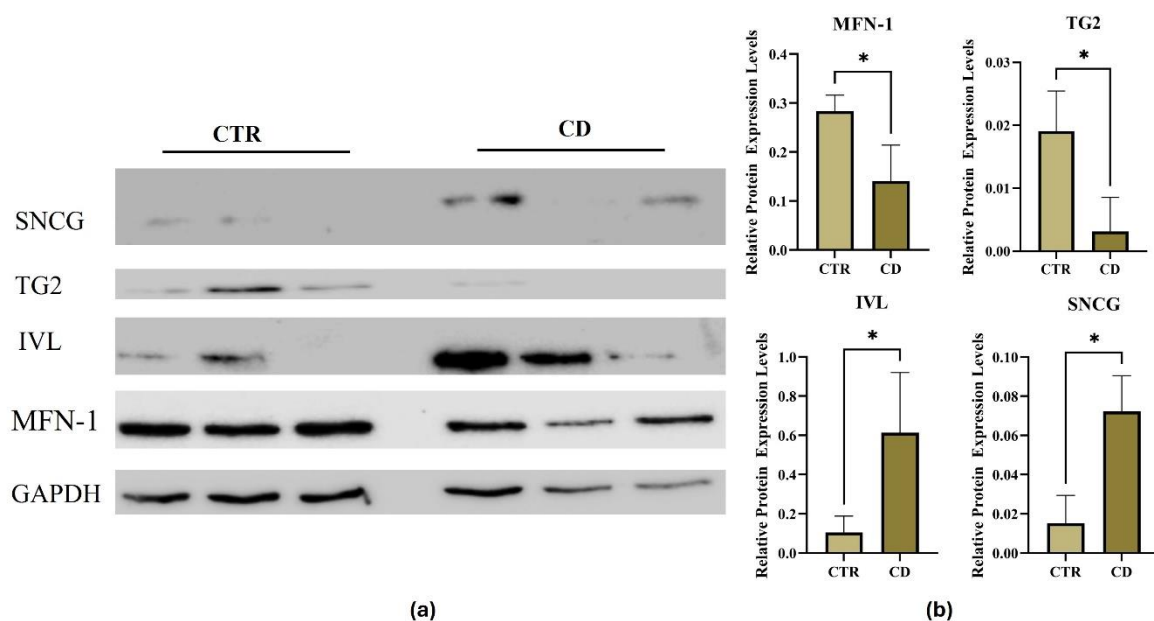


Figure 16. Validation of mass spectrometry analyses. **(a)** Western blot analysis of the validations conducted on three CTR and three CD cultures. **(b)** Densitometric analysis of the validations conducted on three CD and three CTR cultures. 50 μ g for each sample was used. Protein levels are normalized with respect to GAPDH expression. Data are reported as mean $\pm$ SE. * $p < 0.05$ vs. CTR.

Altogether, these data seem to paint a picture in which the differences between CD and CTR were directed toward the cytoskeleton and the link with the extracellular environment, and toward changes in vesiculation phenomena and vesicular trafficking, with many components encompassed by the GO terms relating to the extracellular space/exosomes. This last point is very interesting considering the cellular system under consideration, namely fibroblasts, cells responsible for the deposition of extracellular matrix and thus influencing the microenvironment. Further studies in this direction are certainly necessary to better understand these phenomena.

4.1.4 Phosphoproteome analysis on skin fibroblasts

Previous studies demonstrated that CD subjects exhibited alterations in the phosphorylation level of certain proteins, such as the FAK protein, implicated in the dynamic regulation of the cytoskeleton.⁷⁸ Furthermore, phosphorylation is an important post-translational modification that significantly impacts cellular signalling. Based on this information, analyses of the phosphoproteome of CD and CTR fibroblasts were conducted. Analysis was performed using mass spectrometry after enrichment of phosphoproteins in CTR and CD samples. **Figure 17** shows phosphoproteins enriched samples stained by a fluorescent dye after the separation on SDS-page. We identified 850 proteins, of which **34** were differentially expressed/phosphorylated. Of these, based on selection criteria such as unique peptide >2 and p -value <0.05, **17** are up-, and **17** are down-phosphorylated in CD subjects compared to CTRs. **Supplementary Tables 3-4** list these proteins.

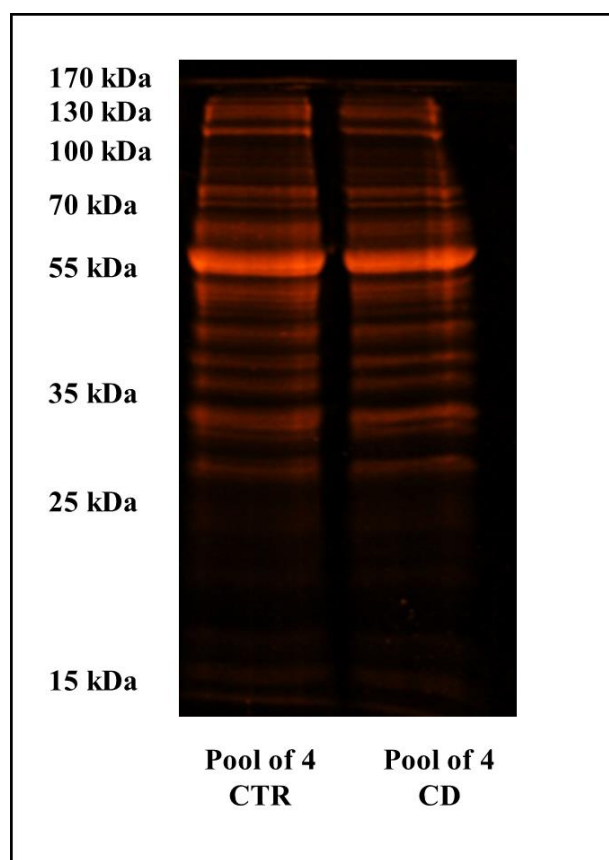


Figure 17. Fluorescence staining phphosphoprotein enrichment in four CD and four CTR cell culture of skin fibroblasts. A total of 30 µg of enriched proteins were used and run on a polyacrylamide gel followed by staining with a fluorescent dye.

Interestingly, one of the down-phosphorylated phosphoproteins identified by mass spectrometry analysis is the **cDNA FLJ58187**, which is highly similar to TG2. An alignment was performed between the amino acid sequence of this transcript and the extracted TG2 sequence using its UniProt accession code **P21980**. Except for a missing segment, the alignment revealed similarities between the two sequences. Furthermore, the three-dimensional structure of this sequence was predicted using Phyre2 software, and the structure appears to be highly correlated with that of TG2. These analyses and predictions were performed accessing to the UniProt database on 08-12-2025. The predictions are shown in **Figure 18**. The reference to cDNA FLJ58187 in databases corresponds to cDNA clones highly similar to transglutaminase 2 (TGM2) and does not represent a distinct or analogous protein, but rather a cDNA clone encoding TGM2 with a highly similar sequence. In addition to its reduced abundance in CD subjects, as revealed by proteomic analyses, TG2 may also exhibit lower phosphorylation levels, potentially affecting its regulation and activity.

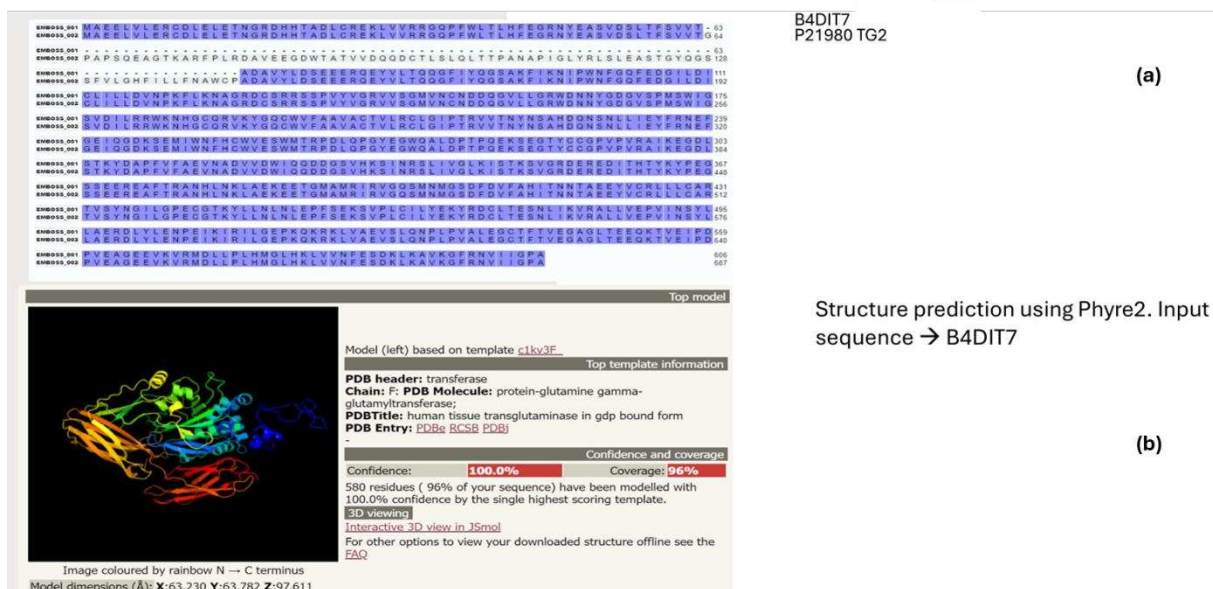


Figure 18. Alignment and structural prediction of the flj58187 transcript identified by mass spectrometry analysis. (a) Sequence alignment between the amino acid sequence derived from the flj58187 cDNA and the sequence of the TG2 protein (UniProt code P21980). (b) Structural prediction of the amino acid sequence using Phyre2 software.

Similarly to the proteome, GO enrichment analysis was carried out for the phosphoproteome. The results are shown in **Figure 19**. A further analysis was performed comparing the proteins identified in the phosphoproteome with those in the proteome. This comparison revealed that **300** proteins were common to both analyses. The next step was to focus attention on proteins with a similar expression level between CD and CTR samples, but with different levels of phosphorylation, considering a p -value <0.05 . This approach allowed us to establish that the differences we found in the phosphorylation level was not due to a different protein expression. **13** proteins met these criteria, of which **5** had a higher level of phosphorylation in CD than in CTR, and **8** had a lower level of phosphorylation. These proteins are shown in **Tables 8-9**.

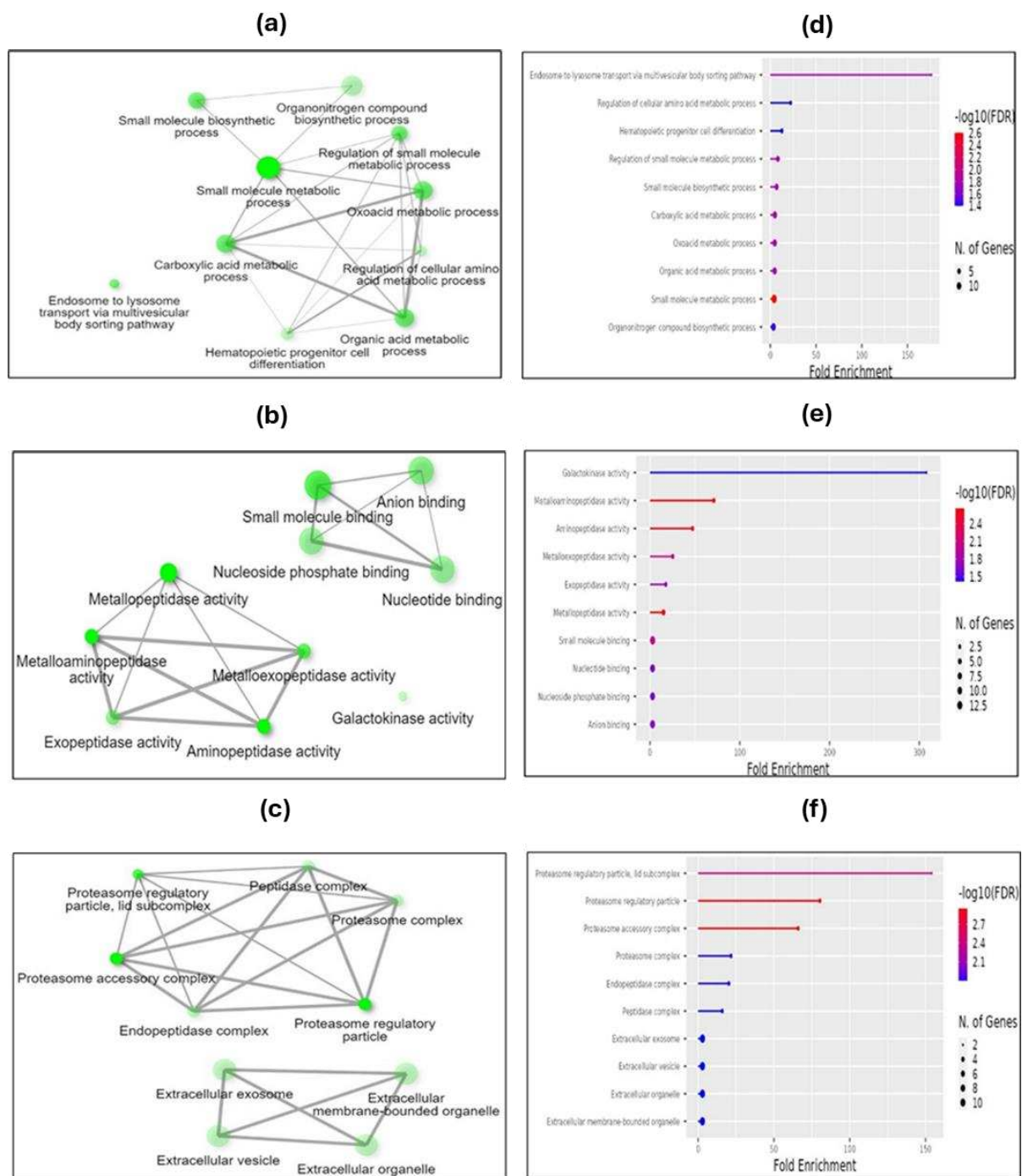


Figure 19. GO enrichment analysis. Enrichment of up- and down-phosphorylated proteins in CD compared to CTR. Clustering and the most representative terms in the protein population of the different GO classes: **(a, d)** biological processes, **(b, e)** molecular functions, **(c, f)** cellular components.

Table 8. List of hyperphosphorylated phosphoproteins in CD skin fibroblasts that have a similar expression in the proteome with respect to the CTR samples.

Accession (UniProt)	Description	Abundance Ratio Phosphoproteome	p-value
Q9H4A4	Aminopeptidase B	2.12	0.02602736
Q99747	Gamma-soluble NSF attachment protein	2.178	0.01885296
P61758	Prefoldin subunit 3	2.315	0.02604454
Q9BZE9	Tether containing UBX domain for GLUT4	2.442	0.01478214
Q99729-3	Isoform 3 of heterogeneous nuclear ribonucleoprotein A/B	2.896	0.00050502

Table 9. List of down-phosphorylated phosphoproteins in CD skin fibroblasts that have a similar expression in the proteome with respect to the CTR samples.

Accession (UniProt)	Description	Abundance Ratio Phosphoproteome	p-value
P48556	26S proteasome non-ATPase regulatory subunit 8	0.08	3.427E-16
P35270	Sepiapterin reductase	0.123	7.5351E-15
O00487	26S proteasome non-ATPase regulatory subunit 14	0.211	0.00019479
Q8WX93	Palladin	0.246	1.6758E-05
O43583	Density-regulated protein	0.26	5.488E-07
P46939	Utrophin	0.371	3.49E-05
O00764	Pyridoxal kinase	0.499	0.00726992
Q96K76	Ubiquitin carboxyl-terminal hydrolase 47	0.504	0.01175294

GO enrichment analysis of the phosphoproteome allowed for gene clustering, thereby enabling the accurate classification and identification of the most representative phosphoproteins differentially abundant in CD compared with CTR samples. These results are reported in **Tables 10-12**, which represent biological process, molecular function, and cellular component, respectively.

Table 10. Phosphoroteome Enrichment analysis. Enrichment of GO terms by biological process.
<http://amigo.geneontology.org/amigo/term>.

Gene Ontology ID	Enrichment FDR	Fold Enrichment	Gene Ontology Term	Number of Genes	Gene Found Associated with the GO term
GO:0043094	1.9E-02	47.1	cellular metabolic compound salvage	3	MTAP PDXK UCK2
GO:0031329	2.0E-02	4.8	reg. of cellular catabolic proc.	9	PSMD10 EIF4A3 HNRNPAB AKT2 PSMD14 GLMN ATP6V1C1 GAPDHS CASP3
GO:0019752	3.0E-02	4.5	carboxylic acid metabolic proc.	9	MTAP GAPDHS ECHS1 LARS1 PSPH GSTP1 BDH2 GALK1 AKT2
GO:0043436	3.2E-02	4.3	oxoacid metabolic proc.	9	MTAP GAPDHS ECHS1 LARS1 PSPH GSTP1 BDH2 GALK1 AKT2
GO:0006082	3.3E-02	4.3	organic acid metabolic proc.	9	MTAP GAPDHS ECHS1 LARS1 PSPH GSTP1 BDH2 GALK1 AKT2
GO:0044281	6.8E-03	3.8	small molecule metabolic proc.	15	MTAP GAPDHS GALK1 SPR ECHS1 LARS1 PSPH PDXK TYMP GSTP1 UCK2 BDH2 AKT2 NLN NADK2
GO:1901566	2.0E-02	3.4	organonitrogen compound biosynthetic proc.	13	MTAP SPR LARS1 DENR PSPH PDXK BDH2 HMBS GSTP1 EIF4A3 UCK2 AKT2 NADK2
GO:1901575	1.9E-02	3.2	organic substance catabolic proc.	15	PSMD8 GAPDHS PSMD14 ECHS1 PSMD10 CHMP3 EIF4A3 BDH2 HNRNPAB TYMP GALK1 CASP3 AKT2 VPS4B GLMN
GO:0044248	2.0E-02	3	cellular catabolic proc.	15	PSMD8 PSMD14 ECHS1 VPS4B PSMD10 CHMP3 EIF4A3 BDH2 HNRNPAB TYMP AKT2 GLMN ATP6V1C1 GAPDHS CASP3
GO:0009056	2.0E-02	2.8	catabolic proc.	16	PSMD8 GAPDHS PSMD14 ECHS1 VPS4B PSMD10 CHMP3 EIF4A3 BDH2 HNRNPAB TYMP GALK1 CASP3 AKT2 GLMN ATP6V1C1

Table 11. Phosphoroteome Enrichment analysis. Enrichment of GO terms by molecular function.
<http://amigo.geneontology.org/amigo/term>.

Gene Ontology ID	Enrichment FDR	Fold Enrichment	Gene Ontology Term	Number of Genes	Gene Found Associated with the GO term
GO:0004645	6.2E-03	139.1	1,4-alpha-oligoglucan phosphorylase activity	2	TYMP MTAP
GO:0070006	5.8E-03	56.2	metalloaminopeptidase activity	3	RNPEP METAP2 NPEPL1
GO:0004177	6.2E-03	38.4	aminopeptidase activity	3	RNPEP METAP2 NPEPL1
GO:0016763	1.3E-02	25.2	pentosyltransferase activity	3	MTAP QTRT1 TYMP
GO:0008235	1.8E-02	21.5	metalloexopeptidase activity	3	RNPEP METAP2 NPEPL1
GO:0008238	4.1E-02	14.6	exopeptidase activity	3	RNPEP METAP2 NPEPL1
GO:0008237	6.2E-03	11.9	metallopeptidase activity	5	PSMD14 NLN RNPEP METAP2 NPEPL1
GO:0036094	3.6E-02	2.5	small molecule binding	14	PLPBP PPP5C AKT2 GALK1 EIF4A3 PDXK BDH2 GAPDHS VPS4B P4HA1 LARS1 UCK2 NADK2 SPR

Table 12. Phosphoroteome Enrichment analysis. Enrichment of GO terms by cellular component. <http://amigo.geneontology.org/amigo/term>.

Gene Ontology ID	Enrichment FDR	Fold Enrichment	Gene Ontology Term	Number of Genes	Gene Found Associated with the GO term
GO:0008541	6.0E-03	121.7	proteasome regulatory particle lid subcomplex	2	PSMD8 PSMD14
GO:0005838	3.1E-03	63.5	proteasome regulatory particle	3	PSMD8 PSMD14 PSMD10
GO:0022624	3.1E-03	52.2	proteasome accessory complex	3	PSMD8 PSMD14 PSMD10
GO:0000502	2.1E-02	17.4	proteasome complex	3	PSMD8 PSMD14 PSMD10
GO:1905369	3.6E-02	13.8	endopeptidase complex	3	PSMD8 PSMD14 PSMD10
GO:0070062	6.0E-03	2.9	extracellular exosome	14	SLC9A3R2 GSTP1 MTAP GALK1 CHMP3 SPR VPS4B NAPG UTRN ATP6V1C1 PDXK BDH2 RNPEP GRB2
GO:1903561	6.0E-03	2.9	extracellular vesicle	14	SLC9A3R2 GSTP1 MTAP GALK1 CHMP3 SPR VPS4B NAPG UTRN ATP6V1C1 PDXK BDH2 RNPEP GRB2
GO:0043230	6.0E-03	2.9	extracellular organelle	14	SLC9A3R2 GSTP1 MTAP GALK1 CHMP3 SPR VPS4B NAPG UTRN ATP6V1C1 PDXK BDH2 RNPEP GRB2
GO:0065010	6.0E-03	2.9	extracellular membrane-bounded organelle	14	SLC9A3R2 GSTP1 MTAP GALK1 CHMP3 SPR VPS4B NAPG UTRN ATP6V1C1 PDXK BDH2 RNPEP GRB2

4.2 Intestinal Fibroblasts

4.2.1 Preliminary characterization

To study the celiac cellular phenotype, a second cellular system was employed: fibroblasts obtained from biopsies of celiac patients with intestinal atrophy and Marsh IIIc severity, the most severe classification on the Marsh scale, and CTR fibroblasts. This second system, therefore, allows us to study the phenotype of different cells, in terms of location and pathological situation, compared to the skin cells previously analyzed. We also analyzed possible protein similarities between these two types of systems to identify common characteristics of CD cells, independent of the site of origin and the state of the disease. Proteins that will be common to the two systems could also be useful for application in diagnostic or monitoring strategies.

Given the interesting findings on defective autophagy in CD skin-derived fibroblasts, we preliminarily investigated basal levels of autophagic markers in intestinal fibroblasts. The results of Western blots of LC3 and p62 are shown in **Figure 20**. The results showed that, even

in this cellular system, the autophagic flux seemed to be delayed in CD subjects: indeed, the LC3-II/LC3-I ratio and p62 levels were higher in CD cells than in CTRs.

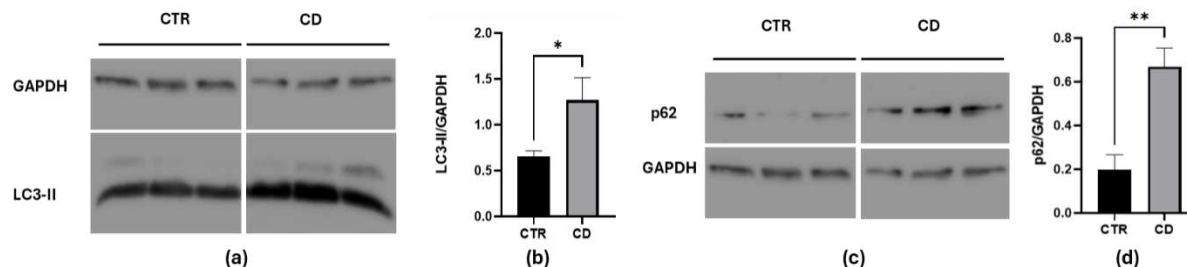


Figure 20. LC3 and p62 protein expression in intestinal-derived fibroblasts. **(a)** Representative Western blot anti-LC3 performed on fibroblasts from three CTR samples and three CD samples. **(b)** Densitometric analysis of blots relative to four CTR samples and four CD samples. Mean values and standard errors are reported **(c)** Representative Western blot anti-p62 performed on fibroblasts from three CTR samples and three CD samples. **(d)** Densitometric analysis of blots relative to four CTR samples and four CD samples. Mean values and standard errors are reported. In all the analyses, 50 µg for each sample was used. GAPDH was used as the internal reference. *p-value< 0.05, **p-value< 0.01 versus CTR samples.

4.2.2 Proteome analysis on intestinal fibroblasts

The next step was to begin an exploratory analysis on this new cell system using the same approach used for the skin system (using 4 CD and 4 CTR samples), evidencing **4,072** proteins. Of these, using an abundance ratio value >1.8 and <0.5 for up- and down-expressed proteins, respectively, and a *p*-value <0.05, **117** proteins were found to be more expressed in CD subjects, and **160** were found to be less expressed in CD subjects compared to CTR samples (**Supplementary Tables 5-6**). As for the skin system, a GO enrichment analysis was then performed on these proteins. ShinyGO software was used, with $FDR \leq 0.05$. **Figure 21** shows the results. The analysis was performed for all three classes of ontologies: Biological Processes (**Figures 21a, d**), Molecular Functions (**Figures 21b, e**), and Cellular Components (**Figures 21c, f**). The results showed a clustering tending towards phenomena including cell adhesion, cell and subcellular component movement, and, to a greater extent, vesiculation towards the extracellular space.

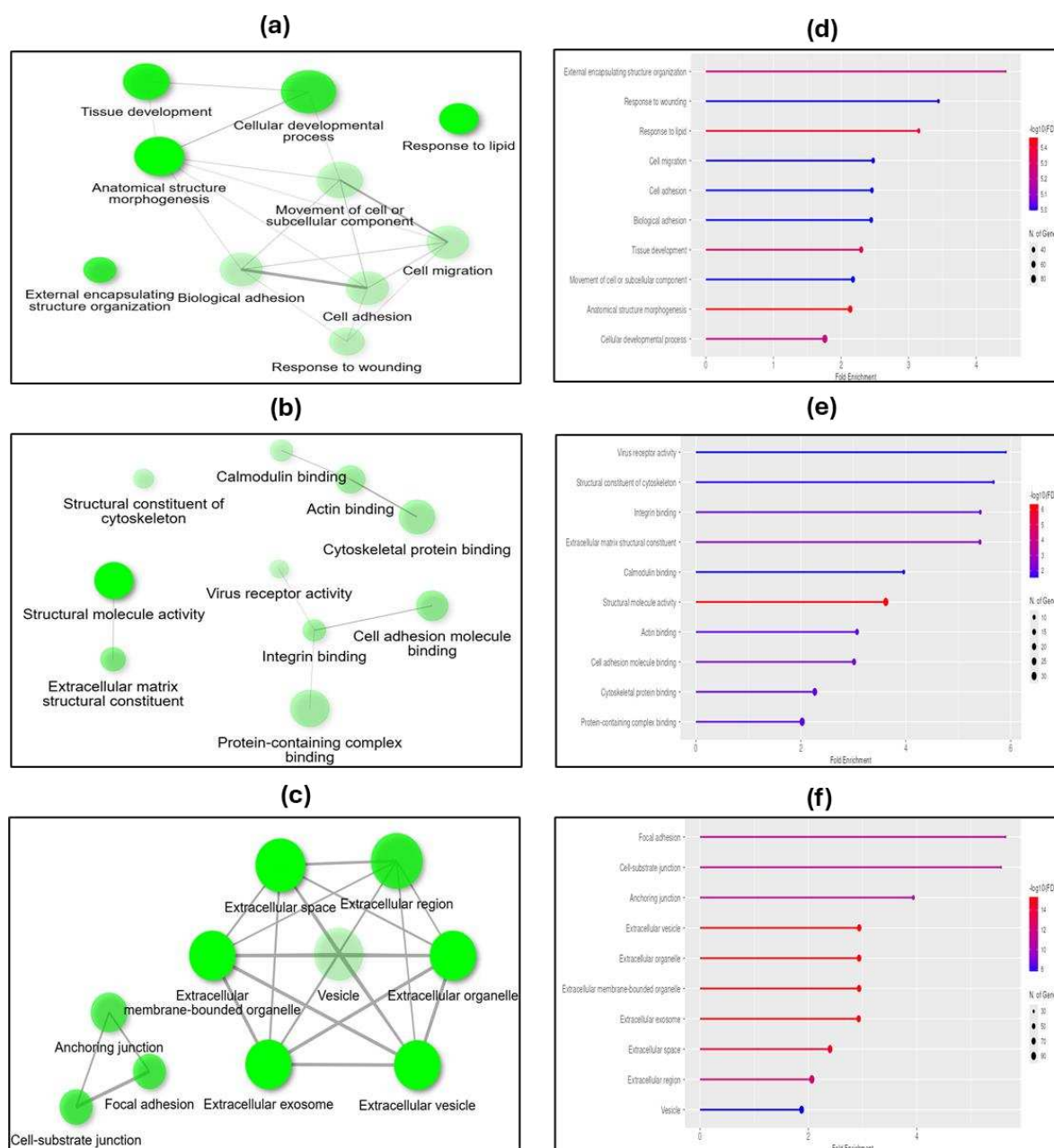


Figure 21. GO enrichment analysis. Enrichment of up- and down-expressed proteins in CD compared to CTR samples. Clustering and the most representative terms in the protein population of the different GO classes: **(a, d)** biological processes, **(b, e)** molecular functions, **(c, f)** cellular components.

Analysis of the individual genes associated with each GO term that best represented our list of proteins also allowed us to define a picture in which fibroblasts obtained from CD subjects appeared to present alterations that primarily affected cellular movement, related to focal adhesions and, more generally, to cytoskeletal components, and finally to vesicular trafficking,

particularly outward, with a greater focus on exosomes and the extracellular space. Furthermore, this was consistent with what was observed in the skin system. The connection appeared significant because, in addition to find similar alterations in a different body region, our observations on skin system regarded cells from subjects on a gluten-free diet, in which the histological, serological, and physiological alterations of CD should be absent. These findings further support the existence of a signature characteristic of the celiac phenotype. Data reported in **Tables 13-15** concern biological process, molecular function, and cellular component, respectively.

Table 13. Intestinal fibroblasts proteome Enrichment analysis. Enrichment of GO terms by biological processes.
<http://amigo.geneontology.org/amigo/term>.

Gene Ontology ID	Enrichment FDR	Fold Enrichment	Gene Ontology Term	Number of Genes	Gene Found Associated with the GO term
GO:0045229	5.6E-06	4.4	External encapsulating structure organization	22	TNC LAMA3 FBLN1 ITGA8 FAP ICAM1 CAV2 SERPINE1 POSTN ITGA11 RB1 HAPLN1 FBLN2 ITGA2 COL1A2 HTRA1 COL3A1 SH3PXD2B BGN HRNR ITGB3 GREM1
GO:0009611	1.0E-05	3.4	Response to wounding	27	TFPI CD9 TNC MYLK FBLN1 FAP NRPI PROCR SERPINE1 WNT5B GNAI1 MAP1B POSTN CHL1 CD36 LACRT DYSF SLC7A11 CLDN1 ITGA2 COL1A2 AXL COL3A1 APOD HBB ITGB3 PRKAR2B
GO:0033993	4.0E-06	3.2	Response to lipid	35	TFPI MGST1 TNC HSD17B2 ICAM1 GGT5 SFRP1 UBR5 SERPINE1 CRYAB CCND1 WNT5B CNOT2 CD274 GNAI1 ALDH1A2 ASS1 AKAP12 MAP1B POSTN CMPK2 CD36 LACRT CASP1 ACTA1 PSPH PID1 CALM3 CLDN1 ITGA2 AXL YES1 RORB CES1 PCK2
GO:0016477	1.0E-05	2.5	Cell migration	43	CD9 LAMA3 HDAC7 MYLK FBLN1 FAP TNS1 ICAM1 NRPI SLC7A5 RAB11A SFRP1 PRKD2 SERPINE1 PDLIM1 WNT5B RND3 CD274 BST2 AKAP12 MAP1B POSTN ARPC5L ITGA11 CDH13 SLC7A11 THY1 CYGB CLDN1 ITGA2 COL1A2 CTHRC1 AXL SLC3A2 COL3A1 PDILT CDH2 CSPG4 YES1 APOD L1CAM ITGB3 GREM1
GO:0007155	1.0E-05	2.5	Cell adhesion	44	CD9 TNC LAMA3 FBLN1 ITGA8 FAP ICAM1 NRPI SFRP1 PRKD2 SERPINE1 PDLIM1 CNTNAP1 KRT18 RND3 CD274 ADGRE5 ASS1 MYH10 POSTN CHL1 CD36 ITGA11 CDH13 HAPLN1 SLC7A11 THY1 CLDN1 FBLN2 ITGA2 AXL COL3A1 CDH2 YES1 CADM1 SPRY4 APOD PCDH18 L1CAM TGM2 HBB PCDHGA6 ITGB3 ZYX
GO:0022610	1.0E-05	2.4	Biological adhesion	44	CD9 TNC LAMA3 FBLN1 ITGA8 FAP ICAM1 NRPI SFRP1 PRKD2 SERPINE1 PDLIM1 CNTNAP1 KRT18 RND3 CD274 ADGRE5 ASS1 MYH10 POSTN CHL1 CD36 ITGA11 CDH13 HAPLN1 SLC7A11 THY1 CLDN1 FBLN2 ITGA2 AXL COL3A1 CDH2 YES1 CADM1 SPRY4 APOD PCDH18 L1CAM TGM2 HBB PCDHGA6 ITGB3 ZYX
GO:0009888	4.6E-06	2.3	Tissue development	55	TNC LAMA3 MYLK PSME4 MPP5 ITGA8 ICAM1 NRPI SFRP1 PRKD2 CAV2 SERPINE1 CCND1 KRT18 WNT5B CENPF KRT17 ALDH1A2 POSTN KRT85 KRT7 RB1 RCN3 ACTA1 SLC7A11 CLDN1 ITGA2 FABP5 COL1A2 CTHRC1 CFL2 PTER KRT86 KRT4 CDH2 KRT15 KRT13 USP19 SH3PXD2B CALML5 MYLPF BGN TMEM119 IQGAP3 KRT6B RNF220 HRNR TGM2 PSMB10 ITGB3 CES1 KRT31 KRT33B GREM1 PCK2
GO:0006928	1.0E-05	2.2	Movement of cell or subcellular component	54	CD9 LAMA3 HDAC7 MYLK FBLN1 FAP TNS1 ICAM1 NRPI SLC7A5 RAB11A SFRP1 PRKD2 SERPINE1 PDLIM1 WNT5B RND3 STMN1 CD274 MYH2 BST2 AKAP12 MAP1B MYH10 POSTN CHL1 ARPC5L ITGA11 CDH13 ACTA1 SLC7A11 THY1 UCHL1 CYGB CLDN1 ITGA2 COL1A2 CTHRC1 CFL2 AXL SLC3A2 COL3A1 ACTBL2 PDILT CDH2 CSPG4 YES1 APOD BLOC1S2 HTT L1CAM ITGB3 MYH4 GREM1
GO:0009653	3.4E-06	2.1	Anatomical structure morphogenesis	67	HSPB6 CD9 TNC DKK3 LAMA3 HDAC7 MYLK PSME4 MPP5 TPD52 FBLN1 ITGA8 FAP EPB41L3 ICAM1 NRPI RAB11A SFRP1 PRKD2 SERPINE1 LIPA CNTNAP1 UGDH CRYAB KRT18 WNT5B CNOT2 RND3 STMN1 KRT17 ALDH1A2 MAP1B NES MYH10 POSTN CHL1 CD36 IGF2BP3 RB1 CDH13 ACTA1 PID1 THY1 UCHL1 DIP2A NAA15 ITGA2 COL1A2 CTHRC1 CFL2 HTRA1 ANPEP AXL ACTBL2 SIN3A

					CDH2 CSPG4 TMEM119 SPRY4 TRPV2 APOD L1CAM TGM2 RORB PSMB10 ITGB3 GREM1
GO:0048 869	5.6E-06	1.8	Cellular development al process	91	TMEM176A MGST1 CD9 TNC LAMA3 HDAC7 PSME4 MPP5 TPD52 FBLN1 ITGA8 EPB41L3 ICAM1 NRP1 RAB11A SFRP1 CAV2 SERPINE1 CNTNAP1 UGDH CCND1 KRT18 WNT5B CNOT2 CTH STMN1 CENPF PRXL2A EEF1E1 HSPA2 KRT17 ALDH1A2 SLC6A6 MAP1B NES POSTN CHL1 HOOK1 CD36 KRT85 KRT7 DYSF ITGA11 RB1 ACTA1 SLC7A11 PID1 THY1 UCHL1 M1AP DIP2A CLDN1 NAA15 ITGA2 CTHRC1 CFL2 PTER HTRA1 ANPEP AXL COL3A1 ACTBL2 PDILT SIN3A KRT86 KRT4 CDH2 KRT15 KRT13 CSPG4 SH3PXD2B YES1 CADM1 TMEM119 KRT6B SPRY4 TRPV2 APOD BLOC1S2 TXNRD3 HRNR L1CAM TGM2 RORB PSMB10 ITGB3 CES1 KRT31 KRT33B GREM1 PCK2

Table 14. Intestinal fibroblasts proteome Enrichment analysis. Enrichment of GO terms by molecular functions.

<http://amigo.geneontology.org/amigo/term>.

Gene Ontology ID	Enrichment FDR	Fold Enrichment	Gene Ontology Term	Number of Genes	Gene Found Associated with the GO term
GO:00016 18	2.8E-02	5.9	Virus receptor activity	6	ICAM1 CLDN1 ITGA2 ANPEP AXL ITGB3
GO:00052 00	2.2E-02	5.7	Structural constituent of cytoskeleton	7	EPB41L3 ACTA1 ACTBL2 KRT15 TUBAL3 KRT6B KRT31
GO:00051 78	8.2E-03	5.4	Integrin binding	9	CD9 LAMA3 FBLN1 FAP ICAM1 THY1 ITGA2 COL3A1 ITGB3
GO:00052 01	2.4E-03	5.4	Extracellular matrix structural constituent	11	TNC LAMA3 FBLN1 PCOLCE POSTN HAPLN1 FBLN2 COL1A2 CTHRC1 COL3A1 BGN
GO:00055 16	2.8E-02	4.0	Calmodulin binding	9	MYLK MYH1 CTH MYH2 CNN1 AKAP12 MYH10 IQGAP3 MYH4
GO:00051 98	4.6E-07	3.6	Structural molecule activity	31	HSPB6 TNC LAMA3 FBLN1 EPB41L3 NUP188 PCOLCE CRYAB KRT18 MRPL18 KRT17 MAP1B POSTN KRT85 ACTA1 HAPLN1 CLDN1 FBLN2 COL1A2 CTHRC1 COL3A1 ACTBL2 KRT15 KRT13 TUBAL3 MYLPF BGN KRT6B MPZL1 KRT31 KRT33B
GO:00037 79	1.1E-02	3.1	Actin binding	16	MYLK TNS1 EPB41L3 PDLIM1 MYH1 MYH2 CNN1 MAP1B MYH10 HOOK1 ARPC5L ITPRID2 CFL2 IQGAP3 AFAP1 MYH4
GO:00508 39	5.1E-03	3.0	Cell adhesion molecule binding	19	CD9 GPRC5A LAMA3 FBLN1 FAP CHMP2B ICAM1 PDLIM1 KRT18 POSTN ITGA11 CDH13 THY1 ITGA2 SLC3A2 COL3A1 CDH2 PPME1 ITGB3
GO:00080 92	1.1E-02	2.3	Cytoskeletal protein binding	26	MYLK TNS1 EPB41L3 RAB11A PDLIM1 MYH1 CRYAB STMN1 CENPF MYH2 HSPA2 CNN1 MAP1B MYH10 HOOK1 ARPC5L ITPRID2 ACTA1 CALM3 CFL2 AXL IQGAP3 BLOC1S2 AFAP1 HTT MYH4
GO:00448 77	1.2E-02	2.0	Protein- containing complex binding	32	CD9 LAMA3 PSME4 FBLN1 FAP ICAM1 PCOLCE MYH1 CRYAB CCND1 CENPF MYH2 GNAI1 APOL2 MAP1B NES MYH10 CD36 LACRT ARPC5L ITGA11 ITPRID2 CDH13 THY1 NAA15 ITGA2 CFL2 COL3A1 SIN3A IQGAP3 ITGB3 MYH4

Table 15. Intestinal fibroblasts proteome Enrichment analysis. Enrichment of GO terms by cellular components.

<http://amigo.geneontology.org/amigo/term>.

Gene Ontology ID	Enrichment FDR	Fold Enrichment	Gene Ontology Term	Number of Genes	Gene Found Associated with the GO term
GO:0005925	4.3E-12	5.6	Focal adhesion	29	CD9 TNC ITGA8 FAP TNS1 ICAM1 NRP1 PROCR CAV2 PDLIM1 RND3 ADGRE5 CNN1 AKAP12 ARPC5L ITGA11 CDH13 THY1 ITGA2 CDH2 CSPG4 YES1 SPRY4 AFAP1 MPZL1 L1CAM TGM2 ITGB3 ZYX
GO:0030055	5.4E-12	5.6	Cell-substrate junction	29	CD9 TNC ITGA8 FAP TNS1 ICAM1 NRP1 PROCR CAV2 PDLIM1 RND3 ADGRE5 CNN1 AKAP12 ARPC5L ITGA11 CDH13 THY1 ITGA2 CDH2 CSPG4 YES1 SPRY4 AFAP1 MPZL1 L1CAM TGM2 ITGB3 ZYX
GO:0070161	1.3E-11	3.9	Anchoring junction	39	CD9 TNC MPP5 ITGA8 FAP TNS1 EPB41L3 ICAM1 NRP1 PROCR CAV2 PDLIM1 CNTNAP1 MYH1 CCND1 KRT18 RND3 ADGRE5 MYH2 CNN1 AKAP12 ARPC5L ITGA11 CDH13 THY1 CLDN1 ITGA2 CDH2 CSPG4 YES1 CADM1 IQGAP3 SPRY4 AFAP1 MPZL1 L1CAM TGM2 ITGB3 ZYX
GO:1903561	4.8E-16	2.9	Extracellular vesicle	75	CD9 GPRC5A LAMA3 MPP5 FBLN1 CHMP2B ICAM1 PTGS1 SUSD2 PROCR SLC7A5 RAB11A SFRP1 PCOLCE SERPINE1 UGDH CRYAB KRT18 WNT5B CTH STMN1 CD274 ACAT2 ADGRE5 CEP250 HSPA2 GNAI1 BST2 ASS1 MYH10 CHL1 PSAT1 KRT7 DYSF ALDH1L2 ARPC5L CDH13 DOP1B ACTA1 GALM PLBD2 THY1 PIGR FBLN2 FABP5 COL1A2 CFL2 PTER HTRA1 CKB ANPEP AXL SLC3A2 ACTBL2 SMR3B KRT15 KRT13 CSPG4 YES1 DPP7 SGSH BGN C11orf54 KRT6B APOD POTEF ARHGEF12 HRNR TGM2 HBB ITGB3 KRT31 KRT33B NAPRT PRKAR2B
GO:0043230	4.8E-16	2.9	Extracellular organelle	75	CD9 GPRC5A LAMA3 MPP5 FBLN1 CHMP2B ICAM1 PTGS1 SUSD2 PROCR SLC7A5 RAB11A SFRP1 PCOLCE SERPINE1 UGDH CRYAB KRT18 WNT5B CTH STMN1 CD274 ACAT2 ADGRE5 CEP250 HSPA2 GNAI1 BST2 ASS1 MYH10 CHL1 PSAT1 KRT7 DYSF ALDH1L2 ARPC5L CDH13 DOP1B ACTA1 GALM PLBD2 THY1 PIGR FBLN2 FABP5 COL1A2 CFL2 PTER HTRA1 CKB ANPEP AXL SLC3A2 ACTBL2 SMR3B KRT15 KRT13 CSPG4 YES1 DPP7 SGSH BGN C11orf54 KRT6B APOD POTEF ARHGEF12 HRNR TGM2 HBB ITGB3 KRT31 KRT33B NAPRT PRKAR2B
GO:0065010	4.8E-16	2.9	Extracellular membrane-bounded organelle	75	CD9 GPRC5A LAMA3 MPP5 FBLN1 CHMP2B ICAM1 PTGS1 SUSD2 PROCR SLC7A5 RAB11A SFRP1 PCOLCE SERPINE1 UGDH CRYAB KRT18 WNT5B CTH STMN1 CD274 ACAT2 ADGRE5 CEP250 HSPA2 GNAI1 BST2 ASS1 MYH10 CHL1 PSAT1 KRT7 DYSF ALDH1L2 ARPC5L CDH13 DOP1B ACTA1 GALM PLBD2 THY1 PIGR FBLN2 FABP5 COL1A2 CFL2 PTER HTRA1 CKB ANPEP AXL SLC3A2 ACTBL2 SMR3B KRT15 KRT13 CSPG4 YES1 DPP7 SGSH BGN C11orf54 KRT6B APOD POTEF ARHGEF12 HRNR TGM2 HBB ITGB3 KRT31 KRT33B NAPRT PRKAR2B
GO:0070062	7.7E-16	2.9	Extracellular exosome	74	CD9 GPRC5A LAMA3 MPP5 FBLN1 CHMP2B ICAM1 PTGS1 SUSD2 PROCR SLC7A5 RAB11A SFRP1 PCOLCE SERPINE1 UGDH CRYAB KRT18 WNT5B CTH STMN1 CD274 ACAT2 ADGRE5 CEP250 HSPA2 GNAI1 BST2 ASS1 MYH10 CHL1 PSAT1 KRT7 DYSF ALDH1L2 ARPC5L CDH13 DOP1B ACTA1 GALM PLBD2 THY1 PIGR FABP5 COL1A2 CFL2 PTER HTRA1 CKB ANPEP AXL SLC3A2 ACTBL2 SMR3B KRT15 KRT13 CSPG4 YES1 DPP7 SGSH BGN C11orf54 KRT6B APOD POTEF ARHGEF12 HRNR TGM2 HBB ITGB3 KRT31 KRT33B NAPRT PRKAR2B
GO:0005615	3.6E-15	2.4	Extracellular space	93	TFPI NDUFAF7 CD9 GPRC5A TNC AIFM2 DKK3 LAMA3 MPP5 FBLN1 FAP CHMP2B ICAM1 PTGS1 NRP1 SUSD2 PROCR SLC7A5 RAB11A SFRP1 CKM PCOLCE SERPINE1 UGDH CRYAB KRT18 WNT5B MRPL18 CTH STMN1 CD274 ACAT2 ADGRE5 CEP250 HSPA2 GNAI1 BST2 ASS1 MYH10 POSTN CHL1 PSAT1 CD36 LACRT KRT85 KRT7 DYSF ALDH1L2 ARPC5L CDH13 DOP1B ACTA1 GALM PLBD2 THY1 PIGR FABP5 COL1A2 CTHRC1 CFL2 PTER HTRA1 CKB ANPEP AXL SLC3A2 COL3A1 ACTBL2 KRT86 SMR3B KRT15 KRT13 CSPG4 YES1 DPP7 SGSH BGN C11orf54

					KRT6B APOD POTEF ARHGEF12 HRNR TGM2 SOGA3 HBB ITGB3 CES1 KRT31 KRT33B GREM1 NAPRT PRKAR2B
GO:0005 576	3.8E-13	2.1	Extracellular region	105	TFPI NDUFAF7 HSPB6 CD9 GPRC5A TNC AIFM2 DKK3 LAMA3 MPP5 FBLN1 FAP CHMP2B ICAM1 PTGS1 NRP1 SUSD2 PROCR SLC7A5 RAB11A SFRP1 CKM PCOLCE SERPINE1 UGDH CRYAB KRT18 WNT5B MRPL18 CTH STMN1 CD274 ACAT2 PRXL2A ADGRE5 CEP250 HSPA2 GNAI1 APOL2 BST2 ASS1 MYH10 POSTN CHL1 GSTM1 GSTM5 PSAT1 CD36 LACRT KRT85 KRT7 DYSF ALDH1L2 ARPC5L CASP1 CDH13 DOP1B ACTA1 GALM NXPE3 HAPLN1 PLBD2 THY1 PIGR FBLN2 FABP5 COL1A2 CTHRC1 CFL2 PTER HTRA1 CKB ANPEP AXL SLC3A2 COL3A1 ACTBL2 KRT86 SMR3B KRT15 KRT13 CSPG4 YES1 DPP7 CALML5 SGSH BGN C11orf54 METTL7A KRT6B APOD POTEF ARHGEF12 CASP4 HRNR TGM2 SOGA3 HBB ITGB3 CES1 KRT31 KRT33B GREM1 NAPRT PRKAR2B
GO:0031 982	1.5E-08	1.9	Vesicle	90	MGST1 CD9 GPRC5A LAMA3 MPP5 FBLN1 CHMP2B ICAM1 PTGS1 NRP1 SUSD2 PROCR SLC7A5 RAB11A SFRP1 CAV2 PCOLCE SERPINE1 UGDH CRYAB KRT18 WNT5B RND3 CTH STMN1 CD274 ACAT2 ADGRE5 CEP250 HSPA2 GNAI1 BST2 ASS1 MYH10 CHL1 PSAT1 CD36 LACRT KRT7 DYSF ALDH1L2 STX17 ARPC5L CDH13 DOP1B ACTA1 GALM PLBD2 THY1 CALM3 PIGR FBLN2 FABP5 COL1A2 CFL2 PTER HTRA1 CKB ANPEP AXL SLC3A2 ACTBL2 SMR3B KRT15 KRT13 CSPG4 YES1 DPP7 CALML5 CHRM2 SGSH BGN C11orf54 METTL7A KRT6B TRPV2 APOD POTEF ARHGEF12 HTT HRNR TGM2 GBP7 HBB ITGB3 KRT31 KRT33B NAPRT PRKAR2B STING1

To confirm the mass spectrometry data, validations were performed using Western blot. Three down-expressed proteins, CD44, CD9, and TG2, were selected. The selection was based on two factors: the first related to CD44 and CD9, which are two important proteins for monitoring external vesiculation/exosome phenomena. TG2, on the other hand, is a key enzyme in the pathogenesis of CD, and it was also found to be down-expressed in the skin proteome; in addition, it is likely down-phosphorylated, as demonstrated in the phosphoproteome. Furthermore, the protein ICAM-1, associated with adhesion phenomena, was validated and found to be upregulated in CD.¹⁰⁴ Its levels can increase following inflammation, a condition present in CD. The results are shown in **Figure 22**. In summary, the data appeared to confirm what was recorded by the mass spectrometry analysis, although there was some inter-sample variability. Further validations of this data are planned.

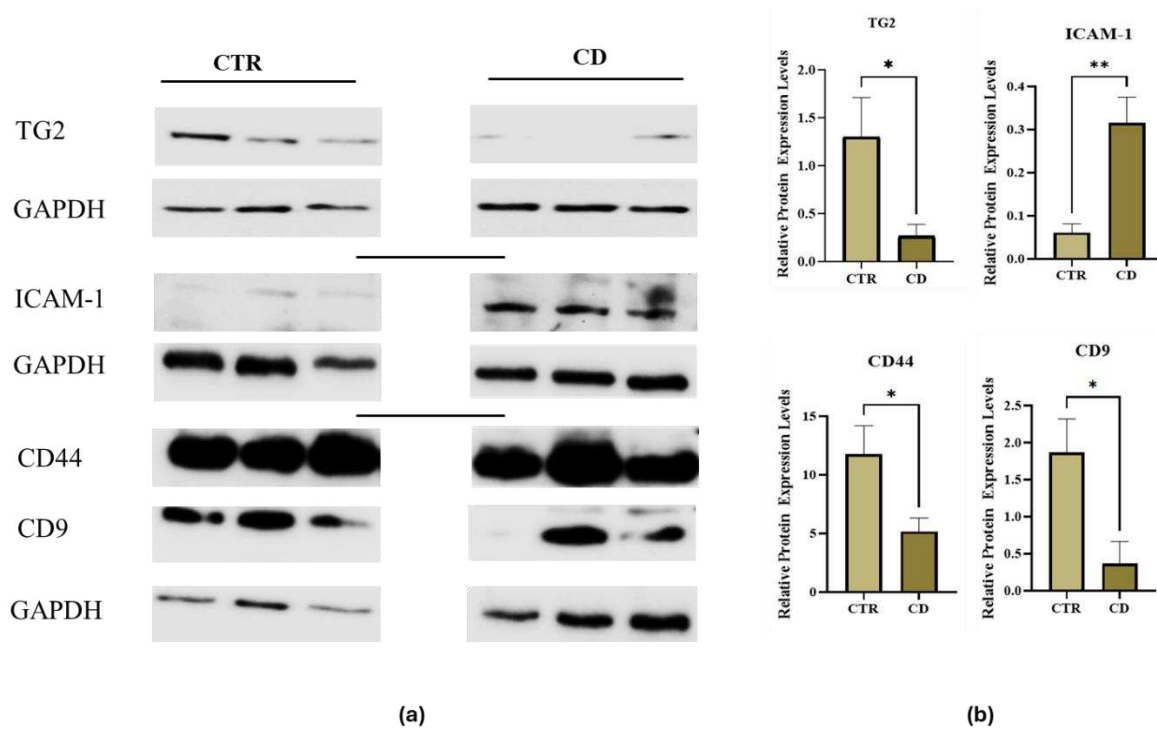


Figure 22. Validation of mass spectrometry analyses. (a) Western blot analysis was conducted on three CTR and three CD cultures. (b) Densitometric analysis of blots conducted on five CD and four CTR cultures. 50 μ g for each sample was used. Protein levels are normalized with respect to GAPDH expression. Data are reported as mean $\pm$ SE. * $p < 0.05$, ** $p < 0.01$ vs. CTR.

Finally, as previously mentioned, we were interested in highlighting the presence of up- and down-regulated proteins in both systems we studied (skin and intestinal fibroblasts), also to open the way to exploring the existence of new potential diagnostic markers. By combining the datasets obtained for proteins with higher expression in CDs compared to CTRs of the two systems and, similarly, the datasets obtained for proteins with lower expression in CDs compared to CTRs of the two systems, the following matches were obtained, shown in **Tables 16-17**, respectively.

Table 16. List of proteins overexpressed in CD subjects compared to CTRs present in both cell systems.

Accession	Description	Gene Symbol	System	Coverage [%]	Abundance Ratio: (Celiac) / (Ctrl)	Abundance Ratio Adj. P-Value: (Celiac) / (Ctrl)
E9PEP6	Neuropilin	NRP1	Dermal	15	1.905	0.042610099
			Intestinal	13	2.64	0.000541645
P02511	Alpha-crystallin B chain	CRYAB	Dermal	54	2.01	0.018901833
			Intestinal	60	3.799	2.49203E-11
P10620	Microsomal glutathione S-transferase 1	MGST1	Dermal	26	4.326	5.52354E-05
			Intestinal	8	100	5.58081E-16
P67936-2	Isoform 2 of Tropomyosin alpha-4 chain	TPM4	Dermal	68	3.624	1.18795E-06
			Intestinal	74	10.149	5.58081E-16
Q96CG8	Collagen triple helix repeat-containing protein 1	CTHRC1	Dermal	33	2.388	0.00103911
			Intestinal	33	2.426	0.000252735

Table 17. List of proteins down-expressed in CD subjects compared to CTRs present in both cell systems.

Accession	Description	Gene Symbol	System	Coverage [%]	Abundance Ratio: (Celiac) / (Ctrl)	Abundance Ratio Adj. P-Value: (Celiac) / (Ctrl)
P21980	Protein-glutamine gamma-glutamyltransferase 2	TGM2	Dermal	19	0.204	2.94534E-10
			Intestinal	50	0.234	4.71465E-14
P30530	Tyrosine-protein kinase receptor UFO	AXL	Dermal	1	0.01	5.14579E-16
			Intestinal	3	0.423	0.013453624

Skin fibroblasts (gluten-free diet) and intestine (acute disease) shared alterations in TG2 and proteins related to cellular stress and ECM remodeling. This confirmed that some characteristics of celiac fibroblasts were constitutive, not merely secondary to active inflammation. TG2, along with proteins such as Alpha-crystallin β chain, Microsomal glutathione S-transferase 1, and Collagen triple helix repeat-containing protein 1, may contribute to a pro-inflammatory microenvironment predisposed to immune response: more vulnerable to stress, more efficient at activating in the presence of immunogenic gluten peptides, and with impaired tissue remodeling. These proteins could represent biomarkers of intrinsic fibroblast susceptibility to stress agents and potential therapeutic targets for modulating cellular stress.

4.2.3 Metabolome analysis of intestinal fibroblasts

The proteomic results obtained so far described a pattern in which CD patients appeared to present alterations in vesiculation and the extracellular environment. Consistent with this observation, cell culture supernatants were analyzed to identify potential differences in the metabolome of these cells compared to CTR ones. Additionally, we also analyzed the intracellular metabolome to increase the magnitude of our analysis. This approach could be interesting as it enables a comprehensive exploratory analysis to identify potential biomarkers for a given condition.^{105,106} Furthermore, given the alteration of outward vesiculation, it is reasonable to hypothesize differences in the metabolome of these patients. An untargeted approach was employed, which is ideal for screening purposes, as it does not direct the research towards a specific target. Furthermore, this approach enables the analysis of as many metabolites as possible, thereby representing the optimal method for analyzing two sets of samples, such as CTR and CD ones, to define their metabolic perturbations. **Figure 23** shows the Partial Least-Squares Discriminant Analysis (hereafter called PLS-DA). This is a multivariate analysis capable of defining global patterns and maximizing class separation. In addition to physically dividing the different classes according to principal components, the model must be tested to determine whether it has been overfit (due to the limited sample size) and whether it is statistically robust and not due to chance. From the data shown, the graphical model indicated a clear separation between classes, especially based on the first component (77.5%). It is evident that the model did not appear to be overfitted, as indicated by the R² and Q² values obtained; however, it did not appear to be completely statistically robust (p -value = 0.1195). This value indicated that the separation was not statistically significant. This suggested a possible overestimation of the model performance due to the small sample size. Therefore, the results should be interpreted with caution and considered indicative of a trend rather than a robust distinction.

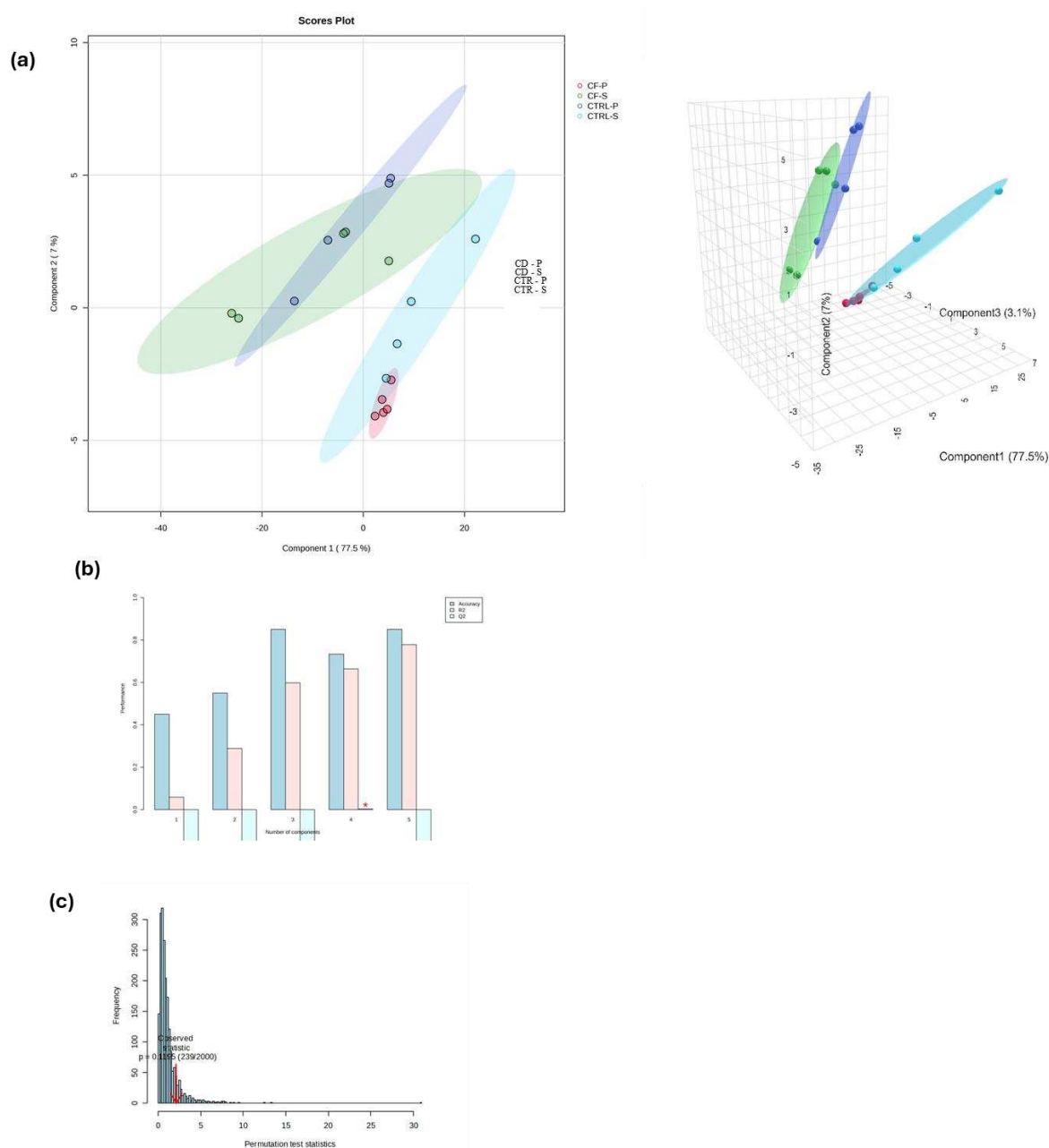


Figure 23. PLS-DA model of metabolomic analysis in intestinal fibroblasts. **(a)** Graphic model of PLS-DA that shows the classes divided by principal component. **(b)** R^2 and Q^2 values for model robustness. **(c)** Permutation test for the statistical validity of the model.

Subsequently, the VIP score was derived from the PLS-DA. This index is useful to measure how much each metabolite contributes to class separation. Generally, VIP values > 1.0 indicate metabolites relevant to the model under consideration. **Figure 24** shows the table containing the metabolites with VIP scores greater than 1.2; furthermore, the heatmap is also shown, which

allows for a clearer graphical visualization of the metabolites. Once the metabolites that best allowed us to discriminate between the different classes have been obtained, we proceeded with the enrichment analysis. This step allowed us to understand which metabolic pathways the metabolites in question might be involved in and, therefore, which pathways appeared to exhibit differences in the classes under examination. The results are shown in **Figure 25**. Metabolic enrichment analysis revealed significant involvement in pathways related to energy metabolism, amino acids, and the tricarboxylic acid cycle. These results suggested a change in cellular energy and redox balance. The impact of each pathway was quantified by considering the number of altered metabolites and their statistical significance. Impact graphs clearly showed the most affected pathways, offering an integrated view of the systemic changes observed. Although the overall statistical robustness of the PLS-DA model was limited ($p = 0.1195$), pathway enrichment supported a consistent biological interpretation of the results. These metabolic pathways appear functionally connected to the observed changes in metabolic profiles, providing a comprehensive picture of the cellular processes most involved and paving the way for further targeted investigations.

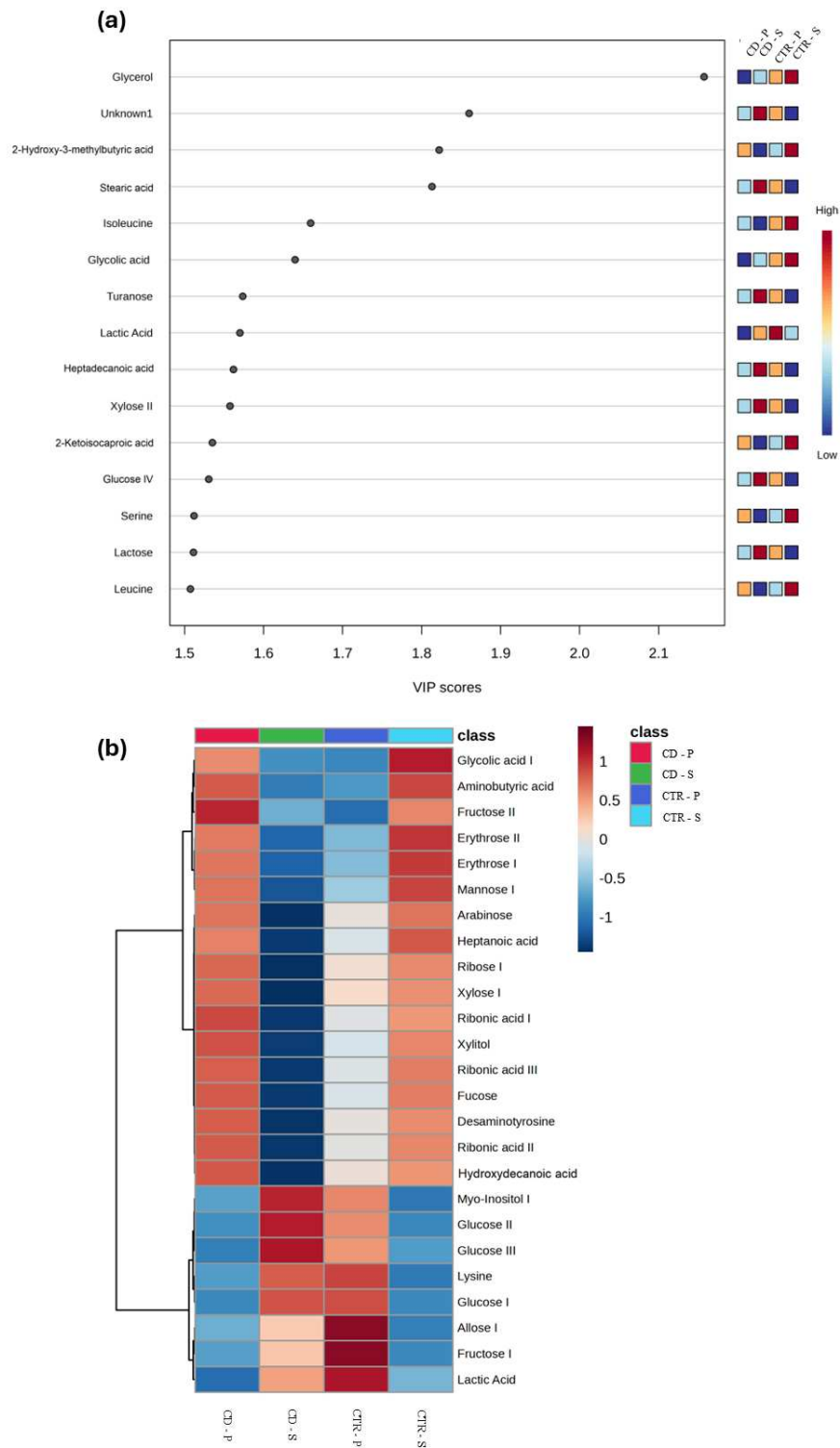


Figure 24. Metabolites that best differentiate the different classes in intestinal fibroblasts. **(a)** VIP scores of the metabolites that best discriminate between the various classes. The selected metabolites have VIP scores > 1.2. **(b)** Heat-map of metabolites with hierarchical classification. The color patterns indicate which metabolites are more/less expressed within the different classes.



Figure 25. Enrichment metabolome pathway analysis in intestinal fibroblasts. The main metabolic pathways that appear to be altered in CD subjects.

Finally, the metabolites best characterized by gas chromatography peak analysis were studied. The latter are shown in **Figure 26**. In the graphs, it is possible to observe the different behaviours between the various classes concerning the detected concentrations of these metabolites. A general impairment of energy production processes emerged. Some studies addressed this issue using samples such as plasma, serum, or urine, but cellular samples are rarely used. In this context, these data may provide further insight into the differences observed in subjects with CD.

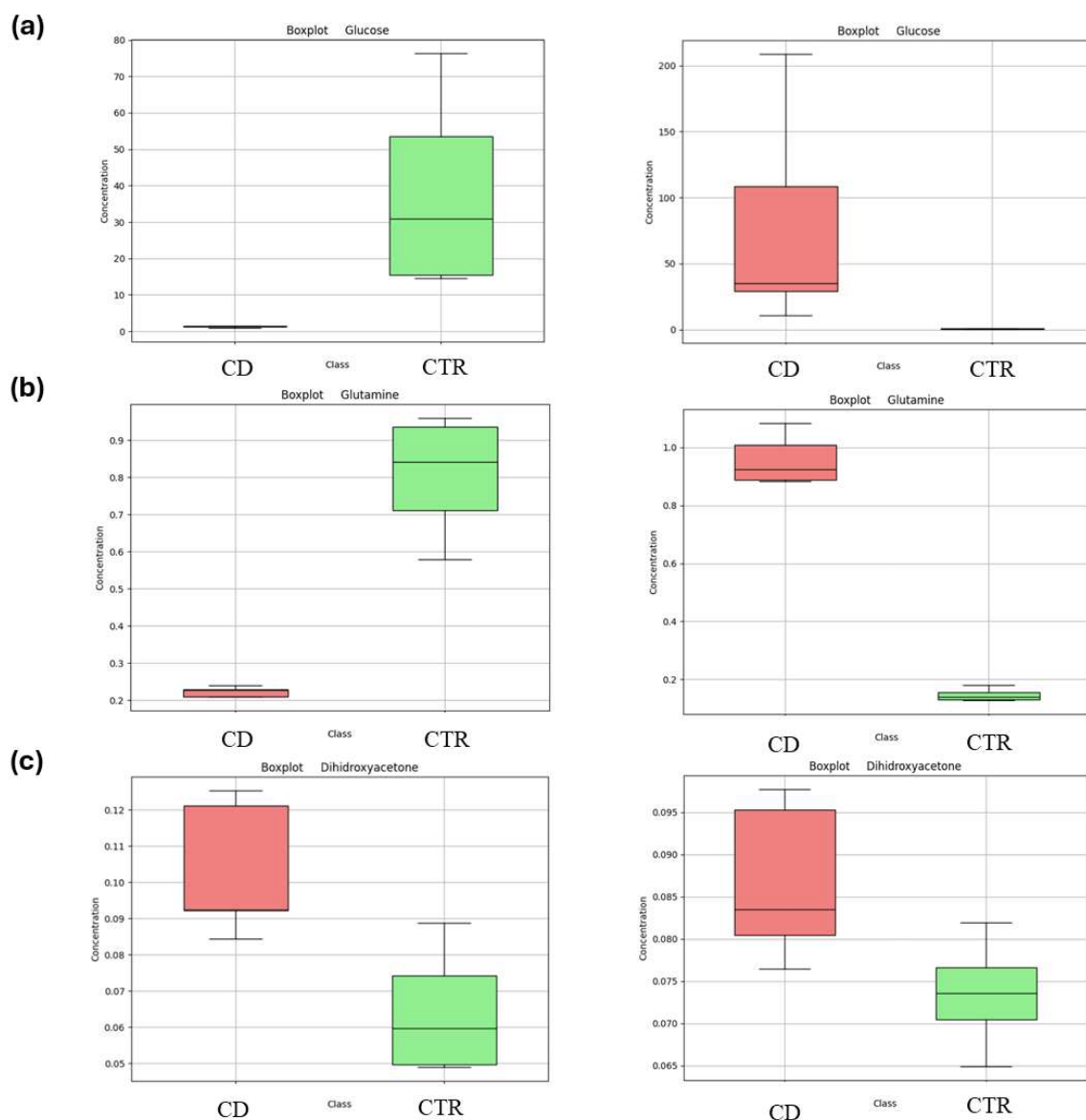


Figure 26. Best characterized metabolites in GS-MS analysis. (a) Boxplots relating to the glucose metabolite. (b) Boxplots relating to the glutamine metabolite. (c) Boxplots relating to the dihydroxyacetone metabolite. On the left, the values for cell pellets. On the right, the values found in cell culture supernatants.

4.2.4 The TG2 inhibitor ZED1227

The analyses conducted on the two cellular systems described in detail here have highlighted an important finding: the reduced level of the TG2 enzyme in CD cells. This finding, together with existing literature regarding the different TG2 subcellular localization and activity (due to

deregulation in Ca^{2+} homeostasis in CD cells⁹⁹), allowed us to confirm an important role of TG2 in determining the celiac cellular phenotype.

Given the key role of TG2 in CD pathogenesis, studies aimed to block its enzymatic activity as a possible therapeutic strategy for CD have been conducted for years, and numerous inhibitors have been developed. In recent years, a promising new inhibitor called ZED1227 has been obtained and entered clinical trials for the treatment of CD (the first TG2 inhibitor to reach this stage). Although the inhibitor is in the advanced stages of testing,¹⁰⁷ there are not many articles in the literature on it.

ZED1227 is a selective, irreversible TG2 inhibitor, characterized by a peptidomimetic chemical structure that allows for high affinity for the enzyme active site. The molecule (see **Figure 27** for the structure) was designed through rational optimization based on knowledge of the three-dimensional structure of TG2 and synthesized using solid peptide chemistry.¹⁰⁷ It contains specific reactive groups capable of forming irreversible covalent bonds with the catalytic residue of TG2, blocking its enzymatic activity. It is a promising candidate for the treatment of active CD¹⁰⁷.

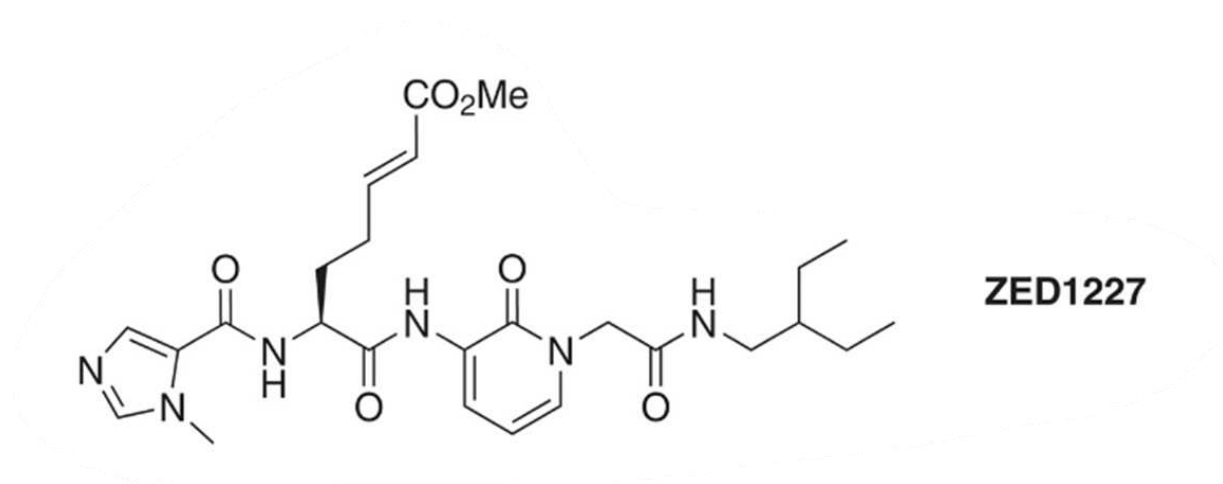


Figure 27. Structure of the ZED1227 inhibitor.¹⁰⁷

As *in vitro* studies, only tests on CACO-2 cells, an intestinal cell model, were conducted by the research team that synthesized the molecule; a clinical trial was then performed on patients by the same research team, published in 2021.⁷⁵ In this thesis, we tried to evaluate the effects of

ZED1227 on the gene expression profile of CD cells. We employed CACO-2 cells and celiac intestinal fibroblasts as study models.

First, we tested the ability of ZED1227 to inhibit the TG2 enzyme in CACO-2 cells. In particular, to test the efficacy of this compound, we analysed its *in vitro* activity at the 300 nM concentration, as employed by *Shuppan et al.*⁷⁵, a concentration that does not cause cytotoxicity. Additionally, in CACO-2 cells, we tested the efficacy of ZED1227 also at higher concentrations, because it appears to be poorly absorbed by cells. The results are reported in **Figure 28**, showing how the inhibitor can counteract the action of enzyme activators. In these experiments, Ca^{2+} was employed for the *in vitro* assays and THP for the *in situ* assays.

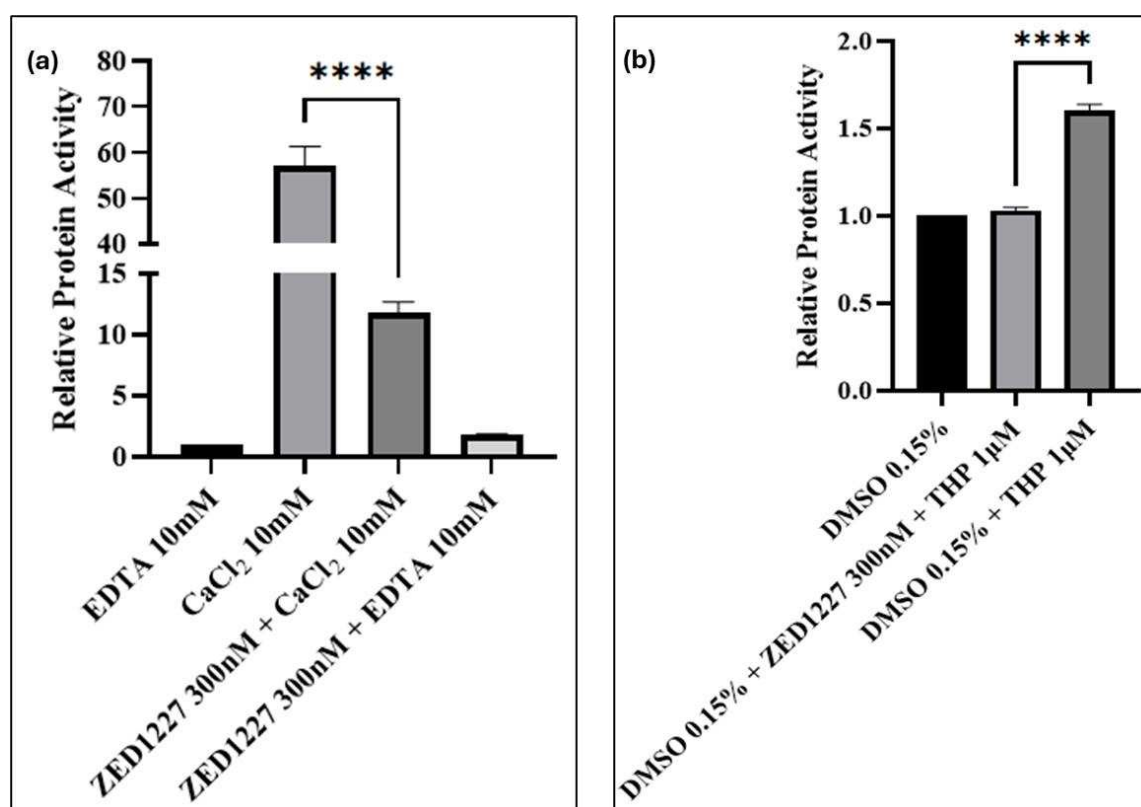


Figure 28. Inhibition of TG2 catalytic activity by the inhibitor ZED1227 in CACO-2. (a) *In vitro* inhibition of TG2 activity using 50 µg of total CACO-2 lysate. CaCl_2 was used as a TG2 enzyme activator. (b) *In situ* inhibition of TG2 in CACO-2 cell culture using 25 µg of protein lysate. THP was used as an activator of Ca^{2+} release, resulting in intracellular TG2 activation. Data are reported as mean $\pm$ SE. **** $p < 0.0001$ vs. respective control.

The inhibitor was then tested on intestinal fibroblasts. Since the cytotoxicity of this inhibitor has been tested only on CACO-2 cells, we first measured the cell viability in a range of concentrations from 1 to 50 μ M of ZED1227. From the results (**Figure 29**), it can be said that, for both CTR and CD cultures, no significant reductions in cell viability were observed at all concentrations tested for 24 h.

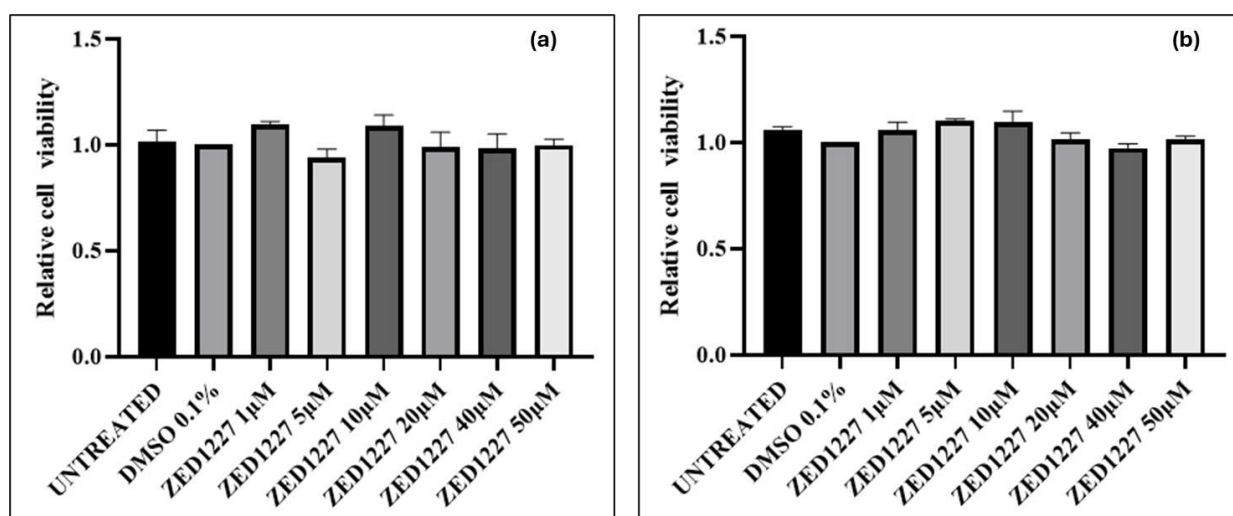


Figure 29. Cell viability in intestinal fibroblasts, FOLD vs DMSO 0.1%. (a) Cell viability of a culture of CTR fibroblasts following stimulation with the inhibitor ZED1227 in a range of concentrations from 1 to 50 μ M for 24 h. (b) Cell viability of a culture of CD fibroblasts following stimulation with the inhibitor ZED1227 in a range of concentrations from 1 to 50 μ M for 24 h. Data are reported as means $\pm$ SE from two independent experiments, each performed in triplicate, on one CTR culture and one CD culture.

Subsequently, the inhibitory effect of ZED1227 on TG2 was evaluated in primary fibroblast cultures. We first evaluated TG2 activity *in vitro* by using a single CD cell culture (**Figure 30a**); in this case, we observed a significant reduction of TG2 activity in the presence of ZED1227. Then we performed an *in situ* assay in both CTR and CD cell cultures (**Figure 30b, c**). We found an important difference in the behaviour of CD cells with respect to CTRs. TG2 catalytic activity was clearly increased by THP treatment in the CTR sample, whereas activity increased in a less evident manner in the CD sample. These findings agree with what was previously observed in CD skin fibroblasts⁹⁹. On the other hand, the TG2 inhibitor had an evident effect on the CTR cultures, but we could not observe a reduction of activity in CD samples. This, in part, could be due to the low sensitivity of the enzymatic assay employed.

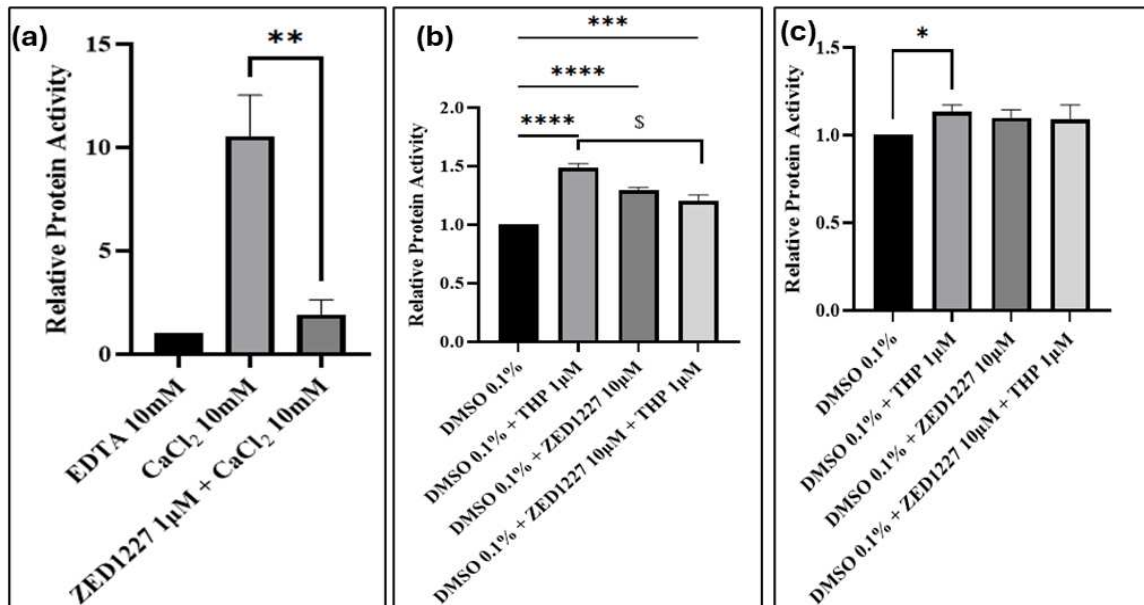


Figure 30. Inhibition of TG2 catalytic activity by the inhibitor ZED1227 in intestinal fibroblasts. **(a)** *In vitro* inhibition of TG2 activity using 30 µg of total CD lysate. One CD culture was tested. CaCl₂ was used as a TG2 enzyme activator. **(b)** *In situ* inhibition of TG2 in a CTR intestinal fibroblast cell culture using 25 µg of protein lysate. TAP was used as an activator of calcium release, resulting in intracellular TG2 activation. **(c)** *In situ* inhibition of TG2 in a CD intestinal fibroblast cell culture using 25 µg of protein lysate. TAP was used as an activator of calcium release, resulting in intracellular TG2 activation. Data are reported as mean ± SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. respective control. \$ $p < 0.001$ vs THP alone.

However, inhibiting TG2 inside the cell or at the membrane surface may result in distinct biological effects, because TG2 is a multifunctional enzyme and displays functions independent from catalytic activities. We were interested in studying the potential global effects of ZED1227 and analyzing the functions modulated by treatment.¹⁰⁸ With this aim, we conducted a transcriptomic analysis on CD intestinal fibroblasts, untreated or treated with this TG2 inhibitor at 50 µM concentration. Four independent CD intestinal cultures were used, cells were collected after 24 h of exposure, and total RNA was extracted. After evaluation of its purity and quality, the RNA samples were analysed by the BMR Genomics (<https://www.bmr-genomics.it/>). Bioinformatics and statistical analyses on the output of the transcriptomics results are ongoing. Preliminary results of these analyses revealed that **425** genes appeared to be dysregulated in CD subjects following TG2 inhibition. Of these, **106** were found to be down-regulated in celiac patients compared to controls, while **319** were found to be up-regulated (**Supplementary Tables 7-8**). A clustering and gene enrichment analysis highlighted the most representative GO terms for this transcript population. The results are shown in **Figure 31**.

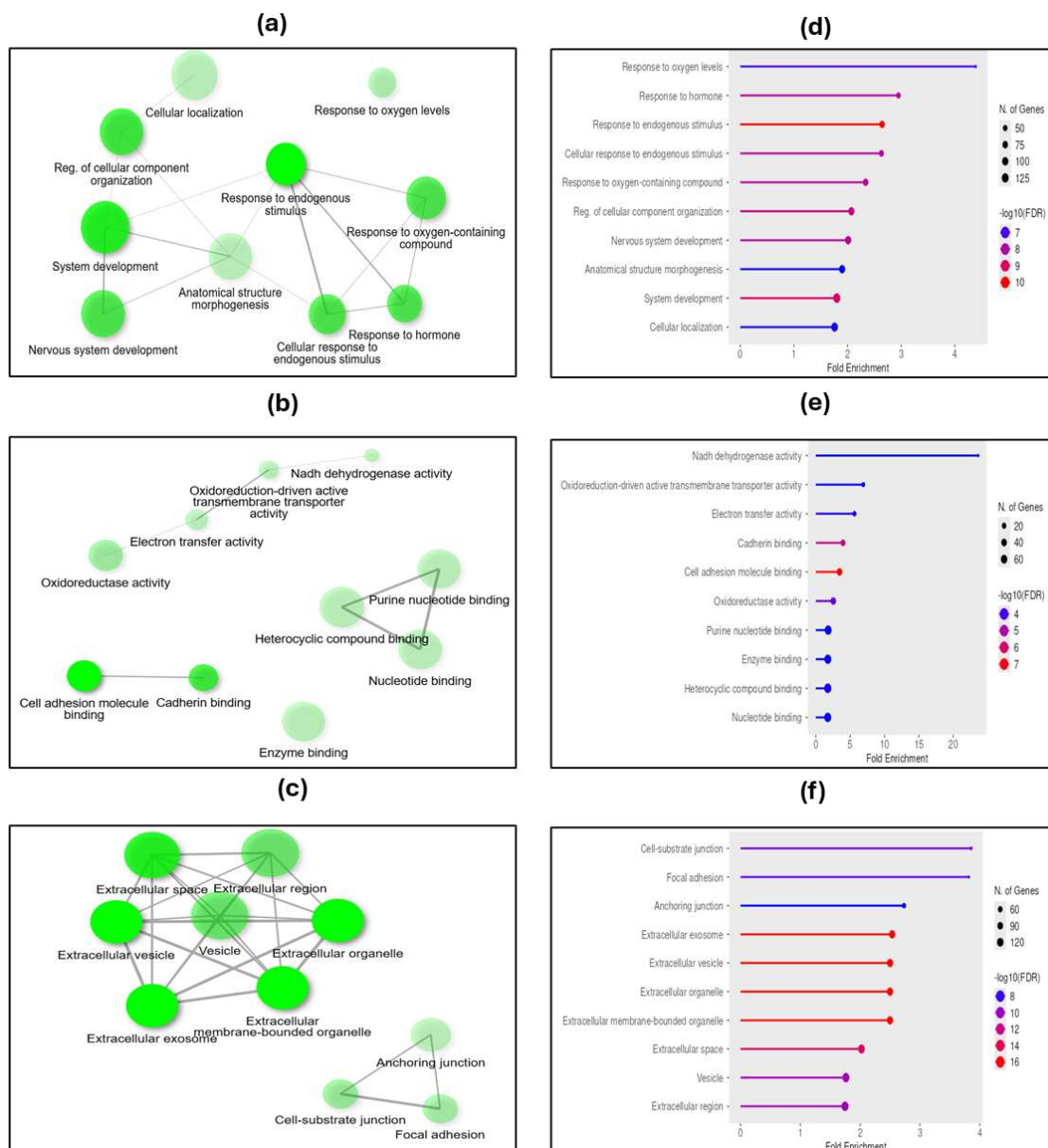


Figure 31. GO enrichment analysis. Enrichment of down and up expressed genes in CD compared to CTR samples after ZED1227 treatments. Clustering and the most representative terms in the protein population of the different GO classes: **(a, d)** biological processes, **(b, e)** molecular functions, **(c, f)** cellular components.

Focusing on transcripts relative to proteins we have previously found differentially expressed by the proteomic investigations, we identified three genes differentially expressed when ZED1227 was used (**Table 18**). These three transcripts will surely be considered among those that will be chosen for the next validations by real-time quantitative Reverse Transcription-Polymerase Chain Reaction.

Table 18. Genes overexpressed in cells treated with ZED1227 and in dermal and intestinal fibroblasts from CD samples analyzed by proteomics.

Accession	Description	Gene Symbol	log2FoldChange: (TG2 inhibition) / (DMSO)	P-Value: (TG2 inhibition) / (DMSO)
ENSG00000164932	collagen triple helix repeat containing 1	CTHRC1	0.107782406280787	0.0226906485866949
ENSG00000008394	microsomal glutathione S-transferase 1	MGST1	0.0773194127075504	0.0447067135303605
ENSG00000167460	tropomyosin 4	TPM4	0.0620811020957792	0.0205342286700108

5. Discussion

The concept of a celiac cellular phenotype is fairly recent. Several studies have been conducted to characterize the possible differences between cells from CD subjects compared to CTR ones. The most significant appeared related to differences in the cytoskeleton, in the vesicular trafficking, in Ca^{2+} homeostasis, and in TG2 enzyme activity and distribution.^{44,78,99,109} These differences could be considered constitutive, even in subjects whose diet no longer includes gluten. Our data indicated that CD cells appeared to respond differently to a stressor. It is important to note that gluten is also a cellular stressor,⁷⁹ so, analyzing cellular responses to stress may be relevant for a better understanding of CD cell responses.

In studies on skin-derived fibroblasts, we employed THP as the stressor agent. Cell viability assays in the presence of THP showed that CD fibroblasts were less sensitive to THP-induced cytotoxicity than CTR cells. This phenomenon could, at least in part, be due to an intrinsic higher level of proliferation in CD cells.⁴⁴ Furthermore, the analysis of the UPR highlighted the

presence of a prolonged response in CD cells; this observation, together with the presence of a lower basal expression of the ER stress marker GRP78 in CD samples, may imply a reduced ability to adapt to noxious stimuli. Indeed, the GRP78 protein is involved in relieving cellular stress, particularly that affecting the ER.¹¹⁰

A sustained UPR signalling appears to be closely related to another cell response, i.e., autophagy. Indeed, the THP-induced ER stress can trigger the autophagic pathway within cells as a defensive response to restore homeostasis.¹¹¹ To study any differences between CD and CTR cells regarding this pathway, two markers widely used for these studies were analyzed LC3 and p62.⁹⁹ The initial results revealed a higher baseline expression of these two markers in CD skin-derived fibroblasts compared to CTR samples. These data suggested a possible delay in the normal progression of the autophagic flux. Furthermore, THP stimulation in CTR cells showed a progressive formation of autophagic compartments, as also demonstrated by the decrease in p62 protein. Instead, in CD subjects, autophagy appears to be delayed or blocked, as shown by a dose-dependent increase in both markers. In this regard, *Bjørkøy et al.*¹¹² have shown that increased p62 protein levels correlated with a blockage of autophagy. For a more in-depth analysis, we conducted experiments using a starvation medium and/or bafilomycin A1, a well-known autophagy inhibitor⁹⁹, which provided evidence supporting this concept. In CTR fibroblasts, a normal autophagic flux was observed, as demonstrated by the increase in LC3-II levels in starved cells and in those treated with bafilomycin A1, with an even greater increase occurring in starved cells treated with bafilomycin A1. Conversely, in CD cells, starvation proved less effective at inducing an increase in the LC3-II isoform, and treatment with bafilomycin A1 caused a comparable increase in LC3-II levels in both non-starved and starved cells. Since the cytosolic Ca^{2+} level is considered a signal to activate autophagy, we hypothesized that a reduced intracellular concentration of Ca^{2+} , previously demonstrated by our group⁹⁹, could contribute to the accumulation of autophagosomes in CD cells treated with THP. Because of low calcium levels in the cytosol and in the ER, TG2 may be scarcely activated in CD cells. Moreover, reduced TG2 activation in CD cells may represent an additional factor contributing to the negative modulation of autophagy, as transamidating activity is essential for the proper completion of autophagic flux.¹¹³ Furthermore, TG2 undergoes degradation within autophagosomes during their maturation. In CD cells treated with THP, the reduction of TG2 was less pronounced compared to CTR cells, potentially due to decreased degradation within autophagosomes. Consistent with the role of TG2 activity in autophagic flux, treatment with the TG2 inhibitor Z-DON notably impeded TG2 degradation in CTR cells. However, this effect

was less pronounced in CD fibroblasts, suggesting that delayed autophagy may occur naturally in these cells.

Interestingly, also in CD intestinal fibroblasts, we observed a higher basal level of LC3-II isoform and p62 than in CTRs, in line with what we found in skin-derived fibroblasts. Moreover, the use of THP to stimulate intracellular TG2 activity highlighted that, also in the intestinal fibroblasts, TG2 was scarcely activable, probably because a deregulated calcium homeostasis occurred also in these cells.

For a better overall understanding of the pathology, we used omics approaches to study the celiac cellular phenotype, we performed analyses both on skin and on intestinal fibroblasts, even if with some differences. The analyses, for both skin and intestinal samples, were conducted using four CD and four CTR cultures. We recognized that we had a small population, and further analyses are planned to provide greater robustness to the results obtained. We performed a proteomic analysis on both cell models, and we compared data obtained from these different cell types. In addition, we employed a phosphoproteomic approach for skin fibroblasts and a metabolomic approach for intestinal fibroblasts, to obtain a wide spectrum of information about differences between the normal and the pathological condition. Finally, we applied a transcriptomic approach on intestinal celiac fibroblasts to study the effect of a TG2 inhibitor, already employed in a clinical trial for CD.

The proteomic analyses conducted on the two cellular systems highlighted the presence of some similarities. One common feature was the identification of proteins involved in the extracellular environment, exosomes, and vesicular trafficking. These terms appeared to be those that best distinguished CD subjects from CTR subjects. Analyses of both models showed reduced levels of the enzyme TG2, a well known key enzyme in CD, also important for vesiculation phenomena.¹¹⁴ Concerning the GO terms that best allowed us to discriminate CD from CTR, in our models, we found as relevant the extracellular space, exosomes, and proteins related to the cytoskeleton, which are important both from a structural/cellular movement point of view and for vesicular trafficking. Interestingly, other omics studies conducted on various matrices, such as serum, urine, and duodenal biopsies, confirmed the presence of multiple alterations and distinct proteomic signatures in CD subjects. Patterns similar to those identified by our analyses appeared to be altered, thus confirming a clear alteration of the cellular vesicular trafficking system in CD subjects^{115,116}. It is worth noting that we found differentially expressed proteins also involved in Ca^{2+} homeostasis and UPR; among them, pro-chloride intracellular channel

protein 4 and calnexin, both upregulated in CD skin fibroblasts, and Heat shock protein beta-6, reticulocalbin-3, and calretinin, upregulated in CD intestinal fibroblasts, as well as calmodulin-3 and other calcium binding proteins, downregulated in CD intestinal fibroblasts.

In addition, in CD intestinal fibroblasts, we found a higher level than in CTRs of ICAM-1; this protein plays key roles in immune cell recruitment, inflammation, and tissue infiltration by leukocytes. It is implicated in many inflammatory diseases and correlates with cell adhesion. *Ensari et al.*¹¹⁷ observed increased expression of adhesion molecules and leukocyte recruitment following gluten ingestion, up to 24 h after ingestion. *Picca et al.*¹¹⁸, however, demonstrated differences in oxidative stress and mitochondrial homeostasis. Specifically, they found an increase in mitochondrial DNA in CD subjects compared to CTR subjects, indicating increased biogenesis of these organelles. In this regard, we found in CD skin fibroblasts a downregulation of MFN-1, a key protein in mitochondrial homeostasis and mitophagy activation. The latter is an important process that allows the release of proinflammatory cytokines such as IL-1 α , IL-1 β , IL-18. In our proteomic analysis, IL-18 was found to be a constitutively overexpressed protein. Together with MFN1 downregulation, the increase of IL-18 suggested that CD skin fibroblasts could present an altered regulation of the inflammatory process. Interestingly, besides TG2, some other proteins appeared up- or downregulated in both skin and intestinal fibroblasts, thus confirming the occurrence of a constitutive phenotype in CD subjects, independent of the inflammatory state or the cell type.

Phosphoproteomics data regarding skin fibroblasts showed differences in compartments such as the extracellular space and in GO terms referring to outward vesiculation, thus consistent with proteomics data. Additionally, differences in protein phosphorylation levels involving the proteasome were present. Notably, an impairment in proteasome regulation can impact various intracellular processes, such as ER stress and the UPR, signaling cascades, apoptosis, proteostasis, and inflammation^{119–123}.

From a metabolomics perspective, studies in the literature regarding CD reported data on serum, urine, or plasma samples. These studies discussed alterations in amino acid metabolism and, more broadly, energy metabolism, including glucose metabolism.^{89,90} To date, our results on intestinal fibroblasts appeared to be the first conducted on primary cultures of celiac subjects, highlighting an energy impairment in CD cells compared to CTR cells. The PLS-DA model showed a clear distinction between the different classes. The model robustness was good, but the permutation test, unfortunately, yielded a p -value > 0.05 , likely due to the small sample size.

Therefore, these results should be considered with caution; however, they were in line with other observations in the literature. The untargeted approach that often characterizes these analyses has allowed us to identify a series of metabolites that better distinguish CDs from CTRs. Among the metabolites best characterized by GS - MS, three were particularly interesting. The levels of these three were altered in CD compared to CTRs. For glucose and glutamine, findings allowed us to outline a picture of altered glycolysis and amino acid metabolism, consistent with data in the literature.^{124,125} The metabolite dihydroxyacetone was of interest as its levels appeared to be increased. This metabolite may derive from its phosphorylated form. Elevated levels may be associated with problems with the enzyme triose phosphate isomerase (TPI). A possible decrease in the enzyme activity may be associated with an imbalance in glycolysis, with involvement of lipid and amino acid metabolism. Additionally, it can lead to the formation of cell-damaging metabolites that can lead to conditions such as increased oxidative stress^{126,127}.

The last part of the research described in this thesis was focused on the use of a new, irreversible TG2-specific inhibitor, ZED1227, in CD intestinal fibroblasts. For the TG2 enzyme, transamidase activity is only one of its catalytic reactions; indeed, TG2 is a multifunctional protein that can act on a broad spectrum of cellular pathways.⁵⁴ Thus, we decided to investigate how the use of these inhibitors could impact the whole expression pathways in CD intestinal fibroblasts. This approach appeared particularly relevant as ZED1227 has already been employed in a phase II clinical trial for CD and is currently under investigation for an advanced clinical trial. Regarding the concluded phase II clinical trial, *Shuppan et al.*⁷⁵ showed that, following controlled gluten ingestion and subsequent administration of the inhibitor at a concentration range of 10-100 mg, the reduction in intestinal villi height decreased by 12-17%, compared to 61% in the placebo group, with a marked improvement in intestinal epithelial histology. Although these results were promising, some critical issues with the study have been considered. First, the treatment duration was only six weeks, thus ignoring possible long-term effects. Furthermore, gluten intake was strictly controlled, which was not often the case in everyday life. Finally, the population tested was between the ages of 18 and 65, not including younger populations. A phase III clinical trial is ongoing. However, studies focused on the effects of ZED1227 in a single celiac cell type are currently lacking. In our model of intestinal primary fibroblasts, we first found that the inhibitor was safe at all tested concentrations (1-50 μ M), then we verified its ability to reduce TG2 activity both in vitro and in situ. After these preliminary tests, we performed a treatment of 24 h with ZED1227 at 50 μ M and analysed the

whole transcriptome in each of four different CD cultures. The data obtained are under analysis to identify the significantly differentially expressed transcripts. Preliminary findings showed us that the inhibition of TG2 by ZED1227 appears to primarily disrupt the vesicular and extracellular compartments, with a focus on exosomes. This inhibition, therefore, may result in impaired vesicle production, cargo loading, and targeting to host cells¹²⁸. Other preliminary findings showed that, after pharmacological inhibition of TG2 with ZED1227, the RNA levels of the three genes whose proteins were upregulated in both dermal and intestinal fibroblasts (i.e., MGST1, TPM4, and CTHRC1) further increased. These genes are involved in matrix remodeling (CTHRC1)¹²⁹, cytoskeletal stabilization (TPM4)¹³⁰, and antioxidant defense (MGST1)¹³¹, which are all processes contributing to CD development. Further investigations are needed to understand biological significance of the differential gene expression we observed in CD fibroblasts treated with ZED1227.

6. Conclusions

The results of this study confirmed the existence of a constitutive cellular phenotype in CD subjects, independent of gluten exposure, characterized by multilevel alterations in both dermal- and intestinal-derived fibroblasts. Many features of this phenotype have been identified.

Alterations in stress response and autophagy: CD cells exhibited a prolonged UPR, elevated basal levels of the autophagy markers LC3 and p62, and delayed or blocked autophagic flux. These phenomena may be correlated with intracellular calcium deregulation and reduced TG2 activity, which appears to play a central role in regulating autophagy and protein degradation.

Structural and vesicular trafficking alterations: both proteomic and phosphoproteomic analyses revealed changes in the cytoskeletal network, extracellular matrix organization, exosomes, and vesicular trafficking. These alterations may influence cell motility, secretion, and intercellular communication, contributing to the pathogenesis of CD.

Metabolic dysfunction: metabolomic differences in intestinal fibroblasts with respect to CTRs indicated altered regulation of energy and amino acid metabolism.

Role of TG2 and therapeutic potential: our experiments targeted the study of expression and activity of TG2 in both cell models, supporting the concept that TG2 has a central importance of TG2 in the celiac phenotype. The use of the irreversible inhibitor ZED1227 induced transcriptional changes in celiac intestinal fibroblasts whose biological significance is under investigation. Future results from transcriptomic analyses will contribute to better understanding which impact the inhibition of TG2 could have into the organisms. Our analyses will eventually give strength to therapeutic approaches targeted to TG2 inhibition or blocking but also highlight possible side effects of the use of TG2 inhibitors.

In summary, this work contributes to the delineation of a complex picture of the celiac cellular phenotype, characterized by systemic alterations in cellular stress, autophagy, vesicle trafficking, metabolism, and inflammation, regardless of the tissue of origin or gluten exposure. These findings not only enrich our understanding of the cellular mechanisms underlying CD but also may contribute to searching, by omics approaches, novel potential biomarkers and therapeutic targets for CD.

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7.0 Appendix

Supplementary Table 1. List of hyperexpressed proteins in CD skin fibroblasts.

Accession (UniProt)	Description	Coverage [%]	#Peptide-Spectrum Matches (PSMs)	Gene Symbol	Abundance Ratio: (Celiac) / (Ctrl)	Abundance Ratio Adj. P-Value: (Celiac) / (Ctrl)
A0A2R8Y5P7	Histidine--tRNA ligase	11	23	HARS2	100	5.15E-16
Q8WUX9	Charged multivesicular body protein 7	2	1	CHMP7	100	5.15E-16
Q96GE4	Centrosomal protein of 95 kDa	1	1	CEP95	100	5.15E-16
G8JLB3	tRNA pseudouridine synthase A (Fragment)	3	1	PUS1	100	5.15E-16
Q9UF33	Ephrin type-A receptor 6	1	1	EPHA6	100	5.15E-16
D6RB85	Calnexin (Fragment)	37	54	CANX	100	5.15E-16
A0A669KBF2	Carbamoyl-phosphate synthase [ammonia], mitochondrial (Fragment)	2	2	CPS1	100	5.15E-16
Q9UKN7	Unconventional myosin-XV	1	1	MYO15A	100	5.15E-16
Q9Y5B8	Nucleoside diphosphate kinase 7	3	1	NME7	100	5.15E-16
A0A3B3ISJ8	CAP-Gly domain-containing linker protein 1 (Fragment)	1	1	CLIP1	100	5.15E-16
K7EPV6	Choline transporter-like protein 2 (Fragment)	5	1	SLC44A2	100	5.15E-16
O76070	Gamma-synuclein	10	2	SNCG	100	5.15E-16
Q15047	Histone-lysine N-methyltransferase SETDB1	1	2	SETDB1	100	5.15E-16
Q14116	Interleukin-18	9	2	IL18	100	5.15E-16
Q5VZ03	Nucleoredoxin-like protein 2	6	1	NXNL2	100	5.15E-16
Q9UI15	Transgelin-3	14	15	TAGLN3	100	5.15E-16
Q9UBF2	Coatomer subunit gamma-2	5	22	COPG2	100	5.15E-16
Q9Y5A9	YTH domain-containing family protein 2	3	5	YTHDF2	100	5.15E-16
P17661	Desmin	51	311	DES	24,99	5.15E-16
E7ENQ1	Mitogen-activated protein kinase kinase kinase kinase 4	1	2	MAP4K4	24,113	5.15E-16
Q9Y2K3	Myosin-15	1	3	MYH15	14,157	1.14E-14
Q9H7D0	Dedicator of cytokinesis protein 5	1	5	DOCK5	12,334	5.15E-16
P47895	Aldehyde dehydrogenase family 1 member A3	21	46	ALDH1A3	8,767	5.15E-16
P39019	40S ribosomal protein S19	41	24	RPS19	7,927	6.88E-12
K7EPJ1	Ubiquitin-conjugating enzyme E2 S (Fragment)	6	1	UBE2S	7,179	1.57E-06
A0A0A0MSY7	Thymidine kinase 2, mitochondrial	3	2	TK2	5,573	6.44E-05
P07476	Involucrin	14	11	IVL	5,16	1.15E-06
C9J719	3-beta-hydroxysteroid-Delta(8),Delta(7)-isomerase (Fragment)	7	5	EBP	4,808	0.000288
V9GYZ1	Integrin beta (Fragment)	7	3	ITGB5	4,679	0.000429
Q96T88	E3 ubiquitin-protein ligase UHRF1	1	2	UHRF1	4,59	0.000506
Q5KU26	Collectin-12	6	12	COLEC12	4,538	2.83E-07
Q16647	Prostacyclin synthase	15	23	PTGIS	4,513	2.99E-09
P21926	CD9 antigen	4	10	CD9	4,373	4.58E-07
P10620	Microsomal glutathione S-transferase 1	26	11	MGST1	4,326	5.52E-05
O14494	Phospholipid phosphatase 1	7	6	PLPP1	4,3	1.81E-06

H0Y320	Kynurenine 3-monooxygenase (Fragment)	8	2	KMO	4,049	0.000435
E5RI94	TBC1 domain family member 31	2	1	TBC1D31	4,02	0.006938
A6NCN2	Putative keratin-87 protein	43	20	KRT87P	3,902	0.00077
D6RBQ6	Ubiquitin carboxyl-terminal hydrolase 17-like protein 17	2	5	USP17L17	3,722	0.001542
Q8ND94	LRRN4 C-terminal-like protein	18	3	LRRN4CL	3,661	0.006141
P67936-2	Isoform 2 of Tropomyosin alpha-4 chain	68	263	TPM4	3,624	1.19E-06
O15040	Tectonin beta-propeller repeat-containing protein 2	1	2	TECPR2	3,604	0.000143
A0A140T8Y4	HLA class I histocompatibility antigen, A alpha chain	28	36	HLA-A	3,564	7.59E-05
A8MT72	Reticulon	8	8	RTN1	3,532	0.009711
Q15121	Astrocytic phosphoprotein PEA-15	15	5	PEA15	3,53	0.00259
B5MCY1	Tudor domain-containing protein 15	0	3	TDRD15	3,403	0.003211
Q9BXJ0	Complement C1q tumor necrosis factor-related protein 5	10	4	C1QTNF5	3,246	0.01523
K7ERM7	DNA repair protein RAD51 homolog 4 (Fragment)	7	1	RAD51D	3,185	0.045805
Q14019	Coactosin-like protein	16	7	COTL1	3,17	0.001036
G8JLA2	Myosin light polypeptide 6	72	94	MYL6	3,136	0.042797
Q9H3Q1	Cdc42 effector protein 4	4	1	CDC42EP4	3,13	0.025858
E7EUN2	Arf-GAP with GTPase, ANK repeat and PH domain-containing protein 1	1	2	AGAP1	3,062	0.033748
Q9BQJ4	Transmembrane protein 47	9	7	TMEM47	3,019	0.006515
J3QTA6	MICOS complex subunit MIC25	3	3	CHCHD6	2,959	0.015566
P62829	60S ribosomal protein L23	44	8	RPL23	2,92	0.022827
Q9UFN0	Protein NipSnap homolog 3A	20	13	NIPSNAP3A	2,76	0.01865
Q14249	Endonuclease G, mitochondrial	3	4	ENDOG	2,746	0.037136
P63096	Guanine nucleotide-binding protein G(i) subunit alpha-1	23	84	GNAI1	2,71	0.049561
Q3ZCM7	Tubulin beta-8 chain	28	374	TUBB8	2,689	0.02424
Q53TN4	Cytochrome b reductase 1	3	5	CYBRD1	2,667	0.024397
Q969H8	Myeloid-derived growth factor	23	14	MYDGF	2,621	0.003267
P35052	Glypican-1	9	14	GPC1	2,536	0.008256
P21964	Catechol O-methyltransferase	38	37	COMT	2,503	0.000748
Q15746	Myosin light chain kinase, smooth muscle	5	19	MYLK	2,468	0.006536
Q96CG8	Collagen triple helix repeat-containing protein 1	33	46	CTHRC1	2,388	0.001039
Q9UBY9	Heat shock protein beta-7	53	24	HSPB7	2,311	0.027698
P35270	Sepiapterin reductase	19	16	SPR	2,263	0.04734
Q9HA77	Probable cysteine--tRNA ligase, mitochondrial	5	6	CARS2	2,25	0.042227
F5GY05	Constitutive coactivator of peroxisome proliferator-activated receptor gamma	1	23	FAM120B	2,177	0.034312
E9PRJ8	Tetraspanin (Fragment)	32	36	CD81	2,164	0.006141
O14495	Phospholipid phosphatase 3	18	49	PLPP3	2,16	0.005472
Q9Y696	Chloride intracellular channel protein 4	74	137	CLIC4	2,156	0.006683
P12277	Creatine kinase B-type	34	66	CKB	2,106	0.009523
P07858	Cathepsin B	25	102	CTSB	2,064	0.012899
P02511	Alpha-crystallin B chain	54	76	CRYAB	2,01	0.018902
P14209	CD99 antigen	13	14	CD99	1,998	0.034312
Q8TF66	Leucine-rich repeat-containing protein 15	18	33	LRRC15	1,945	0.039338
E9PEP6	Neuropilin	15	41	NRP1	1,905	0.04261

Supplementary Table 2. List of lower expressed proteins in CD skin fibroblasts.

Accession (UniProt)	Description	Coverage [%]	#Peptide-Spectrum Matches (PSMs)	Gene Symbol	Abundance Ratio: (Celiac) / (Ctrl)	Abundance Ratio Adj. P-Value: (Celiac) / (Ctrl)
P16401	Histone H1.5	34	147	H1-5	0.478	0.039442
Q9H7C4	Syncoilin	9	14	SYNC	0.476	0.048591
Q96A33	Coiled-coil domain-containing protein 47	8	11	CCDC47	0.47	0.043091
Q76M96	Coiled-coil domain-containing protein 80	6	25	CCDC80	0.467	0.024749
P62736	Actin, aortic smooth muscle	62	841	ACTA2	0.464	0.026936
P32455	Guanylate-binding protein 1	5	11	GBP1	0.461	0.029999
P11047	Laminin subunit gamma-1	11	34	LAMC1	0.458	0.018902
Q9UGI8	Testin	15	18	TES	0.453	0.019702
P23142	Fibulin-1	6	37	FBLN1	0.451	0.018571
P17813	Endoglin	16	62	ENG	0.449	0.017351
P29279	CCN family member 2	18	17	CCN2	0.438	0.020019
P52292	Importin subunit alpha-1	15	19	KPNA2	0.436	0.005096
P16278	Beta-galactosidase	11	12	GLB1	0.421	0.017556
Q9HD26	Golgi-associated PDZ and coiled-coil motif-containing protein	8	6	GOPC	0.412	0.013062
P12111	Collagen alpha-3(VI) chain	28	311	COL6A3	0.406	0.003698
P30419	Glycylpeptide N-tetradecanoyltransferase 1	18	19	NMT1	0.406	0.004248
Q96IZ0	PRKC apoptosis WT1 regulator protein	4	8	PAWR	0.406	0.02918
Q92804	TATA-binding protein-associated factor 2N	12	58	TAF15	0.395	0.004578
Q5T750	Skin-specific protein 32	3	2	KPLCE	0.395	0.045078
B8ZZQ6	Prothymosin alpha	38	30	PTMA	0.389	0.001592
Q12874	Splicing factor 3A subunit 3	12	23	SF3A3	0.385	0.000873
P05114	Non-histone chromosomal protein HMG-14	33	11	HMGN1	0.384	0.021428
B9A062	Methenyltetrahydrofolate cyclohydrolase	5	1	MTHFD2	0.383	0.034512
P07951-3	Isoform 3 of Tropomyosin beta chain	58	207	TPM2	0.38	0.001281
P07951	Tropomyosin beta chain	60	249	TPM2	0.378	0.001142
P50990-2	Isoform 2 of T-complex protein 1 subunit theta	60	204	CCT8	0.376	0.002634
F5GZS6	4F2 cell-surface antigen heavy chain	28	49	SLC3A2	0.373	0.000664
E7ETU9	Procollagen-lysine 5-dioxygenase	17	38	PLOD2	0.373	0.000941
Q3KQV9	UDP-N-acetylhexosamine pyrophosphorylase-like protein 1	9	19	UAP1L1	0.369	0.001837
P07437	Tubulin beta chain	74	801	TUBB	0.364	0.007906
Q8N0X7	Spartin	10	5	SPART	0.353	0.006973
Q8NFK8	Torsin-1 A-interacting protein 2	3	3	TOR1AIP2	0.349	0.006874
Q96P63	Serpin B12	4	3	SERPINB12	0.333	0.037932
P43652	Afamin	1	5	AFM	0.33	0.002579
Q05682-5	Isoform 5 of Caldesmon	62	473	CALD1	0.328	7.59E-05

C9JR82	Complex I assembly factor TIMMDC1, mitochondrial (Fragment)	6	3	TIMMDC1	0.319	0.026903
A0A2R8YE71	Connector enhancer of kinase suppressor of ras 2 (Fragment)	2	2	CNKS2	0.312	0.001402
Q86X55	Histone-arginine methyltransferase CARM1	1	2	CARM1	0.312	0.004859
O60832	H/ACA ribonucleoprotein complex subunit DKC1	2	4	DKC1	0.307	0.003347
P02751-1	Isoform 1 of Fibronectin	44	725	FN1	0.305	1.57E-05
X6R4W8	BUB3-interacting and GLEBS motif-containing protein ZNF207	5	7	ZNF207	0.304	5.79E-05
Q12996	Cleavage stimulation factor subunit 3	2	5	CSTF3	0.303	0.000769
P0DOY2	Immunoglobulin lambda constant 2	9	2	IGLC2	0.301	0.026482
Q9H5N1	Rab GTPase-binding effector protein 2	2	2	RABEP2	0.292	0.000415
Q00534	Cyclin-dependent kinase 6	5	8	CDK6	0.291	0.005326
Q9Y4K0	Lysyl oxidase homolog 2	2	6	LOXL2	0.289	0.000597
Q9ULM0	Pleckstrin homology domain-containing family H member 1	1	4	PLEKHH1	0.284	4.35E-06
Q9NZW5	MAGUK p55 subfamily member 6	2	2	PALS2	0.282	0.004978
A0A0D9SF70	ADP-ribosylation factor GTPase-activating protein 2	3	2	ARFGAP2	0.279	0.001457
Q96I63	ATP-dependent zinc metalloprotease YME1L1	2	2	YME1L1	0.272	0.0012
Q8TED9	Actin filament-associated protein 1-like 1	1	2	AFAP1L1	0.27	0.000664
C9JVG0	Serotransferrin (Fragment)	6	4	TF	0.262	1.03E-05
O15118	NPC intracellular cholesterol transporter 1	1	2	NPC1	0.242	2.65E-09
Q12899	Tripartite motif-containing protein 26	2	1	TRIM26	0.241	0.002126
H3BN14	Calretinin (Fragment)	5	2	CALB2	0.24	0.001716
P16104	Histone H2AX	58	73	H2AX	0.237	5.01E-07
Q16270	Insulin-like growth factor-binding protein 7	12	4	IGFBP7	0.233	0.000143
A0A1W2PSE7	HLA class I histocompatibility antigen, A alpha chain	27	29	HLA-A	0.218	0.000786
P21980	Protein-glutamine gamma-glutamyltransferase 2	19	32	TGM2	0.204	2.95E-10
Q5JP53	Tubulin beta chain	73	778	TUBB	0.2	1.66E-06
Q6UWY5	Olfactomedin-like protein 1	2	5	OLFML1	0.166	4.56E-09
P01876	Immunoglobulin heavy constant alpha 1	3	2	IGHA1	0.091	4.4E-10
Q96T49	Protein phosphatase 1 regulatory inhibitor subunit 16B	1	3	PPP1R16B	0.075	5.15E-16
H3BPD7	U3 small nucleolar RNA-associated protein 4 homolog (Fragment)	7	1	UTP4	0.01	5.15E-16
Q9HBI1	Beta-parvin	8	22	PARVB	0.01	5.15E-16
O00762	Ubiquitin-conjugating enzyme E2 C	7	1	UBE2C	0.01	5.15E-16
Q99567	Nuclear pore complex protein Nup88	1	1	NUP88	0.01	5.15E-16
P83436	Conserved oligomeric Golgi complex subunit 7	3	3	COG7	0.01	5.15E-16
P11387	DNA topoisomerase 1	2	2	TOP1	0.01	5.15E-16
F8VZV1	Protein MON2 homolog	1	1	MON2	0.01	5.15E-16
P56181-2	Isoform 2 of NADH dehydrogenase [ubiquinone] flavoprotein 3, mitochondrial	3	1	NDUFV3	0.01	5.15E-16
Q96SB3	Neurabin-2	1	1	PPP1R9B	0.01	5.15E-16
O96007	Molybdopterin synthase catalytic subunit	8	1	MOCS2	0.01	5.15E-16
P60604	Ubiquitin-conjugating enzyme E2 G2	5	1	UBE2G2	0.01	5.15E-16

P30530	Tyrosine-protein kinase receptor UFO	1	1	AXL	0.01	5.15E-16
J3QRU1	Tyrosine-protein kinase	7	9	YES1	0.01	5.15E-16
Q9Y5N5	Methyltransferase N6AMT1	5	1	N6AMT1	0.01	5.15E-16
Q8NDZ4	Divergent protein kinase domain 2A	2	1	DIPK2A	0.01	5.15E-16
E9PQT1	FYVE, RhoGEF and PH domain-containing protein 4	6	1	FGD4	0.01	5.15E-16
Q8N309	Leucine-rich repeat-containing protein 43	1	1	LRRC43	0.01	5.15E-16
Q9BZ68	Putative FERM domain-containing protein FRMD8P1	2	1	FRMD8P1	0.01	5.15E-16
M0QXN5	Nuclear pore glycoprotein p62	5	1	NUP62	0.01	5.15E-16
Q9Y5J1	U3 small nucleolar RNA-associated protein 18 homolog	3	1	UTP18	0.01	5.15E-16
A0A0A0MSC8	Slit homolog 3 protein	1	1	SLIT3	0.01	5.15E-16
C9J164	Ras-associated and pleckstrin homology domains-containing protein 1	4	2	RAPH1	0.01	5.15E-16
Q9H8H0	Nucleolar protein 11	2	1	NOL11	0.01	5.15E-16
Q9UKG1	DCC-interacting protein 13-alpha	2	1	APPL1	0.01	5.15E-16
A0A2R8YEK8	Ceramide transfer protein	2	1	CERT1	0.01	5.15E-16
Q9UHI8	A disintegrin and metalloproteinase with thrombospondin motifs 1	1	1	ADAMTS1	0.01	5.15E-16
A0A1B0GTK0	Kelch-like protein 33	1	1	KLHL33	0.01	5.15E-16
D6RBV0	LEM domain-containing protein 2	9	1	LEMD2	0.01	5.15E-16
Q5VWQ8	Disabled homolog 2-interacting protein	1	1	DAB2IP	0.01	5.15E-16
Q9UIC8	Leucine carboxyl methyltransferase 1	4	1	LCMT1	0.01	5.15E-16
Q8IWA4	Mitofusin-1	4	4	MFN1	0.01	5.15E-16
K7ENV7	Isochorismatase domain-containing protein 2 (Fragment)	9	1	ISOC2	0.01	5.15E-16
H7C4G9	ERC protein 2 (Fragment)	2	1	ERC2	0.01	5.15E-16
A0A1W2PNP0	GPI transamidase component PIG-T (Fragment)	1	1	PIGT	0.01	5.15E-16
Q8N4B4	F-box only protein 39	2	1	FBXO39	0.01	5.15E-16
P02675	Fibrinogen beta chain	2	1	FGB	0.01	5.15E-16

Supplementary Table 3. List of hyperphosphorylated phosphoproteins in CD skin fibroblasts.

Accession (UniProt)	Description	Coverage [%]	#Peptide-Spectrum Matches (PSMs)	Gene Symbol	Abundance Ratio	Abundance Ratio Adj. P-Value
A0A140VJZ1	Ubiquitin carboxyl-terminal hydrolase	2	5		6.402	4.5E-06
Q0QEN7	ATP synthase subunit beta (Fragment)	9	5	ATP5B	4.261	0.000853
Q05DJ2	Purine nucleoside phosphorylase	46	47	MTAP	4.003	0.000298
A0A0F7G8J1	Plasminogen	6	23	PLG	2.961	0.000114
I3L3H2	RNA helicase	12	23	EIF4A3	2.936	0.043709
Q99729-3	Isoform 3 of Heterogeneous nuclear ribonucleoprotein A/B	23	14	HNRNPAB	2.896	0.000505
A0A3B3IU69	Echinoderm microtubule-associated protein-like 1	4	6	EML1	2.688	0.025771
Q9BZE9	Tether containing UBX domain for GLUT4	22	13	ASPSCR1	2.442	0.014782
P61758	Prefoldin subunit 3	14	4	VBP1	2.315	0.026045
A8K8F9	Phosphoinositide phospholipase C	14	19		2.208	0.015627
Q9BXR0	Queuine tRNA-ribosyltransferase catalytic subunit 1	10	14	QTRT1	2.207	0.015655
Q15599-3	Isoform 3 of Na(+)/H(+) exchange regulatory cofactor NHE-RF2	15	3	NHERF2	2.205	0.021094
Q99747	Gamma-soluble NSF attachment protein	9	2	NAPG	2.178	0.018853
Q9H4A4	Aminopeptidase B	20	24	RNPEP	2.12	0.026027
B2RBH2	Ecto-5'-nucleotidase	26	23		2.039	0.040054
Q5IS54	Caspase-3 OS=Pan troglodytes	8	13	CASP3	2.036	0.040572
Q9BUT1	3-hydroxybutyrate dehydrogenase type 2	30	19	BDH2	2.004	0.049409

Supplementary Table 4. List of down-phosphorylated phosphoproteins in CD skin fibroblasts.

Accession (UniProt)	Description	Coverage [%]	#Peptide-Spectrum Matches (PSMs)	Gene Symbol	Abundance Ratio	Abundance Ratio Adj. P-Value
O00764	Pyridoxal kinase	28	21	PDXK	0.499	0.00727
B4DIT7	cDNA FLJ58187, highly similar to Protein-glutamine gamma-glutamyltransferase 2	6	3		0.463	0.049466
Q9BPW0	Serine/threonine-protein phosphatase (Fragment)	21	20	PPP5C	0.455	0.022119
P46939	Utrophin	2	9	UTRN	0.371	3.49E-05
Q4G0N4	NAD kinase 2, mitochondrial	6	2	NADK2	0.358	0.000222
A8MX94	GST class-pi	13	2	GSTP1	0.341	0.047782
O14556	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	8	22	GAPDHS	0.332	0.00129
Q92990	Glomulin	6	6	GLMN	0.297	7.64E-05
F5H345	Hydroxymethylbilane synthase	12	5	HMBS	0.272	0.001691
O43583	Density-regulated protein	31	14	DENR	0.26	5.49E-07
Q8WX93	Palladin	2	4	PALLD	0.246	1.68E-05
O00487	26S proteasome non-ATPase regulatory subunit 14	7	7	PSMD14	0.211	0.000195
P35270	Sepiapterin reductase	10	2	SPR	0.123	7.54E-15

P48556	26S proteasome non-ATPase regulatory subunit 8	5	4	PSMD8	0.08	3.43E-16
Q6ICN0	Adapter protein GRB2 (Fragment)	21	10	GRB2	0.044	3.43E-16
A0A384MDW7	Enoyl Coenzyme A hydratase, short chain, 1, mitochondrial	12	10	ECHS1	0.019	3.43E-16
Q6FWH3	Adipsin	8	2	DF	0.01	3.43E-16

Supplementary Table 5. List of hyperexpressed proteins in CD intestinal fibroblasts.

Accession (UniProt)	Description	Coverage [%]	#Peptide-Spectrum Matches (PSMs)	Gene Symbol	Abundance Ratio: (Celiac) / (Ctrl)	Abundance Ratio Adj. P-Value: (Celiac) / (Ctrl)
O95873	Uncharacterized protein C6orf47	3	1	C6orf47	100	5.58E-16
P06732	Creatine kinase M-type	8	5	CKM	100	5.58E-16
A0A1W2PSE7	HLA class I histocompatibility antigen, A alpha chain	40	171	HLA-A	100	5.58E-16
Q32P41	tRNA (guanine(37)-N1)-methyltransferase	3	1		100	5.58E-16
P10620	Microsomal glutathione S-transferase 1	8	1	MGST1	100	5.58E-16
C9J236	Protein arginine methyltransferase NDUF7 (Fragment)	6	1	NDUF7	100	5.58E-16
O75923	Dysferlin	2	40	DYSF	100	5.58E-16
F8W145	CCR4-NOT transcription complex subunit 2 (Fragment)	5	3	CNOT2	100	5.58E-16
P02814	Submaxillary gland androgen-regulated protein 3B	66	11	SMR3B	100	5.58E-16
K7ERZ5	DNA ligase 3 (Fragment)	1	1	LIG3	100	5.58E-16
P78357	Contactin-associated protein 1	1	1	CNTNAP1	100	5.58E-16
Q9BYM8	RanBP-type and C3HC4-type zinc finger-containing protein 1	4	1	RBCK1	100	5.58E-16
H0Y2Y8	Zyxin (Fragment)	48	399	ZYX	100	5.58E-16
J3QRU1	Tyrosine-protein kinase	9	30	YES1	100	5.58E-16
Q9Y3R5	Protein dopey-2	0	1	DOP1B	100	5.58E-16
P32929	Cystathionine gamma-lyase	3	4	CTH	100	5.58E-16
E9PHV5	Protein ITPRID2	1	1	ITPRID2	100	5.58E-16
A0A590UJS6	Sarcolemmal membrane-associated protein (Fragment)	4	2		100	5.58E-16
P00966	Argininosuccinate synthase	7	9	ASS1	100	5.58E-16
Q9Y623	Myosin-4	10	57	MYH4	100	5.58E-16
P12110-2	Isoform 2C2A of Collagen alpha-2(VI) chain	13	101	COL6A2	100	5.58E-16
P12882	Myosin-1	14	73	MYH1	100	5.58E-16
Q5T085	Alpha-amylase (Fragment)	7	1		100	5.58E-16
P78386	Keratin, type II cuticular Hb5	19	41	KRT85	100	5.58E-16
H3BPK4	Myosin regulatory light chain 2, skeletal muscle isoform (Fragment)	12	4	MYL11	100	5.58E-16
Q14525	Keratin, type I cuticular Ha3-II	24	160	KRT33B	100	5.58E-16
Q15323	Keratin, type I cuticular Ha1	18	156	KRT31	100	5.58E-16
H0YMG7	Retinal dehydrogenase 2	5	23	ALDH1A2	73.157	5.58E-16
Q9UKX2	Myosin-2	13	69	MYH2	23.919	5.58E-16
O60565	Gremlin-1	34	28	GREM1	14.235	5.58E-16
P21810	Biglycan	6	10	BGN	11.166	5.58E-16
P98095	Fibulin-2	5	18	FBLN2	10.662	5.58E-16

P67936-2	Isoform 2 of Tropomyosin alpha-4 chain	74	845	TPM4	10.149	5.58E-16
P55290	Cadherin-13	5	18	CDH13	6.173	5.58E-16
P23142	Fibulin-1	9	71	FBLN1	5.179	5.58E-16
E9PNK6	Tumor protein D53	17	9	TPD52L1	9.798	1.2E-14
P05121	Plasminogen activator inhibitor 1	21	54	SERPINE1	4.808	3.57E-14
P02751-1	Isoform 1 of Fibronectin	32	599	FN1	4.539	1.25E-13
O14558	Heat shock protein beta-6	71	63	HSPB6	5.546	3.78E-13
P02511	Alpha-crystallin B chain	60	80	CRYAB	3.799	2.49E-11
P01833	Polymeric immunoglobulin receptor	2	1	PIGR	9.542	5.26E-11
A0A2R8Y4T1	Tensin-1	9	67	TNS1	4.592	3.66E-10
P27144	Adenylate kinase 4, mitochondrial	10	8	AK4	7.026	2.17E-09
Q96ST3	Paired amphipathic helix protein Sin3a	1	1	SIN3A	4.382	2.67E-09
Q96D15	Reticulocalbin-3	21	86	RCN3	3.044	7.23E-08
Q8N556	Actin filament-associated protein 1	5	11	AFAP1	3.716	9.62E-08
Q9GZZ8	Extracellular glycoprotein lacritin	8	2	LACRT	5.633	1.49E-07
Q8N3R9	MAGUK p55 subfamily member 5	3	6	PALS1	3.881	3.3E-07
P09936	Ubiquitin carboxyl-terminal hydrolase isozyme L1	58	164	UCHL1	3.008	4.63E-07
A0A0D9SEN1	Prolyl endopeptidase FAP	16	50		3.547	5.22E-07
E9PNQ8	Thy-1 antigen (Fragment)	24	108	THY1	2.819	7.46E-07
P47813	Eukaryotic translation initiation factor 1A, X-chromosomal	25	23	EIF1AX	5.18	8.18E-07
P05362	Intercellular adhesion molecule 1	3	4	ICAM1	4.779	1.08E-06
P08243	Asparagine synthetase [glutamine-hydrolyzing]	28	91	ASNS	2.836	2.64E-06
H3BN14	Calretinin (Fragment)	13	15	CALB2	2.791	3.53E-06
J3QSU6	Tenascin	9	63	TNC	3.324	4.21E-06
Q8TC57	Meiosis 1 arrest protein	1	4	M1AP	3.948	6.92E-06
P15144	Aminopeptidase N	18	110	ANPEP	2.635	7.16E-06
P02452	Collagen alpha-1(I) chain	62	637		2.705	9.88E-06
Q9Y617	Phosphoserine aminotransferase	46	179	PSAT1	2.641	1.87E-05
P30837	Aldehyde dehydrogenase X, mitochondrial	28	68		2.781	1.99E-05
P04114	Apolipoprotein B-100	2	78		2.609	2.29E-05
Q8N1G2	Cap-specific mRNA (nucleoside-2'-O-)-methyltransferase 1	2	7	CMTR1	4.55	2.36E-05
F5GZS6	4F2 cell-surface antigen heavy chain	35	218	SLC3A2	2.591	3.04E-05
P51911	Calponin-1	42	120	CNN1	2.662	3.92E-05
Q92743	Serine protease HTRA1	9	35	HTRA1	3.029	5.84E-05
Q01650	Large neutral amino acids transporter small subunit 1	13	48	SLC7A5	2.883	7.39E-05
P20290	Transcription factor BTF3	38	47	BTF3	3.076	9.28E-05
Q9UKX5	Integrin alpha-11	4	23	ITGA11	2.802	0.000148
B1AHL2	Fibulin-1	6	41	FBLN1	2.71	0.000237
P02461	Collagen alpha-1(III) chain	11	125	COL3A1	2.293	0.000242
Q96CG8	Collagen triple helix repeat-containing protein 1	33	78	CTHRC1	2.426	0.000253
O43790	Keratin, type II cuticular Hb6	20	43	KRT86	2.755	0.000305
O43324	Eukaryotic translation elongation factor 1 epsilon-1	26	34	EEF1E1	2.691	0.000358
E9PEP6	Neuropilin	13	39	NRP1	2.64	0.000542
Q16822	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	23	83	PCK2	2.258	0.000549
Q15113	Procollagen C-endopeptidase enhancer 1	16	24	PCOLCE	2.666	0.000581

A0A087WT A8	Collagen alpha-2(I) chain	42	497		2.273	0.000679
Q96GD3	Polycomb protein SCMH1	1	3	SCMH1	3.053	0.001558
Q9Y5G7	Protocadherin gamma-A6	2	1	PCDHGA 6	3.207	0.001636
C9JBI3	O-phosphoserine phosphohydrolase (Fragment)	34	59	PSPH	2.359	0.002106
P46821	Microtubule-associated protein 1B	13	173	MAP1B	2.126	0.00276
O60701	UDP-glucose 6-dehydrogenase	57	253	UGDH	2.125	0.002782
Q02952	A-kinase anchor protein 12	9	31	AKAP12	2.395	0.002819
A0A494C1M 4	10-formyltetrahydrofolate dehydrogenase	24	139	ALDH1L2	2.083	0.003466
P54687	Branched-chain-amino-acid aminotransferase, cytosolic	12	35	BCAT1	2.134	0.004279
E5RGP0	rRNA-processing protein UTP23 homolog (Fragment)	5	1	UTP23	2.961	0.004816
P09038-1	Isoform 2 of Fibroblast growth factor 2	13	21	FGF2	2.428	0.0051
H7C5K4	Coiled-coil domain-containing protein 80 (Fragment)	13	3	CCDC80	3.041	0.005301
A1X283	SH3 and PX domain-containing protein 2B	7	15	SH3PXD2 B	2.516	0.005402
A0A1B0GV C3	GTP-binding protein Di-Ras2 (Fragment)	10	10	DIRAS2	2.471	0.005754
P0DN79	Cystathionine beta-synthase-like protein	31	63	CBS	2.067	0.005944
Q9UDY4	DnaJ homolog subfamily B member 4	39	49	DNAJB4	2.29	0.006673
Q9H0U6	39S ribosomal protein L18, mitochondrial	11	12	MRPL18	2.387	0.007258
O00469-2	Isoform 2 of Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	29	213	PLOD2	2.012	0.007911
F5GYT8	Methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial	1	3		2.929	0.008136
A0A087X0S 5	Collagen alpha-1(VI) chain	20	190		2.007	0.008317
A0A0C4DG B1	rRNA adenine N(6)-methyltransferase	11	10	DIMT1	2.59	0.009216
Q86V88	Magnesium-dependent phosphatase 1	13	4	MDP1	2.674	0.00988
Q6DKJ4	Nucleoredoxin	22	40	NXN	2.139	0.013071
A0A0A0MT 32	Lysosomal acid lipase/cholesteryl ester hydrolase	10	11	LIPA	2.135	0.013289
A0A0A0MS A4	Band 4.1-like protein 3	2	49	EPB41L3	2.681	0.013789
Q8NHP8	Putative phospholipase B-like 2	14	51	PLBD2	2.156	0.014015
O00425	Insulin-like growth factor 2 mRNA-binding protein 3	25	39	IGF2BP3	2.181	0.015111
Q15063	Periostin	7	16	POSTN	2.106	0.015564
Q9UQN3	Charged multivesicular body protein 2b	5	14	CHMP2B	2.293	0.015724
E9PLT1	Glycoprotein IIb	3	4	CD36	2.706	0.016036
Q92753	Nuclear receptor ROR-beta	3	4	RORB	2.677	0.020215
Q9H7C4	Syncoilin	10	32	SYNC	2.117	0.021963
P19022	Cadherin-2	14	44	CDH2	2.094	0.023255
Q6DKK2	Tetratricopeptide repeat protein 19, mitochondrial	6	4	TTC19	2.645	0.023471
P35580	Myosin-10	21	364	MYH10	2.078	0.023607
Q969Y0	NXPE family member 3	1	1	NXPE3	2.694	0.024905
C9JF17	Apolipoprotein D (Fragment)	3	20	APOD	2.136	0.033005
Q96A00	Protein phosphatase 1 regulatory subunit 14A	4	4	PPP1R14 A	2.295	0.036906
Q00059	Transcription factor A, mitochondrial	3	2	TFAM	2.557	0.0377
I3L1W7	Protein disulfide-isomerase-like protein of the testis (Fragment)	5	3	PDILT	2.334	0.038776

Supplementary Table 6. List of lower expressed proteins in CD intestinal fibroblasts.

Accession (UniProt)	Description	Coverage [%]	#Peptide - Spectrum Matches (PSMs)	Gene Symbol	Abundance Ratio: (Celiac) / (Ctrl)	Abundance Ratio Adj. P-Value: (Celiac) / (Ctrl)
Q96SQ9	Cytochrome P450 2S1	12	20	CYP2S1	0.162	5.58E-16
P32004	Neural cell adhesion molecule L1	5	15	L1CAM	0.153	5.58E-16
A0A0U1RR18	Solute carrier family 12 member 7 (Fragment)	4	9	SLC12A7	0.151	5.58E-16
P02545-2	Isoform C of Prelamin-A/C	75	1232	LMNA	0.139	5.58E-16
Q9BXJ9	N-alpha-acetyltransferase 15, NatA auxiliary subunit	22	132	NAA15	0.119	5.58E-16
Q9NRF9	DNA polymerase epsilon subunit 3	12	12	POLE3	0.083	5.58E-16
Q86VQ6	Thioredoxin reductase 3	3	4	TXNRD3	0.074	5.58E-16
P09488	Glutathione S-transferase Mu 1	47	63	GSTM1	0.031	5.58E-16
A0A3B3IS71	Retinoblastoma-associated protein	1	1	RB1	0.01	5.58E-16
P19012	Keratin, type I cytoskeletal 15	17	467	KRT15	0.01	5.58E-16
P03999	Short-wave-sensitive opsin 1	8	1	OPN1SW	0.01	5.58E-16
O94966	Ubiquitin carboxyl-terminal hydrolase 19	1	1	USP19	0.01	5.58E-16
D6RD48	Claudin domain-containing protein 1 (Fragment)	9	2	CLDND1	0.01	5.58E-16
Q9BZ67	FERM domain-containing protein 8	3	1	FRMD8	0.01	5.58E-16
Q9UK59	Lariat debranching enzyme	2	2	DBR1	0.01	5.58E-16
Q5VTL8	Pre-mRNA-splicing factor 38B	1	1	PRPF38B	0.01	5.58E-16
P42858	Huntingtin	0	1	HTT	0.01	5.58E-16
A0A140T8Y4	HLA class I histocompatibility antigen, A alpha chain	33	151	HLA-A	0.01	5.58E-16
Q96GM8	Target of EGR1 protein 1	2	1	TOE1	0.01	5.58E-16
C9JJ63	Transmembrane protein 176A	6	1	TMEM176A	0.01	5.58E-16
E9PMR6	Rho guanine nucleotide exchange factor 12	1	1	ARHGEF12	0.01	5.58E-16
O95832	Claudin-1	8	3	CLDN1	0.01	5.58E-16
Q9BZL6	Serine/threonine-protein kinase D2	1	2	PRKD2	0.01	5.58E-16
Q5SRE5	Nucleoporin NUP188 homolog	1	1	NUP188	0.01	5.58E-16
P49662	Caspase-4	2	3	CASP4	0.01	5.58E-16
Q8N4X5	Actin filament-associated protein 1-like 2	1	1	AFAP1L2	0.01	5.58E-16
Q9NUU6	Inactive ubiquitin thioesterase OTULINL	2	1	OTULINL	0.01	5.58E-16
Q8IYS1	Peptidase M20 domain-containing protein 2	3	1	PM20D2	0.01	5.58E-16
Q9BVC4	Target of rapamycin complex subunit LST8	2	1	MLST8	0.01	5.58E-16
O60671	Cell cycle checkpoint protein RAD1	3	3	RAD1	0.01	5.58E-16
A0A0A0MSA0	Laminin subunit alpha-3	0	1	LAMA3	0.01	5.58E-16
Q5EBM0	UMP-CMP kinase 2, mitochondrial	3	2	CMPK2	0.01	5.58E-16
M0QYI1	Bifunctional polynucleotide phosphatase/kinase (Fragment)	6	1	PNKP	0.01	5.58E-16
Q9UPY5	Cystine/glutamate transporter	2	2	SLC7A11	0.01	5.58E-16
Q9BRQ8	Ferroptosis suppressor protein 1	3	1	AIFM2	0.01	5.58E-16
F8WF27	Transmembrane 4 L6 family member 1	9	2	TM4SF1	0.01	5.58E-16
A5A3E0	POTE ankyrin domain family member F	11	1004	POTEF	0.01	5.58E-16

F8WAH1	Lysine ketoglutarate reductase	1	1	AASS	0.01	5.58E-16
Q8IUW5	RELT-like protein 1	4	1	RELL1	0.01	5.58E-16
Q96BW5	Phosphotriesterase-related protein	3	1	PTER	0.01	5.58E-16
Q9UHL4	Dipeptidyl peptidase 2	2	1	DPP7	0.01	5.58E-16
Q14689	Disco-interacting protein 2 homolog A	1	7	DIP2A	0.01	5.58E-16
Q9NV56	MRG/MORF4L-binding protein	3	3	MRGBP	0.01	5.58E-16
O75691	Small subunit processome component 20 homolog	0	1	UTP20	0.01	5.58E-16
P21980	Protein-glutamine gamma-glutamyltransferase 2	50	397	TGM2	0.234	4.71E-14
P23141	Liver carboxylesterase 1	9	12	CES1	0.183	4.71E-14
Q9BPX5	Actin-related protein 2/3 complex subunit 5-like protein	16	7	ARPC5L	0.144	9.24E-14
O00533	Neural cell adhesion molecule L1-like protein	4	13	CHL1	0.192	2.34E-13
O00534	von Willebrand factor A domain-containing protein 5A	18	44	VWA5A	0.217	7.57E-13
P08729	Keratin, type II cytoskeletal 7	46	583	KRT7	0.256	1.83E-12
O95050	Indolethylamine N-methyltransferase	8	6	INMT	0.121	1.77E-11
P05783	Keratin, type I cytoskeletal 18	39	241	KRT18	0.281	7.73E-11
P04259	Keratin, type II cytoskeletal 6B	42	974	KRT6B	0.171	7.89E-11
Q9BRX8	Peroxiredoxin-like 2A	18	22	PRXL2A	0.223	9.42E-11
P55327	Tumor protein D52	22	19	TPD52	0.217	1.15E-10
Q7Z2X4	PTB-containing, cubilin and LRP1-interacting protein	3	5	PID1	0.137	3.24E-10
O76074	cGMP-specific 3',5'-cyclic phosphodiesterase	22	113	PDE5A	0.303	7.95E-10
F8VS22	Transmembrane protein 119 (Fragment)	8	4	TMEM119	0.223	9.38E-10
Q10589	Bone marrow stromal antigen 2	6	7	BST2	0.156	1.44E-09
Q9Y570	Protein phosphatase methylesterase 1	43	170	PPME1	0.314	4.11E-09
Q04695	Keratin, type I cytoskeletal 17	32	550	KRT17	0.25	4.29E-09
Q6UVK1	Chondroitin sulfate proteoglycan 4	20	233	CSPG4	0.327	1.67E-08
Q8WWM9	Cytoglobin	64	82	CYGB	0.338	4.11E-08
Q9UGT4	Sushi domain-containing protein 2	3	5	SUSD2	0.186	1.28E-07
P53708	Integrin alpha-8	7	17	ITGA8	0.244	2.22E-07
Q99685	Monoglyceride lipase	47	98	MGLL	0.303	4.34E-07
A0A3B3IRS3	Guanylate-binding protein 7 (Fragment)	3	5	GBP7	0.199	4.67E-07
P01889	HLA class I histocompatibility antigen, B alpha chain	38	177	HLA-B	0.32	1.46E-06
D6RFI7	Hyaluronan and proteoglycan link protein 1 (Fragment)	6	3	HAPLN1	0.196	1.91E-06
Q8N474	Secreted frizzled-related protein 1	17	34	SFRP1	0.273	2.1E-06
P36269	Glutathione hydrolase 5 proenzyme	2	2	GGT5	0.213	4.23E-06
Q01469	Fatty acid-binding protein 5	44	32	FABP5	0.261	4.46E-06
O00151	PDZ and LIM domain protein 1	74	415	PDLIM1	0.395	6.04E-06
P12277	Creatine kinase B-type	32	95	CKB	0.391	6.08E-06
P16070-8	Isoform 8 of CD44 antigen	19	308	CD44	0.408	1.39E-05
P48681	Nestin	45	1046	NES	0.413	2.04E-05
E9PIN5	Tumor protein p53-inducible protein 11 (Fragment)	20	7	TP53I11	0.287	2.45E-05
P05106	Integrin beta-3	17	54	ITGB3	0.378	2.58E-05
Q6UX71	Plexin domain-containing protein 2	4	5	PLXDC2	0.28	5.52E-05
P23219	Prostaglandin G/H synthase 1	11	54	PTGS1	0.446	5.84E-05
P0DP25	Calmodulin-3	61	127	CALM3	0.449	7.12E-05
H7C173	Mitotic-spindle organizing protein 2B (Fragment)	6	1	MZT2B	0.226	7.73E-05
Q6P179	Endoplasmic reticulum aminopeptidase 2	15	87	ERAP2	0.422	8.15E-05

P68871	Hemoglobin subunit beta	63	34	HBB	0.372	8.82E-05
O95071	E3 ubiquitin-protein ligase UBR5	0	5	UBR5	0.355	9.28E-05
P17568	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 7	7	1	NDUFB7	0.219	9.53E-05
A0A140T9U0	HLA class I histocompatibility antigen, C alpha chain	24	120	HLA-C	0.38	9.73E-05
M0QXB5	Persulfide dioxygenase ETHE1, mitochondrial	27	68	ETHE1	0.441	0.0001
Q15746	Myosin light chain kinase, smooth muscle	27	461	MYLK	0.441	0.000103
P21926	CD9 antigen	10	61	CD9	0.455	0.000119
Q13642-1	Isoform 1 of Four and a half LIM domains protein 1	64	325	FHL1	0.445	0.000127
Q96H79	Zinc finger CCCH-type antiviral protein 1-like	11	17	ZC3HAV1 L	0.383	0.00013
P54652	Heat shock-related 70 kDa protein 2	47	682	HSPA2	0.448	0.000148
Q9Y5S1	Transient receptor potential cation channel subfamily V member 2	14	47	TRPV2	0.377	0.000148
Q86WV6	Stimulator of interferon genes protein	23	54	STING1	0.377	0.000191
P51636	Caveolin-2	28	22	CAV2	0.391	0.000191
J3KQL8	Apolipoprotein L2	21	77	APOL2	0.436	0.000192
Q6XQN6	Nicotinate phosphoribosyltransferase	6	8	NAPRT	0.385	0.000241
O43181	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial	19	12	NDUFS4	0.343	0.000293
K7ERE3	Keratin, type I cytoskeletal 13	24	482	KRT13	0.345	0.000318
A0A3B3IRM5	Glutathione S-transferase (Fragment)	51	47	GSTM5	0.298	0.000721
E9PJV6	Syntaxin-17	13	3	STX17	0.29	0.000724
A6NHL2	Tubulin alpha chain-like 3	9	205	TUBAL3	0.367	0.000764
Q9BWD1	Acetyl-CoA acetyltransferase, cytosolic	36	91	ACAT2	0.482	0.000846
Q86YZ3	Hornerin	11	34	HRNR	0.367	0.00086
Q9NZT1	Calmodulin-like protein 5	16	8	CALML5	0.406	0.001104
A8MZA4	Golgin subfamily A member 6-like protein 6	2	2	GOLGA6L 6	0.278	0.001134
P49454	Centromere protein F	0	1	CENPF	0.417	0.001345
Q9HCL0	Protocadherin-18	1	4	PCDH18	0.411	0.00198
Q5TAW7	Calcium-binding protein 39-like (Fragment)	14	20	CAB39L	0.422	0.002024
Q562R1	Beta-actin-like protein 2	20	866	ACTBL2	0.463	0.002057
H3BNN1	Estradiol 17-beta-dehydrogenase 2 (Fragment)	4	5	HSD17B2	0.318	0.002061
Q9UJC3	Protein Hook homolog 1	2	1	HOOK1	0.442	0.002357
P16949	Stathmin	21	10	STMN1	0.35	0.002632
F2Z2E2	Ras GTPase-activating-like protein IQGAP3	6	41	IQGAP3	0.438	0.002672
Q9H0W9	Ester hydrolase C11orf54	9	27	C11orf54	0.463	0.003069
Q9Y281	Cofilin-2	83	115	CFL2	0.461	0.003601
P29466	Caspase-1	19	42	CASP1	0.465	0.003828
Q6QNY1	Biogenesis of lysosome-related organelles complex 1 subunit 2	8	3	BLOC1S2	0.323	0.003938
P68133	Actin, alpha skeletal muscle	64	1943	ACTA1	0.327	0.005005
E7ESP4	Integrin alpha-2	28	266	ITGA2	0.456	0.006551
Q8WUI4	Histone deacetylase 7	2	3	HDAC7	0.431	0.007403
F8VQX6	Methyltransferase-like protein 7A (Fragment)	23	14	TMT1A	0.445	0.009216
Q9BY67	Cell adhesion molecule 1	3	11	CADM1	0.472	0.009739
P61587	Rho-related GTP-binding protein RhoE	33	69	RND3	0.452	0.009857
Q9UNN8	Endothelial protein C receptor	14	27	PROCR	0.47	0.01035
P31323	cAMP-dependent protein kinase type II-beta regulatory subunit	20	27	PRKAR2B	0.481	0.011241

Q14997	Proteasome activator complex subunit 4	2	5	PSME4	0.497	0.011652
Q96KB5	Lymphokine-activated killer T-cell-originated protein kinase	10	24	PBK	0.484	0.011652
H0Y8P3	LIM and calponin homology domains-containing protein 1 (Fragment)	1	2	LIMCH1	0.366	0.011652
P24385	G1/S-specific cyclin-D1	18	31	CCND1	0.496	0.012116
P08172	Muscarinic acetylcholine receptor M2	4	5	CHRM2	0.477	0.012152
F5GWG3	Retinoic acid-induced protein 3 (Fragment)	5	2	GPRC5A	0.358	0.013346
P30530	Tyrosine-protein kinase receptor UFO	3	13	AXL	0.423	0.013454
E9PJP2	Protein SOGA3	1	3	MTCL3	0.463	0.014203
H0YLP8	ER membrane protein complex subunit 4 (Fragment)	31	10	EMC4	0.372	0.014203
O95297	Myelin protein zero-like protein 1	16	17	MPZL1	0.447	0.014257
P19013	Keratin, type II cytoskeletal 4	16	210	KRT4	0.379	0.014785
P40306	Proteasome subunit beta type-10	18	26	PSMB10	0.457	0.015441
Q2L6G2	HLA class I histocompatibility antigen B alpha chain	32	98	HLA-B	0.435	0.015441
Q9H1J7	Protein Wnt-5b	10	6	WNT5B	0.482	0.017856
O75762	Transient receptor potential cation channel subfamily A member 1	7	39	TRPA1	0.488	0.018274
Q96C23	Galactose mutarotase	16	58	GALM	0.479	0.018933
C9J3D7	Peroxisomal carnitine O-octanoyltransferase	6	11	CROT	0.479	0.020215
P48960	Adhesion G protein-coupled receptor E5	8	21	ADGRE5	0.494	0.022426
E9PHT2	Centrosome-associated protein CEP250	1	3	CEP250	0.39	0.023851
E9PLD3	Uncharacterized protein	13	1		0.372	0.024892
Q8NC51-3	Isoform 3 of Plasminogen activator inhibitor 1 RNA-binding protein	43	220	SERBP1	0.432	0.031311
I3L3T3	N-sulphoglucosamine sulphohydrolase (Fragment)	31	4	SGSH	0.41	0.034511
Q92478	C-type lectin domain family 2 member B	19	3	CLEC2B	0.397	0.03761
A0A0C4DFS6	Protein sprouty homolog 4	4	5	SPRY4	0.412	0.038709
H0YBT8	Transforming acidic coiled-coil-containing protein 1 (Fragment)	3	2	TACC1	0.406	0.038963
Q5TDE7	E3 ubiquitin-protein ligase RNF220 (Fragment)	5	1	RNF220	0.388	0.039112
F6SYF8	Dickkopf-related protein 3	3	1	DKK3	0.421	0.03996
K7EKW3	Transmembrane and ubiquitin-like domain-containing protein 2 (Fragment)	11	4	TMUB2	0.415	0.040415
A0A087WYN0	Transporter	7	9	SLC6A6	0.488	0.041895
P63096	Guanine nucleotide-binding protein G(i) subunit alpha-1	33	79	GNAI1	0.489	0.042617
P10646	Tissue factor pathway inhibitor	3	2	TFPI	0.407	0.045862
P62491	Ras-related protein Rab-11A	60	215	RAB11A	0.425	0.046495
Q9NZQ7	Programmed cell death 1 ligand 1	6	11	CD274	0.453	0.048337

Supplementary Table 7. List of downexpressed transcripts in CD intestinal fibroblasts following the ZED1227 treatment.

Gene Symbol	Description	log2FoldChange	P-value
None	novel transcript, antisense to TPT1	-0.94695	0.002263
None	novel transcript, antisense to COL3A1	-0.7791	0.02797
None	novel transcript	-0.62109	0.018649
FAM24B	family with sequence similarity 24 member B	-0.58817	0.015975
None	novel transcript	-0.58013	0.015548
NBPF2P	NBPF member 2, pseudogene	-0.56413	0.020557
RTCA-AS1	RTCA antisense RNA 1	-0.55555	0.037031
BEST1	bestrophin 1	-0.52644	0.024489
ANKRD36	ankyrin repeat domain 36	-0.44939	0.046032
CCDC18-AS1	CCDC18 antisense RNA 1	-0.39592	0.041555
ZSCAN23	zinc finger and SCAN domain containing 23	-0.39529	0.048457
UCN2	urocortin 2	-0.37973	0.038811
MIR5047	microRNA 5047	-0.37085	0.000588
PABPC1L	poly(A) binding protein cytoplasmic 1 like	-0.34075	0.015064
RARB	retinoic acid receptor beta	-0.33342	0.028847
None	novel transcript	-0.33114	0.020637
BMP4	bone morphogenetic protein 4	-0.32808	0.04859
PLEKHG4	pleckstrin homology and RhoGEF domain containing G4	-0.32517	0.030977
KCNQ1OT1	KCNQ1 opposite strand/antisense transcript 1	-0.31955	0.046944
CSAD	cysteine sulfinic acid decarboxylase	-0.31881	0.013091
VAMP1	vesicle associated membrane protein 1	-0.31127	0.007009
FAT3	FAT atypical cadherin 3	-0.30752	0.000756
MIR503HG	MIR503 host gene	-0.29085	0.00494
GPER1	G protein-coupled estrogen receptor 1	-0.28154	0.032068
MEG3	maternally expressed 3	-0.28049	1.25E-12
DNASE1	deoxyribonuclease 1	-0.27714	0.027297
GALNT15	polypeptide N-acetylgalactosaminyltransferase 15	-0.26249	0.048708
PRAG1	PEAK1 related, kinase-activating pseudokinase 1	-0.26124	0.008032
ESPL1	extra spindle pole bodies like 1, separate	-0.25266	0.007531
ZBTB20	zinc finger and BTB domain containing 20	-0.25016	0.031405
FANCG	FA complementation group G	-0.2437	0.03933
RNF215	ring finger protein 215	-0.23388	0.037728
USP31	ubiquitin specific peptidase 31	-0.23145	0.043864
FRY	FRY microtubule binding protein	-0.21896	0.007535
NAV2	neuron navigator 2	-0.20301	0.00077
STARD9	StAR related lipid transfer domain containing 9	-0.19855	0.038942
GPRC5A	G protein-coupled receptor class C group 5 member A	-0.19699	0.006956
EPHA4	EPH receptor A4	-0.19304	0.015246
CPA4	carboxypeptidase A4	-0.19232	0.00241
ZXDC	ZXD family zinc finger C	-0.19125	0.048674
NEURL1B	neuralized E3 ubiquitin protein ligase 1B	-0.1903	0.034614
SNHG1	small nucleolar RNA host gene 1	-0.19	0.008141
LENG8	leukocyte receptor cluster member 8	-0.18442	7.07E-05
CDCA3	cell division cycle associated 3	-0.18208	0.032166
IQGAP3	IQ motif containing GTPase activating protein 3	-0.18064	0.010055
TAF1C	TATA-box binding protein associated factor, RNA polymerase I subunit C	-0.17983	0.013668
TUBGCP6	tubulin gamma complex component 6	-0.17768	0.015364

KIF20A	kinesin family member 20A	-0.17066	0.004601
SGSM2	small G protein signaling modulator 2	-0.16798	0.006848
UXT	ubiquitously expressed prefoldin like chaperone	-0.16609	0.041319
RSRP1	arginine and serine rich protein 1	-0.1645	0.021293
NABP1	nucleic acid binding protein 1	-0.164	0.000275
CCNL2	cyclin L2	-0.16044	0.001747
CCDC14	coiled-coil domain containing 14	-0.15458	0.017158
SPEG	striated muscle enriched protein kinase	-0.15101	0.008829
HOXB3	homeobox B3	-0.14903	0.024984
WSB1	WD repeat and SOCS box containing 1	-0.14226	0.017373
TACC3	transforming acidic coiled-coil containing protein 3	-0.14101	0.017623
ID1	inhibitor of DNA binding 1	-0.13976	0.007824
CAPN5	calpain 5	-0.13936	0.031877
MT-ATP8	mitochondrially encoded ATP synthase membrane subunit 8	-0.13866	0.000789
CIT	citron rho-interacting serine/threonine kinase	-0.13799	0.046972
RBM5	RNA binding motif protein 5	-0.13426	0.010605
MKI67	marker of proliferation Ki-67	-0.12943	0.006695
NRP2	neuropilin 2	-0.12749	0.005881
SMAD3	SMAD family member 3	-0.12684	0.01173
NCAPD2	non-SMC condensin I complex subunit D2	-0.12623	0.010546
PDCD11	programmed cell death 11	-0.12234	0.023996
SRSF5	serine and arginine rich splicing factor 5	-0.12226	0.006993
NCAPG2	non-SMC condensin II complex subunit G2	-0.11742	0.049563
CCDC9B	coiled-coil domain containing 9B	-0.11645	0.020765
MT-ND2	mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 2	-0.11544	0.000258
LDB1	LIM domain binding 1	-0.11493	0.017435
SUGP2	SURP and G-patch domain containing 2	-0.11264	0.027914
POLR1A	RNA polymerase I subunit A	-0.11116	0.031325
MTATP6P1	MT-ATP6 pseudogene 1	-0.11073	0.010472
MT-ND5	mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 5	-0.10908	0.000328
SIK2	salt inducible kinase 2	-0.10878	0.03813
XYLT1	xylosyltransferase 1	-0.10871	0.015185
ARFGAP1	ADP ribosylation factor GTPase activating protein 1	-0.10615	0.02052
UBR4	ubiquitin protein ligase E3 component n-recogin 4	-0.10601	0.001163
SDC1	syndecan 1	-0.10355	0.039411
MAT2A	methionine adenosyltransferase 2A	-0.10293	0.008623
CIRBP	cold inducible RNA binding protein	-0.09617	0.02665
MT-ATP6	mitochondrially encoded ATP synthase membrane subunit 6	-0.09593	0.000861
DHCR24	24-dehydrocholesterol reductase	-0.09491	0.025033
VMP1	vacuole membrane protein 1	-0.0924	0.031115
RBMX	RNA binding motif protein X-linked	-0.09045	0.030937
EPG5	ectopic P-granules 5 autophagy tethering factor	-0.08973	0.042577
SNRNP200	small nuclear ribonucleoprotein U5 subunit 200	-0.08518	0.016368
PTPN14	protein tyrosine phosphatase non-receptor type 14	-0.08372	0.025802
PRKDC	protein kinase, DNA-activated, catalytic subunit	-0.08335	0.018253
MT-CO1	mitochondrially encoded cytochrome c oxidase I	-0.0799	0.003242
SRRM2	serine/arginine repetitive matrix 2	-0.0796	0.008776
DDX5	DEAD-box helicase 5	-0.07902	0.017062
MT-ND4L	mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 4L	-0.07808	0.015679
DDX17	DEAD-box helicase 17	-0.07793	0.021783
FASN	fatty acid synthase	-0.07758	0.033539

HEG1	heart development protein with EGF like domains 1	-0.0762	0.010822
SCD	stearoyl-CoA desaturase	-0.07502	0.041314
CSPG4	chondroitin sulfate proteoglycan 4	-0.07333	0.041494
MT-CO2	mitochondrially encoded cytochrome c oxidase II	-0.06981	0.014288
MT-ND4	mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 4	-0.06886	0.007557
PRPF8	pre-mRNA processing factor 8	-0.06701	0.03666
MT-CO3	mitochondrially encoded cytochrome c oxidase III	-0.06644	0.011308
HUWE1	HECT, UBA and WWE domain containing E3 ubiquitin protein ligase 1	-0.06619	0.039224
MT-CYB	mitochondrially encoded cytochrome b	-0.0636	0.020497
MT-ND1	mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 1	-0.06171	0.047435
SHC1	SHC adaptor protein 1	-0.06127	0.043931

Supplementary Table 8. List of upexpressed transcripts in CD intestinal fibroblasts following the ZED1227 treatment.

Gene Symbol	Description	log2FoldChange	P-value
TPM4	tropomyosin 4	0.062	0.020534229
MORF4L1	mortality factor 4 like 1	0.064	0.049948531
GPC1	glypican 1	0.065	0.042789916
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta	0.066	0.03421969
HSP90B1	heat shock protein 90 beta family member 1	0.066	0.023253893
TPT1	tumor protein, translationally-controlled 1	0.067	0.028981072
COL8A1	collagen type VIII alpha 1 chain	0.067	0.043673145
NPM1	nucleophosmin 1	0.068	0.028565209
COL3A1	collagen type III alpha 1 chain	0.068	0.015854668
EXT1	exostosin glycosyltransferase 1	0.068	0.035074743
IGFBP4	insulin like growth factor binding protein 4	0.068	0.027340276
VAT1	vesicle amine transport 1	0.069	0.037038679
CALM1	calmodulin 1	0.070	0.043590231
PALLD	palladin, cytoskeletal associated protein	0.071	0.021188413
COL6A1	collagen type VI alpha 1 chain	0.071	0.013753655
ITGB1	integrin subunit beta 1	0.071	0.022751156
EID1	EP300 interacting inhibitor of differentiation 1	0.072	0.041466992
FTL	ferritin light chain	0.073	0.020934749
TTYH3	tweety family member 3	0.073	0.029985953
PGD	phosphogluconate dehydrogenase	0.073	0.038779936
COL6A2	collagen type VI alpha 2 chain	0.074	0.013007628
ALCAM	activated leukocyte cell adhesion molecule	0.077	0.020325848
EIF3A	eukaryotic translation initiation factor 3 subunit A	0.077	0.029185183
MGST1	microsomal glutathione S-transferase 1	0.077	0.044706714
RTN4	reticulon 4	0.078	0.005793764
ITGAV	integrin subunit alpha V	0.079	0.039501735
HNRNPA3	heterogeneous nuclear ribonucleoprotein A3	0.080	0.033547072
NREP	neuronal regeneration related protein	0.080	0.045604866
CCNI	cyclin I	0.081	0.022946418

TALDO1	transaldolase 1	0.081	0.021974341
DNAJA1	DnaJ heat shock protein family (Hsp40) member A1	0.082	0.034925016
MAP1LC3B	microtubule associated protein 1 light chain 3 beta	0.083	0.042035134
TRAM1	translocation associated membrane protein 1	0.083	0.017183049
CNN3	calponin 3	0.083	0.010031861
SEC22B	SEC22 homolog B. vesicle trafficking protein	0.083	0.035333888
NHERF2	NHERF family PDZ scaffold protein 2	0.083	0.046057131
SDCBP	syndecan binding protein	0.083	0.034527371
PTPN11	protein tyrosine phosphatase non-receptor type 11	0.083	0.033465587
ITM2B	integral membrane protein 2B	0.085	0.015603632
RAB1A	RAB1A. member RAS oncogene family	0.085	0.038144845
SRP9	signal recognition particle 9	0.087	0.033562891
NAP1L1	nucleosome assembly protein 1 like 1	0.088	0.010985287
NAA50	N-alpha-acetyltransferase 50. NatE catalytic subunit	0.088	0.03544677
RHOQ	ras homolog family member Q	0.088	0.043675576
HYCC1	hyccin PI4KA lipid kinase complex subunit 1	0.088	0.044244047
LPAR1	lysophosphatidic acid receptor 1	0.088	0.040778644
RCN1	reticulocalbin 1	0.088	0.034215889
NECTIN3	nectin cell adhesion molecule 3	0.089	0.037454117
ADAM9	ADAM metalloproteinase domain 9	0.089	0.014490758
SEL1L3	SEL1L family member 3	0.090	0.039108284
ZMPSTE24	zinc metalloproteinase STE24	0.090	0.048277433
ADH5	alcohol dehydrogenase 5 (class III). chi polypeptide	0.090	0.017700425
ASAH1	N-acylsphingosine amidohydrolase 1	0.090	0.046730646
SOD2	superoxide dismutase 2	0.090	0.037337948
ACTR2	actin related protein 2	0.091	0.012774359
NCKAP1	NCK associated protein 1	0.091	0.028601992
RCN2	reticulocalbin 2	0.091	0.046959351
DSTN	destrin. actin depolymerizing factor	0.091	0.001400178
ATP2C1	ATPase secretory pathway Ca2+ transporting 1	0.091	0.043288467
ASPH	aspartate beta-hydroxylase	0.092	0.010614398
GALNT5	polypeptide N-acetylgalactosaminyltransferase 5	0.092	0.026693187
SNAI2	snail family transcriptional repressor 2	0.092	0.039921439
ANGPTL2	angiopoietin like 2	0.092	0.040101431
SPCS3	signal peptidase complex subunit 3	0.093	0.028924686
ARF6	ADP ribosylation factor 6	0.093	0.039524655
RAB2A	RAB2A. member RAS oncogene family	0.093	0.020330504
PPP1CB	protein phosphatase 1 catalytic subunit beta	0.094	0.03852959
PSAT1	phosphoserine aminotransferase 1	0.094	0.03676822
PSMD7	proteasome 26S subunit. non-ATPase 7	0.094	0.044238718
SLC1A5	solute carrier family 1 member 5	0.094	0.008560584
CYBRD1	cytochrome b reductase 1	0.095	0.01364752
RND3	Rho family GTPase 3	0.095	0.015542023
RAB14	RAB14. member RAS oncogene family	0.095	0.040768363
VEGFC	vascular endothelial growth factor C	0.095	0.024000071
NOTCH3	notch receptor 3	0.096	0.045303547
EDIL3	EGF like repeats and discoidin domains 3	0.096	0.008573845
OSTC	oligosaccharyltransferase complex non-catalytic subunit	0.096	0.04216569
TMEM123	transmembrane protein 123	0.096	0.029378355
SERP1	stress associated endoplasmic reticulum protein 1	0.096	0.020628571
TXNDC15	thioredoxin domain containing 15	0.097	0.044122161

EIF5B	eukaryotic translation initiation factor 5B	0.098	0.02047602
VGLL3	vestigial like family member 3	0.098	0.032918112
BNIP3	BCL2 interacting protein 3	0.099	0.038185135
PYGL	glycogen phosphorylase L	0.099	0.030832794
CDC42EP3	CDC42 effector protein 3	0.099	0.00385935
TMX3	thioredoxin related transmembrane protein 3	0.100	0.024964931
MATR3	matrin 3	0.100	0.009426482
CAMSAP2	calmodulin regulated spectrin associated protein family member 2	0.100	0.037870973
YPEL5	yippee like 5	0.101	0.041319095
USP46	ubiquitin specific peptidase 46	0.101	0.04683648
KPNA4	karyopherin subunit alpha 4	0.101	0.027051595
ITGA11	integrin subunit alpha 11	0.102	0.005172318
EIF2S2	eukaryotic translation initiation factor 2 subunit beta	0.102	0.041496667
TXNRD1	thioredoxin reductase 1	0.102	0.002938499
HNRNPH2	heterogeneous nuclear ribonucleoprotein H2	0.102	0.044396925
HIPK3	homeodomain interacting protein kinase 3	0.103	0.038427968
TFPI2	tissue factor pathway inhibitor 2	0.103	0.035151903
NARS1	asparaginyl-tRNA synthetase 1	0.103	0.022495616
C11orf58	chromosome 11 open reading frame 58	0.103	0.018430921
CYCS	cytochrome c. somatic	0.104	0.017537252
ARL8B	ADP ribosylation factor like GTPase 8B]	0.104	0.028393699
ACTR3	actin related protein 3	0.104	0.001995218
RABEP1	rabaptin. RAB GTPase binding effector protein 1	0.104	0.037750558
ERBIN	erbB2 interacting protein	0.104	0.013467667
HDAC2	histone deacetylase 2	0.105	0.017367376
RAB6A	RAB6A. member RAS oncogene family	0.105	0.014964445
TMOD3	tropomodulin 3	0.105	0.024378541
TAX1BP1	Tax1 binding protein 1	0.105	0.021218586
GOLGB1	golgin B1	0.105	0.032871788
FERMT2	FERM domain containing kindlin 2	0.106	0.0186563
STAT1	signal transducer and activator of transcription 1	0.106	0.005250567
KLHL5	kelch like family member 5	0.106	0.028138391
ATP8B1	ATPase phospholipid transporting 8B1	0.107	0.018498634
CTHRC1	collagen triple helix repeat containing 1	0.108	0.022690649
LRRFIP1	LRR binding FLII interacting protein 1	0.108	0.021787856
VPS4B	vacuolar protein sorting 4 homolog B	0.108	0.049467948
TMED7	transmembrane p24 trafficking protein 7	0.108	0.037069977
MTMR2	myotubularin related protein 2	0.109	0.029502992
ZNF638	zinc finger protein 638	0.109	0.042188064
TP53I11	tumor protein p53 inducible protein 11	0.109	0.005681577
GNG11	G protein subunit gamma 11	0.110	0.006273665
CHMP5	charged multivesicular body protein 5	0.110	0.045515637
SMURF2	SMAD specific E3 ubiquitin protein ligase 2	0.110	0.023116632
SUB1	SUB1 regulator of transcription	0.111	0.018374701
GOLGA5	golgin A5	0.111	0.045151742
SEC63	SEC63 homolog. protein translocation regulator	0.111	0.028597515
LHFPL6	LHFPL tetraspan subfamily member 6	0.111	0.009343065
DESI2	desumoylating isopeptidase 2	0.111	0.029994962
SNW1	SNW domain containing 1	0.112	0.037422794
CAB39	calcium binding protein 39	0.112	0.035780413
LIMS1	LIM zinc finger domain containing 1	0.113	0.013515844

ECHDC1	ethylmalonyl-CoA decarboxylase 1	0.113	0.047361447
OSBPL8	oxysterol binding protein like 8	0.113	0.032470917
ROCK1	Rho associated coiled-coil containing protein kinase 1	0.113	0.025596801
EIF3E	eukaryotic translation initiation factor 3 subunit E	0.114	0.007727684
TLK1	tousled like kinase 1	0.114	0.049225852
TM9SF3	transmembrane 9 superfamily member 3	0.115	0.002519573
SWAP70	switching B cell complex subunit SWAP70	0.115	0.042271344
TARS1	threonyl-tRNA synthetase 1	0.115	0.010930399
ABCC4	ATP binding cassette subfamily C member 4 (PEL blood group)	0.115	0.010505927
CALM2	calmodulin 2	0.115	0.000784524
FILIP1L	filamin A interacting protein 1 like	0.116	0.033063628
F2RL2	coagulation factor II thrombin receptor like 2	0.116	0.007837842
CAMK2N1	calcium/calmodulin dependent protein kinase II inhibitor 1	0.116	0.020334785
LRRFIP2	LRR binding FLII interacting protein 2	0.116	0.022437295
GNA13	G protein subunit alpha 13	0.116	0.020900351
SNX9	sorting nexin 9	0.116	0.011193058
SSB	small RNA binding exonuclease protection factor La	0.117	0.011392593
SMARCA5	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5	0.118	0.010938569
CAPZA2	capping actin protein of muscle Z-line subunit alpha 2	0.118	0.010644876
DDAH1	dimethylarginine dimethylaminohydrolase 1	0.119	0.006127031
UBE2V2	ubiquitin conjugating enzyme E2 V2	0.119	0.030186873
IRF2BPL	interferon regulatory factor 2 binding protein like	0.119	0.017262562
P4HA1	prolyl 4-hydroxylase subunit alpha 1	0.119	0.00438273
FGF2	fibroblast growth factor 2	0.119	0.004637849
SBDS	SBDS ribosome maturation factor	0.119	0.003349002
SH3BGR1	SH3 domain binding glutamate rich protein like	0.120	0.011509762
PSMA3	proteasome 20S subunit alpha 3	0.120	0.023081076
HSP90AA1	heat shock protein 90 alpha family class A member 1	0.121	0.000510465
NIPA2	NIPA magnesium transporter 2	0.121	0.020227289
ERP44	endoplasmic reticulum protein 44	0.121	0.030824203
UGDH	UDP-glucose 6-dehydrogenase	0.121	0.010372609
FTTH1	ferritin heavy chain 1	0.122	2.59427E-05
SLC25A46	solute carrier family 25 member 46	0.123	0.035519061
SLK	STE20 like kinase	0.123	0.044753266
AHR	aryl hydrocarbon receptor	0.124	0.030927971
F3	coagulation factor III. tissue factor	0.124	0.001376522
UBL3	ubiquitin like 3	0.124	0.024440424
TBC1D23	TBC1 domain family member 23	0.125	0.046423564
VAPA	VAMP associated protein A	0.126	0.00345717
SCP2	sterol carrier protein 2	0.126	0.008954315
PRKACB	protein kinase cAMP-activated catalytic subunit beta	0.126	0.027188195
RIMOC1	RAB7A interacting MON1-CCZ1 complex subunit 1	0.126	0.046244618
CMPK1	cytidine/uridine monophosphate kinase 1	0.126	0.006043855
GBE1	1,4-alpha-glucan branching enzyme 1	0.126	0.005850627
DNAJC21	DnaJ heat shock protein family (Hsp40) member C21	0.127	0.049213615
PHLDA1	pleckstrin homology like domain family A member 1	0.127	0.00318508
ME1	malic enzyme 1	0.127	0.010395239
GCLM	glutamate-cysteine ligase modifier subunit	0.127	0.018243136
C4orf3	chromosome 4 open reading frame 3	0.128	0.002958197
SERINC1	serine incorporator 1	0.128	0.001457621

RAB18	RAB18, member RAS oncogene family	0.129	0.008096736
PTGR1	prostaglandin reductase 1	0.131	0.009373878
TCEAL9	transcription elongation factor A like 9	0.131	0.009586413
ANXA1	annexin A1	0.132	0.000282082
STRN3	striatin 3	0.132	0.043162405
MYO1B	myosin IB	0.132	0.002728396
RAP1A	RAP1A, member of RAS oncogene family	0.132	0.025465097
DYNLT3	dynein light chain Tctex-type 3	0.132	0.045611158
SNX18	sorting nexin 18	0.134	0.008844035
RNF6	ring finger protein 6	0.134	0.045786823
NQO1	NAD(P)H quinone dehydrogenase 1	0.134	1.70631E-05
BNIP2	BCL2 interacting protein 2	0.135	0.008161603
HK2	hexokinase 2	0.135	0.028587265
TNFRSF11B	TNF receptor superfamily member 11b	0.136	0.000556943
SEPTIN7	septin 7	0.136	0.001839672
CNOT6	CCR4-NOT transcription complex subunit 6	0.137	0.038785435
GALNT1	polypeptide N-acetylgalactosaminyltransferase 1	0.137	0.00724359
CBR1	carbonyl reductase 1	0.138	0.013713279
SEC62	SEC62 homolog, preprotein translocation factor	0.138	0.00690256
FAM8A1	family with sequence similarity 8 member A1	0.138	0.022155403
PCDH9	protocadherin 9	0.138	0.046586409
MINPP1	multiple inositol-polyphosphate phosphatase 1	0.138	0.048638499
ATG12	autophagy related 12	0.139	0.015229501
YES1	YES proto-oncogene 1, Src family tyrosine kinase	0.140	0.012144627
GREM1	gremlin 1, DAN family BMP antagonist	0.140	0.000137892
RALA	RAS like proto-oncogene A	0.141	0.003897807
ADSS2	adenylosuccinate synthase 2	0.141	0.016649645
PAWR	pro-apoptotic WT1 regulator	0.142	0.00835495
NAMPT	nicotinamide phosphoribosyltransferase	0.142	0.023265604
RAB21	RAB21, member RAS oncogene family	0.142	0.015584245
ZEB1	zinc finger E-box binding homeobox 1	0.143	0.009512071
ZC3H15	zinc finger CCCH-type containing 15	0.143	0.016570671
MEMO1	mediator of cell motility 1	0.143	0.040767296
VPS26A	VPS26 retromer complex component A	0.144	0.009220542
VBP1	VHL binding protein 1	0.144	0.030111109
ROCK2	Rho associated coiled-coil containing protein kinase 2	0.145	0.003176137
AK5	adenylate kinase 5	0.145	0.003455802
DEK	DEK proto-oncogene	0.146	0.006519446
LUM	lumican	0.147	0.000872231
CFAP97	cilia and flagella associated protein 97	0.149	0.012714771
CSGALNAC T2	chondroitin sulfate N-acetylgalactosaminyltransferase 2	0.149	0.015708398
DCAF13	DDB1 and CUL4 associated factor 13	0.149	0.010052254
UPF2	UPF2 regulator of nonsense mediated mRNA decay	0.150	0.024432131
NFE2L2	NFE2 like bZIP transcription factor 2	0.152	0.000449704
PCNP	PEST proteolytic signal containing nuclear protein	0.154	0.002164682
TXN	thioredoxin	0.155	4.99362E-05
PCGF5	polycomb group ring finger 5	0.155	0.01647766
SOCS6	suppressor of cytokine signaling 6	0.156	0.018726823
FGFR1OP2	FGFR1 oncogene partner 2	0.157	0.043361434
GMFB	glia maturation factor beta	0.157	0.003173144

TAF13	TATA-box binding protein associated factor 13	0.157	0.003930496
AASDHPPT	aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase	0.157	0.018806604
SMC3	structural maintenance of chromosomes 3	0.158	0.009502022
SHOC2	SHOC2 leucine rich repeat scaffold protein	0.158	0.007162735
ZZZ3	zinc finger ZZ-type containing 3	0.159	0.015281203
GNAI1	G protein subunit alpha i1	0.159	0.005263312
TDP2	tyrosyl-DNA phosphodiesterase 2	0.159	0.018788926
RMDN1	regulator of microtubule dynamics 1	0.160	0.016898218
OXR1	oxidation resistance 1	0.160	0.046838575
GCLC	glutamate-cysteine ligase catalytic subunit	0.161	0.011752431
SMIM15	small integral membrane protein 15	0.162	0.007683986
ETNK1	ethanolamine kinase 1	0.164	0.028394803
DNAJB4	DnaJ heat shock protein family (Hsp40) member B4	0.164	0.004951511
PRELID3B	PRELI domain containing 3B	0.165	0.009618205
NMD3	NMD3 ribosome export adaptor	0.165	0.006042595
MIER1	MIER1 transcriptional regulator	0.166	0.012196047
SUCLA2	succinate-CoA ligase ADP-forming subunit beta	0.168	0.008790842
FOXF1	forkhead box F1	0.169	0.000708937
COPS2	COP9 signalosome subunit 2	0.170	0.001453447
GOLGA4	golgin A4	0.171	0.001676665
ANKRD12	ankyrin repeat domain 12	0.171	0.03603653
CPOX	coproporphyrinogen oxidase	0.171	0.040724229
SLC2A1	solute carrier family 2 member 1	0.172	7.00663E-05
COMMD2	COMM domain containing 2	0.172	0.0164494
TWF1	twinfilin actin binding protein 1	0.173	0.000908752
PITX1	paired like homeodomain 1	0.173	0.000573309
TVP23B	trans-golgi network vesicle protein 23 homolog B	0.174	0.034474516
NIFK	nucleolar protein interacting with the FHA domain of MKI67	0.178	0.013576379
SNX4	sorting nexin 4	0.179	0.022748427
WFDC1	WAP four-disulfide core domain 1	0.179	0.00378042
DNAJB9	DnaJ heat shock protein family (Hsp40) member B9	0.180	0.041865558
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha	0.180	0.009325152
NEXN	nexilin F-actin binding protein	0.180	0.000515881
AMIGO2	adhesion molecule with Ig like domain 2	0.181	0.001318478
BEX1	brain expressed X-linked 1	0.182	0.020689467
ARMT1	acidic residue methyltransferase 1	0.185	0.015648559
LAMTOR3	late endosomal/lysosomal adaptor, MAPK and MTOR activator 3	0.188	0.01031518
IL1RAP	interleukin 1 receptor accessory protein	0.189	0.047687178
GTF2F2	general transcription factor IIF subunit 2	0.189	0.00351438
SYF2	SYF2 pre-mRNA splicing factor	0.189	0.011565643
JAZF1	JAZF zinc finger 1	0.190	0.009091678
AP5M1	adaptor related protein complex 5 subunit mu 1	0.191	0.00639293
RHOT1	ras homolog family member T1	0.192	0.007286736
STK38L	serine/threonine kinase 38 like	0.192	0.046486193
POSTN	periostin	0.193	0.006196245
HINT3	histidine triad nucleotide binding protein 3	0.194	0.016482254
LURAP1L	leucine rich adaptor protein 1 like	0.195	0.016050855
VPS54	VPS54 subunit of GARP complex	0.199	0.014509441
INSIG2	insulin induced gene 2	0.200	0.032946858
ACAN	aggrecan	0.202	0.002793855

NCK1	NCK adaptor protein 1	0.203	0.007385858
PRKAR2B	protein kinase cAMP-dependent type II regulatory subunit beta	0.207	0.002868373
PTX3	pentraxin 3	0.212	0.005608348
SPRYD7	SPRY domain containing 7	0.214	0.019641825
RASSF2	Ras association domain family member 2	0.219	0.000916392
TBX2	T-box transcription factor 2	0.220	0.000537704
NTN4	netrin 4	0.221	0.014857313
LRRC2	leucine rich repeat containing 2	0.223	0.027291867
RGS5	regulator of G protein signaling 5	0.223	0.016004071
CCDC91	coiled-coil domain containing 91	0.226	0.018248072
TTC39C	tetratricopeptide repeat domain 39C	0.227	0.01309731
ALDH1A1	aldehyde dehydrogenase 1 family member A1	0.233	0.003794662
PIR	pirin	0.234	0.018751418
SHISAL1	shisa like 1	0.234	0.000527334
MSC	musculin	0.239	0.013416966
ADAMTS14	ADAM metalloproteinase with thrombospondin type 1 motif 14	0.242	0.045879665
INPP4B	inositol polyphosphate-4-phosphatase type II B	0.243	0.018568354
SECTM1	secreted and transmembrane 1	0.247	0.031204344
COL5A3	collagen type V alpha 3 chain	0.250	0.017288621
FOXC2	forkhead box C2	0.253	0.034116516
STC2	stanniocalcin 2	0.254	5.87643E-10
BMP2	bone morphogenetic protein 2	0.256	0.027315497
SSTR1	somatostatin receptor 1	0.285	0.034637847
EFNA5	ephrin A5	0.295	0.045139283
TSPAN2	tetraspanin 2	0.307	0.043606306
EREG	epiregulin	0.335	0.020090837
IL6	interleukin 6	0.350	0.049214265
FAM43A	family with sequence similarity 43 member A	0.351	0.00196052
TIPARP	TCDD inducible poly(ADP-ribose) polymerase	0.360	3.79777E-07
CYP1B1	cytochrome P450 family 1 subfamily B member 1	0.369	0.007397885
ENOX1	ecto-NOX disulfide-thiol exchanger 1	0.374	0.015723207
RN7SL1	RNA component of signal recognition particle 7SL1	0.381	0.048600871
PLAC9	placenta associated 9	0.446	0.041385234
ST6GALNAC3	ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 3	0.469	0.047504846
NPTX1	neuronal pentraxin 1	0.727	6.36276E-08

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“Questa è per te che mi hai capito, sostenuto, sopportato

Lo ripeto all'infinito (sei unica al mondo)”